TRANSFOROMING GROWTH FACTOR-β1 ACTIVATION AND TRANSFORMING GROWTH FACTOR-β RECEPTOR 2 EXPRESSION LEVELS IN THE REGULATION OF THE TGF-β SIGNALING PATHWAY

By

Andrés Rojas

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Approved:

Professor William M. Grady

Professor Robert J. Matusik

Professor Ambra Pozzi

Professor Mark P. de Caestecker

Professor Neil A. Bhowmick

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ACF: Aberrant Crypt Foci

ALK: Activin Receptor-Like Kinase

AOM: Azoxymethane

APC: Adenomatous Polyposis Coli

BCA: Bicinchoninic Acid

BMP: Bone Morphogenetic Protein

BrdUrd: Bromodeoxyuridine

CDK: Cyclin-dependent Kinase

DN2R: Dominant Negative Transforming Growth Factor-β type 2 Receptor

ECM: Extracellular Matrix

ELISA: Enzyme-Linked Immunoabsorbent Assay

EMT: Epithelial-to-Mesenchymal Transition

ERK: Extracellular Signal-Regulated Kinase

FAP: Familial Adenomatous Polyposis

FoxO: Forkhead Box O

GAP: GTP-ase Activating Protein

JNK: Jun-N terminal Kinase

LAP: Latency-Associated Peptide

LOH: Loss of Heterozygosity

MAPK: Mitogen-Activated Protein Kinase

MMP: Matrix Metalloproteinase

NF-κB: Nuclear Factor-κB

PIK3CA: Phosphatidylinositol 3-Kinase, Catalytic, Alpha

PTEN: Phosphatase and Tensin Homolog

RB: Retinoblastoma

RIPA: RadioImmunoPrecipitation Assay

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SARA: Smad Anchor for Receptor Activation

SBE: Smad-binding Element

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SFM: Serum Free Medium

SMURF: Smad Ubiquitin Regulatory Factor

TCF/LEF: T-Cell Factor/Lymphoid Enhancer Factor

TGF-β: Transforming Growth Factor-β

TGFBR: Transforming Growth Factor-β Receptor

TGFBR2: Transforming Growth Factor-β Receptor 2

VEGF: Vascular Endothelial Growth Factor

Wnt: Wingless-type

CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

Colorectal cancer is the third most common cancer in the United States. In 2007, approximately 154,000 new cases of colorectal cancer will be diagnosed, and it is estimated that more than 52,000 people will die from the disease. (1)

1. Molecular basis of colorectal cancer

Colorectal cancer develops as result of the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelial cells to colon cancer cells. Like other types of cancers, the majority of cases of colon cancer are sporadic, while a small proportion are caused by genetic mutations inherited in an autosomal dominant fashion (2). Genomic instability, a common event observed in colon cancer, facilitates the accumulation of mutations in tumor suppressor genes and oncogenes which can be detected in early adenomas.

Colorectal cancer originates as a result of histological and genetic events that drive the process known as the ‘‘adenoma-to-carcinoma progression sequence.’’ This sequence of events results in the neoplastic transformation of normal colonic epithelium into adenocarcinoma, a process that may take between 10 to 15 years. The most commonly affected genes in this process include *APC, CTNNB1, KRAS, BRAF, SMAD4*, *TP53*, *PIK3CA*, and TGF-β receptor 2 (*TGFBR2)* (**Figure 1.1**). Additionally,

environmental factors may accelerate the occurrence and progression of colon cancer (3, 4).

1.1. Genomic instability

In early neoplastic lesions, including colorectal adenomas, it has been observed that DNA damage induces the activation of checkpoint systems involved in DNA repair, which prevents the transformation from adenoma to adenocarcinoma. However, if the DNA repair system fails for some reason, the loss of this barrier facilities the progression of adenoma to adenocarcinoma. This process leads to genomic instability, which induces cell proliferation and cell survival (5). At least three forms of genomic instability have been identified in patients with colon cancer: (a) microsatellite instability (MSI), (b) chromosomal instability (CIN), and (c) chromosomal translocations. MSI and CIN are typically seen after adenoma formation, but before progression to adenocarcinoma.

The frequency of CIN in colorectal cancer is very low, and has only been observed in few cases. However, MSI, which results from genetic and epigenetic alterations in genes required for DNA repair, seems to play an important role in the progression of colon cancer.

Genomic instability is a characteristic feature of neoplastic cells which provides an interesting area of investigation aimed at developing new therapies to prevent the progression from adenoma to adenocarcinoma (6).

1.2. Epigenetic changes and colon cancer

Colon cancer progresses as result of the accumulations of sequential events that either activate oncogenes or inhibit the action of tumor suppressor genes. It is accepted that one of the key events in the progression of this type of cancer is the gain of genetic alterations in the components of different intracellular signaling pathways, including Wingless/Wnt, *KRAS2, TP53,* and TGF-β (7).

In addition to mutations in the genes that comprise the pathways mentioned above, the presence of epigenetic changes such as the aberrant DNA methylation of CpG islands (CGI) seems to be an important factor in the development and progression of colon cancer. The presence of methylated DNA is not exclusive to tumors, but actually it is a common molecular process that is present throughout the genome and its patrons are maintained during development in a relatively stable manner. The CpG islands are regions of DNA of 0.2-3 kb that are present in the 5' prime end of the promoter. These islands are under-represented in the genome, since they are consider as hot spots to gain mutations which results in the inhibition of the gene (7, 8). It has been established that DNA methylation commonly targets tumor suppressor genes, resulting in the inhibition of translation of these proteins that normally function as a barrier that prevents the progression of neoplasia.

The exact origin and consequences of epigenetic changes in the initiation and progression of colon cancer are controversial. Some investigators have proposed that these events are passenger events accompanying genetic changes, while others recognize epigenetic changes as pathogenic events (7).

In order to clarify these possibilities, mice genetically deficient in methyltransferase were created. These animals proved to be resistant to colorectal tumorogenesis initiated by mutations of the *APC* tumor suppressor gene. These results, in addition to reports describing aberrant methylation in breast cancer metastases in bone, brain or lungs, support the hypothesis that epigenetic changes may favor the progression of colon cancer as well as other types of cancers (9, 10).

Interestingly, the methylation pattern detected in metastases differs from the pattern observed in the original tumor. It has been demonstrated that the reexpression of some of the genes silenced by aberrant methylation increases the susceptibility of metastatic tumors to treatment (11). In addition, Lee et al detected a group of genes (*RASSF1A, CDKN2A, GSTP1, THBS1* and *TIMP*) that were more commonly methylated in colon cancer than in adenomas, suggesting that a least some of these genes might be involved in the process of the development of colon cancer (7, 12). In a report by Kane et al, the aberrant methylation of the promoter region of the gene *MLH1* (mutL homologue 1) was sufficient to suppress the expression of this protein, creating genomic instability (4, 7, 13).

Another example of the importance of aberrant methylation in cancer has been observed in hepatocellular carcinomas, in which the expression of the gene *RUNX3* (an important regulator of the TGF-β pathway) is repressed, disrupting the existing balance between cell proliferation and apoptosis in the liver that is normally under the control of TGF-β (14).

In the pancreatic cancer cell line, MIA PaCa-2, it has been reported that low activity of the transcription factor *Sp1* results in defective *TGFBR2* expression, thereby

altering the tumor-suppressive action of TGF-β. In cells treated with 5-azadeoxycytinde, an inhibitor of DNA methyltransferase 2, an increase in the expression *TGF-β* was detected. This has been correlated with increased expression mediated by *Sp1*, which ultimately resulted in functional TGF-β tumor suppressor activity (15).

Another component of the TGF-β signaling pathway whose expression is altered by the action of aberrant methylation is the *TGFBR1*. Aberrant methylation of *TGFBR1* has previously been detected in patients with gastric carcinoma (Reviewed in (16)).

Recent progresses in the understanding of the mechanisms through which DNA methylation may affect transcription have shown that aberrant DNA methylation impedes the interaction between promoters and transcription factors, such as AP-2, CREB, E2F, CBF, and NF-κB, thereby preventing the expression of proteins normally regulated by these factors. For example, methylation of the CCAAT box present in the promoter of the gene *MLH,* prevents the binding of the transcription factor CBF, resulting in the inhibition of *MLH1* transcription (8, 17).

Due to the importance of methylation in the initiation and progression of cancer, several investigations are underway to identify genes that are commonly methylated in colorectal as well as other types of cancer. Additionally, some methylated genes might be used as markers for early detection of tumors, staging of the tumors. Certain methylated genes are associated with hereditary cancer syndromes such as Lynch syndrome, where methylation of *MLH1* in adenomas might play a role in the initiation of this familial colon cancer syndrome (3, 4, 18).

In addition to delineating the role of aberrant DNA methylation in colon cancer, these studies have the potential to provide useful information that is being used to

develop new cancer therapies. In fact, various pharmaceutical or biological agents that might control the progression of normal epithelium to adenocarcinoma are currently being evaluated in clinical trials (7, 18).

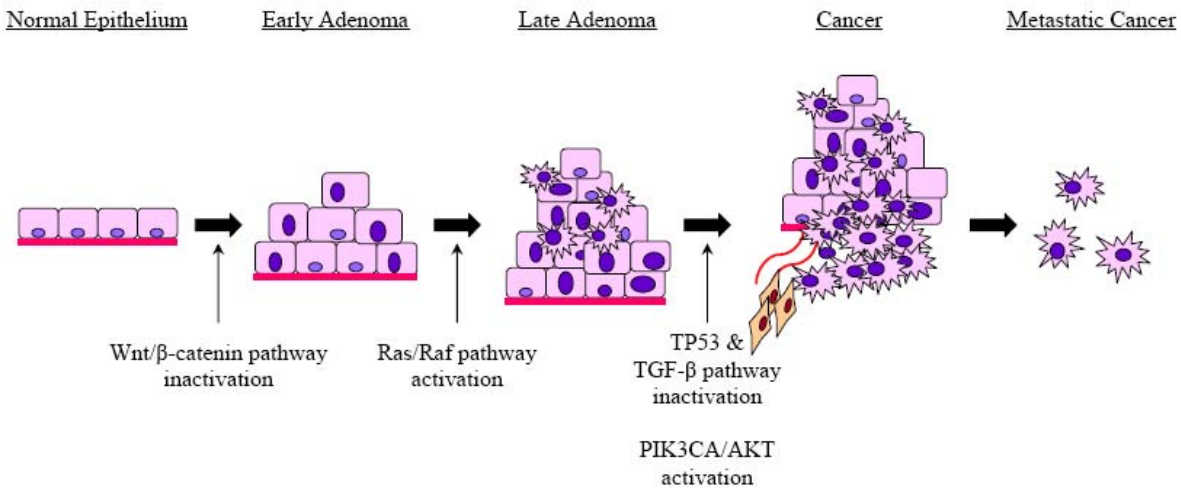


Figure 1.1 Progression of Colon cancer. Adapted from Muñoz (2006) (19)

1.3. APC/Wnt signaling pathway and colon cancer

Prior studies of the family cancer syndrome familial adenomatous polyposis (FAP) suggested the association of genetic alterations with the formation of colorectal cancer. FAP is characterized by the development of hundreds to thousands of intestinal adenomatous polyps. It has been shown that mutations in the gene adenomatous polyposis coli (*APC*) are responsible for this syndrome. *APC* is a gene that normally plays an important role in the regulation of cytoplasmic β-catenin by forming a multiprotein complex with Casein Kinase 1α (CK1α), Axin, and glycogen synthase kinase-3β (GSK-3β). Once β-catenin is bound to this complex, it is phosphorylated by CK1α and GSK-3β, which promotes its ubiquitin-mediated proteasomal degradation.

On the other hand, when secreted ligands of the Wnt family bind and activate their membrane receptors, which are encoded by the *Frizzled* genes, cytoplasmic Dishevelled induces Axin phosphorylation and posterior degradation. As result of this degradation, β-catenin phosphorylation is prevented and thus it is free to translocate into the cell nucleus. Nuclear β-catenin is a co-activator of the T-Cell Factor (TCF)/Lymphoid Enhancer Factor (LEF) family of transcription factors that induce the transcription of several target genes that control the progression of normal epithelium to adenocarcinomas, including *CCND1* and *CMYC* (reviewed in (20)).

In addition other studies have demonstrated that the *APC* gene is one of the most common targets for mutations in colon cancer. Approximately 85% of all sporadic colorectal adenocarcinomas have truncations and missense mutations in *APC,* which results in the stabilization and accumulation of β-catenin in the nucleus of a cell.

It is accepted that APC is implicated in the initiation of adenomas, while other gene mutations, such as those involving *TP53*, appear to mediate the malignant transformation of adenomas into adenocarcinomas.

Several genetically engineered mouse models of intestinal tumorigenesis have corroborated the importance of the Wnt/β-catenin pathway in carcinogenesis. The $Apc^{Min/+}$ mouse, the first genetic model of gastrointestinal neoplasia to be generated, harbors a nonsense mutation in codon 850 that results in a truncated protein. Due to the large number of tumors in these animals, is so high that their lifespan is very short and therefore most lesions only reach the stage of adenoma (21). Due to this characteristic of the Min mouse, additional *APC* mutations have been developed, and interaction of APC with intracellular signaling pathways such as TGF-β have been established. These models have demonstrated, the critical role that TGF-β and APC play in the initiation and progression of colon cancer (22).

1.4. The Ras pathway

Ras is a protein that plays an important role in controlling the activity of several signaling pathways that are responsible for the regulation of cell proliferation. It has been shown that the expression of mutated Ras in human tumors is very frequent (~20% of all human tumors has undergone an activating mutation in one of the *Ras* genes) (23). The presence of mutant Ras is responsible for the deregulation of cellular processes such as programmed cell death, angiogenesis, and tumor invasion.

The Ras GTPase functions as a transducer of cell signals that originate in membrane receptors and are then transmitted via intracellular pathways to control cell

growth, survival and differentiation. Three different members of the Ras family are found to be mutated in human cancer: *H-Ras*, *K-Ras* and *N-Ras*. The protein products of these genes share 85% homology in their amino acid sequences. (23).

The Ras protein that is bound to GTP is able to interact with and activate effector enzymes that regulate various cellular processes. One of the best studied targets of Ras is Raf, a serine/threonine kinase. Once phosphorylated, Raf activates mitogen-activated protein kinase (MAPK) cascades, including Mek1/2 and Erk1/2. The activation of Erk induces its translocation to the cell nucleus, where it promotes the formation of the AP-1 transcription factor (Fos/Jun) that regulates the expression of proteins such as D-type cyclins (23). Other pathways, including AKT/PKB, c-JunN-terminal kinase/stress activated protein kinase (JNKs/Sapks), and p38, also are induced by Ras. It is known that in the JNK and p38 pathways, the interaction between Ras and Rho pathways seems to play a key role (24).

Several reports have established that the particular effects of the Ras signaling pathway are strongly influenced by the cellular context. For instance, it has been shown that the presence of activated Ras in immortalized cells promotes an oncogenic transformation, whereas in primary cells, activated Ras can induce cell cycle arrest (24, 25). It is unclear if there is a correlation between TGF-β signaling and mutant Ras during the progression of cancer, as different studies have reached disparate conclusions (26-28)

1.5. The AKT signaling pathway

AKT is involved in the regulation of several types of cellular process and activities such as cell proliferation and survival, cell size and response to nutrient

availability, intermediary metabolism, angiogenesis, and tissue invasion (Reviewed in (29)). Several studies have demonstrated that in over 25% of cancers involving the colon, liver, breast, stomach and glioblastomas, the catalytic subunit of PIK3 (also known as PIK3CA) is mutated. These mutations lead to elevated enzymatic activity, which is a crucial event in the process of cell growth (in the absence of growth factors) and invasion (30, 31).

The activation of the PI3K/AKT pathway can be triggered by diverse types of extracellular stimuli that are transmitted intracellularly via growth-factor receptor and/or integrin-receptor pathways. The aberrant induction of this pathway is an important factor in the development of different types of cancers (32). It is also known that AKT is able to regulate cell growth and translation of different through pathways that induce the activation of mTor (32, 33).

A sequence of phosphorylation of PIP2 and PDK1/2 is required to induce the phosphorylation of AKT on threonine 308 and serine 473, events which are required for its complete activation. Once activated, AKT translocates to the cytoplasm and to the nucleus, where it phosphorylates a vast variety of targets. The activity of PI3K is regulated by a group of lipid phosphatases, including PTEN (Phosphatase-and-Tensin-Homolog) and SHIP (SH2-containing inositol phosphatase) which are involved in the dephosphorylation of PIP3, inducing its conversion to PIP2 (34).

Several proteins are targets of the active form of AKT including FoxO1. FoxO1 is a member of the family of proteins that is important in a variety of cellular processes, including cellular differentiation, tumor suppression, metabolism, cell-cycle arrest, cell death, and protection from stress (35). Once FoxO1 is phosphorylated by AKT, it is

forced out of the nucleus, preventing the expression of genes regulated normally regulated by FoxO1 (35). One such gene is *p21* which together with the protein products of other genes, including *p27* and *p15* (cyclin-dependent kinases) regulate the cell cycle (32).

Finally, several reports have demonstrated the crucial role of AKT in the process of tumor invasion and metastasis, related to its ability to promote the secretion of matrix metaloproteinases (MMPs) , and to induce of the transition of epithelia to mesenchyme (TMA) (32, 36-38) (**Figure. 1**). Indeed, it has been observed that mutations in *PIK3CA* occur at the stage in which colorectal tumors become invasive (39).

Since AKT is regulated by PTEN, mutations of *PTEN* are other means to up-regulate the activity of AKT. This can lead to the formation of hamartomatous polyps in the gastrointestinal tract, mucocutaneous lesions, and an increased risk of developing neoplasms (40). Moreover, it is known that *Pten*-null mice die during embryonic development, and animals heterozygous in *Pten* develop tumors in a variety of organs, which are associated with loss of heterozygosity in the *Pten* locus (41).

Finally, extensive work is still in progress in order to understand the complexity of the AKT pathway, and to establish potential interactions with other pathways that might be important in the progression of cancer. One such interaction of the AKT pathway might be with the TGF-β pathway (35).

2. The TGF-β pathway

2.1 TGF-β ligands

The TGF-β family is a group of cytokines characterized by the presence of six conserved cysteine residues. This family is subdivided between two subfamilies defined by their sequence similarity and the specific signaling pathways that they are able to activate. These two subfamilies are the TGF-β/Activin/Nodal subfamily and the BMP (bone morphogenic protein)/GDF (growth and differentiation factor)/MIS (Muellerian-inhibiting substance) subfamily. The active form of TGF-β cytokine is a dimer stabilized by hydrophobic interactions which is typically supported by disulfide bridges (42). All of the TGF-β superfamily members transduce their signal through heterotetrameric complexes including two types of serine-threonine kinase receptors known as TGBR1 and TGFBR2 (43).

2.2. TGF-β1 activation

Among the three different TGF-βs (β1, β2 and β3) that have been described in mammalian cells, TGF-β1 is the most frequently upregulated in cancer, and its role in carcinogenesis has been extensively evaluated (44).

The TGF-β1 protein is secreted as an inactive "latent" complex, which is not able to interact with its receptor. It has been reported in experiments *in vivo* and *in vitro* that the activity of the mature TGF-β is blocked by its noncovalent association with a dimer in the N-terminal propeptide called latency-associated protein (LAP) (45, 46). The LAP component of the TGF-β:LAP complex is typically disulfide-linked to the protein known

as latent TGF-β1-binding protein (LTBP), resulting in a larger form of latent TGF-β (45, 46).

The presence of the TGF-β inhibitory complex protects TGF-β from degradation, thereby enhancing the stability of the protein and preventing undesired binding with its receptor (44, 45). Once the latent complex is secreted, it is sequestered in the extracellular matrix, which acts as a reservoir of TGF-β. This reserve provides the nearby cells rapid access to this important cytokine without the need for new synthesis (47, 48). It is known that proteins like decorin and α-2-macroglobulin bind to and sequester free TGF-β to prevent receptor activation (42).

Secretion of the latent ligand requires a regulated activation process involving various proteases that specifically target the inhibitory proteins LAP and LTBP, thus releasing active TGF-β1 into the extracellular space where it is recognized by TGFBR2. Several methods of activation have been established, and it is known that *in vitro,* TGF-β can be activated by extremes pH, heat or chaotropic agents. Other more physiological methods have been proposed for TGF-β activation, including deglycosylation, or enzymatic activation of calpain or cathespin. However, the most accepted methods for TGF-β1 activation are currently those mediated by the proteins plasmin and thrombospondin-1 (TSP1) (44-46).

Plasmin, which activates TGF-β1 by cleaving LAP, seems to be an important activator of TGF-β1 *in vivo*; however, experiments performed using plasminogen-null animals do not recapitulate the pathology observed in TGF-β null mice, suggesting the existence of another major activator of TGF-β1 (45, 49). A prior investigation by Crawford et al (45) proposed that TSP1 is a key player in the activation of TGF-β1.

TSP1 is a trimer of disulfide-linked 180 kD subunits which is secreted by several types of cells. In cell-free systems, it has been reported that TSP1 is able to bind to both small and large latent forms of TGF-β1. Other studies have demonstrated that region of TSP1 involved in the activation of TGF-β is localized in a domain that consists of three type 1 repeats (3TSR) (46, 50, 51). The TSRs of TSP1 are able to activate TGF-β1 by binding with the N-terminal region of LAP, forming a trimolecular complex that alters the conformation of TGF-β and makes it accessible to its receptor (51).

2.3. The TGF-β receptors

TGF-β, originally named for its ability to stimulate proliferation of normal rat fibroblasts in soft agar, is part of the TGF-β superfamily that includes TGF-βs (-β1, -β2 and -β3), BMPs, activins and related proteins. The signaling pathways regulated by the members of this superfamily are involved in numerous varied biological processes that occur during embryonic development. These proteins also play a crucial role in adult organisms by maintaining tissue homeostasis between apoptosis and cell survival and proliferation (43, 44, 52-54).

The balance between these numerous cellular events is crucial to many physiological processes, and its deregulation may precede the initiation or progression of various diseases. The TGF-β signaling pathway transduces its signal through type I (TGBR1) and type II (TGFBR2) serine/threonine kinase transmembrane receptors.

Five type II and seven type I receptors, also known as activin-receptor-like kinases (ALKs), have been identified in vertebrates (55). In the absence of ligand, TGBR1 and TGFBR2 exist as homodimers at the cell surface. The binding of ligand

with the TGFBR2 induces its phosphorylation and its assembly with the TGBR1. The formation of the TGFBR2/TGBR1 complex results in the phosphorylation of TGBR1 in the GS domain, which is rich in glycine and serine residues, ultimately initiating a downstream signaling cascade. The presence of both receptors is required and sufficient for TGF-β signaling (43, 56-59).

2.4. Mutations in the TGF-β receptors

The presence of genetic alterations in the *TGFBR2* gene has been reported as one of the most common mechanism through which colon cancer cells acquire TGF-β resistance (58). It has been shown that mutations in the *TGFBR2* are commonly found in colon cancer cell lines with microsatellite instability (MSI), which develops following inactivation of the complex of proteins that repairs basepair mismatches that arise during DNA replication. The target for mutation in MSI cells is a region in exon 3 of *TGFBR2* that consists of a 10 base pair polyadenine repeat named BAT-RII (big adenine tract in TGFBR2). This type of mutation is characterized by an insertion/deletion of one or two adenines that results in the introduction of non-sense mutations, leading to a truncated protein lacking both the transmembrane domain and the intracellular serine-threonine kinase domain. BAT-RII mutations found in 100 of 110 MSI from patients with colon cancers were biallelic; however, point mutations in the serine-threonine kinase domain of the *TGFBR2* have been reported in one of the alleles, suggesting its function as a tumor suppressor gene mediated by TGF-β in normal colon (43).

In addition to the genetic alterations that are present in *TGFBR2* that are responsible for the inhibition of the TGF-β pathway, the loss of expression of *TGFBR2*,

which has been reported in several types of cancer, represents another event that blocks the TGF-β pathway (43). This event is either characterized by a loss of TGFBR2 protein or loss of mRNA expression in tumor cells, which suggests that reduction in the expression levels of the TGFBR2 may be an important event in the initiation and progression of colon cancer (43, 52).

However, no alterations in TGBR1 or the type III TGF-β receptor (TGFBR3) have been observed in studies performed in colon cancer cell lines that are resistant to TGF-β. These results suggest that mutations that inactivate the *TGFBR2* are highly favorable in the process of tumor formation.

2.5. The Smad signaling pathway

The activated type I receptor initiates intracellular signaling by phosphorylating the receptor-regulated Smads (Smad2 and Smad3) in a process that is mediated by the presence of the protein known as Smad anchor for receptor activation (SARA) (60). The Smads are modular proteins with conserved N-terminal Mad-homology (MH1), and intermediate linker and C-terminal MH2 domains. The MH1 domain is involved in the process of nuclear localization, DNA binding and protein-protein interactions (16). The receptor-phosphorylated Smad2 and Smad3 demonstrate high affinity for Smad4 (Co-Smad) binding. This induces the formation of a functional trimeric protein complex that has a high affinity for the cell nucleus, contrasting with the monomeric proteins that continuously shuttle in and out of the nucleus (Reviewed in (16)).

Once the complex Smad2/3-Smad4 is formed, it translocates to the nucleus where it regulates the transcription of genes regulated by TGF-β. The Smad complexes bind to

the promoter regions of target genes and in presence of other transcription factors induce the expression of genes in a cell-context and cell-type dependent manner (**Figure 2**) (42, 55).

In order to regulate the activity of the TGF-β pathway mediated by Smad proteins, the Smad signal has to be negatively regulated. Inhibitory (I)-Smads (Smad-7 and 6) function in this capacity. By binding to the activated receptor, they compete with the R-Smads to bind with TGBR1, thus preventing the phosphorylation of the R-Smads. Smad7 is also able to recruit phosphatases that dephosphorylate and inactivate the receptor complex. Finally, Smad7 is able to bind and activate the E3-ubiquitin ligases Smurfs1 and 2 to induce the ubiquitination of the TGBR1, resulting in the degradation of the receptor (16)..

2.6. TGF-β Smad-independent signaling

As previously mentioned, TGF-β has the ability to regulate both cell proliferation and apoptosis at different stages of colon cancer development. These qualities have led to intensive investigations in order to understand the complexity of the TGF-β. Currently, it is accepted that complex protein interactions are required at multiple levels in the TGF-β signal cascade in order to regulate such processes (61). In fact, it is known in addition to Smad-mediated transcription, TGF-β is also able to activate other signaling pathways, including MAPK, ERK1 and ERK2, c-Jun, PI3k and p38. These groups of pathways are designated the "Smad-independent" pathways (16, 44, 52). The activation of these pathways has been associated with regulation of the Smad pathway and with the mediation of signals induced by other growth factors, such as EGF (16). An example of

Smad pathway regulation mediated by the Smad-independent pathway was reported by Kretzschmar et al (62). This group observed that the phosphorylation of the linker domain of R-Smad, which is mediated by Erk, can block the nuclear translocation of R-Smad, thus preventing the transcription of genes mediated by TGF-β. Additionally, the activation of the Ras/Erk MAPK signaling pathway can induce the expression of TGF-β1, which can result in the amplification of TGF-β-mediated cell responses inducing secondary effects (63). Additionally, JNK activation has been associated with repression of Smad2 transcription activity by inducing the interaction of Smad2 with its co-repressor protein, TGIF (TG interacting factor) (64). Finally, it has been observed that the activation of PI3K in glioblastoma cells is responsible for the phosohprylation of FoxO which prevents the formation of the Smad-FoxO complex, preventing the expression of *p21* (35, 65) .

Additionally, the activation of Smad-independent pathways have been associated with several TGF-β mediated morphogenetic responses, including cell migration and epithelial-to-mesenchymal transition (EMT), which are important events in embryogenesis, fibrotic diseases, and advanced carcinomas (Reviewed in (64)).

Despite the knowledge that TGF-β can activate proteins other than Smad, the nature of the biochemical link between the TGF-β receptors and MKK and why this link is present in some conditions and not in others, is not fully understood (66). Previous reports have proposed several proteins that might function as linkers, including TGF-β activated kinase (TAK-1), that has also been observed to play a role in the interlukin-1 and Wnt pathways. Additionally, members of the Rho family have been associated with TGF-β–mediated JNK activation and have also been proposed as linkers (**Figure 1.2**). In

addition to the proteins mentioned above, MEKK1 may also function upstream of TGF-β-mediated activation of MAPKKs, and in cooperation with TAK-1 may be responsible for p38 and IκB kinase activation, resulting in the stimulation of NF-κB signaling (63).

The interaction between the Smad-dependent and Smad-independent pathways determines how a cell responds to TGF-β, and may explain the observation that the cell state and cell type, referred to as the "cellular context," plays a significant role in determining TGF-β's effects (63, 67, 68).

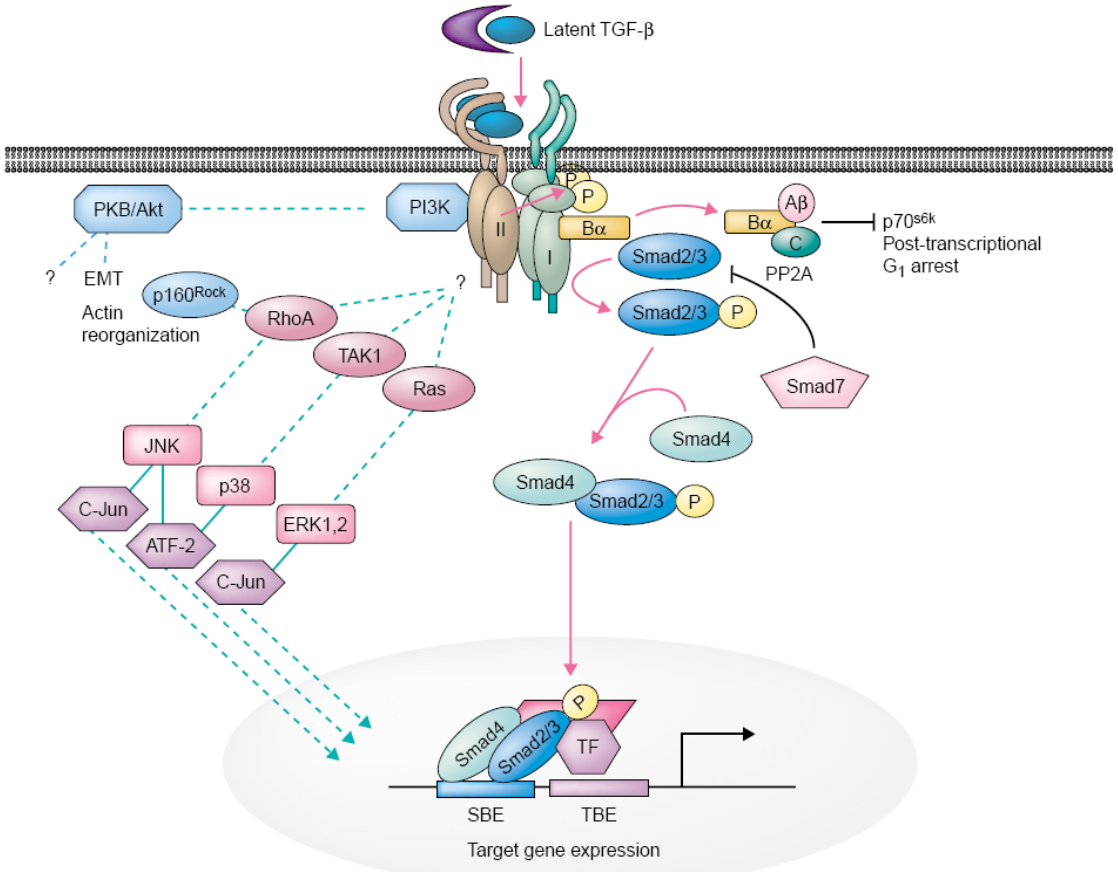


Figure 1.2. The TGF-β pathway: See text for details. Adapted from Wakefield and Roberts 2002 (52)

2.7. TGF-β and Cancer Formation

The results of several studies have demonstrated that the process of tumorogenesis is a multistep process, involving both genetic and epigenetic alterations, that drives the transformation from normal cells into a highly malignant tumor cells (69).

In order to understand the origin and consequences of these genetic and epigenetic alterations in the initiation and progression of cancer, intense investigation is currently underway. A prior article by Hanahan and Weinberg proposed a model describing a common set of seven biological attributes that cancers typically possess in order to undergo full transformation (69). In order to acquire these seven characteristics of cancer (which include resistance to growth inhibitors; growth factor independence; invasion and metastasis; angiogenesis; immortalization; resistance to apoptosis; and evasion of immune surveillance), colorectal cancers progressively accumulate genetic and epigenetic alterations that lead to the transformation of normal colonic epithelial cells to colon cancer cells (69). For example, it has established that rodent cells required at least two genetic events to acquire neoplastic characteristics, whereas human cells appear to be more difficult to transform thus one would expect additional genetic and/or epigenetic alterations (Reviewed in (69)).

Intensive investigation of animal models and various forms of human cancers has demonstrated that the process of tumor development is preceded by a selection process (analogous to the Darwinian evolution) in which the accumulation of genetics changes confers upon a cell a growth advantage, resulting in the transformation of a normal cell into a cancer cell (69). Once a cell or group of cells has undergone transformation, they posses the ability to invade other tissues or organs.

Genomic instability seems to be a key feature of the tumorogenesis process observed in colon cancer (70). It has been demonstrated that certain mutations are more common in some types of cancer versus others. For instance, it has been observed that in colorectal cancer, the TGF-β signaling pathway is frequently altered, and mutations in the *APC* gene are common (71).

Mutations in the *APC* gene in general give rise to colon adenomas. Following this, additional sequential events are thought to be responsible for the activation of oncogenes or inactivation of tumor suppressor genes, which plays a key role in the progression of adenomas to adenocarcinomas. Mutations of *KRAS, TP53* and members of the transforming growth factor (TGF-β) signaling pathway have been implicated in mediating these progression events (22, 72, 73).

More recently, epigenetic alterations, including aberrant CpG island DNA methylation, have been described in colon adenomas and adenocarcinomas. Aberrant DNA methylation has been proposed to be as an alternate mechanism for inactivating tumor suppressor genes. Aberrant DNA methylation induces the transcriptional repression of tumor suppressor genes and is generally believed to promote tumor formation through this effect. Some of the genes that have been reported to be methylated during the initiation and/or progression of colon cancer include *MLH1, CDKN2A/p16* and *TIMP*3 (4, 74).

Mutation events in elements of the TGF-β pathway, including *TGFBR2, SMAD2*, and *SMAD4,* have been identified in approximately half of colon cancers, and these mutations appear to affect the tumor-suppressor activities of TGF-β. Other cellular functions, such as apoptosis, cell proliferation, and cell cycle arrest can also be affected

by mutations in these proteins (44, 75, 76), which result in resistance to the tumor-suppressor effects of transforming growth factor TGF-β (59, 77). The transforming growth factor β (TGF-β) was originally named for its ability to transform normal fibroblasts in culture. Shortly thereafter, other reports demonstrated that this cytokine also had the ability to inhibit the growth of normal epithelial cells. In addition to these tumor suppressor functions, TGF-β seems to have the potential to mediate tumor-promoting effects through the induction of epithelial-to-mesenchymal transition (EMT), angiogenesis, and extracellular matrix remodeling, features that would promote the invasive behavior of cancers (52, 78-80).

TGF-β functions as a tumor suppressor gene in the normal colon and in the early stages of colorectal carcinogenesis by inhibiting cell growth in epithelial and lymphoid cells. However, in advanced tumors, cells become resistant to growth inhibition mediated by TGF-β, and overexpression of the cytokine has been associated with EMT, tumor invasion, immunosuppression, and angiogenesis (44, 81).

The inhibitory response on the cell cycle mediated by TGF-β is associated with critical regulators of the G1 phase. Expression of G1-phase regulators has been associated with the activation of the Smad-dependent pathways (64). Regulators such as p15, p21 and p57 are induced by TGF-β, and in conjunction with other pathways (such as FoxO in the case of p21), TGF-β is able to regulate cellular proliferation (35, 52). Another example of the role of the Smad-dependent pathway in the regulation of cell proliferation is the downregulation of *c-myc,* which results from the binding of the Smad complex to the TGF-β-inhibitory elements presents in the *c-myc* promoter.

However, previous reports have shown that the cell growth inhibition mediated by TGF-β in certain cells lines can be affected by the Smad-independent pathway, since p21 and p15 can be upregulated upon activation of the MAPK pathway (82). Additionally, it has been demonstrated that the presence of Smad-4 is dispensable for certain TGF-β-mediated cellular responses, including cell growth inhibition (52, 83, 84).

Due to the dual role of TGF-β as a tumor suppressor gene and oncogene, it would appear that the TGF-β pathway is very sensitive to minor changes in the expression of TGF-β. For example, in mice it has been observed that the loss of one allele of TGF-β1 is sufficient to affect its role as a tumor suppressor gene (85). Based on this model, it is worth considering that small modifications in the expression of TGF-β might affect the propensity to develop cancer in humans (52, 85). An example of this concept was reported by Kushiyama et al., who noted that TGF-β1 production in colonic mucosa was lower in the distal colon versus the proximal colon, and concluded that development of sporadic colon adenomas is associated with reduced levels of TGF-β1 (86). Additionally, low expression levels of TGFBR2 are associated with more aggressive phenotypes of different types of cancer. This may be due to the activation of a catalytic pathway which amplifies low input of the "normal" TGF-β signaling pathway which is regulated by the Smad-dependent pathway (52). Mutations, epigenetic changes and activation of oncogenes like *HRAS*, *SKI* and *SNO* likely play a role in the reduction of TGF-β receptor expression, as well as, inactivation of *SMAD4*, *SMAD2*, or attenuation of TGF-β signals. These events have been observed in tumors of the breast, pancreas, lung, and in melanomas (87, 88).

Interestingly, overexpression of TGF-β1 or TGBR1 has been associated with an increased rate of breast cancer metastasis in mice (89). These results are specific examples demonstrating the complexity of this pathway. It has been accepted that in normal tissue or in early stages of cancer development, the tumor-suppressor activities of TGF-β predominate, yet during carcinogenesis these qualities are supplanted by oncogenic functions that favor processes such as cell growth, angiogenesis and immunosuppression.

The results mentioned above make clear the importance of the context in which the TGF-β pathway is activated. It is apparent that both the cellular and molecular context will strongly influence the ultimate cellular response.

The use of knockout animals or cell lines lacking particular components of the TGF-β pathway have been important tools that have demonstrated the existence of two major TGF-β-regulated pathways: the "Smad-dependent pathway" and the "Smad-independent pathway" (**Figure 1.2**) (64). In order to demonstrate the existence of the Smad-independent pathway, embryonic fibroblasts from Smad4 knockout animals have demonstrated that these cells conserve a certain- number of genes and cellular responses that are controlled by TGF-β (83). Several of these cellular responses are mediated by different MPAK pathways, including Erk, JNK, and p38 MPAK. Typically the activation of such pathways occurs between five and thirty minutes after the signaling cascade is induced by the binding of the ligand with its receptor (i.e. EGF, IGF or TGF-β), demonstrating independence from transcriptional processes; however, the biological consequences of such activation is poorly characterized (63, 66). Conversely, in other cell types and under particular conditions such activation takes place very slowly,

suggesting that the activation of the MAPK pathway may be secondary effects (44). Other pathways that are modulated by TGF-β have been identified, and it is known that depending on the cell line evaluated, TGF-β can activate Rho-like GTPases (including RhoA, Rac and Cdc42), inducing changes in cytoskeletal organization or the process of epithelial-to-mesenchymal transition (63, 90).

Several studies with mouse models and cell lines have demonstrated that TGF-β1 is a potent tumor suppressor in colorectal cancer. It has been established that some degree of resistance to the effects of TGF-β, via inactivating mutations of *TGFBR2* (25%), *SMAD4* (20%), and *SMAD2* (<5%), has been found in approximately 50% of colorectal cancer cases (35). Moreover, it has been shown that *TGFBR2* mutations correlate with the progression of microsatellite unstable adenomas to cancer (72). Finally, it has been demonstrated that restoration of a functional TGFBR2 into several colon cancer cell lines reverses some characteristics of their transformed phenotype (91). However, it has also been shown that expression of a dominant-negative TGFBR2 in a mouse colon cancer cell line prevents EMT, reduces cell motility, and decreases a cell's ability to form metastases (91).

3. **Research Objectives**

The cell signaling mediated by TGF-β is very complex and has been involved in important and divergent cellular processes such apoptosis and cell proliferation. This paradoxical role of TGF-β has been the source of several investigations, and although important information has previously been generated, many of the mechanisms involved in the pathways that are regulated by TGF-β are unknown. In order to better understand the role of TGF-β in the initiation and progression of colon cancer, we have performed studies utilizing both cellular and animal models. In our previously published studies, we have shown the dramatic consequences that the absence of TGF-β mediated pathways (due to mutations or deletions of the *TGFBR2* gene) has on the progression of colon cancer (22, 92). These reports and others clearly show the relevance of genetic alterations in the deregulation of pathways mediated by TGF-β.

Recently, increased attention has been focused on the role of epigenetic changes (such as DNA methylation) in different types of cancers. Epigenetic changes in tumors mostly result in inappropriate gene silencing as a consequence of a sequence of alterations in chromatin structure, including CpG island hypermethylation and histone modification (reviewed in (93). Some types of cancer, such breast, lung and renal carcinoma demonstrate reduced TGFBR1 and TGFBR2 expression, presumably due to the presence of epigenetic changes (Reviewed in (16)).

Although there are reports describing the presence and importance of epigenetic changes in colorectal cancer (GRADY LAB), no prior correlation between epigenetic changes and deregulation of the TGF-β signaling pathway has been established in

colorectal cancer. We strongly believe that the information provided will help to understand the role of epigenetic changes in the deregulation of the TGF-β signaling pathway and the potential consequences of such changes in the initiation and progression of colon cancer.

There are some cases in which cells do not suffer mutations in the TGF-β pathway, yet they do not respond to the tumor-suppressive effects of TGF-β. Instead, these cells demonstrate higher rates of proliferation and increased invasiveness, increasing the chances metastases. The exact mechanisms responsible for this phenomenon are not clear, and several hypotheses have been proposed (52, 94, 95). In order to better understand the TGF-β pathway, we decided to study how changes in the expression levels of the TGFBR2, as well as changes in the activation levels of TGF-β1, may be responsible for TGF-β resistance developed by some cells.

One of the changes that have been observed in cancer cells is the presence of epigenetic modifications such as DNA methylation, which leads to gene silencing. Taking advantage of this event in cancer cells, we decided to examine how the absence of TSP1 (a mayor activator of TGF-β1) affects levels of TGF-β, and the consequences of these changes on the activation of TGF-β signaling pathways. Additionally, this report will provided new knowledge regarding the role of epigenetic changes in the initiation and progression of colon cancer (Chapter II).

Finally, we conducted a series of experiments aiming to regulate TGFBR2 expression levels, since it is believed that TGFBR2 expression levels may be a mechanism that can alter the activation patterns of the TGF-β signaling pathways, and may be responsible for the paradoxical activity observed in established colorectal cancer

(CRCs) (52). The pathways proposed to be involved in this paradoxical activity are the Smad-dependent pathway (apoptosis) and the Smad-independent pathway (cell growth, motility, etc.) (63). In order to assess this model, we regulated the expression of the TGFBR2 using a modified version of the classic Ecdysone inducible system. Preliminary data obtained in our lab has shown that this system can precisely regulate the expression of the HA-tagged TGFBR2 transgene in the V-400 CRC cell line. Taking advantage of this system, we have generated important results that may contribute to the understanding of the dual role of TGF-β in colon cancer (Chapter III).

CHAPTER II

ABERRANT METHYLATION OF *TSP1* SUPPRESSES TGF-β1 ACTIVATION IN COLORECTAL CANCER

Abstract

Colorectal cancer arises from the progressive accumulation of mutations and epigenetic alterations in colon epithelial cells. Such alterations often deregulate signaling pathways, such as the RAS-MAPK and TGF-β pathways, which promotes tumor growth. The tumor promoting effects of mutations in genes, such as *APC,* have been demonstrated in cancer cell lines and in mouse models of intestinal cancer; however, the biological effects of most epigenetic events identified in colorectal cancer remain unknown. Consequently, we assessed whether the aberrant methylation of *TSP1* (Thrombospondin 1), a protein that regulates TGF-β activation, is an epigenetic mechanism for inhibiting the TGF-β signaling pathway. We determined the frequency of aberrantly methylated *TSP1* and its effects on TGF-β signaling in colon cancer. We observed methylated *TSP1* in colon cancer cell lines (28%), colon adenomas (14%) and colon adenocarcinomas (21%). We demonstrated that methylation-induced silencing of *TSP1* expression reduces the concentration of secreted active TGF-β1 and alters TGF-β signaling. Reversal of *TSP1* methylation results in increased TSP1 mediated activation of the latent TGF-β:LAP complex and subsequent TGF-β receptor activation. Our results provide evidence that the aberrant methylation of *TSP1* is a novel epigenetic mechanism for suppressing TGF-β signaling in colorectal cancer.

Introduction

Colon cancer arises from the accumulation of DNA alterations in colon epithelial cells, which mediate the initiation and progression of this cancer. Mutations in genes such as *KRAS, TP53* and members of the transforming growth factor (TGF-β) signaling pathway play a pathogenic role in the polyp-carcinoma sequence (22, 72, 73).

TGF-β is a pluripotent cytokine that has a multitude of effects on epithelial cells including the inhibition of proliferation, the induction of apoptosis and the stimulation of differentiation. Inactivating mutations in central members of the TGF-ß signaling pathway, *TGFBR2* and *SMAD4,* have been identified in approximately half of colon cancers (44, 75, 76). The biological effects of TGF-β and the identification of inactivating mutations in *TGFBR2* and *SMAD4* in colon cancers have demonstrated that the TGF-β signaling pathway can act as a colon cancer tumor suppressor pathway. The TGF-β signaling pathway consists of the TGF-β ligand; the heteromeric TGF-β receptor composed of the type I and type II TGF-β receptors (TGFBR1 and TGFBR2); and the post-receptor signaling proteins, such as SMAD2, 3, and 4. TGF-β1 is the most commonly expressed TGF-β ligand in adult epithelial tissues and is secreted in a latent form, which is noncovalently associated with the latency-associated peptide (LAP). The N-terminal region of LAP is the domain that interacts with TGF-β1 and mediates the latency of the complex (96). The dissociation of TGF-β:LAP is induced *in vivo* by plasmin or cathepsin as well as by thrombospondin 1 (*TSP1*) and is a required step for the activation of secreted TGF-β (45, 97). The activation of TGF-β1 is one of the primary mechanisms for regulating the activity of the TGF-β signaling pathway (96). Once TGF-β1 is activated, it can bind to and activate the TGF-β receptor, composed of TGFBR1 and

TGFBR2, inducing post-receptor signaling pathways (98, 99). The biological events induced by TGF-β, such as apoptosis or inhibition of proliferation, then mediate the tumor suppressor effects of this signaling pathway in the colon (75).

The identification of mutations in *TGFBR2* and *SMAD4* in primary colorectal cancer and of intestinal adenocarcinomas in animal model studies with mice that have inactivated TGFBR2 or SMAD4 demonstrates that genetic alterations can disrupt this pathway and promote the neoplastic behavior of intestinal epithelial cells (22, 75). Recently, genes in signaling pathways implicated in cancer formation have also been shown to be targets of aberrant CpG island DNA methylation, which represses gene expression. For example, silencing of *SFRP2* by methylation can promote the Wnt signaling pathway in colorectal cancer and is an alternate and complementary mechanism to *APC* mutation for activating this pathway (100). In addition to *SFRP2*, other aberrantly methylated genes are found commonly in colon adenomas and colon adenocarcinomas, and it has been suggested that they also promote tumor formation through signal pathway deregulation (101). *TSP1* is one of the genes that has been found to be aberrantly methylated in some colorectal cancers as well as in neuroblastomas, and gastric cancers. It has been suggested this event may promote tumorigenesis through its effects on angiogenesis (12, 102, 103). We hypothesized that in light of TSP1's known role in mediating TGF-β1 activation that the aberrant methylation of *TSP1* may promote tumor formation through inhibiting the TGF-β signaling pathway. We now present results that demonstrate the aberrant methylation of *TSP1* is a novel epigenetic mechanism for inhibiting the TGF-β signaling pathway in colorectal cancer.

Material and methods

Cell lines

SW-48, RKO, LoVo, V411, Moser, V241, FET and Raji cells were used in these studies and were cultured in DMEM+10% FBS (Gibco/Invitrogen, Carlsbad, California) The SW-48 cell line was obtained from ATCC (CCL-231, Manassas, VA). The RKO and LoVo cell lines were provided by Dr. Sanford Markowitz (Case Western Reserve University, Cleveland, OH). The human Burkitt lymphoma cell line Raji was kindly provided by Jennifer Pietenpol (Vanderbilt University, Nashville, TN). The Moser and FET cells were a generous gift from Michael Brattain (Roswell Park Cancer Institute, Buffalo, NY).

With regards to the treatment with 5-Aza 2' deoxycytidine (5Aza) treatment, the cells were grown to 70% confluence and then treated with 1 μM of 5Aza (Sigma, A3656) or vehicle only (DMSO) overnight. After an overnight incubation, the media was replaced with serum free DMEM. The cells were grown for an additional 48, 96 and 144 hours. The cells and conditioned media were harvested at these time points. The peptide LSKL was obtained from Genemade Synthesis, Inc, (San Francisco, CA).

Plasmids

The pBLAST49-hTSP1 expression vector was obtained from InvivoGen, (San Diego, California,). The pRC-CMV-TGFBR2 expression vector has been previously published (75). The p3TP-Lux vector was kindly provided by Joan Massague (Memorial

Sloan Kettering Cancer Center, New York, New York). The pRL-TK vector was purchased from Promega (Madison, WI).

Tissue samples

Colon adenomas and adenocarcinomas were collected from patients treated at Vanderbilt University Medical Center, the Department of Veterans Affairs Tennessee Valley Health Care System, and Meharry Medical Center (Nashville, TN) and at University Hospitals of Cleveland (Cleveland, OH) following protocols approved by the Institutional Review Board of each institution. All tissue samples were formalin-fixed and paraffin-embedded tissue blocks obtained from the pathology archives and were collected randomly based on tissue availability. The cases included 42 colon adenomas, 22 primary colon adenocarcinomas, and 15 samples of normal colon mucosa obtained from colon resection specimens from patients who had diverticular disease. In addition, two tissue microarrays (TMA) derived from 38 patients' resections of metastatic colon cancer resections to the liver were constructed. Four 1.5mm cores from each lesion of routinely processed formalin-fixed paraffin embedded adenocarcinoma were used. The TMAs were constructed using a Beecher Instruments Manual Tissue Arrayer 1.

DNA extraction and sodium bisulfite treatment

DNA extraction from the cell lines and tissue samples was performed as previously published. DNazol (Invitrogen) was used to extract DNA from the cell lines (72). The DNA was modified with sodium bisulfite for use in methylation specific PCR (MSP) assays as previously described (104).

Methylation Specific PCR (MSP)

MSP primers were designed to amplify the methylated and unmethylated alleles for *TSP1* and methylation-specific PCR assay conditions were determined so that specific reaction products were obtained from each respective set of primers. Each PCR reaction mix consisted of a total volume of 20 µl composed of PCR buffer (Qiagen, Valencia, CA), 200 pM deoxynucleotide triphosphate mix (Applied Biosystems, Foster City, CA), 500 nM of each primer (Sigma Genosys, The Woodlands, TX), 1 unit of HotStar Taq DNA polymerase (Qiagen), and bisulfite-modified DNA. The primers sequences and reaction conditions have been previously published (4). All the samples were subjected to at least two independent rounds of sodium bisulfite treatment and MSP assays. Control samples from cells with known methylated and unmethylated *TSP1* were included in each MSP assay to confirm the technical success of the assays. The MSP products were subjected to horizontal gel electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized with UV transillumination using an Eagle Eye Imaging system (Stratagene, La Jolla, CA).

RT-PCR

RNA was extracted from cells from the cell lines using TRI reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's recommended protocol. One microgram of total RNA from each sample was reverse transcribed using oligo d(T) priming and Superscript-II reverse-transcriptase (Invitrogen). The sequence of the *TSP1* primers is forward primer: 5'-CCA-GCTGTACATCGACTGTGA-3' and reverse primer:

5’-GCAGATGGTTAACTGAGTTCTGA-3’. The RT-PCR reaction mix components were $MgCl_2$ 1.5 mM, 1X PCR buffer, dNTPs 0.2mM, primers 2μM, and Taq polymerase 2.5 Units (AmpliTaq, Applied Biosystems, Foster City CA). The thermocycler conditions were (94°C x 45 seconds, 64°C x 45 seconds and 72° x 2 minutes) X 25; 72°C for 7 minutes. RT-PCR for GAPDH was performed as a loading control for the amount of mRNA used in each reaction. The primer sequences and reaction conditions used for the GAPDH RT-PCR are as follows: forward primer: 5’-CTGGCATGGCCTTCCGTG-3’ and reverse primer: 5’-GAAATGAGCTTGACAAAG-3’. The thermocycler conditions were (94°C x 45 seconds, 60°C x 45 seconds and 72° x 2 minutes) X 25; 72°C 7 minutes.

Quantitative RT-PCR

TaqMan On-Demand primers and probes were used to determine the relative expression levels of TSP1 (Assay number Hs00170236_m1, Applied Biosystems, Foster City, CA) and 18S in all samples (Assay number Hs99999901_s1, Applied Biosystems,). 18S RNA was used as an internal control. The reactions were run in triplicate in the ABI Prism 7700 detection system (Applied Biosystems) and results were analyzed with SDS 2.1 software.

Immunostaining

For TSP1 and pSmad2 immunostaining, tissue sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked with H_2O_2 treatment using standard methods (92). Antigen unmasking was achieved by incubation in boiling sodium

citrate buffer pH 6.0 (Biogenex, San Ramon, CA) for 15 minutes. Non-specific binding was blocked by treating the sections with 1.5% horse normal serum for 10 minutes, and the TSP1 immunostaining was performed with the mouse monoclonal antibody anti-TSP1 (ab2 Lab Vision, Fremont, CA) diluted 1:2000 incubated at 4°C overnight. For pSmad2 immunostaining a plyclonal antibody (cat # 3101 Cell Signaling, Danvers, MA) The Vectastain ABC Kit (PK-4002, Vector labs, Burlingame, CA) was used to obtain the final stain. The tissue sections were then coverslipped and analyzed with an Eclipse 80i compound microscope (Nikon). Photomicrographs were obtained using a CCD camera (Micropublisher, Coolpix 3.3). The evaluation of positive TSP1 expression in tumors was performed using a semiquantitative scoring of the percentage of positive tumor cells. TSP1 expression was scored positive when >40% of the tissue analyzed was immunoreactive; similar scoring system has been used by Zlobec et al, showing better prognostic value of several tumor markers and they have shown that this scoring method is reproducible among pathologists in the study of colorectal cancer (105).

siRNA transfection

1-2 x10^5 FET cells were cultured in a 24 well plate in complete media and then transfected 24 hours after the cells were seeded. The media was replaced by serum free media and the cells were transfected with the siRNA for TSP1 (cat # L-019743-00, Dharmacon) and control siRNA (cat # D-001810-03-05, Dharmacon), using Lipofectamine 2000 (cat # 11668-019, Invitrogene) following the manufacturer's protocols. Forty-eight hour after the transfection, activated TGF-β1 or the TGF-β:LAP

complex was added to the media. The media was collected 24 hours later. RNA from the cells was extracted at this time as well.

TGF-β1 ELISA

Media was collected from cells treated with 5-aza-2'-deoxycytidine or transfected with the pBLAST49-hTSP1 expression vector described above. The concentration of TGF-β1 was measured with the TGF-β1 Quantikine ELISA assay which uses an antbosdy that reconszes the active form of TGF-β1 (DB-100, R&D Systems, Minneapolis, MN). The manufacturer's protocol was followed with the next modification. In order to measure the concentration of active and latent TGF-β, the media was divided. The first portion of the media was activated with hydrochloric acid and remaining portion of the same media was assessed without any hydrochloric acid treatment. The ELISA assay results were measured using a VERSAmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) set at 450 nm.

Formation of the TGF-β:LAP complex

TGF-β1 (2ng) (101-B1-010, R&D Systems) was added to recombinant human LAP (30ng) (246-LP, R&D Systems). The complex was allowed to form at room temperature for at least 2 hours before being added to the media.

3TP-Lux luciferase reporter assay

SW-48 cells were transfected with the pBLAST49-hTSP1 vector using FuGene (11814443001, Roche) following the manufacturer's protocol or treated with 5-aza 2' deoxycitidine in a six well plate as previously published (75).

Results

Loss of TSP1 expression and aberrantly methylated TSP1 is present in both colon adenomas and adenocarcinomas.

The overall frequency of TSP1 loss in colorectal cancer was assessed by immunostaining normal colon (N=5), colon adenomas (N=4), and colon adenocarcinomas (N=51). TSP1 immunoreactivity was observed in 80% (N=4/5) of normal colon mucosa, 50% (N=2/4) of colon adenomas, 33% of primary cancers (N=4/12), and 25% (N=10/39) of liver metastases, demonstrating that TSP1 loss is common in colon cancers (**Figure 2.1**).

In light of the frequent loss of TSP1 expression in colon adenomas and adenocarcinomas, we next determined the frequency of aberrantly methylated *TSP1* in colorectal cancer cell lines, primary colon adenomas and adenocarcinomas. The methylation status of *TSP1* was assessed in a region that is 30 bp upstream from the transcription start site, which has been demonstrated to be involved with methylation-induced transcriptional repression (4, 102). We found aberrantly methylated *TSP1* in 33% of colon cancer cell lines (N=3/9), 14% of colon adenomas (N=6/42) and 21% of adenocarcinomas (N=18/96) (**Figure 2.2**). (Note: A portion of the adenomas and cancers used in this study have been reported previously (4)) Importantly, no methylated *TSP1*

was detected in normal colon mucosa samples, providing evidence that *TSP1* hypermethylation is a cancer specific epigenetic event. In order to assess the correlation between *TSP1* methylation and TSP1 expression, TSP1 immunostaining was performed in a subset of the cancer cases described above. We observed that none of the tumors that carried methylated *TSP1* (N=5) expressed TSP1 in the neoplastic tissue and that 78% of tumors with unmethylated

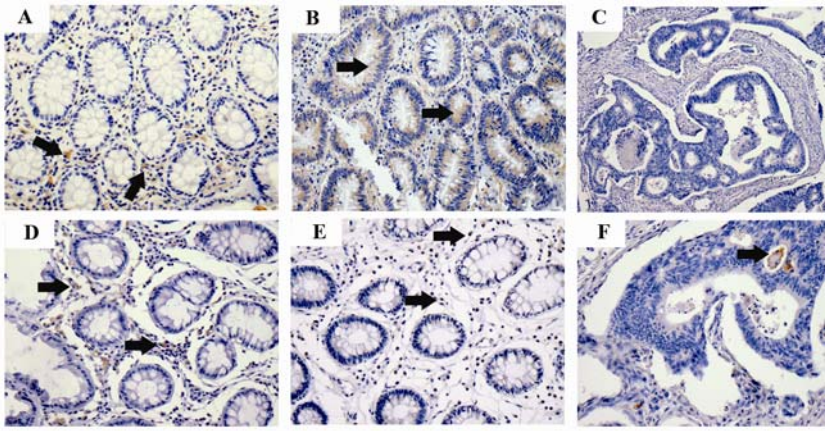


Figure 2.1. Results of immunostaining for TSP1 in colon tissue samples. Photomicrographs of representative samples are shown (magnification 40X and 100X). The expression of TSP1 in samples of normal colon (**A, D**), adenomas (**B, E**) and adenocarcinoma (**C, F**) is observed by the brown staining. Note that in the adenomas the expression is reduced in one adenoma **(E)** but not in the other **(B)**. TSP1 expression in adenocarcinomas is lower that in normal tissue and adenomas (**C, F**). The arrows indicate the areas of TSP1 immunoreactivity.

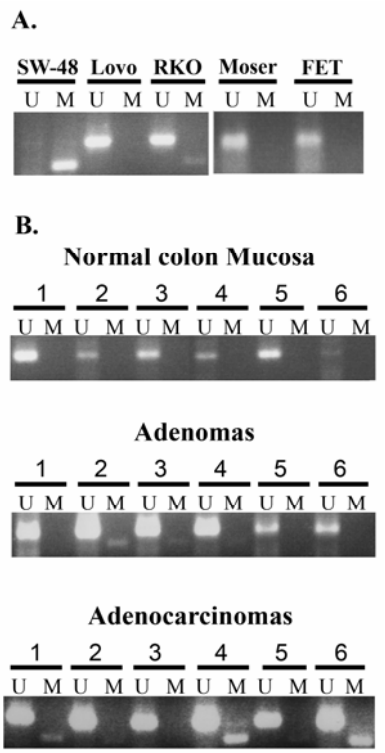


Figure 2.2. **A. Representative *TSP1* MSP assay results for cell lines.** **U**, PCR product from the MSP assay using primers specific for the unmethylated allele; **M**, PCR product from the MSP assay using primers specific for the methylated allele. **B.** Representative results from the *TSP1* MSP analysis of normal colon mucosa, adenomas and adenocarcinomas using primers for methylated (M) and unmethylated (U) alleles of *TSP1*. No methylated *TSP1* was detected in any of the normal colon mucosa samples. Approximately 20% of colon neoplasms were found to have methylated *TSP1*. The unmethylated allele present in the tumors that were shown to have methylated TSP1 is presumably from intermixed normal tissue.

TSP1 (N=14) expressed TSP1. Of interest, some of the tumors with absent TSP1 expression in the tumor cells did show TSP1 expression in the adjacent stroma.

The aberrant methylation of TSP1 induces silencing of TSP1 expression

We next evaluated the effect of aberrant methylation of *TSP1* on TSP1 mRNA expression and protein secretion. Treatment of SW-48 cells with 5-aza-2'deoxycytidine (5-Aza) resulted in the demethylation of *TSP1* and a 1.5-2 fold increase in expression of *TSP1* that was detected after 96 hours of treatment (**Figures 2.3A and 2.3B).** Moreover, secreted TSP1 was present in the conditioned media of SW-48 after treatment with 5-Aza, consistent with the effects of 5-Aza observed on mRNA expression and demonstrating increased levels of secreted TSP1 secondary to demethylation of the gene (**Figure 2.4**). These results suggest the aberrant methylation of *TSP1* suppresses *TSP1* mRNA expression and TSP1 protein secretion in colorectal cancer.

TSP1 inactivation inhibits the activation of secreted TGF-β1

Upon providing evidence that the aberrant methylation of *TSP1* inhibits the expression of TSP1, we assessed the effect of loss of TSP1 secretion on TGF-β1 activation. Demethylation of *TSP1* with 5-Aza resulted in a two fold increase in activated TGF-β at 96 hours (**Figure 2.5A**). The concentration of total TGF-β1 did not change after treatment with 5-Aza (data not shown), demonstrating that the effect of 5-Aza on TGF-β activation was not simply a consequence of increased production of total TGF-β1 (**Figure 2.5A**).

In order to provide evidence for the specificity of the 5-Aza treatment on TGF-β1 activation through the induction of TSP1, we also assessed the effect of *TSP1* reconstitution on

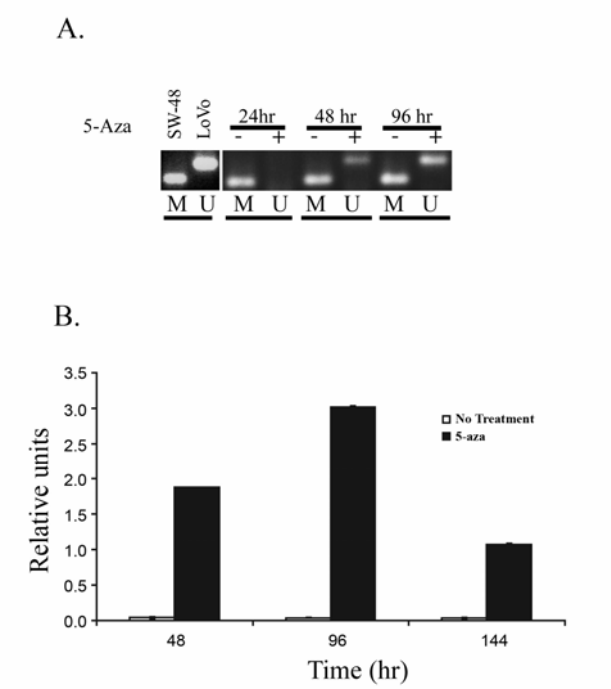


Figure 2.3. **Re-expression of TSP1 in SW-48 cells after treatment with 5-aza-2'deoxycytidine (5-Aza). A.** *TSP1* MSP results from cells that were treated for 24, 48 and 96 hours with 5-Aza. The presence of unmethylated DNA can be seen after 48 hours of treatment. **B.** TSP1 mRNA levels assessed by quantitative RT-PCR for TSP1 expression, treatment with 5-Aza resulted in an increment in the levels of TSP1 mRNA in SW-48 cell which normally do not make this mRNA due to DNA methylation.

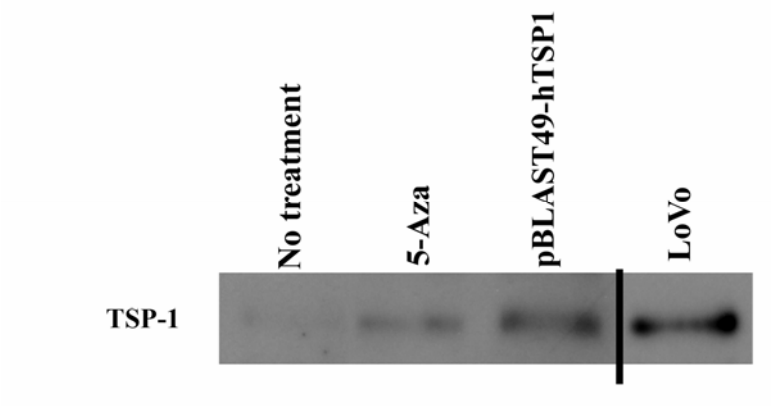


Figure 2.4. TSP1 is secreted from SW-48 cells after treatment with 5-Aza. Western blot analysis for TSP1 in conditioned media from SW-48 and LoVo colon cancer cells. Conditioned media from SW-48 cells transfected with *TSP1* or after treatment with 5-Aza was collected and analyzed. The amount of TSP1 observed in conditioned media from the LoVo colon cancer cell line, which has unmethylated *TSP1*, is similar to that from the SW-48 cells after *TSP1* transfection or treatment with 5-Aza.

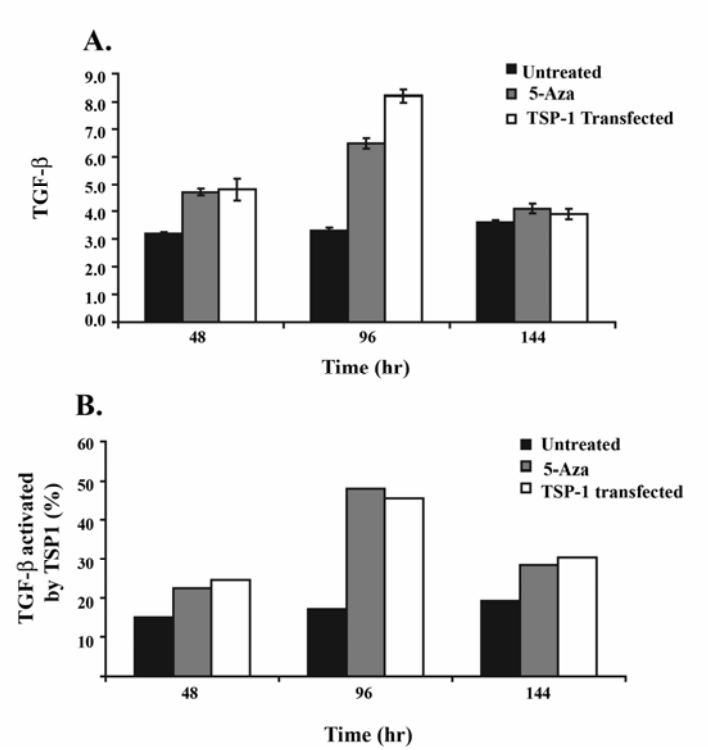


Figure 2.5. Increased secreted activated TGF-β1 is present in conditioned media of SW-48 cells after treatment with 5-Aza or transfection with *TSP1*. Results from TGF- β1 ELISA for secreted active and total TGF-β1 in conditioned media from SW-48 after treatment with 5-Aza or transfection with *TSP1*. **A.** Increased activated TGF-β1, compared to the basal levels, is present 48 and 96 hours after the initiation of treatment with 5-Aza or after transfection with *TSP1*, with the most significant increase observed at 96 hours. **B**. The percentage of activated TGF-β1 is also increased at 48, 96, and 144 hours after initiation of treatment with 5-Aza or transfection with *TSP1*.

TGF-β1 activation. Interestingly, we observed similar effects on TGF-β1 activation in the SW-48 cells reconstituted with *TSP1* compared to the 5-Aza treatment, although the magnitude of the effect was greater at 96 hours in the TSP1 reconstituted SW-48 cells compared to the 5-Aza treated cells, consistent with the increased amount of secreted TSP1 observed in SW-48 cells transfected with TSP1 (**Figure 2.5A**). These results suggest ~25% of the total activated TGF-β1 present in the media is activated by TSP1 (**Figure 2.5B**).

To further assess if TSP1 silencing can affect the activation of TGF-β1, we knocked down TSP1 expression with siRNA in the FET colon cancer cell line, which carries unmethylated TSP1 and expresses the gene. We observed decreased *TSP1* expression and reduced activation of TGF-β1 in FET transfected cells with siRNA against TSP1 demonstrating that silencing of TSP1 can attenuate the activation of TGF-β1 (**Figures 2.6A-C).**

Aberrant methylation of TSP1 can suppress TGF-β signaling by impairing the activation of TGF-β.

After demonstrating that *TSP1* methylation can affect the concentration of secreted, activated TGF-β1 in colon cancer, we assessed the functional consequences of this effect on TGF-β mediated signaling. Using the 3TP-Lux reporter assay, we observed a ~ 30% increase in 3TP-Lux activity after *TSP1* transfection (**Figure 2.7A**). As TSP1 is known to activate TGF-β1 by a specific mechanism, which is the disassociation of LAP from TGF-β1, we also assessed the effect of *TSP1* reconstitution on activating pre-formed TGF-β1:LAP complexes and inducing TGF-β signaling. We observed increased

3TP-Lux activity at 96 hours after *TSP1* reconstitution (**Figure 2.7B**). Additionally in cells in which the TGF-β1:LAP complex was added but that were not transfected with *TSP1*, no increase in 3TP-Lux reporter activity was observed.

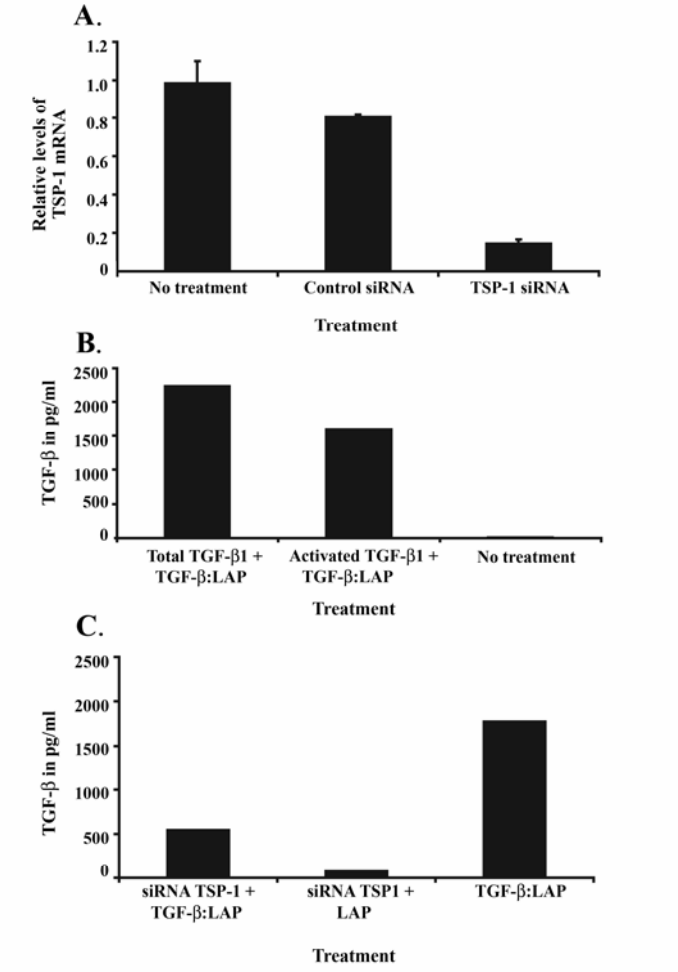


Figure 2.6. A. Assessment of *TSP1* mRNA levels by quantitative RT-PCR after transfection with siRNA against TSP1. *TSP1* expression is reduced to approximately 15% of basal levels by siRNA. No effect of the control siRNA on *TSP1* expression is observed. **B.** Assessment of total and activated TGF-β in FET cells treated with TGF-β:LAP. Activated TGF-β1 is detected in FET cells that were treated with the TGF-β:LAP complex, such activation represents ~50% of the total TGF-β present in the media. **B.** Assessment of TGF-β:LAP complex activation in FET cells after treatment with siRNA against TSP1. A substantial reduction in activated TGF-β is observed. No effect on TGF-β activation is observed in the FET cells treated with the control siRNA. These experiments were done by duplicate, and they were repeated at least three times, here we are showing the must representative.

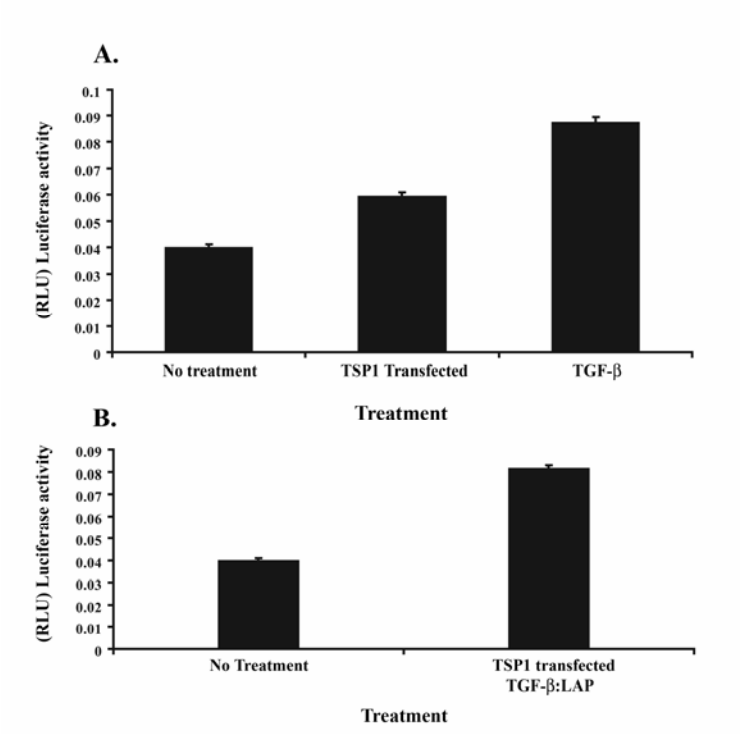


Figure 2.7. **TSP1 activates TGF-β present in the TGF-β1:LAP complex and induces Smad signaling.** **A**. 3TPLux luciferase reporter activity in the SW-48 cell line 48 hours after transfection with *TSP1*. The 3TP-Lux reporter activity is increased approximately 1.2X in the *TSP1* transfected SW-48 cells and 1.8X in the SW-48 cells treated with activated TGF-β (2ng/ml). (*p=0.0005, t-test analysis). **B.** 3TPLux luciferase reporter activity in the SW-48 cell line after addition of preformed TGF-β1:LAP and transfection with *TSP1*. The 3TP-Lux reporter activity is increased approximately 1.3 fold at 48 hours and 1.6 fold at 96 hours compared to the baseline level. (**p= 0.003 t-test analysis).

The region of TSP1 that is responsible for the activation of TGF-β1 is localized in the amino end of the TSR2 domain. This portion of the protein interacts with the LSKL sequence present in the LAP inducing the conformational change of LAP that results in the dissociation of TGF-β from the TGF-β1:LAP complex. In order to confirm that the effect we observed on TGF-β pathway activation was due to TSP1 activating the latent TGF-β:LAP complex and not through a nonspecific mechanism, we assessed the effect of an inhibitory peptide that contains the LSKL motif on TGF-β pathway activation. The LSKL peptide inhibits the ability of TSP1 to induce the dissociation of LAP from TGF-β. We observed that the LSKL peptide inhibited the activation of the TGF-β signaling pathway reporter 3TP-Lux in SW-48 cells transfected with *TSP1* (**Figure 2.8**).

TSP1 expression is associated with the nuclear localization of Smad2 in primary colon neoplasms

Our studies in the SW-48 and FET cell lines demonstrated *in vitro* that the absence of TSP1 inhibits TGF-β pathway activation. We next assessed the correlation between TSP1 expression and TGF-β pathway activation in primary colon cancer by determining the correlation between phosphorylated SMAD2 and TSP1 in normal colon, colon adenomas, and colon adenocarcinomas. We found that TSP1 expression correlated with phosphorylated Smad2 in the majority of the cases (N=17/20) (Pearson rank test, p=0.032) **(Figure 2.9).** Of note, we also observed phosphorylated Smad2 in the absence of TSP1 expression in three tumors, which suggests alternate mechanisms can activate TGF-β in these tumors, such as plasmin or cathepsin. We also observed that in one of the cases cells that express TSP1, did not have detectable phosphorylated Smad2. In this

case, we presume that the TGF-β signaling pathway has been disrupted at the level of the receptor or post-receptor elements.

In summary, we have demonstrated TSP1 expression is often reduced in colorectal neoplasms and the aberrant methylation of *TSP1* is one mechanism responsible for the silencing of *TSP1*. We have further shown the loss of TSP1 attenuates the activation of secreted TGF-β and the SMAD signaling pathway in vitro systems. Our assessment of primary colon neoplasms is also consistent with the aberrant methylation of *TSP1* attenuating TGF-β signaling.

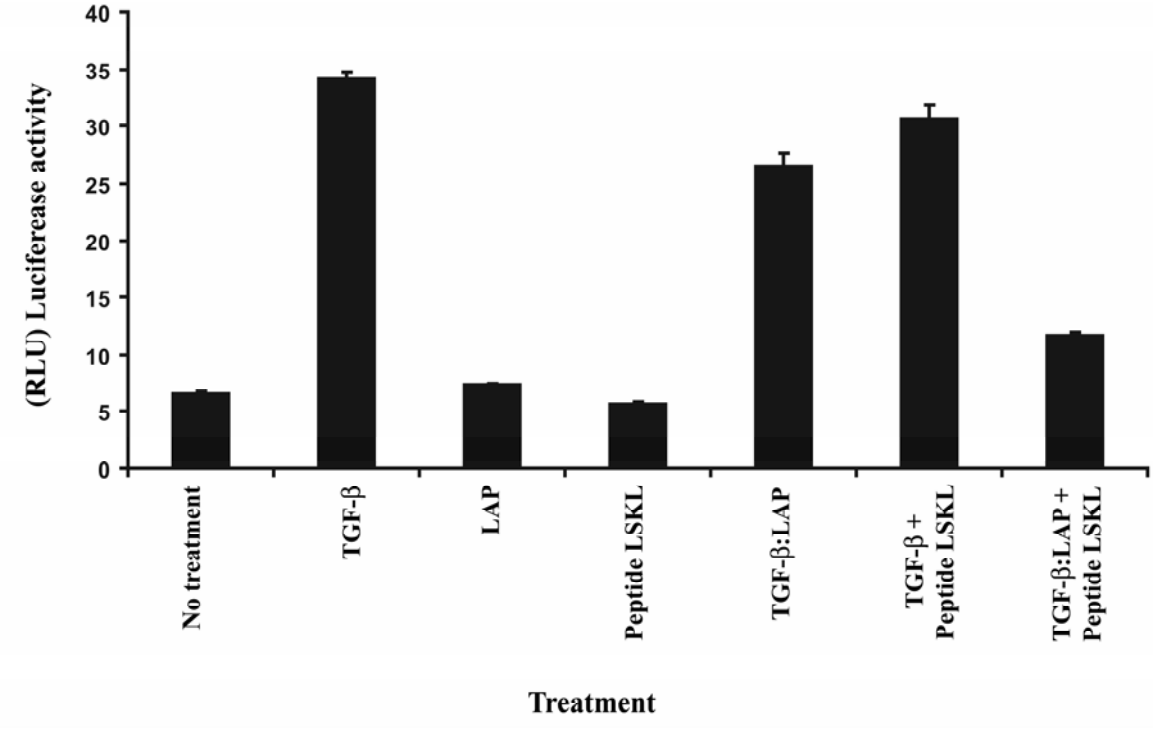


Figure 2.8. **TGF-β pathway activation measured by luciferase activity using the 3TP-Lux reporter assay.** The SW-48 cell line was transfected with the 3TP-Lux reporter, TSP1, and then treated with the agents noted. The addition of active TGF-β induces the highest activity of the pathway. When the complex TGF-β:LAP is added in presence of TSP1 (transfected cells) the pathway is activated. The inhibitory peptide LSKL attenuates the 3TP-Lux activity in the cells with and without the TGF-β:LAP complex. These results confirm that TSP1 is inducing the activation of the TGF-β pathway through effects on activation of TGF-β1.

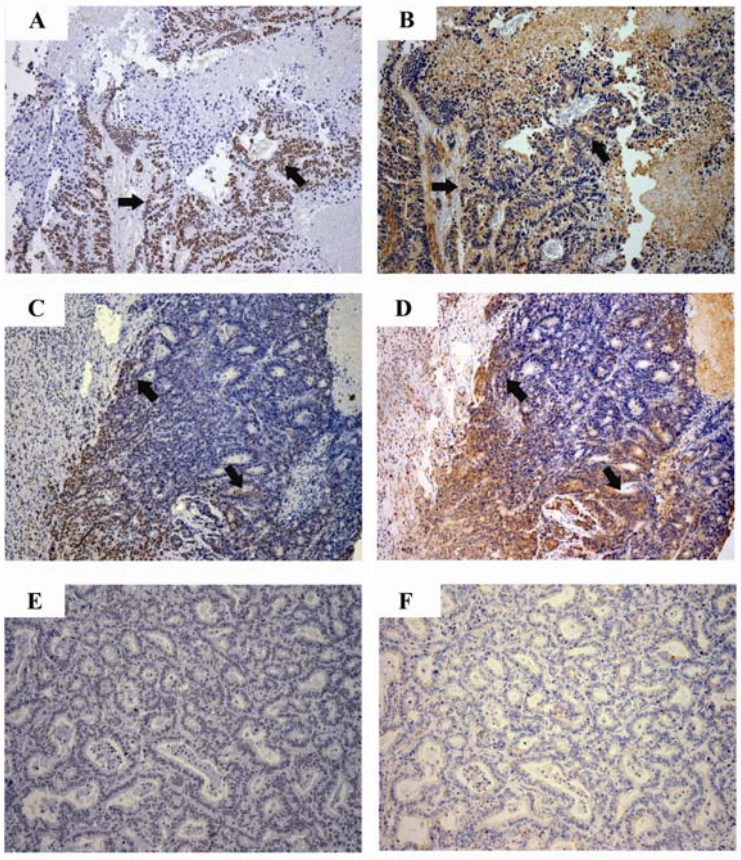


Figure 2.9. Results of immunostaining for phosphorylated Smad2 (pSmad2) (A, C, and E) and TSP1 (B, D, and F) in primary colorectal cancers. A-B. Nuclear pSmad2 is present in the most of the epithelial cells and TSP1 expression is increased. **C-D.** Heterogeneity of pSmad2 and TSP1 expression is present in this tumor. Colocalization of pSmad2 and TSP1 can be appreciated. **E-F.** No expression of pSmad2 or TSP1 is present. (Magnification: 100X).

Discussion

TGF-β can inhibit cell proliferation, induce apoptosis, and induce the terminal differentiation of normal colon epithelial cells and colon adenoma cells (106, 107). The TGF-β pathway is inactivated or impaired in >50% of colon adenocarcinomas through a variety of mechanisms including the mutation of *TGFBR2, SMAD4*, and *SMAD2*, the overexpression of inhibitory Smad7, and the increased expression of Smad signaling repressors such as Ski or SnoN (22, 73, 92, 108-110). We now provide evidence that an epigenetic mechanism, the aberrant methylation of *TSP1*, can impair TGF-β signaling in colorectal cancer, providing evidence for a tumor promoting biological effect of the methylation of *TSP1* and for a novel mechanism for impairing TGF-β signaling in colorectal cancer.

The aberrant methylation of tumor suppressor genes has been shown to affect many genes in colon cancer, including *MLH1, HLTF, CDKN2A, SLC5A8*, and is believed to contribute to the clonal progression of the tumors since DNA aberrant methylation can inactivate the function of genes (4, 12, 111-113). Indeed, the aberrant methylation of *SFRP2, TIMP3, HIC1*, and *ITGA4* have all been shown to have tumor promoting effects in cancer cell lines (100, 114-116). Nonetheless, it is also clear that some of the aberrantly methylated genes are hitchhiker or passenger events that do not influence the pathogenesis of the tumors (117, 118). Thus, the functional consequences that DNA hypermethylation and hypomethylation have on the behavior of the cancer cells remains to be determined for the majority of the genes that have been shown to be aberrantly methylated in cancer (114, 119, 120)

We have demonstrated that the aberrant methylation of *TSP1* results in loss of TSP1 expression and in attenuation of TGF-β activation and signaling pathway activity. These results provide support that epigenetic alterations can play a pathogenic role in colon cancer formation by inhibiting TGF-β signaling. In approximately 75% of colon cancer cell lines, there is resistance to TGF-β mediated growth inhibition, and in many colorectal cancers, the TGF-β signaling pathway is inhibited by mutational inactivation of the TGF-β receptor or Smads (75). However, in approximately 30-40% of colon cancers, the mechanism(s) resulting in impaired TGF-β signaling is not known. Some investigators have shown that epigenetic alterations can affect *KM23, TGFBI* (Betaig-h3 gene), and *RUNX3*, which are all genes involved with regulation of the TGF-β signaling pathway, but none of these studies have demonstrated that the aberrant methylation of these genes directly alters the TGF-β signaling pathway (121-123). We have provided evidence that TGF-β activation is impaired when *TSP1* is aberrantly methylated resulting in the suppression of TGF-β receptor activation. These results provide the first evidence of which we are aware that an epigenetic event can attenuate TGF-β responsiveness in colon cancer. It is also possible that the aberrant methylation of *TSP1* may promote angiogenesis through effects on VEGF or through effects on extracellular matrix remodeling, which was not investigated in this study (102, 124).

The demonstration of aberrant methylation and functional inactivation of TSP1 also provides support that *TSP1* is a true tumor suppressor gene that is subject to epigenetic inactivation in cancer. Prior to the discovery of aberrant methylation of *TSP1*, there was no genetic or epigenetic evidence that supported the role of *TSP1* as a bona fide tumor suppressor gene (124). Earlier studies have shown TSP1 is a downstream target of

TP53 mutations and a regulator of VEGF. Which suggests *TSP1* is a tumor suppressor gene (125, 126). The identification of aberrantly methylated *TSP1* in colorectal cancer, neuroblastomas, and gliomas demonstrates, that like *CDKN2A*, *TSP1* is susceptible to epigenetic alterations even though it does not appear to be commonly mutated in cancer (102) (124, 127). It is not clear whether the stroma can be a source for TSP1 in the setting of a cancer that has silenced *TSP1* expression. The immunostaining results we have obtained demonstrate a strong correlation between *TSP1* methylation and lack of TSP1 expression as well as between TSP1 expression and the presence of phosphorylated Smad2. These results suggest that the tumor cells are a major source of TSP1 and that methylation of *TSP1* substantially reduces TSP1 activity in the tumors.

In summary, we have identified a novel epigenetic mechanism for suppressing the TGF-β signaling pathway. We have provided evidence that the aberrant methylation of *TSP1* impairs the activation of TGF-β1 in a colon cancer cell line system and propose that the effect is similar in primary human colon cancer. Furthermore, these results provide evidence that the aberrant methylation of *TSP1* is an epigenetic event that silences this tumor suppressor gene and results in biological events that would promote the development of colon cancer. These results suggest that targeting therapy towards aberrant DNA methylation could inhibit cancer cells at least in part by inducing the TGF-β signaling pathway. Additional studies should help clarify the ultimate effects of the aberrant methylation of *TSP1* and the effects of therapy directed towards this epigenetic event on colon cancer formation given the complex effects of TSP1 and TGF-β on cancer cells and the cancer-associated stroma.

CHAPTER III

EVALUATION OF THE EFFECTS OF *TGFBR2* EXPRESSION LEVELS ON SIGNALING PATHWAY ACTIVATION IN COLORECTAL CANCER

Abstract

TGF-β is a pluripotent cytokine that is involved in the maintenance of normal tissue homeostasis in different organs as well as in a variety of disease processes. TGF-ß is a secreted protein that mediates its effects on cells through the activation of a transmembrane receptor complex consisting of TGF-β receptor type II (TGFBR2) and type I (TGFBR1) and the subsequent induction of post-receptor signaling pathways. The TGF-β signaling pathway(s) is initiated when the TGF-β1 ligand binds to the TGF-β receptor type II initiating a sequence of events that results in the activation of different cell signaling pathways that result in the induction of different cellular processes ultimately directed by TGF-β. The best characterized of such pathways is the Smad pathway, which has been implicated in the tumor suppressor activity of TGF-β. TGF-ß can also induce Smad independent pathways, although the mechanisms involved in the activation of the Smad-independent pathways are not well understood. There is some evidence that the expression levels of TGF-β receptor type II may play a role in determining whether the Smad dependent or independent pathways are activated by TGF-ß. In order to determine whether the expression level of TGFBR2 is one mechanism through which cells can regulate the activation of Smad dependent and Smad independent signaling pathways, we have used a modified version of the Ecdysone inducible TGFBR2 expression system to assess the effects of differences in the expression levels of TGFBR2

in the activation of the Smad, PIK3/AKT, MAPK/ERK pathways and in the expression of p21. We have also assessed the effects of TGFBR2 expression levels and signal pathway activation on the induction of TGF-β mediated apoptosis.

Introduction

The canonical TGF-β pathway

The canonical TGF-β pathway is initiated when TGF-β1 binds to and activates TGFBR2, which then forms a heteromeric complex with TGFBR1. TGFBR1 is phophorylated by TGFBR2 in the GS box and then induces the activation of Smad2 and Smad3 through phosphorylation. The phosphorylated Smad2 and Smad3 complex with each other and with Smad4 to form a transcription factor complex that translocates to the nucleus (42, 43, 128). Interestingly, Smads do not appear to be static proteins, but rather are in constant exchange from the cytoplasmic compartment to the nucleus once activated by TGF-β. In cells that are not stimulated by TGF-β, Smad2 and 3 are predominantly cytoplasmic, whereas Smad 4 is localized in both compartments (Reviewed in (55)). As demonstrated by Schmierer et al (129), the phosphorylation of Smad2 by the activated receptor is the rate-limiting step in the nuclear accumulation process of Smads, where the nucleus acts as a trap due to the high affinity that pSmad2 has for nuclear binding elements. In unstimulated cells very low accumulation of Smad2 occurs, and its export rate is higher that its import rate, confirming the importance of the presence of active TGFBR2 in the translocation of Smad proteins in cells (129).

In TGF-β stimulated cells once the complex pSmad2/3 binds to Smad 4, it is translocated into the cell nucleus, and in conjunction with other transcription factors, co-

activators, and co-repressors, the Smad complex modulates the transcription of several TGF-β-responsive genes in a cell type specific manner.

The expression of certain proteins induced by TGF-β has been associated with the promotion of apoptosis, cell cycle arrest, cell growth and differentiation in epithelial cells. (16). For example, it is known that the activation of the TGF-β Smad-dependent pathway induces the expression of the CDK inhibitors p21 and p15 which play a key role in regulating the cell cycle and apoptosis (130). TGF-β can induce growth inhibition and apoptosis in this way and thus mediate some of its tumor suppressor activities through these mechanisms. These effects on cell cycle control genes are modulated in part through signal pathway interactions that occur downstream of the TGF-β receptor. For example, in neuroepithelial cells, the induction of p21 is mediated by a transcription factor complex composed of pSmad2/3 and FoxO. This complex can be disrupted if FoxO1 is phosphorylated by AKT, which triggers the nuclear export of FoxO1, resulting in the loss of p21 expression (82, 131-133). Thus, nonSmad pathways can interact with the TGF-β induced Smad pathway to regulate the final response mediated by TGF-β.

The non-canonical TGF-β pathways and oncogenic activity mediated by TGF-β

It is well known that TGF-β, TGFBR2, SMAD4, and SMAD2 are tumor suppressor genes that play a central role in maintain normal tissue homeostasis in different organs, including the colon. However, in a subset of colorectal cancers that have not mutated genes in the TGF-β pathway, TGF-β appears to have the potential to act as an oncogene as well as a tumor suppressor gene. This paradoxical role of TGF-β has been associated with its ability to activate Smad independent pathways and with

modulation of TGF-β signaling pathway activation through interactions with other signaling pathways induced by mutated oncogenes and tumor suppressor genes. For example, activation of pathways such as MAPK, PI3K and Rho mediated by TGF-β is associated with the induction of EMT, cell proliferation, and resistance to apoptosis (35, 52, 63, 67). In addition, in the presence of aberrantly activated Ha-Ras or Ki-Ras, the growth inhibitory actions regulated by TGF-β in prostate and colon cancers can be transformed into Smad-independent mitogenic responses (134). Similar observations have been reported in other cell types; in kidney cells for example, the activation of Raf confers epithelial cells protection against the pro-apoptotic activity of TGF-β, while enhancing its pro-invasive effects (52). Additionally, the induction of EMT by TGF-β in breast cancer seems to be dependent on the presence of activated Ras, (52). It is also clear that activation of Smad independent pathways can also induce apoptosis and thus mediate tumor suppressor activities, demonstrating the complexity of TGF-β mediated effects on cells (135, 136).

Despite the knowledge that TGF-β is able to activate the pathways mentioned above, it is not clear what mechanisms cells use to determine how they will respond to TGF-β in such diverse manners. The understanding of the mechanisms involved in the activation of pathways that regulate these paradoxical cellular responses, are under active investigation. It has been proposed that one of the mechanisms involved in the regulation of pathways mediated by TGF-β is the expression levels of TGFBR2. It is hypothesized that cells expressing low levels of TGFBR2 have a relatively low activation levels of the Smad dependent pathway which may lead to more Smad independent pathway activation compared to Smad dependent pathway activation (52, 91). Some of the Smad

independent pathways that are activated by TGF-β are the MAPK/ERK, PIK3/AKT and JNK signaling pathways, which in the proper context could result in the amplification of the oncogenic characteristics induced by TGF-β (62-64).

Dosage effects of TGFBR2 in the TGF-β pathway

The components of the TGF-β signaling pathway, including TGFBR2, are expressed in the majority of epithelial tissues (137). While it is clear that inactivation of the TGF-β-SMAD signaling pathway occurs commonly in many types of cancer and that complete inactivation of the pathway silences potent tumor suppressing activities of TGF-β signaling, TGF-β can also have paradoxical effects on tumor cells that retain complete or partial TGF-β responsiveness (99, 138). There are several possible mechanisms though which TGF-β may mediate these opposing effects on cells with the most likely being the following: 1) modulation of the degree of TGF-β pathway activation (e.g. strong vs. weak) alters the ultimate signaling pathways that are activated by the TGF-β receptor, 2) modulation of the pathway output occurs through signaling pathway crosstalk (e.g. MAPK can phosphorylate Smad3 and promote its degradation.), or 3) paracrine effects of TGF-β result in stromal cell behaviors and interactions with adjacent epithelial cells that favor tumorigenesis.

With regards to the first proposed mechanism, the modulation of TGF-β signaling pathway output through alterations in the activation level of the receptor has been suggested by several lines of indirect evidence. First, it is clear that there is a dose-response effect on cells with increasing concentrations of the activated TGF-β ligand (139). Low concentrations of TGF-β can induce growth inhibition in hepatocytes

whereas higher concentrations (>5ng/ml) induce apoptosis (140). Further evidence has been provided by a study by Shimanuki et al in which the investigators used an inducible system to demonstrate dosage effects of TGFBR2 expression levels on proliferation (81). The second line of indirect evidence that supports the concept that TGF-β receptor activation levels could be responsible for the differences in cell responses to TGF-β that have been observed is related to differences between the Smad dependent and Smad independent signaling pathways. There is a fundamental difference between the Smad dependent and Smad independent signaling pathways in that the Smad pathway is a noncatalytic pathway as opposed to the Smad independent pathway, which transduce their signals through sequential activation of kinases. This difference raises the possibility that there may be differential pathway activation in the setting of TGF-β receptor activation, especially at low levels of TGF-β receptor activation, which would be predicted to yield low levels of Smad dependent pathway activity compared to the Smad independent pathways (52).

With regards to this hypothesis that TGF-β signaling output varies depending on the degree of receptor activation, this model has potentially important implications in certain cancers in which the TGFBR2 gene is transcriptionally repressed rather than mutated. Tumor types that display low levels of TGFBR2 but infrequent TGFBR2 mutations include small cell lung cancer, esophageal cancer, hepatocellular carcinoma, breast cancer, endometrial carcinoma, bladder cancer, osteosarcoma and non-small cell lung adenocarcinomas (NSCLC) (109, 141, 142). Indeed, several lines of evidence suggest that transcriptional repression of the TGFBR2 gene may be a major mechanism to attenuate TGFBR2 function (109). It is plausible that low levels of TGFBR2 may

permit the activation of tumor promoting pathways, which would lead to a state in which the transcriptional repression of TGFBR2 not only suppresses TGF-β's anti-tumor effects but also permits its oncogenic activities.

In light of the implications for understanding the role of TGF-β signaling deregulation in cancer formation and for determining whether TGF-β receptor activation is one mechanism for regulating the myriad of TGF-β regulated responses, we have developed an inducible system to regulate the expression of TGFBR2 and have assessed the effect of modulation of TGFBR2 levels on signal pathway activation and on the biological effects of the cell, specifically apoptosis.

Generation of an inducible system of TGFBR2 expression

In order to control the temporal expression and protein expression levels of genes, several inducible systems have been developed that permit the generation of in vitro systems in which the expression of transgenes in mammalian cells can be temporally regulated (143, 144). One such system that is commonly used takes advantage of the Ecdysone receptor, which is present in insects and other related invertebrates (145). Since the Ecdysone receptor and its ligands are not present in vertebrates, this system is usually tightly regulated gene expression in mammalian cells with low levels of unstimulated gene expression.

The Ecdysone receptor is a nuclear receptor that heterodimerizes with the retinoid X receptor, activating the transcription of genes controlled by Ecdysone response elements in various cell types, including mammalian cells (145). Although inducible systems based on the ecdysone receptor have lower levels of unstimulated gene

expression compared to other systems based on tetracycline, etc, it is known that some nonspecific gene expression is typically detected and that the precise regulation of gene expression is difficult to achieve even with this system (145). In order to improve the original Ecdysone receptor-based inducible gene regulation system, a modified and more precisely regulated system (RheoSwitch, New England BioLabs) has been generated to obtain a system in which the expression of the transgene can be precisely adjusted (RheoSwitch system, NEB (originally developed by RheoGene, Norristown, PA). Modifications made to the original ecdysone receptor based system include the fusion of the Ecdysone receptor from *C. fumiferana*, the retinoid receptor from *Mus musculus,* and the VP16 activation domain in order to decrease the amount of uninduced gene expression and also to create a tighter dose-response curve compared to the original Ecdysone system (143). The combination of these elements has been shown to provide this system with better results in terms of low basal expression in the absence of ligand, and precise and robust expression in the presence of ligand (145). Based on this information and the data published by Palli et al (145), we established a collaboration to use this system to regulate the expression of TGFBR2, to evaluate how changes in TGFBR2 levels affect the activation of TGF-β regulated pathways, including the Smad dependent and Smad independent pathways.

Materials and methods

Cell culture

Vaco-400 (V-400), a cell line derived from a liver metastases of a human colon cancer, and YAMC, a cell line derived from the normal mouse colonic epithelium of the Immorto mouse, were used for the studies described below (146). V-400 is a microsatellite stable (MSS), TGF-ß resistant colon cancer cell line, which was kindly provided by James K.V. Willson (UT Southwestern Medical School, Dallas, TX). It carries biallelic missense *TGFBR2* mutations and has an intact Smad signaling pathway (75). The cell lines derived the mouse colon epithelium are designated YAMC (Young Adult Mouse Colonocytes) and Y-R2 null and were derived from the colon of the Immorto mouse and the Immorto;Tgfbr2$^{flx/flx}$ mouse, respectively (146). The Y-R2 null cell line was generated by treating a colon epithelial cell line established from an Immorto;Tgfbr2$^{flx/flx}$ mouse with a Cre expressing adenovirus (147), which was provided kindly by Frank Graham (McMaster College, Montreal, CA). The YAMC cells express a temperature-sensitive mutant of the SV-40 large-T antigen under the control of an Interferon γ-responsive promoter; they retain most of the characteristics of normal intestinal epithelium and are conditionally immortalized (146). The V-400 cell line and a second colon cancer cell line, FET, were grown in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY). The YAMC cells were cultured in media consisting of HAMS 12 F12 (50%), 500 ml RPMI 1640 (50%), and was supplemented with transferring, insulin, and selenium (100X ITS, Gibco BRL), HEPES, and 25 ml of FBS.

In addition, interferon gamma (1 unit/ml) and hydrocortisone were added to the media in which the YAMC cells were grown as published previously (146)

Inducible system

This inducible system was developed by the RheoGene company (Norristown, PA) and it is a modified version of the classic ecdysone receptor use in other inducible gene systems. The nuclear receptor of this system is a fusion of the Ecdysone receptor from *C. fumiferana*, the retinoid receptor from *Mus musculus* and the VP16 activation domain. The combination of these elements provide to this system better results in terms of low basal expression in absence of the ligand, and precise and robust expression in the presence of the ligand.

Plasmids

The plasmid that was used to evaluate the activity of the inducible systems, was provided by RheoGene (Norristown, PA). This vector uses the β-Gal gene as a reporter gene and is under the control of responded elements of the nuclear receptor mentioned above.

The vector p5004 in which the TGFBR2 was inserted, also responded to the nuclear receptor made by the fusion of the Ecdysone receptor from *C. fumiferana*, the retinoid receptor from *Mus musculus* and the VP16 activation domain.

The TGFBR2 transgene in the vector pRC/CMV that was used in this project was present in a construct provided by Harold Moses (Vanderbilt University, Nashville, TN).

Generation of V-400R2

V-400 cells were infected with a lentivirus which was constructed by cloning an HA tagged TGFBR2 cDNA transgene, provided by Sanford Markowitz (Case Western Reserve University Medical School, Cleveland, OH) into the p5004 vector provided in collaboration by RheoGene (Norristown, PA). An important characteristic of the HA-tag is that it is localized in the extracellular region of the TGFBR2, however it does not interfere with the interaction ligand-receptor, the activation of the pathways mediated by TGF-β is not affected either (this has been demonstrated by a series of experiments performed in the lab). To clone the TGFBR2 into the plasmid p5004, we made a digestion using the enzymes SwaI and PstI. Using these enzymes we released the β-Gal gene and the TGFBR2 was cloned. The TGFBR2 gene was released from its original backbone (kindly provided by Harold Moses) using the enzymes NotI and EcoRI. Since the DNA ends formed by the enzymes used to open the recipient plasmid and those used to release the TGFBR2 are not compatible, we had to treat the DNA using Klenow (Cat # M0210S, New England Biolabs, Ipswich, MA) in order to create blunt ends. To establish the orientation of the TGFBR2 gene, we sequenced the fragment and clones were selected. No mutations were detected.

Using the vector p5004+TGFBR2 a lentivirus carrying the TGFBR2 transgene was produced in the Gene Therapy core lab at the Fred Hutchinson Cancer Research Center using a 293T-based packaging cell line. V-400 and YRII null cells were infected with .5 ml of virus (viral supernatant). This volume showed the best infection levels in our set of cells. The viral supernatant in presence of polybrene was incubated with the

cells for 24 hours. The viral supernatant was then removed and 48 hours later Blasticidin (6 μg/ml) was added to the media. The transduced cells were then subjected to cloning by limiting dilution. The clonal populations were then subjected to evalution to identify clones that displayed minimal expression in the baseline state but showed adequate induction of TGFBR2 with treatment with Rheochem. Several clones were evaluated to establish they response to the treatment with TGF-β and different concentrations of Rheochem. The control cells were treated with DMSO vehicle alone.

Luciferase reporter assays

In order to evaluate TGF-β-mediated transcription, the cell lines were transiently transfected with the p3TP-lux reporter (kindly provided by Joan Massagué, Memorial Sloan-Kettering Cancer Center, New York, NY) or with the CAGA reporter assay (kindly provided by Bert Vogelstein, Johns Hopkins University, Baltimore, MA) concomitantly with the pRL-TK reporter construct (Promega, Madison, WI). The cells were treated with TGF-β1 (10ng/ml), and luciferase activity was evaluated 48h after transfection using the Dual Luciferase Reporter Assay System (Promega) with a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). The p21 expression was evaluated using a luciferase reporter assay (kindly provided by Dr. Xiao-Fan Wang's Laboratory). To evaluate the transfection efficiency the pRL-TK reporter construct was used. The luciferease activity was evaluated 24 and 48 hr post-transfection using the luminometer mentioned above.

FACS analysis

V-400R2 and V-400 cells treated for 48 hours with Rheochem and TGF-β1 were used for these studies. The cells were collected after being treated with trypsin 0.125% to detach them from the flasks. They were washed in media with 20% FBS (4ºC) and then 1X PBS (4ºC). They were then fixed using the buffer 3% formaldehyde in PBS, which permitted the detection of only cell surface proteins. The cells were then incubated with 10% normal horse serum for 30 minutes, washed with PBS X 3, incubated with an anti HA antibody in order to track HA-TGFBR2 expression (1:100,Cat # 2367 Cell Signaling, Danvers, MA), followed by incubation with a goat-anti mouse secondary antibody labeled with Fluorescein (FITC) in a concentration 1:100 (Cat # 115-095-146 Jackson ImmunoResearch, West Grove, PA) . The cells were washed and then subjected to FACS analysis using FACScan (Becton Dickenson, Franklin Lakes, NJ). The results were analyzed using CellQuest software (Becton Dickenson, Franklin Lakes, NJ).

Western blotting

Cell lysates were prepared using RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich), The lysates were then used for SDS-PAGE 10% acrylamyde The proteins were transferred onto PVDF membranes (Pierce, Rockford, IL). Immunoblots were performed using the antibodies of interest following the manufacturer's recommendations. The following antibodies were used: p-ERK1/2 (Cat # 9101 Cell Signaling, Danvers, MA 01923), ERK1/2 (Cat # 9102 Cell Signaling, Danvers, MA 01923), pAKT (Cat # 9271 Cell Signaling, Danvers, MA 01923), AKT (Cat # 9272, Cell Signaling, Danvers, MA

01923), pFoxO1 (Cat # 9464 Cell Signaling, Danvers, MA 01923), FoxO (Cat # 2488 Cell Signaling, Danvers, MA 01923) and p21(Cat # OP68 EMD San Diego California). All secondary antibodies were from Santa Cruz Biotecnologies (Santa Cruz, CA), HRP goat anti-mouse (Cat # SC 2031) and donkey anti-rabbit (Cat # SC 2317). Commercial ECL, was used to detected positive signals (RPN 3004, GE Healthcare, Piscataway, NJ)

PIK3/AKT specific ELISA

Phosphorylated AKT was detected using a commercially generated ELISA assay (SuperArray, CA) following the manufacturer's recommendation. Ten thousand cells/well were seeded in a 96 well plate. The V-400R2 cells were treated with different concentrations of Rheochem in order to differentially induce the expression of the TGFBR2. 10 ng/ml of TGF-β (10 ng/m;) was then to the cells and the cells were harvested after 48 hours. The ELISA was performed following the manufacturer's recommendations (cat # FE-001 SuperArray, CA). The ELISA was read in a plater reader at 450 nm (VERSAmax, Molecular Devices, Sunnyvale, CA).

Other Reagents

n-Butyric Acid Sodium salt (cat# B-5887), Luminol (cat# A8511), and p-Coumaric acid (cat# C9008) were purchased from Sigma-Aldrich (St. Louis, MO). The MEK1/2 inhibitor U0126 (cat# 662005), and the PIK3 inhibitor LY294002 (cat# 440204) were obtained from Calbiochem (San Diego, CA).

Results

Precise inducible transgene expression can be achieved through the use of the RheoGene expression system.

In order to study the role of TGFBR2 expression levels on the regulation of TGF-ß pathway activation and biological outputs, we believed we would need to generate a model system that would permit the precise regulation of transgene levels and that would have essentially no unstimulated expression. We identified the RheoGene expression system (RheoGene, Norrristown, PA) as having characteristics that could potentially satisfy these criteria. (145). This inducible system is a modified version of the Ecdysone receptor which has been used to induce the expression of several genes for *in vitro*, *in vivo* and in gene therapies (148).

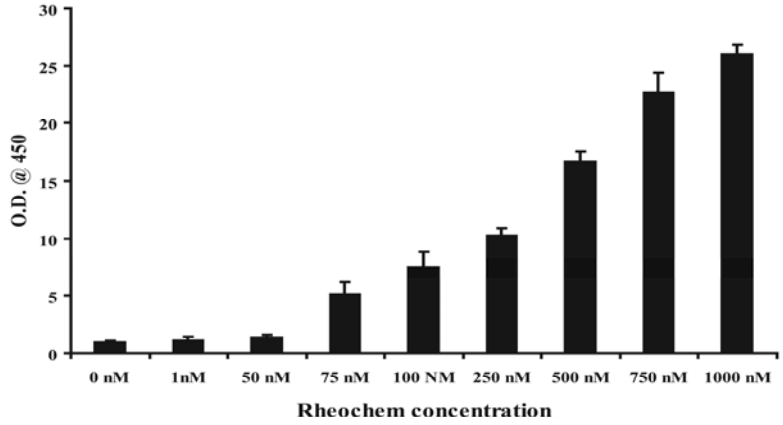
After determining that the RheoGene inducible expression system had the potential to be a precisely regulatable expression system in colon cancer cell lines, we assessed whether it would behave appropriately in the V-400 and Y-RII null cell lines. The performance characteristics of the RheoGene system were initially assessed using an inducible reporter gene (β-galactosidase) transiently transfected into both the V-400 and YRII null cells. The cells were transfected with the fsEcR plasmid, the inducible β-galactosidase reporter plasmid, and a pRL-TK plasmid (used as reference for transfection efficiency). The cells were then treated for 48 hours with different concentrations of Rheochem (the diacylhydrazine ligand provided by RheoGene). We observed that 1 μM of Rheochem induced the highest β-galactosidase activity in V-400 (26 times higher than

V-400 without treatment) and that 750 nM promoted the highest expression in the YRII null cells inducing a 70X increased in β-galactosidase activity compared to the basal untreated state. We also demonstrated a linear dose response relation that was present over a broad concentration range and that there was essentially no β-galactosidase activity in the setting of no ligand. (**Figure 3.1**).

After establishing that the RheoGene system had the potential to be a precisely regulatable system in the V-400 and YRII null cells, an HA-tagged TGFBR2 transgene was cloned into a lentiviral expression vector provided by the RheoGene Company (vector described previously in Materials and Methods section). A lentivirus shuttle vector was used because of difficulties we experienced with transgene silencing with conventional expression vectors. After cloning the TGFBR2 transgene into the expression vector, it was subjected to sequencing to confirm that the sequence was wild-type.

The V-400 and Y-RII cells were then infected with lentivirus generated by the Gene Therapy core at FHCRC. The selection of clones expressing the Rheochem receptor and the exogenous *TGFBR2* was performed using the selection marker Blasticidin (2.5 μg/ml) as described in the methods section. The clones that displayed Blasticidin resistance were transiently transfected with the 3TP-lux or CAGA reporters and then treated with 10 ng/ml of TGF-β in order to identify clones that displayed a linear dose response of 3TP-Lux activity in relation to Rheochem dose. A total of 4 clones were tested for each cell line. The V-400R2 cells displayed a higher amount of 3TP-Lux luciferase activity than did the Y-RIInull-R2 cells. (**Figure 3.2**).

A.



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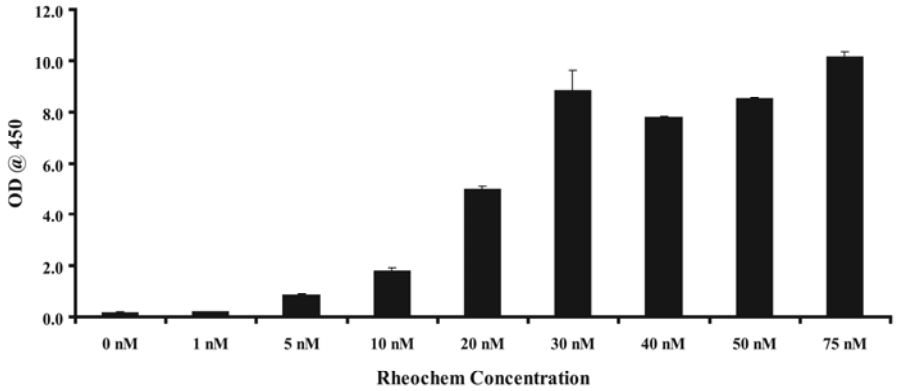

Figure 3.1. Activation of β-Galactosidase in V-400 and YRII null cells after treatment with Rheochem. V-400 and YRII null were treated with different concentrations of Rheochem and transfected with the reporter assay β-Gal. **A.** V-400 cells show strong induction of the β-Gal reporter assay that is linear from 50-750 nM of Rheochem. B-Gal activation was detected in cells treated with 1nM and 50 nM of Rheochem that was no different from the untreated cells **B.** B-Gal activity also was induced in YRII null cells bay adding different concentrations of Rheochem. These clones are induced at lower concentrations of Rheochem but do not display the same amount of reporter activity as seen in the V-400 cells.

Ultimately, we observed that the original clones of TGFBR2 reconstituted Y-RII null cells became TGF-β resistant. While the mechanism responsible is not known, it is possible that aberrant DNA methylation led to silencing of the transgene (149-151).

Precise regulation of TGFBR2 in the V-400 R2 cell line

In order to determine if the expression of the *TGFBR2* transgene was being regulated by the presence of Rheochem in the V-400R2 cells, FACS analysis was performed using clone 1 and clone 2. which showed a higher level of 3TP-Lux induction with Rheochem Rheochem (**Figure 3.2**). FACS analysis on formalin fixed cells was performed to determine if the transgenic TGFBR2 was being properly localized to the cell surface, where it would have the potential to bind TGF-β. FACS was performed using an anti-HA antibody that can detect the HA tag present in the amino-terminal domain of the receptor (extracellular domain) **(Figure 3.3**).

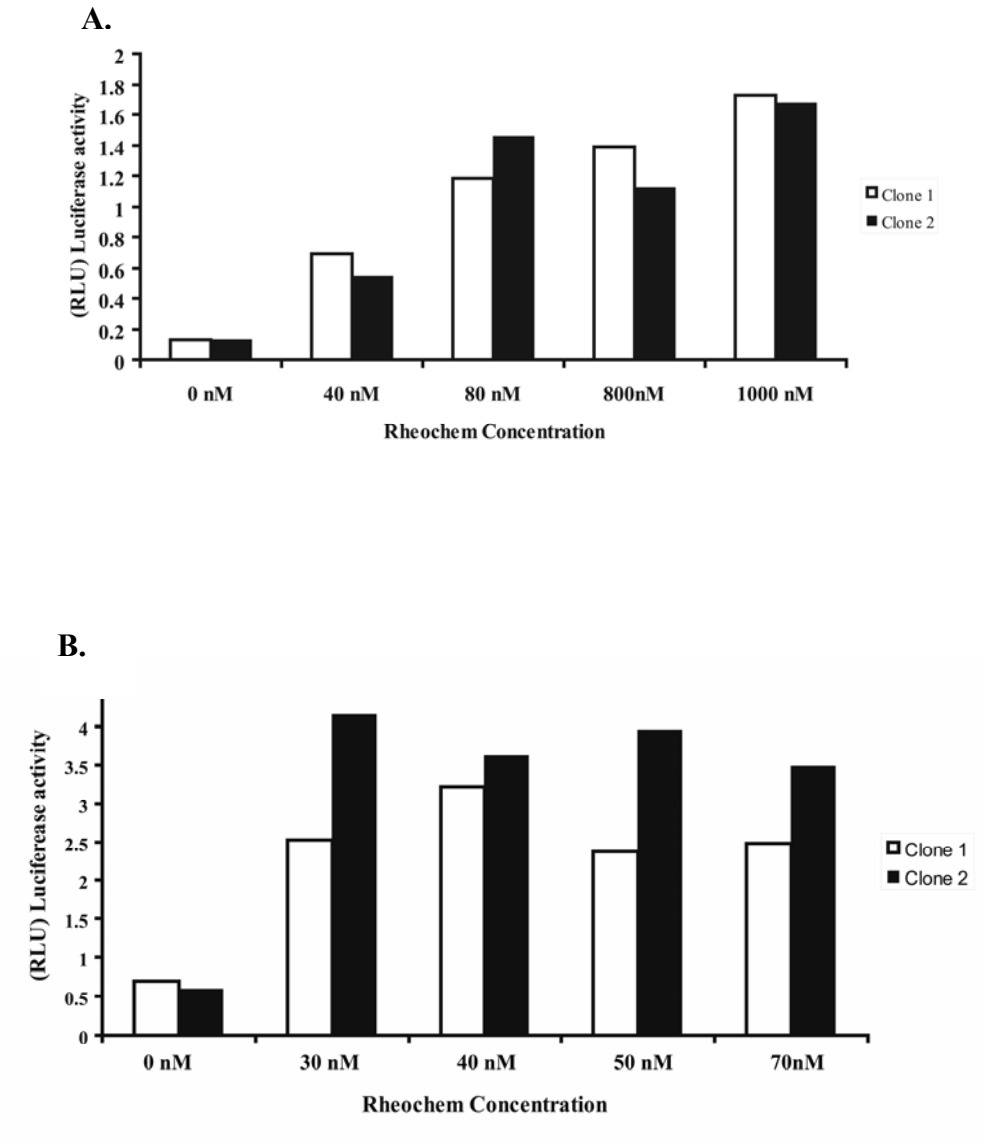

Figure 3.2. Assessment of TGF-β signaling in representative clones of V-400 after transduction with the TGFBR2 transgeneA. V-400R2 clones 1 and 2 display a linear dose response from 0-100nM with the greatest increase in 3TP-lux activity occurring between 0nM and 80nM. B. 3TP-Lux activity measured at low concentrations of Rheochem. Note that clone 1 had a more linear response when compared with clone 2.

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The FACS results support the data obtained using the CAGA reporter assay in which we established that cells treated with 1 μM of Rheochem had higher activity of TGF-β pathway and 0.04 μM the lowest (**Figure 3.3**).

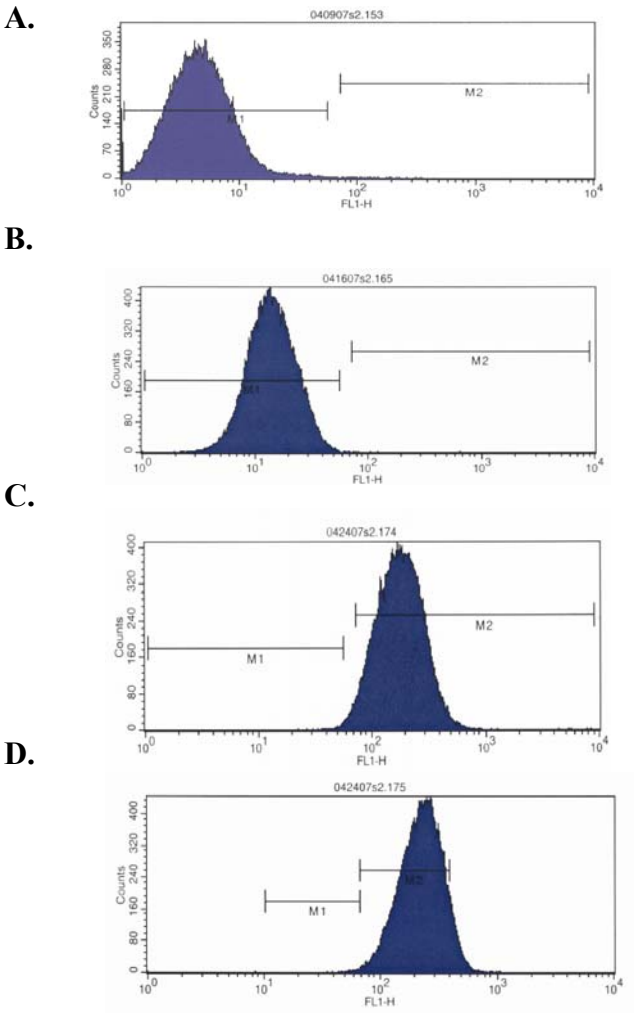


Figure 3.3. The expression of cell surface TGFBR2 in V-400 after treatment with Rheochem. FACS analysis using an anti-HA antibody was performed after treatment with different doses of Rheochem (A. 0 μM, B. 0.04 μM, and C. 0.5 μM D. 1 μM). An increased amount of TGFBR2 is observed with the increasing Rheochem doses.

The regulation of TGFBR2 expression levels may be a mechanism for controlling the specificity of TGF-β effects on cells.

TGF-β can regulate the expression of a variety of genes that control a myriad of cell responses including proliferation, apoptosis, differentiation, etc. The myriad of effects that TGF-β can have on a cell has raised interest in determining the mechanisms through which a cell determines how it will respond to TGF-β stimulation. The mechanisms through which specificity of the TGF-β response is determined are largely unknown, although at least some level of regulation occurs at the level of signal pathway crosstalk. One of the TGF-β regulated genes that highlights the issues related to control of the specificity of the TGF-β response is the cdk inhibitor p21, which plays an important role in several cellular processes including apoptosis, cell cycle arrest, induction of cell differentiation, and cellular senescence (152). Thus, we assessed the regulation of p21 expression in the setting of different levels of TGFBR2 expression to determine if receptor level regulation may be a mechanism through which TGF-β mediated responses are regulated. We postulated that the level of TGFBR2 expression and activation may be one mechanism through which cells may determine whether p21 will be induced by TGF-β.

Thus, in order to determine how changes in the expression levels of TGFBR2 affect the expression of *p21*, V-400R2 cells were treated for 24 and 48 hours with four different concentrations of Rheochem (0.04, 0.08, 0.4 and 1 μM) in the presence of 10 ng/ml of TGF-β (10ng/ml). The induction of p21 expression was assessed using a luciferase reporter that contained the p21 promoter (153). No significant differences in p21 expression were detected at 24 hours (**Figure 3.4 A**); however, differences in p21

expression were present at 48 hours consistent with TGFBR2 expression levels affecting the ability of TGF-β to stimulate p21 expression (**Figure 3.4A**). No differences in p21 expression were detected between V-400R2 without Rheochem and the parental cell line V-400. Western blots were also performed to establish if the differences observed with the p21 luciferase reporter assay also were reflected in changes in protein levels. (**Figure 3.4 B**).

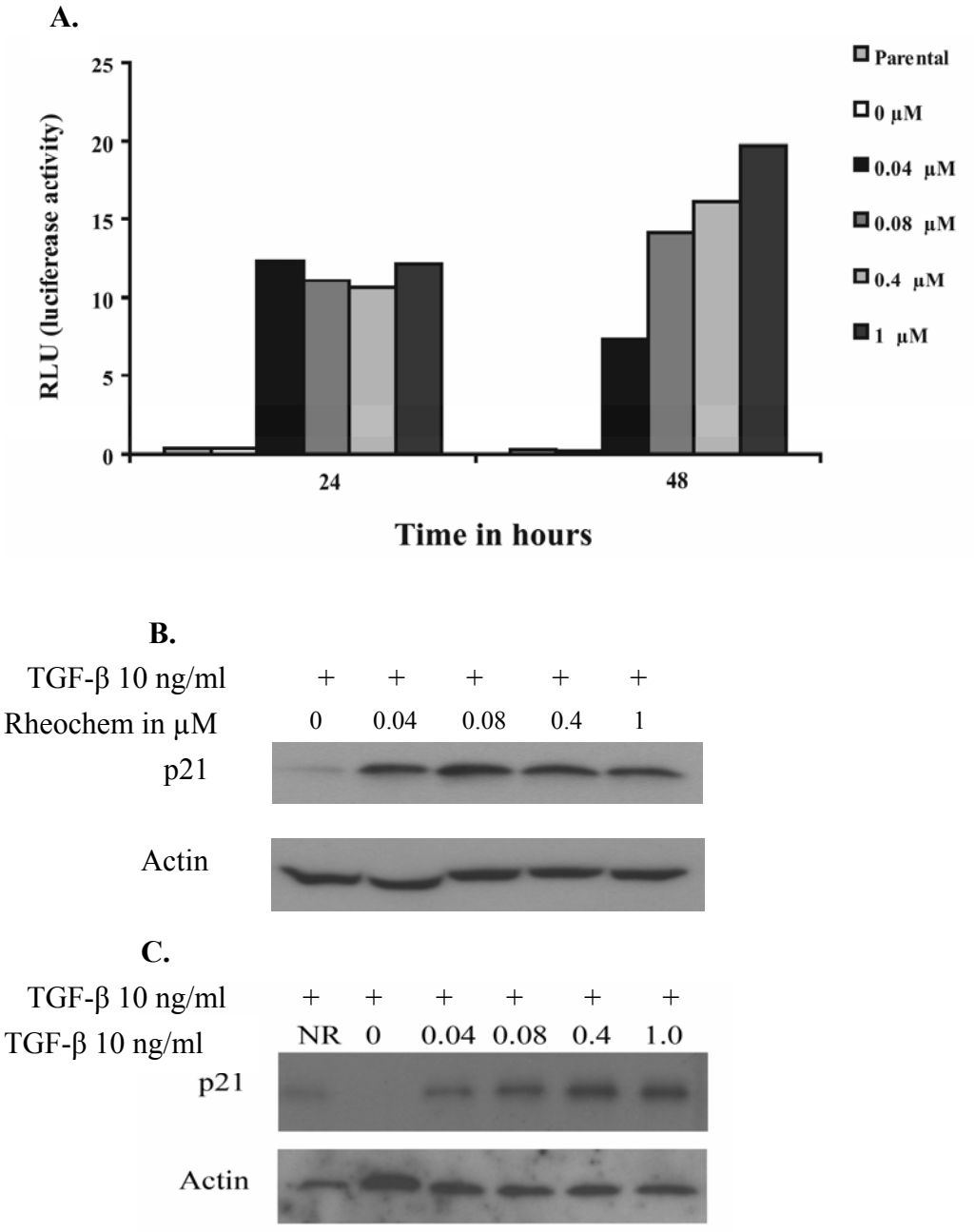


Figure 3.4. p21 expression in V-400R2 cells after treatment with different concentrations of Rheochem. V-400R2 Clone 1 was treated with four different concentrations of Rheochem 0.04; 0.08; 0.4 and 1 µM and then p21 expression was assessed. **A.** p21 luciferase reporter activity after 24 and 48 hours of RheoChem. Induction of p21 is present at 24 and 48 hours with a linear dose-response being evident at 48 hours. **B (24 hr) and C (48 hr).** Immunoblotting for p21 after 24 hr and 48 hours of treatment. After 48 hours more p21 expression is present in the cells expressing high levels of TGFBR2.

TGFBR2 levels can differentially affect AKT pathway activation, which may be a mechanism for regulating TGF-β mediated effects on p21 expression.

In a report by Seoane et al (35), evidence was presented that demonstrated that active AKT can inhibit *p21* expression. Thus we decided to evaluate if changes in the expression levels of TGFBR2 may differentially activate Smad independent signaling pathways and affect the activation of AKT, which could suppress *p21* expression. V-400R2 cells were treated for 24 and 48 hours with the same concentrations of Rheochem that were used in previous experiments in the presence of TGF-β (10 ng/ml). Interestingly no changes in the activation of AKT, as assessed by measuring the phosphorylation state of S473 were observed after 24 hours in which activation of AKT was detected in cells expressing different levels of TGFBR2 (data no shown). In contrast at 48 hours under the same conditions mentioned above, the levels of p-AKT were higher in those cells expressing low levels of TGFBR2 compared to the cells with high levels of TGFBR2 (**Figure 3.5**). Total AKT was evaluated and no differences were detected in the V-400R2 cells treated with different concentrations of Rheochem (**Figure 3.5 A**). The immunoblotting results were confirmed using an ELISA-based assay **(Figure 3.5 B**).

TGFBR2 levels can differentially affect ERK pathway activation, which may be a mechanism for regulating TGF-β mediated effects on p21 expression.

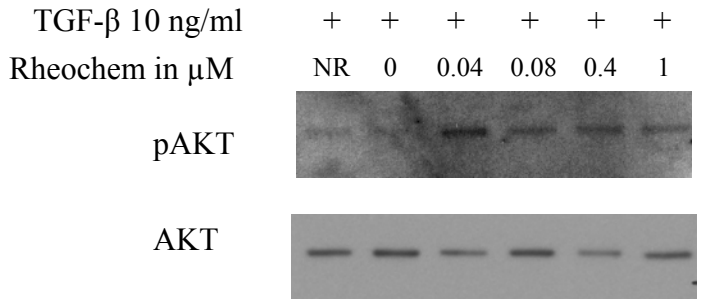
As presented by Park et al a study done in colon cancer cell lines, ERK activation plays a key role in the regulation of p21 expression which results in a reduction of cell proliferation (154). Since ERK is one of the proteins that are regulated by TGF-β, we wanted to determine a possible correlation between TGFBR2 expression levels, ERK

activation and p21 expression. V-400R2 cells were treated with 4 different concentrations of Rheochem at two different time points, 24 hours and 48 hours. Interestingly, we found that ERK activation was not affected after 24 hours of treatment with Rheochem; however, after 48 hours of treatment, ERK activation was strongly regulated by TGF-β. We observed an increment in the levels of pERK in V-400R2 cells that expressed high levels of TGFBR2 (**Figure 3.7**). Moreover, we found that in such cells, the expression of p21 was higher that in cells expressing low levels of TGFBR2.

Assessment of mechanisms through which TGFBR2 mediated activation of AKT and/or ERK regulate the expression of p21.

After observing that low levels of TGFBR2 associate with the highest amount of pAKT, we hypothesized that the differences in p21 expression observed at different TGFBR2 expression levels may be a consequence of differences in the activation of the AKT pathway observed with the different TGFBR2 expression levels. It has been shown that activated AKT can phophosphylate FoxO1 (S219), which is a transcription factor that can participated in a transcription factor complex with Smad2/3 to regulate p21 expression (35) AKT negatively affects the assembly of this transcription factor complex by phosphorylating FoxO1, which promotes its nuclear export. (35, 155) Thus we assessed whether the activation of AKT induces the phosphorylation of FoxO1 on Ser 219. Interestingly, we did not find significant differences in the phosphorylation levels of FoxO1 in cells that were treated with different doses of Rheochem, demonstrating that this is unlikely to be a mechanism responsible for regulating p21 expression by TGF-β in colon epithelial cells (**Figure 3.6**).

A.



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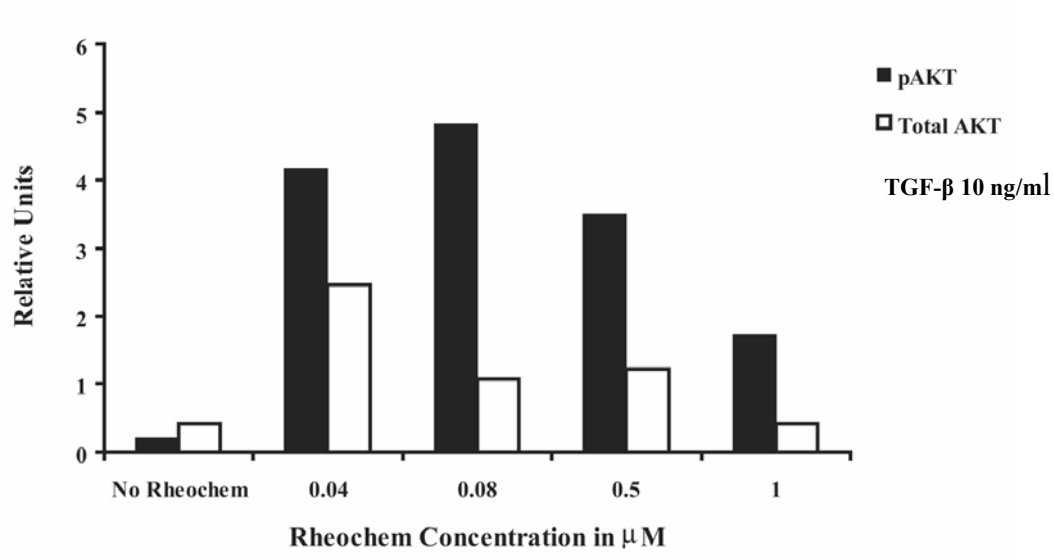

Figure 3.5 AKT activation correlates with the expression levels of TGFBR2. V-400R2 cells were treated with different concentrations of Rheochem for 48 hours. Phosphorylated Akt was assessed by Western Blot (**A.**) and by an ELISA that detects pAKT S473 and total AKT (Superarray) a specific ELISA to detected pAKT (**B.**). Higher pAKT levels were observed in cells treated with 0.4 μM of Rheochem NR: No Rheochem.

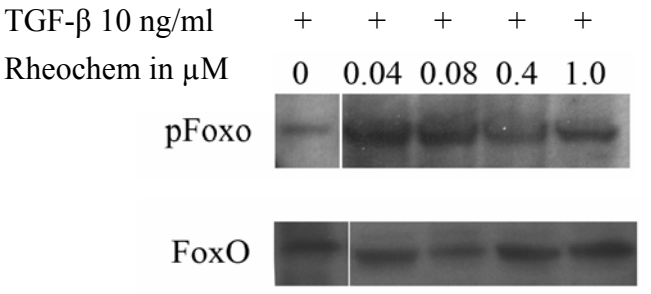

Figure 3.6. Phosphorylation of FoxO1 (Ser 219) is not affected by the expression levels of TGFBR2. V-400R2 cells were stimulated with Rheochem in presence of TGF-β, after 48 hours of treatment and increment pFoxO1 was detected in cells expressing high levels of TGFBR2; showing the independence between the TGF-β pathway and FoxO1 phosphorylation

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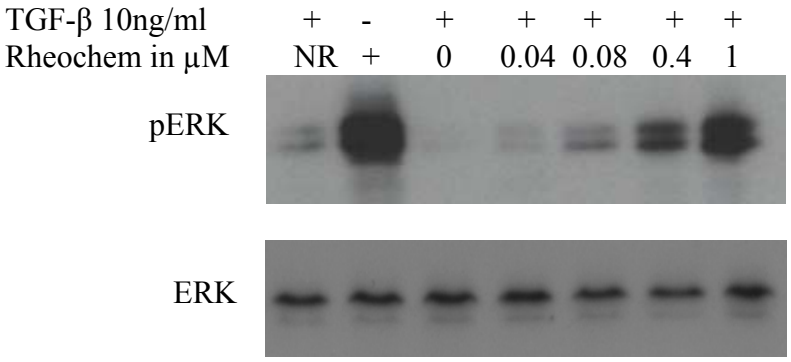


Figure 3.7. ERK activation is induced in cells expressing high levels of TGFBR2. V-400R2 were treated by 48 hours with different concentrations of Rheochem, the presence of pERK was assessed by western blot. Increased pERK is present in the cells treated with higher levels of Rheochem. A linear dose response is present.

Expression levels of p21 in V-400R2 cells are regulated by ERK but not by AKT

Our initial attempt to determine whether the mechanism that determines the specificity of TGF-β mediated induction of p21 though a candidate approach based on published studies did not reveal any significant positive findings. Thus, in light of the fact that we observed that the activation of both the AKT and ERK pathways are differ between low and high levels of TGFBR2, we assessed the role these pathways have on modulated TGF-β mediated induction of p21 though pharmacologically inhibited the pathways in the setting of TGFBR2 activation. The V-400R2 cells were treated with different concentrations of Rheochem, 10mM LY294002 (PIK3/AKT inhibitor), 5mM U0126 (MAPK/ERK inhibitor) and 10ng/mL TGF-β1. We found that inhibition of the AKT pathway did not affect TGF-β mediated induction of *p21* expression (**Figure 3.8**). Additionally using the PIK3/AKT inhibitor we have confirmed that in V-400R2 the phosphorylation of FoxO1 is independent of AKT activation. However, when ERK was inhibited with U0126, the differences previously observed in p21 expression were not detected in cells cultured in presence of the inhibitor U0126 which suggests that the ERK pathway is a Smad independent TGF-β mediated signaling pathway that regulates the expression of p21 (**Figure 3.9**)

Smad pathway activation is not affected by TGFBR2 expression levels.

It has been proposed that in cells in which the TGFBR2 has been activated there is higher accumulation of Smad 2 in the nucleus (129). With the purpose of evaluating if the expression levels of the TGFBR2 affect the degree of activation of the Smad signaling pathway, we measured the accumulation of Smad 2 in the nucleus, which is a

reflection of Smad pathway stimulation. This analysis revealed that when the cells were treated with TGF-β, the majority of Smad2 is located the nucleus of the cells. No significant differences were detected among cells expressing different levels of TGFBR2, suggesting that even low levels of TGFBR2 are sufficient to maximally activate the Smad signaling pathway. (**Figure 3.10**).

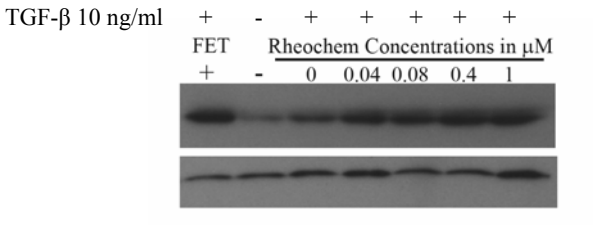
Thus, using an inducible system we have established that the expression levels of TGFBR2 associate with the activation state of the Smad independent pathways but not the Smad dependent pathway, which appears to be maximally stimulated even at low TGFBR2 expression levels. In summary, these results suggest that changes in the expression of the TGFBR2 have direct consequences in the regulation of the PIK3/AKT and MAP/ERK pathways in V-400R2 cells. On the other hand, no significant changes in the phosphorylation of Smad2 were detected in V-400R2 cells treated with different concentrations of Rheochem, showing that the activation of the Smad dependent pathway is independent of the levels of TGFBR2 present in the membrane of the cell.

TGFBR2 expression levels and their effects on biological processes regulated by TGF-β.

As noted above, TGF-β is known to mediate a wide variety of effects on cells. The mechanisms though which specific effects are induced is largely unknown. In light of our results showing that differences in TGFBR2 expression correlate with differences in AKT and ERK activation, we carried out a series of studies to determine whether the expression levels of TGFBR2 may also have effects on TGF-β mediated apoptosis in these cells. Indeed, using specific inhibitors to suppress the activation of these the ERK

and AKT pathways, we have demonstrated that the MAPK/ERK pathway seems to be an important regulator of *p21* expression, which would be predicted to effect TGF-β mediated apoptosis. Consequently, we assessed the effect of TGF-β on inducing apoptosis in V-400R2 cells that expressed different levels of the TGFBR2.

A.



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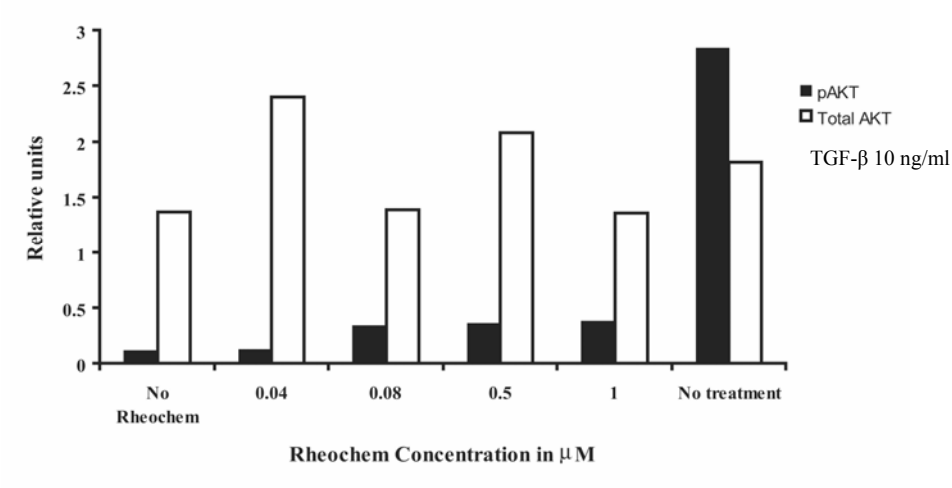


C.

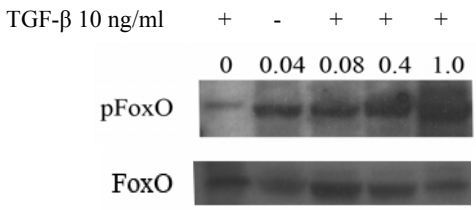
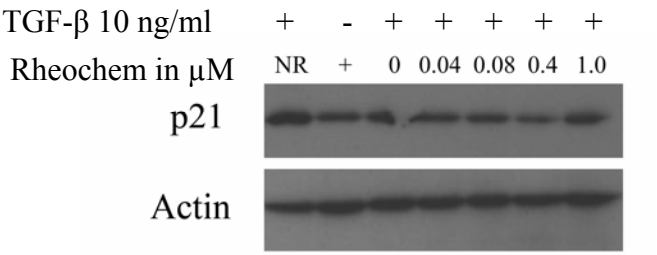


Figure 3.8. Inhibition of PIK3/AKT pathway does not induce changes in the expression levels of p21 in V-400R2 cells. V-400R2 cells were treated with TGF-β, and different Rheochem concentrations in presence of the PIK3/AKT inhibitor LY294002 (2 nM). pAKT was completely inhibited by LY294002. **A**. p21 expression with treatment with TGF-β, Rheochem, and LY294002). p21 is modestly induced with higher receptor levels, similar to what was observed in the setting of no LY294002 treatment **(-)** Rheochem nor TGF-β were added. **(+)** Protein extracted from FET cells which are known to express high levels of p21. **B.** Levels of pAKT were assessed using a specific ELISA for this active form of AKT. A reduction of almost 3 times less in the levels of AKT was detected when we added the inhibitor LY294002 to V-400R2 stimulated with Rheochem and TGF-β. **C.** pFoxO1 levels did not changes after treatment with LY294002, which demonstrates that in V-400R2 AKT activation induced by TGF-β does not regulate the expression of p21 via FoxO1.

A



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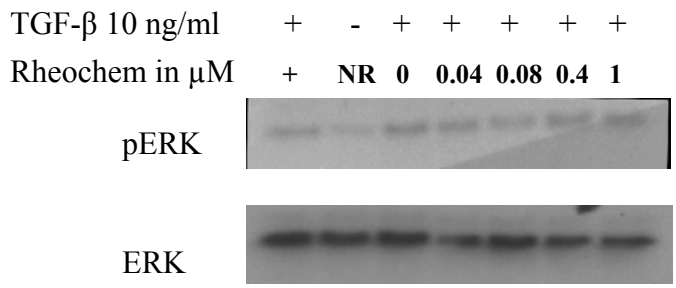

Figure 3.9: p21 expression levels are regulated by MAPK/ERK in V-400R2 cells V-400R2 were cultured in presence of Rheochem, TGF-β and the MAPK/ERK inhibitor U0126 for 48 hours. **A.** p21 expression was detected in V-400R2 cells that were treated with Rheochem and TGF-β in presence of the MAPK/ERK inhibitor, however the regulation of the expression levels of p21 is lost, indicating that its expression is regulated by ERK in a TGFBR2 dependent manner. **B.** The activation of ERK is considerably reduced due to the presence of the inhibitor, importantly total levels of ERK were not altered

V-400R2 cell line that express higher levels of TGFBR2 demonstrate increased TGF-β mediated apoptosis indicating a clear correlation between apoptosis and TGFBR2 expression levels (**Figure 3.11**). These results suggest that increased ERK activation and p21 expression that occurs secondary to increased TGFBR2 expression may, at least in part, be a mechanism through which TGFBR2 expression levels can regulate the specificity of TGF-β effects on cells.

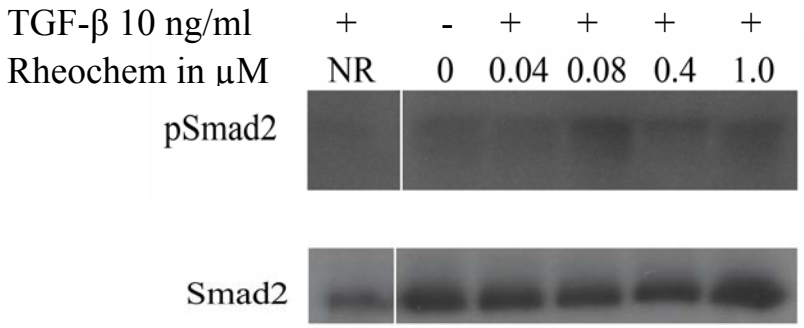

Figure 3.10 SMAD2 phosphorylation is affected by changes in the expression of TGFBR2. V-400 cell were tested with different concentrations of Rheochem, and nuclear pSmad was assessed by western blot, not significant differences in pSmad2 levels were detected.

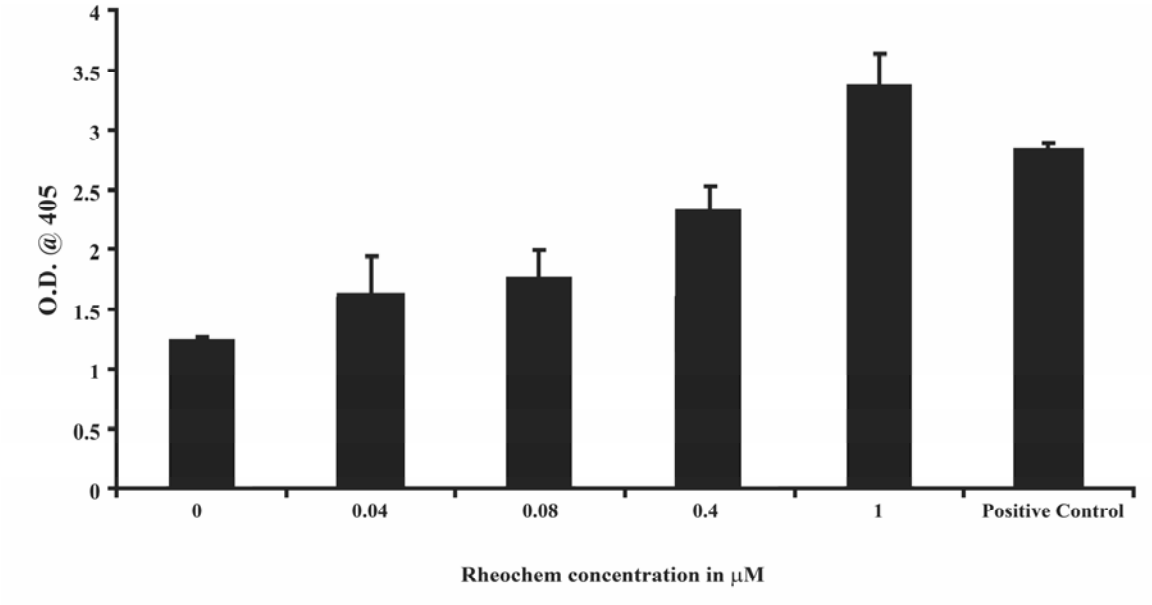

Figure 3.11. High expression levels of TGFBR2 induces apoptosis in V-400R2 cells. V-400R2 where treated with different concentrations of Rheochem in presence of 10 ng/ml of TGF-β. Apoptosis was measured using a specific ELISA to detect apoptosis. As shown, cells that express high levels of TGFBR2 are more susceptible to suffer apoptosis when they express high levels of TGFBR2.

Discussion

TGF-β is a complex cytokine that is able to induce the activation of several pathways that are involved in the regulation of both apoptosis and cell proliferation (16, 44, 94, 156). Numerous studies have examined the mechanisms involved in TGF-β mediated regulation of programmed cell death and proliferation, yet the processes involved in determining the specificity of the TGF-β response remain largely uncertain (52).

In this project we have utilized an inducible system (Rheochem) to precisely regulate the expression of TGFBR2 in V-400 and YRII null cells. Interestingly, we observed that the expression of the TGFBR2 in YRII null cells was silenced in cells that were cultured for more than five passages, precluding them from further assessment. The V-400 cells maintained expression of the inducible TGFBR2 and thus were used in the studies described above. The regulation of TGFBR2 expression and activity was evaluated by FACS analysis and with the CAGGGA reporter assay (CAGA), which reports Smad mediated transcription. This allowed us to confirm the presence of the receptor in the cell membrane, a crucial requirement in designing experiments aimed at evaluating TGFBR2 expression levels in the activation of various pathways.

After establishing that the V400R2 cell line was a tractable model for assessing the effect of TGFBR2 expression levels on the regulation of TGF-β mediated effects, we used this cell line system to investigate potential mechanisms through which cells determine how they will specifically respond to TGF-β. The regulation of the specificity of the response is important because of the myriad of potential effects that TGF-β can have on cells and subsequent need to limit the responses to those appropriate to the

biological situation. The V400R2 system permitted the evaluation of the regulation of TGFBR2 levels, a mechanism through which at least some of this specificity could be achieved. Since TGF-β is able to induce apoptosis, and since p21 plays a key role in the regulation of apoptosis, we chose to evaluate how changes in the TGFBR2 expression affect p21 induction. Importantly, p21 is also regulated by a variety of factors, such as p53, Sp1/Sp3, Smads, Ap2, signal transducers and activators of transcription (STAT), BRCA1 and E2F-1/E2F-3 (Reviewed in (132, 157)).

Using V400R2, we found that cells expressing higher levels of TGFBR2 express increased levels of p21 when compared to cells that expressed no or low levels of TGFBR2. Despite these findings, it was not clear how TGFBR2 expression was involved in the regulation of p21 expression. These results clearly showed that the TGF-β receptor does not induce binary responses.. Rather, we have demonstrated that it is not only the activation of the TGFBR2, but the also the intensity of its activation, that determines which genes are expressed in TGF-β1 stimulated cells.

In order to elucidate the pathways responsible for p21 expression in V-400 cells expressing various levels of TGFBR2, we assessed the activation state of Smad-dependent and Smad-independent pathways at low and high levels of TGFBR2. Interestingly, we observed small differences in the amount of pSmad2 between cells that expressed various levels of TGFBR2, demonstrating that the activation of this pathway appears to be modestly affected of the amount of TGFBR2. It is possible that other factors, such as overexpression of Smad 7, or changes in the internalization of the receptor 2, might be the key elements in the regulation of pSmad2 levels in V-400 cells. Despite the small differences in the activation of Smad 2 detected by Western blot, an

analysis performed using the CAGA reporter assay (specific to detect the activity of the Smad-dependent pathway) showed important differences in the activation of this pathway in cells expressing different levels of the TGFBR2. The low levels of pSmad 2 detected in V400R2 cells could be due to limited precision of immunoblotting to measure biologically relevant differences in phosphorylated Smad2.

Another potential mechanism to regulate the biological specificity of the transduced signal from TGFBR2 is via differential activation of the Smad-independent pathways, including MAPK/ERK and PIK3/AKT (16, 64, 158-162). Thus, we assessed changes in levels of pAKT and pERK in V-400 cells expressing various levels of TGFBR2 and correlated the expression levels of the phosphorylated proteins with the expression levels of the transgenic TGFBR2. Interestingly, our results showed that cells that expressed high levels of TGFBR2 had an increase in the amount of pERK when compared with control cells or cells that expressed low levels of TGFBR2. These results demonstrate that TGF-β can affect the activation of ERK, and that the levels of TGFBR2 affect the amount of activation of the ERK pathway, which is involved in several cellular processes, including cell proliferation, EMT, and apoptosis (158, 163).

Since significant changes in p21 expression levels were detected in cells treated with Rheochem, and due to the role that this CDK inhibitor plays in apoptosis and cell proliferation, we studied the potential interaction between p21 expression and TGF-β mediated ERK activation. Interestingly, we established that in cells with high levels of pERK, more p21 was expressed, and vice-versa. In order to prove that the changes in pERK had a causal effect on p21 expression, we used U0126, an inhibitor of the MAPK/ERK pathway to determine the consequence of inhibiting this pathway on TGF-

β's regulation of p21. Cells that were treated with the inhibitor showed that when the MAPK/ERK signaling pathway was inhibited, p21 was still expressed. However, the modulation of levels of p21 expression detected in cells that expressed differing amounts of TGFBR2 were not present in cells treated with the MAPK/ERK inhibitor. Thus, we propose that although the MAPK-ERK pathway appears to be important regulator of p21 expression, it is not the mechanism for inducing p21 expression. As mentioned previously, p21 is regulated by several factors, and it is possible that one of these other factors is responsible for the induction of basal p21 expression in V-400R2 cells. Our results are consistent with previous reports that demonstrated a correlation between ERK activation and p21 expression (154, 164).

A second Smad-independent signaling pathway that was evaluated using our inducible system was the PIK3/AKT pathway. In general, the activation of this pathway is associated with an increase in the rate of cell proliferation, which in certain cases might promote tumor formation (162, 164). As observed with the MAPK/ERK pathway, we also detected differences in AKT activation in cells that were treated with various concentrations of Rheochem. However, the pattern of AKT activation was in the opposite direction of the pattern observed in ERK. In the case of AKT, higher levels of phosphorylation were detected in cells with lower levels of TGFBR2. In order to determine the effects of TGF-β-induced PIK3/AKT activation on p21 expression, we assessed the phosphorylation state of FoxO1 in these same cells, FoxO1 has been shown to be involved in TGF-β mediated regulation of p21 in neuroepithelial cells via interactions of Smad proteins with FoxO1 (165). Thus, we studied the phosphorylation of FoxO1, an AKT-downstream protein in the setting of different levels of TGFBR2. As

previously described by Seoane et al, FoxO1 is phosphorylated when p-AKT is present, and once FoxO1 is phosphorylated, it induces its nuclear export. This prevents its interaction with Smad2/3, and the lack of the Smad-FoxO1 complex prevents the binding of these transcription factors with the p21 promoter (35). Using our in vitro system, we established that although differences in the expression of the TGFBR2 resulted in changes in AKT phosphorylation, they did not affect the phosphorylation state of FoxO1. Thus, the effects we observed on p21 expression are independent of FoxO1 (35).

In light of our results with FoxO1, we next assessed the causal role of AKT pathway activation in the regulation of p21 in the V400R2 cells. In order to determine if the PIK3/AKT pathway regulates p21 expression we used the inhibitor LY294002 to assess the effect of AKT activation on p21 expression. We observed that despite reduced levels of pAKT, the inhibitor did not have an effect on p21 expression levels. Additionally, we did not detect changes in the levels of pFoxO1, which has been reported to be one of the targets of AKT. Based on these results, we propose that the regulation of p21 mediated by TGF-β in V-400R2 cells is independent of AKT.

In order to demonstrate the functional role of the Smad-dependent pathway in the activation of p21, we plan to carry-out a set of studies using Smad7 to inhibit the Smad pathway. If no changes in the expression of p21 are detected when the Smad pathway is inhibited, this result will be interpreted to be consistent with a model in which TGF-ß primarily regulates p21 through an ERK dependent mechanism in the V400R2 cells.,

In order to establish if the changes in p21 expression induced by various TGFBR2 levels have biological consequences, we assessed the correlation between p21 expression and the induction of apoptosis in the V-400R2 cells in the setting of different levels of

TGFBR2 expression. We found that cells expressing higher levels of TGFBR2, and consequently higher levels of p21, underwent more frequent apoptosis than cells that either did not express TGFBR2 or expressed lower levels of TGFBR2. In light of p21's role in both cell growth inhibition and apoptosis, we are also studying the effect of p21 on cell proliferation in the setting of different expression levels of TGFBR2. We anticipate finding that the amount of p21 will directly correlate with the amount of cell growth inhibition, which will provide evidence that regulation of the expression level of TGFBR2 can modify the biological output from the TGF-β receptor

In summary, we have shown that changes in TGFBR2 expression affect the activation of Smad-independent pathways, and that these changes are involved in the regulation of p21, ultimately resulting in differences in the effect of TGF-β on the induction of apoptosis in the V-400R2 colon cancer cell line. These findings suggest that the regulation of TGFBR2 expression can modulate the specificity of TGF-β mediated cellular responses. These results have implications for the paradoxical effects of TGF-β observed for some types of cancer. Based on the results presented here, we propose that low levels of TGFBR2 will result in a loss of the apoptotic, tumor suppressor effects of TGF-β and may favor the oncogenic effects of this cytokine. Additionally, if this hypothesis is correct, the level of TGFBR2 expression might be used as a marker to help determine the behavior of a cancer. Moreover, novel therapies aimed at increasing TGFBR2 expression in tumor cells with reduced levels of the TGFBR2 could be developed. Thus, through the use of an inducible cell line system we have provided evidence that the regulation of different levels of TGFBR2 can result in differential activation of signaling pathways, p21 expression, and apoptosis. These results suggest

that the regulation of TGFBR2 may be a mechanism through which specificity of the biological response of a cell to TGF-β is determined.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Colon cancer is a disease that kills approximately 55,000 people each year in the United States alone, and although there have been advances in the treatment of colorectal cancer in the last decade; these advances have translated into relatively small gains in the 5-year relative survival rate of patients diagnosed with stage III disease. The modest success in improving the clinical care of patients with colorectal cancer has led to a focus on the investigation of the molecular basis of colorectal cancer. It is hoped this approach will identify potential targets for therapeutic intervention. These studies have led to the discovery of a number of tumor suppressor genes and oncogenes in colon cancer, and some of these have the potential to be used in targeted therapies (94, 166).

It is now known that one of the most common genes affected by mutations in colon cancer is *APC*; 85% of all sporadic tumors carry mutant *APC* (2). Additionally, mutations in genes that are part of the TGF-β pathway are present in a large percentage of colorectal tumors. One of the main targets in the TGF-β pathway that is affected by genetic alterations is TGFBR2. Mutations in this receptor result in the inactivation of the TGF-β pathway. Interestingly, the activity of TGF-β as a tumor suppressor gene is lost in approximately 75% of colorectal neoplasms and this inactivation event appears play a role in the transition of adenomas to adenocarcinomas (22, 72, 92). However, it also appears to be true that in some cancers, such as breast cancer and gliomas, TGF-β can act to promote the progression of cancer by inducing EMT and metastatic behavior in the

cancer (91, 167, 168). The paradoxical effects of TGF-β pathway in cancer has promoted interest in understanding the mechanisms involved in determining the regulation of the specificity of the biological effects TGF-β. It has been proposed that factors that control the ultimate biological effects include differences in activation of Smad dependent and Smad independent pathways, alterations in signal pathway crosstalk caused by concurrent gene mutations, and by the nature of the genetic and/or epigenetic events in the TGF-β pathway (e.g. mutations in *TGFBR2* vs. *TGFBR1* vs. *SMAD4*, etc.) (43).

Epigenetic events affect TGF-β pathway activation

In general, certain genetic alterations prevent the expression of the proteins, whereas others affect the activity of the gene product, resulting in deregulation of the TGF-β pathway (43). In recent years, increased attention has been devoted to epigenetic changes, which are characterized by specific DNA methylation and chromatin modification patterns resulting in changes in the expression of a large variety of genes. The importance of epigenetic changes in colon cancer has been previously demonstrated, and it is known that large number of genes such as *MLH1*, *MGMT* and *TIMP3* are commonly methylated in colon adenomas and adenocarcinomas (4, 18). One of the genes that has been shown to be methylated in approximately 15% of colon cancers is *TSP1*, the gene encoding thrombospondin 1 (169). TSP1 has several functions including the regulation of the expression of VEGF and the activation of secreted, latent TGF-β. Thus, the biological consequences of TSP1 methylation on TGF-β activation have been unclear and led us to conduct a series of experiements that have defined the consequences of the aberrant methylation of TSP1 on TGF-β activation. We sought to establish whether

the activity of the TGF-β pathway was affected by the methylation of *TSP1*. In order to determine the role of *TSP1* methylation in colon cancer, we performed experiments in an *in vitro* model using the colon cancer cell line SW48, which contains methylated *TSP1*. We demonstrated that in the absence of *TSP1* there is a reduction in the level of active TGF-β1, which resulted in a significant reduction in the activation of the TGF-β pathway as measured by the CAGA reporter assay.

The *in vitro* studies were complemented with studies of primary human colon adenomas and adenocarcinomas. *TSP1* expression was assessed in normal colon, colon adenomas, and colon adenocarcinomas, and was found to be absent in a substantial proportion of these neoplasms. We also demonstrated that methylated *TSP1* was present in both primary colon adenomas and in the adenocarcinomas and that the tumors that did not express *TSP1* had reduced phosphorylated Smad2, which correlates with a lack of TGF-β pathway activity in these tumors. Our results obtained in studies of both colorectal cancer cell lines and primary tumors have demonstrated that epigenetic modification of *TSP1* is responsible for the deregulation of the TGF-β pathway in the colon in approximately 15-20% of tumors.

Based on the results obtained in our *in vitro* studies with SW48 cells and on animal models, we conclude that deregulation of TGF-β induced by epigenetic modifications could play a role in the process of adenoma to adenocarcinoma transformation. This model illustrates a novel mechanism by which the TGF-β pathway can be inactivated, and in conjunction with alterations in other pathways could induce the progression of colon cancer. Since the TGF-β pathways is complex and context dependent, the precise mechanisms involved in the regulation of apoptosis and cell

proliferation, both TGF-β-regulated, are not yet clear. In order to confirm the importance of *TSP1* methylation in events such as invasion, metastasis and angiogenesis, additional studies will be needed and could include the assessment of tumor xenograft growth in xenografts derived from cell lines that carry methylated *TSP1* vs. unmethylated TSP1. The completion of these experiments would help elucidate the role of *TSP1* methylation in the deregulation of the TGF-β pathway, and consequently how it can affect the activity TGF-β as a tumor suppressor gene.

TGF-β receptor expression and TGF-β mediated effects

After providing evidence that deregulation of the processes involved in TGF-ß ligand activation through epigenetic mechanisms can affect the activation of the TGF-β pathway in colon cancer, we sought to determine whether alterations in the expression of levels of the TGF-ß receptor could also affect the activation of the post-receptor pathways in a way that might alter the biological effects of TGF-β. As with other signaling pathways, there are additional proteins, such as receptors, scaffold proteins, etc, that are important in the transduction of extracellular signals to the cell nucleus raising the question of whether simple regulation of TGF-β receptor levels would be sufficient to alter the receptor output. In the particular case of the TGF-β pathway, TGFBR2 is an important member of the pathway that is frequently mutated in colon cancer, preventing the activation of the pathway. However, it is also clear that in approximately 40% of colorectal cancers, there are no inactivating mutations in *TGFBR1, TGFBR2*, or any of the SMAD genes. Despite the lack of mutations in the genes encoding for the TGF-β receptor and SMAD pathways, the majority of these tumors are not responsive to the

growth inhibitory effects of TGF-β (75). The mechanism(s) responsible for this resistance are not clear in most cases. Our studies of methylated *TSP1* suggest that this is one mechanism responsible for attenuating the TGF-β signaling pathway that is independent of gene mutations. In addition, in recent years, several groups have suggested that differences in TGFBR2 expression correlate directly with the type of cellular response to TGF-β. It has been proposed that those cells with low levels of cell surface TGFBR2 have different biological responses to TGF-β compared to cells with high levels of TGFBR2 and it has been shown that cells that express low levels of TGFBR2 do not respond to the TGF-β-mediated tumor suppressor activity, whereas cells with high levels of TGFBR2 are more susceptible to the pro-apoptotic stimulus induced by TGF-β (52).

In order to determine if TGFBR2 expression levels lead to activation of distinctive TGF-β-mediated pathways, we have used a modified version of the Ecdysone inducible system as reported by Palli et al (145) to regulate the expression of TGFBR2 in the V-400 colorectal cancer cell line, which lacks a functional TGFBR2. Using this system, we were able to generate clones in which four well-defined expression levels of TGFBR2 could be induced. Of interest, we observed modest differences in the levels of phosphorylated Smad2 among cells treated with different concentrations of Rheochem, which precisely altered the expression level of TGFBR2. Of significance to our model, we did observe basal phosphorylated Smad2 (pSmad2), which we believe reflects a low level of TGFBR2 expression even in the untreated cells. However, this low expression did not induce the activation of the 3Tp-Lux reporter assay which suggests that such low levels of pSmad2 are not sufficient to promote the expression of genes regulated by TGF-

β. We believe that the low TGFBR2 expression could be due to an nonspecific activation of the Rheochem inducible system; however, as mentioned above, the basal levels of phosphorylated Smad2 were not sufficient to induce the activation of the 3Tp-Lux reporter assay, which clearly indicates that the theses low levels pSmad are not sufficient to induce the expression of genes, such as p21. Another potential explanation of the presence of pSmad2 in untreated cells is that the Smad pathway also could be activated by ERK, it has been shown that in cells that are treated with EGF or HGF, Smad2 is phosphorylated. It is possible that V400R2 cells culture in absence of Rheochem, were stimulated by other agents different that TGF-β1 which results in the phosphorylation of Smad2 (26, 128). In order to prove if this is the case an antibody against TGF-β1 can be used to block the activation of the TGFBR2, if an activation of Smad2 is detected in these cells, we could assume that the presence of pSmad2 in untreated cells is due to activation promoted by other growth factors than TGF-β1, like activin.

Based on the results mentioned above, we determined that the activation of the Smad-dependent pathway is not highly affected by the expression levels of the TGFBR2, and it is possible that this pathway is regulated by other proteins that interact with elements in this post-TGF-β receptor pathway, such as Smad7 or Smurfs. Another protein that also could be involved in the regulation of the Smad dependent pathway is SARA, which plays an important role in the phosphorylation of Smad 2 and 3 which affects the localization of Smad complex and the control of gene expression regulated by TGF-β. (42, 63, 66, 170). Lastly, although Smad2 and Smad 3 are typically considered to function as a complex, there is accumulating evidence that these proteins have the potential to induce specific effects. It is possible that different expression levels of

TGFBR2 could differentially activate Smad2 vs. Smad3, which could result in altered cellular responses to TGF-β (170).

Also, as with virtually all signaling pathways, it is known that the Smad-dependent pathway is not a linear pathway, but instead it interacts with other pathways such as MAPK/ERK and PIK3/AKT (52, 64), which could serve as another mechanism for modulating Smad signal pathway output. For example, it has been shown that ERK is able to promote the phosphorylation of serines present in the linker domain of Smad 2 and 3 and that when those serines are replaced by negatively charged residues the nuclear translocation of the R-Smads is inhibited (reviewed in (128)).

In order to address the hypothesis that the expression levels of TGFBR2 are responsible for changes in the activation of the TGF-β Smad-independent pathways, which could not only affect output from the Smad pathway but also have direct effects on gene trasnscription, we treated V-400 cells with various concentrations of Rheochem in order to induce different expression levels of TGFBR2 and then assessed the activation state of the MAPK-ERK and PI3K-AKT pathways. Interestingly, we observed that the MAPK/ERK and PIK3/AKT pathways, that are known to be activated by TGF-β, were highly affected by changes in the expression of the TGFBR2 (35, 44, 52). Moreover, we observed that these pathways were not necessarily affected in the same way in relation to the TGF-β receptor levels; AKT activation was detected in cells expressing low levels of TGFBR2, whereas higher levels of phosphoryalted ERK1/2 (pERK1/2) were present in V-400 cells expressing high levels of TGFBR2. In order to determine the consequences of the differential activation of these pathways on cellular responses, we examined the effect on *p21* expression. Using several methods to evaluate potential differences in *p21*

expression, we observed that when pERK levels were high, increased levels of *p21* were expressed in V-400R2 cells. Since p21 is an important regulator of the cell cycle and apoptosis, we assessed how high levels of p21, promoted by high levels of TGFBR2, affected cell death in V-400 cells. We observed that the presence of high levels of TGFBR2 in the cell membrane appeared to play a role in induction of the tumor suppressor activity mediated by TGF-β potentially through modulating the expression and activity of p21. Thus, we have provided evidence from a precisely controlled system that demonstrates a direct correlation between TGFBR2 expression and apoptosis.

In addition, we have also shown that although differences in TGFBR2 expression levels promote changes in the activation of the PIK3/AKT pathway. However, these differences do not correlate with changes in p21 expression suggesting that in colon cancer, TGF-β does not regulate p21 expression though an AKT dependent mechanism. Studies in which the V-400 cells were treated with various concentrations of Rheochem in the presence of either a PIK3/AKT or MAPK/ERK inhibitor demonstrated this lack of correlation. Furthermore, using ERK and AKT kinase inhibitors, we established that in the presence of LY294002, the *p21* expression was not affected, clearly demonstrating the independence of p21 expression from TGF-β mediated activation of the AKT pathway. In contrast, when the MAPK/ERK inhibitor was used, we found that TGF-β could no longer induce ERK phosphorylation regardless of the level of TGFBR2 expression. These results suggest that p21 expression levels are dependent on ERK activation and not AKT activation.

Finally, our results suggest that although the Smad-dependent pathways are the best understood TGF-β-activated pathways, the TGF-β-mediated cellular response is

likely the result of a close interaction between the Smad-dependent and the Smad-independent pathways.

Conclusions

In summary, we have shown that, as with genetic alterations, epigenetic alterations can attenuate or ablate the activation of the TGF-β signaling pathway. We have shown that the aberrant methylation of *TSP1* can affect the activation of the TGF-β pathway due to the reduction of activated TGF-β1. Lower levels of activated TGF-β1 are reflected in a diminished response to TGF-β in SW48 cells. We propose that such reduction in these levels could be an important event in the behavior of colon cancers that carry methylated *TSP1*.

In order to confirm the importance of these changes in colon cancer, additional *in vivo* experiments are necessary. The completion of *in vivo* experiments will be important to establish if cells that do not express TSP1 are different from cell that express this protein in terms of TGF-β regulated cell proliferation and invasion. Also, these experiments may improve our understanding of the role of TGF-β in metastasis since it has been shown that this cytokine regulates the expression and activity of MMPs and the expression of chemokines and chemokine receptors which could favor the metastasis process in addition to the resistance to apoptotic signals (22, 171-173). Other process such as angiogenesis that also are regulated by TSP1 could also be the subject of these proposed studies as well. Additionally, we have shown that alterations in TGFBR2 expression levels affect the response of cells to TGF-β. Our findings regarding the effect of TGFBR2 expression levels on the activation of Smad dependent and independent

pathways may lead to a better understanding of the paradoxical role of TGF-β in some cancers.

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