CONTRIBUTION OF TRANSFORMING GROWTH FACTOR-β SIGNALING

TO INTESTINAL CANCER DEVELOPMENT

By

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LIST OF ABBREVIATIONS

ACF: Aberrant Crypt Foci

ALK: Activin Receptor-Like Kinase

AOM: Azoxymethane

APC: Adenomatous Polyposis Coli

ARTS: Apoptosis-related protein in the TGF-β Signaling pathway

BAD: Bcl-2 Antagonist of cell Death

BAT: Big Adenine Tract

BCA: Bicinchoninic Acid

BCL-2: B-Cell Lymphoma-2

BMP: Bone Morphogenetic Protein

BrdUrd: Bromodeoxyuridine

CBP: CREB-Binding Protein

CDK: Cyclin-dependent Kinase

Chr3:   Chromosome 3

CK1α: Casein Kinase 1α

CTLA-4: Cytotoxic T Lymphocyte-Associated-4

DAPK: Death-associated Protein Kinase

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

E2flx: Exon 2 floxed

ECM: Extracellular Matrix

EGFR: Epidermal Growth Factor Receptor

eiF: Eukaryotic translation Initiation Factor

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

GEF: Guanine nucleotide Exchange Factor

GSK-3β: Glycogen Synthase Kinase-3β

H&E: Hematoxylin and Eosin

HGF: Hepatocyte Growth Factor

HRAS: Harvey RAS

IEKO: Intestinal Epithelium Knock-Out

IGF: Insulin-Like Growth Factor

IL: Interleukin

JNK: Jun-N terminal Kinase

KRAS: Kirsten RAS

LAP: Latency-Associated Peptide

LOH: Loss of Heterozygosity

MAPK: Mitogen-Activated Protein Kinase

MDM2: Mouse Double Minute 2 Homolog

MHC: Major Histocompatibility Complex

MMP: Matrix Metalloproteinase

MTOR: Mammalian Target of Rapamycin

NF-κB: Nuclear Factor-κB

NRAS: Neuroblastoma RAS

PALA: N-phosphonoacetyl-L-aspartate

PDGF: Platelet-derived Growth Factor

PDK: Phosphoinositide-dependent protein Kinase

PIK3CA: Phosphatidylinositol 3-Kinase, Catalytic, Alpha

PIP$_2$: Phosphatidylinositol (3,4)-bisphosphate

PIP$_3$: Phosphatidylinositol (3,4,5)-trisphosphate

PTEN: Phosphatase and Tensin Homolog

PTHrP: Parathyroid Hormone-related Protein

RAL: Ras-like Protein

RB: Retinoblastoma

RHOA: Ras Homolog Gene Family, Member A

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

SHIP: SH2-containing Inositol Phosphatase

SMURF: Smad Ubiquitin Regulatory Factor

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

TERT: Telomerase Reverse Transcriptase

TGF-β: Transforming Growth Factor-β

TGFBR: Transforming Growth Factor-β Receptor

TGIF: Transforming Growth Factor-induced Factor

TIEG: Transforming Growth Factor-inducible Early Gene

TP53: Tumor Protein 53

VEGF: Vascular Endothelial Growth Factor

Wnt: Wingless-type

ZO-1: Zona Occludens-1

CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

According to the American Cancer Society, colorectal cancer is the third most common type of cancer in both men and women in the United States. It is estimated that 106,680 cases of colon cancer and 41,930 cases of rectal cancer will occur in the United States during 2006. Moreover, it is expected that in the same year this type of cancer will cause 55,170 deaths, 10% of all cancer deaths (1).

1. Molecular basis of colorectal cancer

Colorectal cancer is caused by exposure to environmental carcinogens, by inherited factors and/or by gene-environment interactions. Like in the majority of cancers, most cases of colorectal cancer are sporadic, and a small proportion are caused by genetic mutations inherited in an autosomal dominant fashion (2).

Sporadic colorectal cancer is a pathological condition that results from the accumulation of multiple genetic alterations that lead to the aberrant regulation of several biochemical pathways in the intestinal epithelial cells. **Figure 1-1** represents the adenoma-carcinoma sequence of colorectal cancer, a model that describes the accrual of clonally selected genetic changes associated with the transformation of normal to dysplastic epithelium, and then to colorectal adenocarcinoma (3).

Signaling cascades that play central roles in the processes of initiation and progression of this disease have been identified through analysis of both hereditary and sporadic human

Normal Epithelium   Early Adenoma   Late Adenoma   Cancer   Metastatic Cancer

Wnt/β-catenin pathway inactivation

Ras/Raf pathway activation

TP53 & TGF-β pathway inactivation

PIK3CA/AKT activation
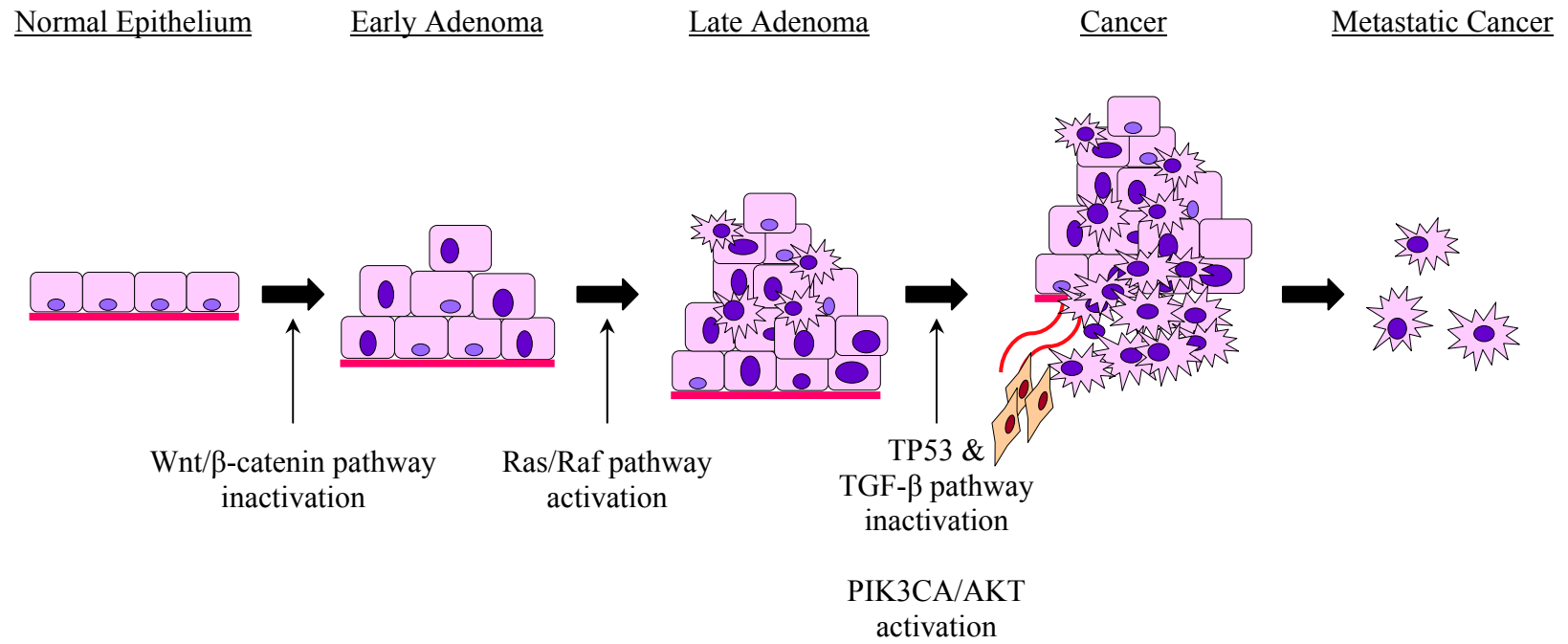
Figure 1-1. Colorectal adenoma-carcinoma sequence.

tumors. Furthermore, their functions have been validated with *in vivo* as well as *in vitro* model systems. Thus, it has been recognized that among the signaling pathways that drive the development and progression of colorectal cancer are the Wnt/β-catenin, MAPK/ERK, PIK3/AKT, and TGF-β pathways.

1.1. The Wnt/β-catenin signaling pathway

The canonical Wnt/β-catenin signaling pathway, a highly conserved pathway between insects and vertebrates, is implicated in the control of several cellular processes in developing embryos as well as in adult organisms. As depicted in **figure 1-2**, in non-stimulated cells, cytoplasmic β-catenin is destabilized by a multiprotein complex formed by Casein Kinase 1α (CK1α), Axin, Glycogen Synthase Kinase-3β (GSK-3β), and Adenomatous Polyposis Coli (APC). β-catenin bound to this complex is sequentially phosphorylated by CK1α and GSK-3β and, as a result, it is targeted for ubiquitin-mediated proteasomal degradation. Conversely, when secreted ligands of the Wnt family stimulate their cognate membrane receptors, which are encoded by the Frizzled genes, cytoplasmic Dishevelled induces Axin phosphorylation and degradation. As a consequence, β-catenin phosphorylation is prevented and this allows it to translocate into the cell nucleus. Nuclear β-catenin then acts as a co-activator of the T-Cell Factor (TCF)/Lymphoid Enhancer Factor (LEF) family of transcription factors, which induce transcription of a variety of target genes such as *CCND1* and *CMYC* that promote cell proliferation (reviewed in (4)).

It has been shown that the adenoma-carcinoma sequence that takes place in most cases of sporadic colorectal cancer is initiated by deregulation of the Wnt/β-catenin signaling
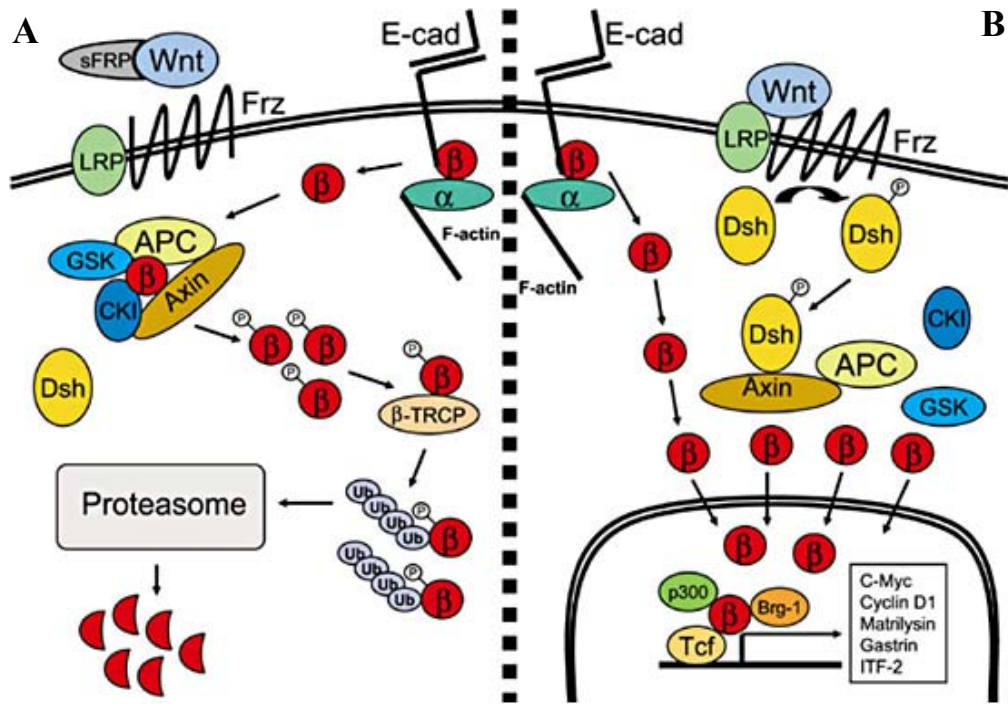
Figure 1-2. The Wnt/β-catenin pathway

A In unstimulated cells, β-catenin (β) is associated with E-cadherin (E-cad) and with α-catenin (α), thus linking the cell membrane to the actin cytoskeleton. Free β-catenin is associated with a complex consisting of CKI, APC, Axin and GSK-3β. Following the priming phosphorylation by CKI, β-catenin is phosphorylated by GSK-3β. Subsequently, it binds to β-TRCP, an E3 ligase, that transfers ubiquitin to β-catenin and marks it for proteasomal-mediated degradation.

B Binding of Wnt ligands to a Frizzled (Frz) receptor results in phosphorylation of Dishevelled (Dsh) which causes the disruption of the Axin-dependent complex. β-catenin can then be translocated into the nucleus where it functions as a co-activator of Tcf transcription factors. c-myc, Cyclin D1, and Matrilysin are some of β-catenin target genes. Soluble Frizzled-related proteins (sFRP) bind to Wnt ligands and inhibit Wnt pathway activation.

Adapted from Kolligs F *et al.*, 2002 (5)

pathway (6). Truncating and missense mutations targeting the *APC* gene are found in the early stages of about 85% of all sporadic intestinal tumors, and similar alterations are also the cause of Familial Adenomatous Polyposis or FAP, a hereditary condition characterized by the formation of hundreds to thousands of colorectal adenomas beginning in late childhood (2). APC, a protein of ~312kDa with several domains, is involved in multiple cellular functions. Two attributes of APC that make it important for colorectal tumorigenesis are its ability to interact with the microtubules during chromosome segregation in mitosis, and also, its role in the negative regulation of the Wnt/β-catenin cascade (4). As described above, nuclear β-catenin functions as co-activator of the TCF/LEF family of transcription factors, which induce transcription of genes involved in cell proliferation. Remarkably, it has been shown that sporadic intestinal tumors that lack mutations in can *APC* carry activating mutations in *CTNNB1* (7).

Several genetically engineered mouse models of intestinal tumorigenesis have corroborated the relevance of the Wnt/β-catenin pathway in this process. 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 (8). At an age of four months, the animals develop an average of thirty adenomas in the small intestine and five in the colon. The tumor burden in these animals is so high that their lifespan is very short and, probably because of that, most tumors only reach the stage of adenoma. Two additional models based on *Apc* mutations are the $Apc^{\Delta716/+}$ (9) and the $Apc^{1638N/+}$ (10, 11). The $Apc^{\Delta716/+}$ mouse develops ten times more tumors than the $Apc^{Min/+}$ animals of a similar age; nevertheless, the distribution of the adenomas is very similar and most

neoplasms arise in the small intestine. Conversely, $Apc^{1638N/wt}$ mice have a life span of sixteen months and develop an average of four tumors at approximately one year of age. Carcinomas are observed more frequently in these animals than in the other two models, and liver metastases have also been reported (10). In addition, these animals also show some of the extracolonic manifestations observed in FAP, i.e. desmoid tumors of the abdominal cavity and cutaneous cysts (12). Animal models of intestinal cancer based on the disruption of β-catenin function have also been generated. One model based on the expression of a constitutively active mutant of β-catenin showed that deregulation of the Wnt/β-catenin pathway through this approach results in the disruption of the tissue architecture in the intestine (13). Also, mutant mice expressing stable β-catenin in the intestine, $Catnb^{+/lox(ex3)};Krt1-19^{+/cre}$ and $Catnb^{+/lox(ex3)};Tg.Fabpl^{cre}$, develop multiple intestinal neoplasms that are reminiscent of the phenotype of the $Apc^{\Delta716/+}$ mouse (14). Taken together, the findings derived from the analysis of human tumors and from animal models emphasize the crucial role that the Wnt/β-catenin pathway plays in the maintenance of homeostasis in the intestinal epithelium and the significance of its disruption in intestinal carcinogenesis.

1.2. The Ras signaling pathway

The Ras signaling cascade, a central regulator of eukaryotic cell proliferation, is normally activated in response to extracellular stimuli like growth factors and hormones. There are three mammalian isoforms of *RAS*, *Kirsten-RAS* (*KRAS*), *Harvey-RAS* (*HRAS*), and *Neuroblastoma-RAS* (*NRAS*). The biological activity of RAS, a 21KDa protein with intrinsic GTP-ase activity, is tightly modulated by its interaction with GTP-ase Activating

Proteins (GAPs) and Guanine nucleotide Exchange Factors (GEFs), which strictly control the cycle between the GTP- and GDP-bound states of RAS. Mutations that affect RAS amino acids 12, 13 and 61 impair its ability to interact with GAPs, and lock RAS in a GTP-bound or active state that result in abnormal stimulation of RAS effectors. Among the downstream targets of RAS activity are the RAF/MEK/ERK1/2, the RalGDS/RAL, and the PIK3/AKT signaling cascades, which are implicated in the regulation of cell proliferation, actin cytoskeleton remodeling, and survival, respectively (reviewed in (15)).

The Ras signaling pathway is aberrantly activated in approximately 30% of all types of cancers due to genetic changes affecting the activity of several components of the cascade, particularly that of *RAS* itself (15). Accordingly, the *RAS* genes are the most frequently mutated oncogenes in all human malignancies. *KRAS* in particular is altered in approximately 90% of the cases of pancreatic cancer, in 35% of non-small-cell lung cancer, and in 50% of the cases of colorectal cancer. Additionally, it has been reported that about 15% of colorectal cancer cases contain mutations in BRAF (15, 16).

Sophisticated murine models in which constitutively active mutants of *Kras* are targeted to specific adult tissues have demonstrated that deregulation of this pathway drives cellular transformation and tumor formation. In the context of intestinal cancer, studies by Tuveson and co-workers showed that *in vivo* expression of the oncogenic mutant *Kras*[G12D] in colonic epithelial cells causes hyperplasia and dysplasia (17). Moreover, Janssen and collaborators reported that expression of human *Kras*[G12V] in the epithelium of both small and large intestines results in the formation of Aberrant Crypt Foci (ACF) in the colon, and in the development of invasive adenocarcinomas in the small intestine.

The results of this study also suggest that in this particular model oncogenic activation of KRAS may cooperate with TP53 abrogation, rather than with Wnt/β-catenin activation, in order to promote tumor progression (18). Interestingly, the same group recently demonstrated that compound transgenic $Apc^{1638N/wt}$; $Kras^{V12G}$ mice have a ten-fold increase in tumor multiplicity and accelerated tumor progression compared to control animals (19). These observations support the idea that deregulation of Kras can also cooperate with aberrantly activated Wnt/β-catenin to drive tumor progression.

1.3. The PIK3/AKT signaling pathway

The Phosphatidylinositol-3-Kinase (PIK3)/AKT pathway is an important regulator of cell growth, proliferation, survival and glucose metabolism. Activation of receptor tyrosine kinases, like EGFR or the insulin receptor, induces the activation of PIK3. Activated PIK3 phosphorylates PIP_2 into PIP_3, which promotes the recruitment of AKT to the inner leaflet of the cell membrane, and induces AKT phosphorylation by Phosphoinositide-dependent protein Kinases (PDKs). AKT is a serine/threonine kinase that promotes cell survival by inactivating the pro-apoptotic proteins BAD, Caspase-9, and the Forkhead Box O (FOXO) transcription factors. In addition, AKT-mediated phosphorylation causes the activation of survival proteins Nuclear Factor-Kappa B (NF-κB) and Mouse Double Minute 2 Homolog (MDM2). AKT also induces cell proliferation and growth due to its regulatory function over GSK-3β and the Mammalian Target of Rapamycin (MTOR), respectively. PIK3 activity is counterbalanced by the PIP_3 phosphatases PTEN (Phosphatase-and-Tensin-Homolog) and SHIP (SH2-containing inositol phosphatase) (reviewed in (20)).

Recent comprehensive screenings have demonstrated that the catalytic subunit of PIK3, also known as PIK3CA, is mutated in more than 25% of cancers of colon, stomach, liver, and breast as well as in glioblastomas (21). Thus, the elevated frequency of mutation indicates that *PIK3CA* is one of the most highly mutated oncogenes identified to date. Not surprisingly, functional analyses of the PIK3CA mutants found in human tumor tissues showed that the altered proteins have increased enzymatic activity, which facilitates tumor cell proliferation in the absence of growth factors as well as invasion (22). Indeed, it has been observed that mutations in *PIK3CA* occur at the stage in which colorectal tumors become invasive (23).

Up-regulation of the PIK3/AKT pathway in human cancers is also achieved through genetic alterations that impair the function of other components of the cascade such as PTEN (24). Accordingly, inherited *PTEN* mutations cause Cowden Disease, a hereditary syndrome characterized by the development of hamartomatous polyps in the gastrointestinal tract, mucocutaneous lesions, and increased risk of developing neoplasms (24). Furthermore, *Pten* null mice die during embryonic development, and heterozygous animals have an increased incidence of tumors in a variety of organs, which are associated with loss of heterozygosity in the *Pten* locus (25).

1.4. The TGF-β signaling pathway

The TGF-β superfamily comprises a large number of cytokines with fundamental roles in the regulation of cellular processes such as proliferation, differentiation, motility and programmed cell death. Accordingly, the activities of these cytokines are essential for

adequate tissue homeostasis as well as organ development, and their disruption is implicated in numerous pathological processes (26).

Three TGF-β isoforms have been found in mammals, TGF-β1, -β2 and -β3. These cytokines signal through transmembrane receptors that have serine-threonine kinase activity. The receptor complex consists of type I and type II receptors, and both are required for the activation of the heteromeric receptor complex upon ligand binding. There are three type I receptors, ALK1, ALK2 and ALK5/TGFBR1, and one type II TGF-β receptor, TGFBR2. Intracellular signaling is initiated when a TGF-β dimer binds to a homodimer of TGFBR2, which consequently recruits a homodimer of TGFBR1, and form a heterotetrameric complex. As a result, the activated TGFBR2 phosphorylates the type I receptor in the glycine-serine rich sequence located on the amino terminal side of the kinase domain. This phosphorylation activates the kinase activity of the type I receptor, which then mediates the phosphorylation of downstream target proteins (reviewed in (27, 28)) (**figure 1-3**).

A variety of different proteins binds to TGF-β and regulates its ability to interact with and activate the receptor complex. The pro-region of TGF-β, the so-called Latency-Associated Peptide or LAP, binds in a non-covalent fashion to the active ligand and together they form a latent complex. This complex is only activated after interaction with αvβ6 integrin or after cleavage by proteases like plasmin and thrombospondin. Also, proteins like decorin and α-2-macroglobulin bind to and sequester free TGF-β, and prevent receptor activation. In addition, cell membrane proteins or co-receptors like endoglin and betaglycan, also known as TGFBR3, facilitate ligand binding to the TGFBR1 and TGFBR2, respectively (reviewed in (29)).
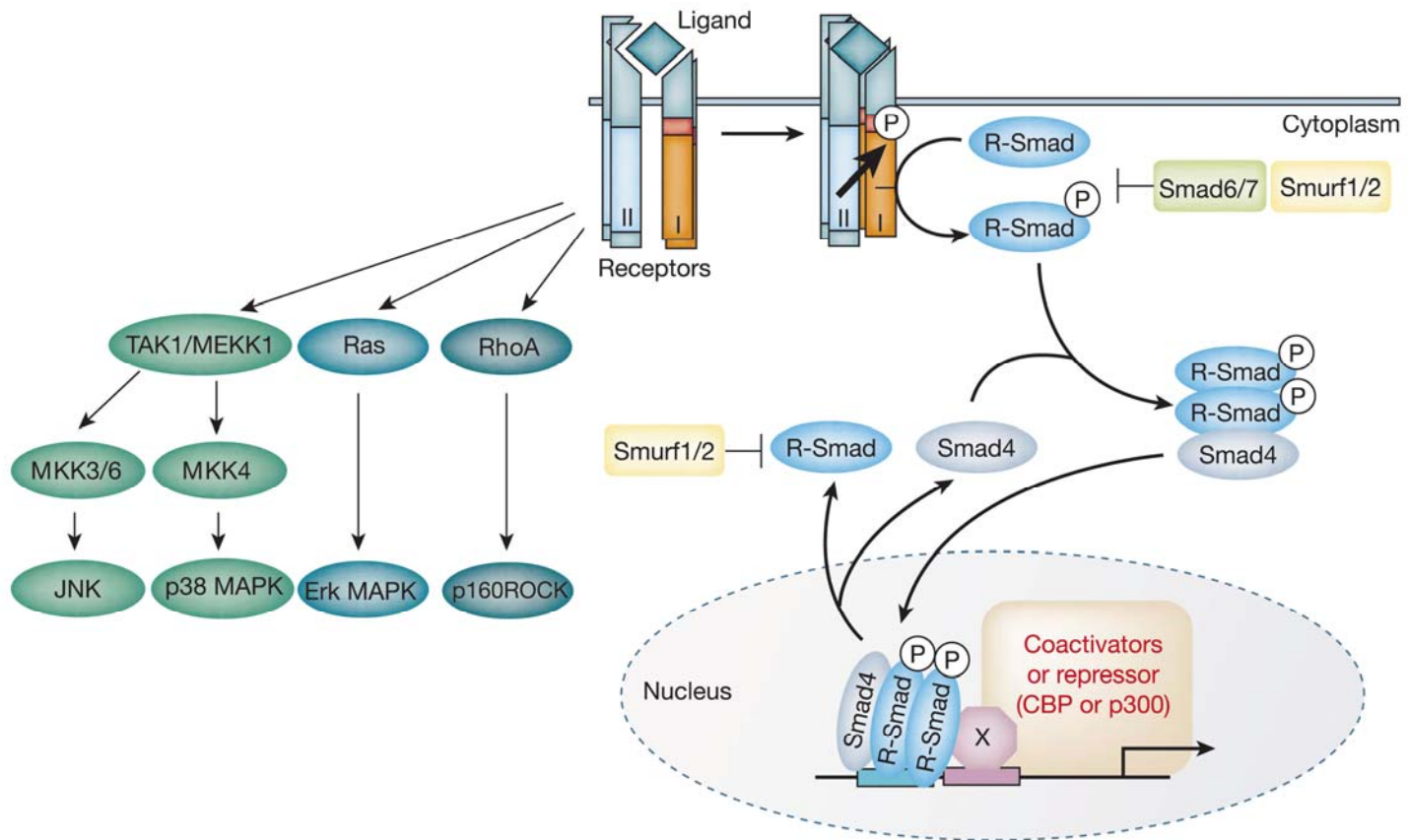
Figure 1-3. The TGF-β signaling pathway.
See text for details
Adapted from Derynck and Zhang, 2003 (30)

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Two members of the evolutionary conserved Smad family, Smad2 and Smad3, are activated via carboxy-terminal phosphorylation of the SSXS motif by the type I TGF-β receptor. The interaction between the receptor complex and the receptor-activated Smads or R-Smads is facilitated by a membrane protein named SARA, Smad Anchor for Receptor Activation. Thus, SARA tethers Smad2 to the inner leaflet of the cell membrane and facilitates receptor-mediated phosphorylation. In the active state, Smad2 and Smad3 form heterotrimeric complexes with the common mediator Smad4 or Co-Smad. These complexes translocate into the nucleus where they function as transcriptional activators or repressors of target genes. Activated Smad3 and Smad4, but not Smad2, have the ability to bind CAGA sequences in the DNA, which are known as Smad-Binding Elements or SBE; however, the affinity of such interaction is rather low. Therefore, the association of the Smad complex with other transcription factors is required for effective DNA binding and control of gene expression. Such interactions in conjunction with the cell type and cell state specify the transcriptional activation or repression of target genes (27, 28). The transcriptional activity of Smads is negatively modulated by proteins like TGIF, c-SKI and SNO-N, which bind to the Smad complex and block its interaction with the transcriptional co-activators p300 and CBP (31-33). TGF-β signals are also negatively regulated by inhibitory Smads or I-Smads. Hence, Smad7 competes with R-Smads for binding to the receptor complex, and induces the internalization of the receptor in caveolin-coated vesicles, which is followed by protein degradation through the proteasomal pathway. Interestingly, TGF-β signaling induces expression of Smad7, which provides a TGF-β-induced negative feedback loop (34). Also, an additional mechanism implicated in the control of TGF-β signals is the

ubiquitin-mediated proteasomal degradation of effector Smads and receptor complex induced by Smad ubiquitin regulatory factor or Smurfs.

Besides activating the Smad2/3 proteins, the receptor complex can modulate other non-Smad signaling cascades like the PIK3/AKT, RhoA, MAPK/ERK, p38MAPK, and JNK pathways; however, the molecular mechanism by which these pathways are activated by TGF-β is not completely understood. It has been established that in some systems the activation of these kinases occurs very rapidly, which indicates that such events may be direct and not secondary to transcriptional events. Conversely, in other cell types and under given conditions such activation takes place very slowly, suggesting that these may be secondary effects (27).

Epidemiological as well as mouse model data have shown that TGF-β1 has a potent tumor suppressor role in colorectal cancer, which is selected against in approximately 50% of the cases through inactivating mutations of *TGFBR2* (25%), *SMAD4* (20%), and *SMAD2* (<5%) (35). Moreover, it has been shown that *TGFBR2* mutations correlate with the progression of microsatellite unstable adenomas to cancer (36). Additionally, it has been demonstrated that restoration of a functional TGFBR2 into several colon cancer cell lines reverses some characteristics of their transformed phenotype. However, it has also been found that expression of a dominant negative TGFBR2 in a mouse colon cancer cell line prevents EMT, reduces its motility and its ability to form metastasis (37). Furthermore, some studies have indicated that patients with colon cancer with high levels of microsatellite instability and *TGFBR2* BATRII mutations have an increased 5-year survival as compared to those that do not have such mutations (38, 39).

In contrast to the animal models based on Wnt pathway deregulation, *Tgfb1* null mice develop both cecal and colonic carcinomas that, remarkably, do not show evidence of aberrant activation of the Wnt signaling pathway (40). It has also been established that conditional inactivation of *Tgfbr2* in the colonic epithelium promotes the development and progression of AOM-induced tumors (41). On the other hand, it has been shown that *Smad3* null and *Smad4* hemizygous animals develop colonic and intestinal neoplasias, respectively (42, 43). In these two models there is no deregulation of the Wnt signaling pathway either. Interestingly, recent studies have shown that chronic inflammation of the colon caused either by infection with *Helicobacter pylori* (44) or *Helicobacter hepaticus* (45) is necessary for *Smad3* null and *Tgfb1* null mice, respectively, to develop intestinal neoplasms. Additionally, the generation of compound heterozygous Cis-*Apc*$^{\Delta716}$(+/-); *Dpc4*(+/-) animals, which develop large and invasive tumors not observed in the simple *Apc*$^{\Delta716}$(+/-) heterozygous animals, has suggested that deregulation of the Wnt and TGF-β/BMP signaling pathways cooperate in the formation of intestinal neoplasms (46). Furthermore, the observation that *Apc*$^{Min/+}$; *Smad3*$^{-/-}$ mice develop intestinal tumors with an earlier onset and higher tumor multiplicity as compared to *Apc*$^{Min/+}$, *Smad3*$^{+/+}$ or *Smad3*$^{+/-}$, reinforces the idea of the cooperation between these signaling pathways during the progression of colon cancer (47).

2. TGF-β signaling and the hallmarks of cancer

The process of tumor development and progression has been found to be an evolutionary process in which normal cells gradually acquire genetic and epigenetic changes that give them growth advantage over intact cells. Furthermore, if these few cells are allowed to

propagate uncontrolled, their genetic alterations are perpetuated in their progeny, which will be more susceptible to acquire more genetic changes. Tumor cells in different organs and from diverse origins share some fundamental acquired capabilities that allow them to continuously proliferate avoiding the normal constraints their microenvironment and the organism impose. Those attributes of tumor cells have been classified in terms of the biological processes they impact (48), and quite remarkably, TGF-β has been found to play important roles in most of them. Relevant data that support the role of TGF-β in each one of the so-called hallmarks of cancer is presented next.

2.1. Insensitivity to anti-growth signals

TGF-β is a potent growth inhibitory cytokine that exerts this effect over normal epithelial, endothelial, myeloid and lymphoid cells. The TGF-β growth inhibitory effect involves two distinct types of immediate gene responses. On the one hand, TGF-β promotes the expression of inhibitors of cyclin-dependent kinases 4 and 6 (cdk4 and cdk6), which are implicated in the progression through the G1 phase of the cell cycle. Thus, TGF-β induces the expression of *CDKN2B* (p15^{INK4B}) and *CDKN1A* (p21^{CIP1}) that result the accumulation of the cdk substrates RB, p107 and p130, which in turn prevent the E2F transcription factors from inducing cell cycle progression. On the other hand, TGF-β causes transcriptional repression of *CMYC*, gene that promotes cell cycle progression because it induces the expression of *CCND2* (Cyclin D2), *CDK4* and the translation initiation factors *EIF4E* and *EIF2S1*, among others (reviewed in (49)).

Resistance to the TGF-β growth inhibitory effect is strongly correlated with the malignant progression of different types of neoplasms. Insensitivity to the TGF-β anti-

proliferative effect is commonly due to disruption of the function of the components of the TGF-β signaling cascade in the tumor cells (50). *TGFBR2*, for instance, is frequently inactivated in colon and gastric cancers through mutations that affect a sequence of 10 Adenines, the Big-Polyadenine-Tract or BAT RII, which is highly susceptible to mutations caused by defects in the DNA mismatch repair system. Such mutations originate truncated receptors that lack the kinase domain that cannot propagate TGF-β signals. However, alteration of the TGF-β signaling pathway by this mechanism is not usually found in other type of malignancies that also show remarkable resistance to the TGF-β growth inhibitory effect. Instead, deletion or decreased expression of the TGF-β receptors, inactivation of *SMAD4*, *SMAD2*, or attenuation of TGF-β signals by the activation of oncogenes like *HRAS*, *SKI* and *SNO* are observed in tumors of the breast, pancreas, lung, and in melanomas (50, 51).

2.2. Evasion of the immune system

TGF-β is the most potent immunosuppressive factor described to date. It plays a fundamental role in maintaining adequate immune responses because it modulates the proliferation and function of several cell types of the immune system (reviewed in (52)). Accordingly, *Tgfb1* knock-out mice die few weeks after birth due to severe defects of the immune system; these animals develop multifocal infiltration of inflammatory cells in several organs, enlarged lymph nodes and undersized thymus (53).

As expected, TGF-β plays an important role in the regulation of tumor-induced immune responses, and deregulation of the TGF-β signaling cascade in tumor or immune cells has major implications in tumor progression. It has been shown that most cancer cells

produce higher amounts of TGF-β in comparison to their less malignant counterparts, and

that the excess of this cytokine inhibits the immune response that tumor-specific antigens

should normally trigger (52). Indeed, cancer patients commonly present high levels of

TGF-β in the plasma and are immunosuppressed (54). In agreement with this

observation, Leach *et al.* reported that blockade of the TGF-β signaling cascade in T-cells

with antibodies for CTLA-4 increases the immune response against the tumor cells (55).

TGF-β secreted by tumor cells impairs the immune response by preventing the

proliferation and function of immune cells, and also by downregulating the expression of

Major Histocompatibility Complex (MHC) molecules by the tumor cells themselves,

which further weakens the immune response elicited by transformed cells (56).

Moreover, it has been established that the immunogenicity of cancer cells can be

importantly reduced by increasing their production of TGF-β1 (57). Interestingly, recent

evidence has emphasized the importance of the status of TGF-β signaling in the immune

cell themselves in the context of development and progression of intestinal cancer in two

mouse models. Becker *et al.* showed that defects in the immune system of mice, caused

by inactivation of TGF-β signaling through the expression of a dominant negative

TGFBR2 in T-cells, increases tumor growth and multiplicity in the AOM colon cancer

model through an Interleukin-6 or IL-6-dependent mechanism (58). Furthermore, Kim

and collaborators established that partial or complete disruption of Smad4 expression in

T-cells promotes the development of gastrointestinal hyperplasia and cancer,

respectively. The tumors arisen in these mice are characterized by a dramatic expansion

of the stromal compartment possibly due to the increase in the production of IL-4, -5, -6

and -13 by T-cells (59). These results clearly indicate that Smad-mediated signaling in

immune cells is implicated in the maintenance of intestinal tissue homeostasis, and that it can contribute importantly to the development and progression of tumors of the intestine.

2.3. Sustained angiogenesis

An essential role for TGF-β signaling in the repair and remodeling of blood vessels was recognized with the finding that inherited mutations in *ENG* (the gene for endoglin) and *ALK-1*, two endothelial-specific TGF-β receptors, result in defects in the vascular system that cause hereditary hemorrhagic telangiectasia (60, 61). Furthermore, mouse models in which *Tgfb1* (62), *Tgfbr2* (63), *Alk-1* (64) and *Alk-5* (65) are disrupted die during embryonic development with severe defects in endothelial differentiation, which are reflected in abnormal vasculogenesis. In addition, the absence of *Eng* also results in embryonic lethality in a mouse model because of major defects in angiogenesis (66).

With respect to the role of TGF-β in the control of tumor angiogenesis, there is evidence that TGF-β can either promote or inhibit the formation of new blood vessels. It has been reported that TGF-β1 significantly induces the expression of Vascular Endothelial Growth Factor (VEGF), an important regulator of new vessel development, in different types of tumor cells maintained *in vitro* (67); and that overexpression of TGF-β1 in a mouse model of head and neck cancer results in increased angiogenesis (68). Accordingly, blockade of TGF-β activity in a xenograft model reduces tumor size because of poor vascularization (69). Interestingly, *in vitro* studies that replicate the conditions of hypoxia and high concentration of TGF-β observed in most tumors demonstrate that these two factors synergize to induce the expression of VEGF in cancer cells (70). Therefore, these reports support a pro-angiogenic role for TGF-β signaling.

In marked contrast, Kim *et al.* showed that inhibition of TGF-β signaling in the MH129F mouse hepatoma cell line, which is sensitive to the TGF-β cytostatic effect, augments the production of VEGF, which results in a significant increase in vascularization and rapid progression of the tumors originated from that cell line (71). Also, Go *et al.* reported that blockade of the TGF-β cascade in the epidermis of mice exposed to skin carcinogens results in accelerated tumor progression, due in part to increased angiogenesis (72). Thus, these data support an anti-angiogenic role for TGF-β signaling in these model systems. The contrasting results of studies of this type may be due to intrinsic differences between the model systems used, such as alternative tumor types; but also to divergences in biochemical contexts, i.e. signaling pathway deregulation, in which the effects of TGF-β signaling are evaluated.

2.4. Genomic instability

Instability of the genome facilitates the establishment of cancers by serving as a mechanism through which they can become hypermutable and able to tolerate the alteration of a variety of biochemical pathways. TGF-β has been implicated in the regulation of the integrity of the genome by its role in different pathways that respond to genotoxic stress. For instance, Ewan *et al.* provided evidence that supports the notion that TGF-β plays a protective role of the genome in the context of cellular stress (73). They found that the status of TGF-β signaling is positively correlated to the occurrence of p53-induced apoptosis and cell cycle arrest in mammary gland epithelium and embryonic tissues of whole-body irradiated mice. In addition, it has been shown that *Tgfb1* null keratinocytes have higher frequencies of chromosomal abnormalities when transduced

with oncogenic *Kras* (74), and increased gene amplification when treated with the drug

N-phosphonoacetyl-L-aspartate or PALA (75) in comparison to *Tgfb1* wild type cells.

Conversely, the results from Kanamoto and collaborators implicate TGF-β signaling in

the promotion of genomic instability through inhibition of Rad51-mediated DNA repair

in Mv1Lu cells (76). Also, Dubrovska *et al*. showed that TGF-β signals mediated by

Smad3 suppress BRCA1-dependent DNA repair in response to mitomycin C, a DNA

damaging agent (77). Therefore these two studies provide evidence of a mechanism by

which this cytokine may promote tumor progression *in vivo*. In aggregate, these reports

suggest that the effects of TGF-β on genomic instability might be modulated by other

factors that ultimately lead to paradoxical effects.

2.5. Tumor invasion and metastasis

TGF-β is implicated in the regulation of several proteins involved in cytoskeletal

organization, cell-cell and cell-extracellular matrix (ECM) adhesion, as well as in the

control of the synthesis of ECM components and the enzymes that degrade them.

Moreover, TGF-β is also involved in the regulation of several chemokines and cytokines

that play important roles during the process of metastasis (78). Consequently, TGF-β has

direct influence on the ability of tumor cells to invade surrounding tissues and colonizing

secondary sites.

It has been established that TGF-β signaling induces a phenotypical change known as

Epithelial-to-Mesenchymal Transition or EMT in epithelial cells of various origins

(reviewed in (78)). The transition is associated with downregulation of epithelial markers

like E-Cadherin and ZO-1, upregulation of mesenchymal markers like α-smooth muscle

actin and vimentin, and the reorganization of the actin cytoskeleton into stress fibers (79).

As TGF-β induces these changes in several types of tumor cells, it increases the motility

and invasiveness of the cells both *in vitro* and *in vivo* (80). Also, through upregulation of

integrin α3β1, TGF-β promotes the invasiveness of cells derived from hepatocellular

carcinoma *in vitro*. Interestingly, analysis of human tumor tissue reinforces such positive

correlation between the expression TGF-β1 and α3β1 by tumor cells with the occurrence

of metastatic lesions (81).

The potential that TGF-β has to enhance invasiveness of solid tumors is based on its

effects on tumor cells and also on components of the tumor stroma. Stromal fibroblasts

become "activated" by cytokines like TGF-β1, Platelet-derived Growth Factor (PDGF)

and IL-4 produced by tumor cells. The fibroblasts then begin to express α-smooth

muscle actin, secrete several ECM proteins as well cytokines and chemokines, and

proliferate at higher rates (82). These cells, known as myofibroblasts, are thought to play

a very important role in the process of tissue invasion by the tumor cells mainly because

of the cytokines and ECM proteins they secrete into the microenvironment (82). It has

been established that TGF-β-activated fibroblasts produce elevated amounts of growth

factors like Hepatocyte Growth Factor or HGF, which in turn stimulates epithelial tumor

cells to invade through basement membrane proteins *in vitro*. Furthermore, these cells

are exclusively found in the invasive edge of squamous cell carcinomas, which strongly

argues in favor of their role in promoting tumor invasion (83).

With respect to the role of TGF-β in the process of metastasis, several reports have

shown that TGF-β promotes the formation of metastases for certain tumor types, and

consequently, systemic blockade of the pathway prevents the formation of tumors in

secondary sites (80). Some of the molecular mechanisms that implicate TGF-β in the colonization of new microenvironments have been elucidated (reviewed in (78)). In the case of breast cancer, there is evidence that indicates that TGF-β produced by the bone matrix upregulates the production of Parathyroid-Hormone related Protein or PTHrP by breast tumor cells, and this promotes bone destruction and facilitates the development of bone metastases (84). Furthermore, recent reports from Deckers *et al.* and Kang *et al.* indicate that Smad4-dependent TGF-β signals promote the production of IL-11 by breast cancer cells, which also facilitates the establishment of bone metastases in a mouse xenograft model (85, 86). Nevertheless, Forrester *et al.* showed that the early loss of TGF-β signaling in tumor cells derived from mouse mammary gland leads to a substantial reduction in the number of lung metastasis observed in the transgenic mice (87).

2.6. Limitless replicative potential

The role of TGF-β signaling in the acquisition of indefinite replicative potential has not been studied in great detail. Nevertheless, it has been demonstrated that TGF-β is implicated in repression of transcription of TERT, the catalytic unit of telomerase, the enzyme involved in the lengthening of the ends of the chromosomes. Furthermore, TGF-β-mediated control over *TERT* expression seems to be mediated by Smad3, given its capacity to bind the *TERT* promoter, and to require c-myc inhibition, since c-myc causes TERT upregulation (88, 89). The aberrant expression of telomerase facilitates the immortalization of transformed cells and contributes to the process of carcinogenesis

(78); therefore, the negative regulation of this enzyme by TGF-β supports another tumor suppressor function of this cytokine.

2.7. Evasion of apoptosis

Tissue homeostasis is the result of a precise balance between cell proliferation and cell death. Maintenance of the equilibrium between these two processes is especially important in high-turnover tissues, such as the epithelium that lines the gastrointestinal tract. Disruption of this balance is observed in pathological conditions like autoimmune disorders and cancer.

TGF-β signaling induces programmed cell death to epithelial cells from liver, prostate, pancreas, and colon as well as to lymphocytes (78); and some of the molecular mediators of the TGF-β apoptotic program have been identified. Among them, TIEG or TGF-β-inducible early gene, a Zinc-finger transcription factor that is dramatically upregulated in pancreatic epithelial cells by TGF-β treatment, has been recognized as an important component of the cascade that mediates the apoptotic response in these cells (90). Also, DAPK or death-associated protein kinase, another immediate response gene whose transcription is increased by TGF-β-induced Smad activity in Hep3B cells, is essential for the apoptotic response of this hepatoma cell line (91). Another effector of the apoptotic signals of TGF-β has been described based on observations made in hematopoietic cells. Hence, SHIP, an enzyme implicated in phospholipid metabolism, has been found to be upregulated in hematopoietic cells in response to TGF-β or activin treatments. Furthermore, since SHIP functions as a negative regulator of AKT activation, its expression is associated with a marked decrease in cell survival (92). Finally, ARTS

or <u>A</u>poptosis-<u>r</u>elated <u>p</u>rotein in the <u>T</u>GF-β <u>s</u>ignaling pathway, a mitochondrial protein that translocates into the nucleus upon apoptotic stimuli, has been found to be necessary for transduction of the TGF-β apoptotic signals in rat prostate epithelial cells (93).

Paradoxical to the data just presented, several reports have provided evidence of a protective effect of TGF-β against apoptosis triggered by different stimuli. Huang *et al.*, for instance, established that TGF-β1 blocked cell death caused by serum withdrawal in A549 human lung carcinoma cells (94). Such protective effect was shown to be caused by TGF-β1-induced phosphorylation of c-Jun, which in turn results in decreased JNK activation. Additionally, Shin *et al.* demonstrated that in HaCaT cells (keratinocytes) TGF-β1 induces AKT-dependent phosphorylation of the transcription factor FOXO3A (FKHRL-1). In such way, TGF-β1 prevents FOXO3A nuclear translocation, reduces its transcriptional activity and partially suppresses programmed cell death caused by serum starvation in NMuMG cells (mammary epithelial cells) (95). Furthermore, Saile *et al.* reported that TGF-β1 causes a decrease in the expression of CD95L in activated rat hepatic stellate cells. As a result of that, apoptosis caused by growth factor deprivation was significantly reduced in these cells(96). Finally, Sachsenmeier *et al.* have showed that after loss of anchorage, human normal keratinocytes increase the steady states levels of TGF-β1 mRNA and the production of the active cytokine. Hence, TGF-β1-treated cells show reduced DNA fragmentation and consequently have an increased potential to form colonies in soft agar (97).

Importantly, the work from Sachsenmeier and coworkers draws attention to the role of TGF-β signaling in cell death induced by the lack of anchorage. Signals transmitted to the cells by the extracellular matrix or adjacent cells induce the expression of genes

involved in cytoskeletal structure, differentiation and survival, and inadequate cell-matrix and/or cell-cell contacts trigger apoptosis in normal cells (98). This form of cell death is known as *anoikis*, from the Greek for 'homelessness' (reviewed in (99, 100)). To become resistant to anoikis is a selective advantage for tumor cells because it confers them the ability to survive without attachment and, consequently, it facilitates the colonization of secondary sites. Indeed, tumor xenograft models have shown that some anoikis-resistant cancer cell lines have increased survival in blood circulation and increased capacity to form metastases (101, 102).

In summary, the pluripotency and complexity of TGF-β signaling are reflected in the capacity of this cytokine to exert a wide variety of tumor suppressive and tumor promoting functions. Its capacity to arrest the proliferation of tumor cells, to induce apoptosis and senescence, and to prevent genomic instability seem to be the central activities of its tumor suppressive program. In contrast, its ability to inhibit the proliferation and function of immune cells, to promote angiogenesis as well as invasion and metastasis, are crucial to its tumor promoting nature. The data collected from animal models and human cancers suggest that the balance between the contrasting phases of this signaling cascade depend on the stage of the disease. Thus, the anti-tumorigenic functions of TGF-β predominate during early phases of tumor development, whereas its pro-tumorigenic characteristics prevail in late stages, most likely because most tumor cells gain resistance to TGF-β-induced cell cycle arrest and apoptosis but remain responsive at levels that eventually promote tumor progression. Moreover, the ultimate effect that TGF-β signals, or the lack of them, have over the process of tumor development depends on the status of other signaling pathways at any given tumor stage.

3. Research Objectives

In order to better understand the effects of TGF-β signaling in the context of colon cancer development, our laboratory has conducted studies with colon cancer cell line systems and mouse models of intestinal cancer. Studies with novel mouse models have shown that TGFBR2 can suppress the progression of intestinal tumors in the AOM colon cancer model, consistent with *TGFBR2* acting as a tumor suppressor gene (41). Nevertheless, tumors induced in animals through the use of chemical carcinogens are likely to carry multiple undefined genetic alterations that mask the relationship between tumor stage with the underlying mutations, as well as our ability to study the cooperation between TGF-β signaling inactivation and specific signaling pathways commonly deregulated in colon cancer. Therefore, in order to better elucidate the role of TGF-β signaling deregulation in the development of intestinal neoplasia, we generated a mouse model that replicates two common genetic events observed in human colorectal cancer, *APC* mutation and *TGFBR2* inactivation. Previously, Takaku and collaborators crossed genetically engineered mice that harbor mutations that affect the Wnt and TGF-β pathways, and generated the Cis-*Apc*$^{\Delta716}$(+/-), *Dpc4* (+/-) compound heterozygous mice. Their study revealed that simultaneous deregulation of these two signaling cascades cooperate to drive tumor progression (46). However, disruption of the TGF-β pathway in that animal model was constitutive rather than restricted to the tumor epithelial cells, as it occurs in humans, and recent findings have clearly shown that the status of TGF-β signaling in immune and stromal cells is critical to the progression of cancer in mouse models (58, 103). Furthermore, disruption of Smad4 function in that model system not only affected TGF-β but also BMP signals, which have been shown to play an important

role in intestinal homeostasis (104). Thus, in order to circumvent these issues and generate a model that better reflects the conditions observed in human colorectal cancer, we mated $Tgfbr2^{E2flx/E2flx}$;*Villin*-Cre mice with $Apc^{1638N/wt}$ mice, in order to characterize the effects of disrupting TGF-β signaling exclusively in intestinal epithelial cells in the context of Wnt pathway deregulation (chapter 2).

In addition to the studies in mouse models of intestinal cancer, we also conducted a series of *in vitro* studies with the aim of evaluating cell-autonomous effects of TGF-β signaling in human colorectal cancer lines. Our preliminary data indicated that, although exogenous TGF-β1 inhibits proliferation of the human colon cancer cell line HCT116 plus chromosome 3, TGF-β also protects this cell line from undergoing anoikis. This paradoxical observation clearly illustrates the dual character of TGF-β signaling in colon cancer, because it arrests the proliferation of malignant cells and at the same time facilitates their survival in hostile environments. Hence, we investigated how general this protective effect is and also the biochemical mechanisms underlying it (chapter 3).

CHAPTER II

INACTIVATION OF TRANSFORMING GROWTH FACTOR β RECEPTOR TYPE II INDUCES THE MALIGNANT TRANSFORMATION OF INTESTINAL NEOPLASMS INITIATED BY *APC* MUTATION

Abstract

The Transforming Growth Factor (TGF-β) signaling pathway is a tumor-suppressor pathway that is commonly inactivated in colon cancer. TGF-β is a secreted ligand that mediates its effects through a transmembrane heteromeric receptor complex, which consists of type I (TGFBR1) and type II subunits (TGFBR2). Approximately 25% of colon cancers carry *TGFBR2* mutations, demonstrating that it is a common target for mutational inactivation in this cancer. To assess the functional role of TGFBR2 inactivation in the multistep progression sequence of colon cancer, we generated a mouse model that recapitulates two common genetic events observed in human colon cancer by mating $Apc^{1638N/wt}$ mice with mice that are null for *Tgfbr2* in the intestinal epithelium, *Villin*-Cre;$Tgfbr2^{E2flx/E2flx}$ mice. In this model, we observed a dramatic increase in the number of intestinal adenocarcinomas in the $Apc^{1638N/wt}$;*Villin*-Cre;$Tgfbr2^{E2flx/E2flx}$ mice (referred to as $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$) compared to those mice with intact *Tgfbr2*, ($Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$). Additionally, *in vitro* analyses of epithelial tumor cells derived from the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice showed enhanced expression and activity of MMP-2 and MMP-9, as well as increased TGF-β1 secretion in the conditioned medium. Similarly, primary tumor tissues from the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice also showed elevated amounts of TGF-β1 as well as higher MMP-2 activity in comparison to $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$-derived tumors. Thus, loss of TGFBR2 in intestinal epithelial

cells promotes the invasion and malignant transformation of tumors initiated by *Apc* mutation, providing *in vivo* evidence that Wnt signaling deregulation and TGF-β signaling inactivation cooperate to drive the initiation and progression, respectively, of intestinal cancers *in vivo*.

Introduction

The Transforming Growth Factor-β (TGF-β) superfamily of proteins is a family of dimeric secreted ligands that includes the TGF-βs, Bone Morphogenetic Proteins (BMPs), activins, and inhibin, among others. TGF-β regulates tissue homeostasis and development through affecting fundamental biological processes such as cell proliferation, differentiation, motility and programmed cell death. TGF-β exerts its effects on the cells by binding to the transmembrane TGF-β type II receptor (TGFBR2), which causes the recruitment of the TGF-β type I receptor (TGFBR1) with subsequent activation of the receptor complex. SMAD2 and SMAD3 are direct substrates of the activated TGF-β receptor complex, and once phosphorylated they form a heterotrimeric complex with the common mediator Smad, SMAD4. This trimeric complex translocates into the nucleus where it functions as a member of different transcription factor complexes that regulate the expression of a variety of genes (27, 105). In addition, TGF-β receptor activation can induce a variety of non-SMAD signaling pathways including Phosphoinositol 3-kinase, Mitogen-Activated Protein Kinase, and RhoA (27).

The role of TGF-β signaling in the process of tumor formation is complex and its effects appear to be context-dependent as they vary between tumors arising from different organs and between tumors in different stages of progression (105). Components of the TGF-β

signaling pathway, including *TGFBR2, SMAD2*, and *SMAD4*, have been shown to be altered frequently in colon cancer, consistent with the idea that TGF-β signaling prevents tumor development, presumably through the induction of growth arrest, differentiation, or apoptosis (106-109). However, evidence from *in vitro* tissue culture systems shows that, paradoxically, TGF-β signaling may be able to induce tumor progression due to its ability to promote Epithelial-to-Mesenchymal Transition, its capacity to inhibit cell death caused by growth factor deprivation, and its immunosuppressant function (37, 40, 95).

To investigate the complex effects of TGF-β signaling deregulation in primary colon cancer, several genetically engineered mouse models have been generated that harbor alterations in components of the TGF-β signaling pathway; specifically *Tgfbr2, Tgfb1, Smad2, Smad3*, and *Smad4* have been altered *in vivo* (reviewed in (110)). *Tgfb1$^{-/-}$;Prkdc$^{scid/scid}$* mice develop colonic carcinomas (40). In addition, constitutive abrogation of *Smad3* and *Smad4* leads to the development colonic and intestinal neoplasms, respectively (42, 111). Of interest, the *Tgfb1$^{-/-}$;Prkdc$^{scid/scid}$* and *Smad3$^{-/-}$* mice require intestinal colonization with *Helicobacter* to develop colon neoplasms (44, 45). Furthermore, conditional inactivation of *Tgfbr2* in the colonic epithelium promotes the development and progression of Azoxymethane-induced tumors (41). Interestingly, compound heterozygous Cis-*Apc^{Δ716}* (+/-)*;Dpc4* (+/-) mice develop large and invasive tumors not seen in the *Apc^{Δ716}* (+/-) heterozygous mice; however, *Smad2$^{+/-}$* mice do not develop intestinal neoplasms spontaneously and it does not appear that *Smad2* haploinsufficiency has a dramatic effect on the formation of intestinal tumors in the *Apc^{Δ716}* or *Apc^{580D}* mice (112, 113). These studies show that deregulation of the Wnt and SMAD pathways can cooperate in the development of intestinal neoplasia. However,

because SMADs regulate BMP, activin, and inhibin signaling and because TGF-β activates non-SMAD pathways, these studies do not permit a precise assessment of the effect of deregulation of TGF-β-mediated signaling in intestinal cancer formation *in vivo* (46, 112, 113). In addition, except for the *Fabp$^{4xat-132}$*-Cre*;Tgfbr2$^{flx/flx}$* mouse, these models abrogate TGF-β signaling in all cell types raising the question of whether the neoplasms observed in these mouse models are a consequence of direct loss of TGF-β signaling in the intestinal epithelial cells or a consequence of loss of TGF-β signaling in the immune or stromal cells (103, 114).

With the aim of understanding the implications of TGF-β receptor inactivation in the intestinal epithelium in the process of tumor development, we generated a mouse model that replicates two common genetic events observed in human colorectal cancer, *APC* mutation and TGFBR2 inactivation. Thus, we mated *Tgfbr2$^{E2flx/E2flx}$;Villin*-Cre mice (referred to as *Tgfbr2^{IEKO}*) with *Apc$^{1638N/wt}$* mice in order to elucidate the effect of disrupting TGF-β signaling exclusively in intestinal epithelial cells in the context of Wnt pathway deregulation. We observed that the *Apc^{1638N};Tgfbr2^{IEKO}* mice develop intestinal adenocarcinomas at a substantially higher incidence than mice that carry the *Apc^{1638N}* mutation alone. Furthermore, potential cell autonomous and nonautonomous mechanisms through which the inactivation of *Tgfbr2* in intestinal epithelial cells may promote rapid tumor progression were identified.

Experimental procedures

Generation and characterization of *Villin*-Cre, *Tgfbr2*$^{E2flx/E2flx}$, *Apc*$^{1638N/wt}$ mice

The generation of transgenic *Tgfbr2*$^{E2flx/E2flx}$, *Villin*-Cre and *Apc*^{1638N} mice has been previously described (11, 12, 115). The animals were mated in order to generate the following compound genotypes: *Villin*-Cre;*Tgfbr2*$^{E2flx/E2flx}$ (called *Tgfbr2*^{IEKO}), *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$, or *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} and were fed *ad libitum* with a standard rodent diet and water. The mice were genotyped using PCR-based assays with visualization of the PCR products by UV transillumination after staining with ethidium bromide. The following primer sequences were used for genotyping: *Tgfbr2* primer 1: 5'-GCAGGCATCAGGACCTCAGTTTGATCC-3' and primer 2: 5'-AGAGTGAAGCCGTGGTAGGTGAGCTTG-3', which generate a 556bp amplicon from the wild type *Tgfbr2* allele and a 711bp amplicon from the *Tgfbr2*^{E2flx} allele; *Villin*-Cre primer 1: 5'-GTGTGGGACAGAGAACAAACCG-3' and primer 2: 5'-TGCGAACCTCATCACTCGTTGC-3', which generate a 1000bp product from Cre positive DNA (115); and *Apc*^{1638N} primer 1: 5'-TGCCAGCACAGAATAGGCTG-3', primer 2: 5'-TGGAAGGATTGGAGCTACGG-3', primer 3: 5'-GTTGTCATCCAGGTCTGGTG-3' that generate a 300bp amplicon from the wild type *Apc* allele and 400bp amplicon from the *Apc*^{1638N} mutant allele (10). Animals with the genotypes *Tgfbr2*$^{E2flx/E2flx}$ or *Tgfbr2*^{IEKO} were harvested at 3.5, 6, 10, 17 and 24 months of age in order to evaluate the morphology of the intestinal mucosa and the development of spontaneous tumors. Similarly, mice with the genotypes *Apc*$^{1638N/wt}$;*Tgfbr2*$^{E2flx/E2flx}$ and *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} were harvested at 9 and 12 months of age.

Tissue harvesting

Two hours before sacrifice, the mice were injected intraperitoneally with Bromodeoxyuridine (BrdUrd, Sigma-Aldrich, St. Louis, MO) at a dose of 100mg/kg of weight. Immediately after sacrifice, the small intestine and colon were dissected, flushed with PBS, opened flat, and assessed for macroscopically visible lesions. Tissue samples were fixed in 10% neutral buffered formalin overnight at 4°C, and subjected to standard histological processing and H&E staining. The histological evaluation of all lesions was performed by a gastrointestinal pathologist, who was unaware of the genotype of the mice.

Immunostaining

For BrdUrd immunostaining, tissue sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked with H_2O_2 treatment. Antigen unmasking was achieved by incubation with Target Retrieval Solution (DAKO, Carpinteria, CA) for 20 minutes. Non-specific binding was blocked by treating the sections with 1.5% rabbit normal serum for 10 minutes, and the immunostaining was performed with the rat monoclonal antibody anti-BrdUrd (clone BU1/75, Accurate Scientific, Westbury, NY) diluted 1:2000. For β-catenin immunostaining, the sections were treated similarly; but the antigen was retrieved using boiling sodium citrate buffer pH 6.0 for 15 min. A mouse monoclonal antibody anti-β-catenin (clone 14, BD Transduction Laboratories, San Diego, CA) was used in a 1:200 dilution with a DakoCytomation ARK system (DAKO).

Assessment of Cre-mediated recombination

Genomic DNA was extracted from tissues and cell cultures using a standard phenol-chloroform extraction protocol as previously described (116). Cre-mediated recombination of $Tgfbr2^{E2flx}$ allele was assessed using a PCR-based assay that only generates an amplicon if the $Tgfbr2^{E2flx}$ allele has undergone Cre-mediated recombination. The primer sequences are as follows: primer 1: 5'-AGGGATGAATGGGCTTGCTT-3', and primer 2: 5'-CTCACCTCAGAGCCTGATTA-3'.

Tumor-derived cells isolation and long term culture

Tumor cells were isolated following a modification of a protocol previously described (117). In summary, pieces of the tumors of interest were rinsed several times in PBS with 10ug/mL gentamicin (10μg/mL), penicillin (20 units/mL), and streptomycin (20μg/mL) [referred to as PBS-A]. Subsequently, the samples were incubated for 20 minutes in a 0.04% Sodium Hypochlorite Solution (Sigma-Aldrich) at room temperature and handled as sterile specimens afterward. The tissues were rinsed with PBS-A and minced in small pieces, which were then centrifuged at 100 x g for 5 minutes in PBS-A. The pellet was resuspended in Liver Digest Medium (Gibco, Grand Island, NY) with the antibiotics above mentioned, and incubated for 90 minutes at 37°C with occasional shaking. The samples were centrifuged at 100 x g for 5 minutes; the pellet resuspended in Liver Digest Medium with antibiotics, and then incubated overnight at 4°C. Subsequently, the samples were centrifuged and the pellet washed twice with tissue culture medium [RPMI-1640, 10% fetal bovine serum (FBS), 10ng/mL Epidermal

Growth Factor (EGF, Sigma-Aldrich), 1mL/L of ITS-X (Gibco-Invitrogen), 0.5μg/mL Fungizone (Gibco-Invitrogen), and the same antibiotics used previously (see above)]. The cell suspension was plated in six-well plates coated with Matrigel (10μg/mL) (BD Biosciences, Bedford, MA). When the cells reached confluence, they were disaggregated with 0.25% Trypsin/EDTA (Gibco/Invitrogen) and sub-cultured using standard technique. To remove tumor-associated fibroblasts, the cultures were subjected to several rounds of differential trypsinization and the medium was changed to keratinocyte SFM supplemented with bovine pituitary extract and recombinant EGF (Gibco/Invitrogen) until no further fibroblasts were observed. After cultures of epithelial cells demonstrated stable growth patterns, they were grown in CellBind (Corning, Corning, NY) culture vessels.

Western blotting

For evaluation of TGF-β-induced Smad-2 phosphorylation, tumor-derived cell cultures at 70% confluence were treated with 10ng/mL TGF-β1 (R&D Systems, Minneapolis, MN) for 24 hours in keratinocyte SFM. Protein lysates were prepared in radioimmunoprecipitation assay buffer supplemented with a complete protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich), and then used for SDS-PAGE. The mouse monoclonal antibodies anti-phospho-Smad-2 (Ser$^{465/467}$, Cell Signaling, cat. # 3101, Beverly, MA) and Smad-2 (Cell Signaling, cat. # 3102) were used for Western blot analyses.

Luciferase reporter assays

To evaluate TGF-β-mediated transcription, tumor-derived cells were transiently transfected with the p3TP-lux or CAGA reporters (kindly provided by Joan Massague- Memorial Sloan-Kettering Cancer Center, New York, NY-, and Bert Vogelstein- Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, respectively) concomitantly with the pRL-TK reporter construct (Promega, Madison, WI). Subsequently, the cells were treated with 10ng/mL TGF-β1, and luciferase activity was evaluated 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega) with a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). Similarly, for the analysis of β-catenin-mediated transcription, the cells were transfected with the superTOPFLASH (118) and pRL-TK reporters, and examined 48 hours later with the Dual Luciferase System.

Gelatin zymography

The activity of matrix metalloproteases MMP-2 and MMP-9 produced by tumor-derived cells *in vitro* was assessed through gelatin zymography as previously reported (119). Briefly, 70% confluent cultures were switched from FBS-containing medium to Keratinocyte SFM, and the supernatant collected 24, 48, and 72 hours after serum deprivation started. The supernatants were concentrated with Microcon YM-10 Centrifugal Devices (Millipore, Bedford, MA) and equivalent amounts of all samples were loaded into the gel. To evaluate metalloprotease activity in the tumors, proteins were extracted from fragments of frozen tissue (30-80 mg) using lysis buffer [0.5M Tris pH7.5, 0.2M NaCl, 10mM $CaCl_2$, 1% Triton X-100] supplemented with a complete

protease inhibitor cocktail (Roche). The protein lysates were homogenized by sonication, clarified by centrifugation, and quantified with the BCA Protein Assay Kit (Pierce, Rockford, IL). Fifty micrograms of protein per sample were loaded in 10% Zymogram Gels (BioRad, Hercules, CA) and processed as described above (119).

In situ gelatin zymography

In situ gelatin zymography was performed as previously published (120). Briefly, OCT-embedded tissues were sectioned (10 μm), and incubated at 37°C for 20 hours with reaction buffer [50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 2mM NaN_3] containing 40 μg/mL FITC-labeled DQ-gelatin (Molecular Probes, Eugene, OR) and 0.02 μg/mL 4',6-diamidino-2-phenylindole. At the end of the incubation period, the samples were evaluated by fluorescence microscopy without prior washes or fixation processes.

Quantitative RT-PCR

TaqMan Gene Expression Assays (Assays-on-Demand from Applied Biosystems) for Serpine1 or PAI-1 (Mm00435860), Tgfb1 (Mm00441724), Mmp2 (Mm00439508) and 18s (4333760F) were used for quantitative RT-PCR. mRNA was extracted from 70% confluent tumor-derived cultures that were serum deprived for 24h, or from snap-frozen tumor tissue samples using Trizol (Invitrogen) and following the manufacturer's instructions.   cDNA was obtained through standard techniques. The assays were performed using an ABI 7900 Real-time PCR system and 18s values were used for normalization.

The amount of TGF-β1 present in the conditioned media of tumor-derived epithelial cells was evaluated using the TGF-β1 Quantikine kit (R&D Systems). The supernatants were obtained similarly to those used for gelatin zymography, after 48, 72 and 96h after serum removal from the medium. Alternatively, fragments of frozen tissue (80-100 mg) were lysed using 20mmol/L Tris (pH 7.5), 140mmol/L NaCl, 1mmol/L EDTA (pH 8.0), 1mmol/L EGTA pH (8.0), 1% Triton X-100, supplemented with a complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 1 and 2 (Sigma). These protein lysates were homogenized by sonication, clarified by centrifugation, and quantified with the BCA Protein Assay Kit (Pierce). One hundred twenty-five micrograms of protein per tumor were used in the assay. Latent TGF-β1 present in the samples was acid-activated before developing the ELISA assay according to the indications of the manufacturer.

Results

Villin-Cre;*Tgfbr2*$^{E2flx/E2flx}$ (*Tgfbr2*^{IEKO}) mice show *Tgfbr2* recombination in the intestinal mucosa

It has been previously demonstrated that Cre expression in *Villin*-Cre transgenic mice occurs exclusively in the epithelial cells of the small intestine and colon, and that Cre-mediated DNA recombination is continuous in the crypt-villus and longitudinal axes of these organs (115). To confirm that the *Tgfbr2*^{E2flx} allele was undergoing Cre-mediated recombination in the intestinal epithelium, genomic DNA from intestinal mucosa obtained through a blunt dissection was assessed using a PCR assay that specifically

amplifies the recombined $Tgfbr2^{E2flx}$ allele. The recombined $Tgfbr2^{E2flx}$ allele could only be detected in the intestinal epithelium from the mice carrying the *Villin*-Cre transgene, providing evidence that the $Tgfbr2^{E2flx}$ allele is being deleted in the $Tgfbr2^{IEKO}$ mice (**figure 2-1**).

<u>Inactivation of *Tgfbr2* in the intestinal epithelium neither increases proliferation of the epithelial cells nor induces the development of spontaneous tumors</u>

TGF-β has been shown to be a potent inhibitor of intestinal epithelial cell proliferation *in vitro* (121, 122). In order to evaluate the effect of TGF-β signaling abrogation on the basal proliferation rate of the intestinal epithelium, BrdUrd incorporation in the intestines of $Tgfbr2^{IEKO}$ and $Tgfbr2^{E2flx/E2flx}$ mice was analyzed at 3.5, 6 and, 10 months of age (n=5-14 mice per time point). The organs of the animals from both genotypic groups were grossly and histologically normal at each time point. In addition, evaluation of the proliferation indices of the intestinal epithelial cells using BrdUrd incorporation by the intestinal mucosa revealed similar distribution of the indices in the $Tgfbr2^{IEKO}$ and $Tgfbr2^{E2flx/E2flx}$ mice at all the time points considered (P > 0.05 as determined by the Nonparametric Wilcoxon's Rank Sum Test).

With regard to spontaneous neoplasm formation, elderly $Tgfbr2^{IEKO}$ and $Tgfbr2^{E2flx/E2flx}$ mice (17 and 24 months of age) developed small duodenal adenomas. Specifically, at 17m of age 71% (n=10 of 14) $Tgfbr2^{IEKO}$ and 78% (n=7 of 9) $Tgfbr2^{E2flx/E2flx}$ mice developed duodenal adenomas. Similarly, at 24 months of age 57% (n=8 of 14) $Tgfbr2^{IEKO}$ and 62% (n=5 of 8) $Tgfbr2^{E2flx/E2flx}$ animals had this type of lesion.
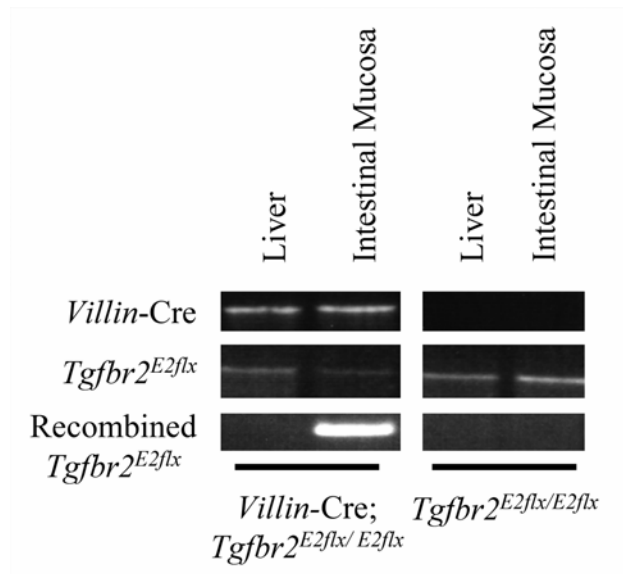
Figure 2-1. Status of *Tgfbr2* in the intestines of *Villin*-Cre;*Tgfbr2$^{E2flx/E2flx}$* and *Tgfbr2$^{E2flx/E2flx}$* mice. Results of PCR-based assays showing evidence of recombination of the *Tgfbr2^{E2flx}* allele in the intestinal epithelial layer of the *Villin*-Cre;*Tgfbr2$^{E2flx/E2flx}$* but not in the *Tgfbr2$^{E2flx/E2flx}$* mice. The *Tgfbr2^{E2flx}* product generated from the intestinal mucosa of the *Villin*-Cre;*Tgfbr2$^{E2flx/E2flx}$* mouse is presumably derived from contaminating submucosa. The liver was used as a negative control and has no detectable recombined *Tgfbr2^{E2Flx}* allele.

Furthermore, we observed invasive intestinal adenocarcinomas in 24-month-old $Tgfbr2^{IEKO}$ and $Tgfbr2^{E2flx/E2flx}$ mice (n=2 of 14 and n=1 of 8, respectively). Of note, the three colonic adenocarcinomas observed in the $Tgfbr2^{IEKO}$ animals did not show evidence of β-catenin nuclear localization, while the only invasive neoplasm observed in the $Tgfbr2^{E2flx/E2flx}$ mouse displayed nuclear localization of β-catenin. Thus, our findings indicate that deletion of $Tgfbr2$ in the epithelial cells does not affect basal cell proliferation in the intestinal epithelium and that suppression of TGF-β signaling in intestinal epithelial cells alone is not sufficient to promote the formation of intestinal tumors. This observation is consistent with those of previous studies of $Fabp^{4xat-132}$-Cre;$Tgfbr2^{E2flx/E2flx}$ mice, which lack $Tgfbr2$ in the colonic epithelium (41).

Tgfbr2 inactivation promotes the malignant transformation of intestinal adenomas initiated by Apc mutation.

In light of the infrequent formation of spontaneous tumors in $Tgfbr2^{IEKO}$ mice, we reasoned that $Tgfbr2$ inactivation may have its primary effect on tumorigenesis in the background of other gene mutations and deregulated signaling pathways. Consequently, we evaluated the effect of $Tgfbr2$ inactivation in the context of mutations in Apc, the most common initiating genetic event observed in colon neoplasms (35). Hence, we mated $Tgfbr2^{IEKO}$ mice with $Apc^{1638N/wt}$ mice, which develop 3-4 intestinal adenomas during their first year of life (11, 12), and evaluated the mice for tumor development at 9 and 12 months of age (**figure 2-2**). As shown in **table 2-1**, the total number of neoplastic lesions in the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ and $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ mice at 9 and 12 months of age was similar; however, the number of advanced tumors (i.e. adenomas with

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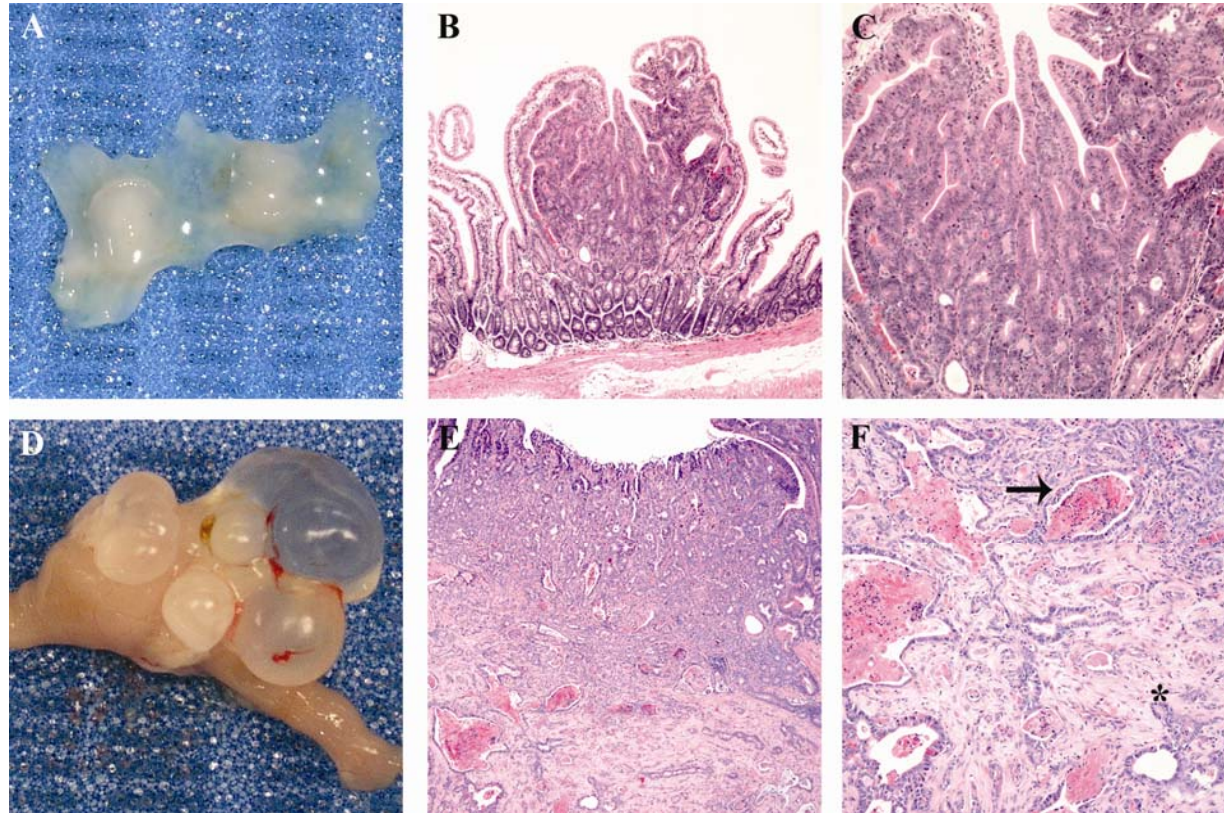
Figure 2-2. Gross and histological features of the intestinal neoplasms initiated by the *Apc^{1638N}* mutation. Gross appearance of representative intestinal adenomas (**A**) and adenocarcinoma (**D**) observed in *Apc$^{1638N/wt}$;Tgfbr2$^{E2flx/E2flx}$* and *Apc$^{1638N/wt}$;Tgfbr2^{IEKO}* mice, respectively. Photomicrographs of H&E-stained sections of a representative adenoma, 40X (**B**) and 100X (**C**), and an adenocarcinoma, 40X (**E**) and 100X (**F**). Cystic structures filled with mucin (arrow) were frequently observed in the invasive neoplasms, as well as a dramatic desmoplastic response (asterisk) in the invasive component of such tumors (**F**).

Table 2-1. **Comparative tumor incidence in** $Apc^{1638N/wt}$**;**$Tgfbr2^{E2flx/E2flx}$ **and** $Apc^{1638N/wt}$**;**$Tgfbr2^{IEKO}$ **mice.**

Genotype	Number of mice with tumors	Average number of tumors per mouse	Total number of adenomas	Total number of adenomas w/HGD*§	Total number of adenocarcinomas§	Total number of tumors
9 months						
$Apc^{1638N/wt}$ $Tgfbr2^{E2flx/E2flx}$ (n=10)	8	2.62	21	0	0	21
$Apc^{1638N/wt}$ $Tgfbr2^{IEKO}$ (n=11)	10	2.70	9	4	14	27
12 months						
$Apc^{1638N/wt}$ $Tgfbr2^{E2flx/E2flx}$ (n=12)	12	4.92	44	11	4	59
$Apc^{1638N/wt}$ $Tgfbr2^{IEKO}$ (n=12)	12	5.17	19	17	26	62

* HGD=high grade dysplasia
§ OR of developing high-grade lesions for the $Apc^{1638N/wt}$, $Tgfbr2^{IEKO}$ mice is 10.2 compared to $Apc^{1638N/wt}$, $Tgfbr2^{E2flx/E2flx}$ mice (95% confidence interval 4.9-21.3)

high-grade dysplasia and adenocarcinomas) was significantly greater in the $Apc^{1638N/wt};Tgfbr2^{IEKO}$ than in the $Apc^{1638N/wt};Tgfbr2^{E2flx/E2flx}$ mice. Accordingly, the odds ratio of probabilities for developing high-grade lesions for the $Apc^{1638N/wt};Tgfbr2^{IEKO}$ mice is 10.2 compared to $Apc^{1638N/wt};Tgfbr2^{E2flx/E2flx}$ mice (95% confidence interval 4.9-21.3, logistic regression with age adjusted). The increase in the number of advanced neoplastic lesions in the $Apc^{1638N/wt};Tgfbr2^{IEKO}$ mice suggests that suppression of TGF-β signaling, in the context of *Apc* mutation and aberrant activation of the Wnt pathway, promotes the transformation of benign neoplasms into malignant neoplasms. Our observations are similar to those made in the Cis-$Apc^{\Delta716}$ (+/-), *Dpc4* (+/-) mouse model previously described (46). In these mice, deregulation of both the Wnt and Smad pathways leads to a rapid progression of intestinal adenomas to invasive adenocarcinomas. Further, the invasive lesions of both the $Apc^{1638N/wt};Tgfbr2^{IEKO}$ and Cis-$Apc^{\Delta716}$ (+/-), *Dpc4* (+/-) animals show significant desmoplasia, and in neither of the two models metastatatic lesions are observed. However, it is important to note that deregulation of the Smad pathway in the Cis-$Apc^{\Delta716}$ (+/-), *Dpc4* (+/-) mice is constitutive, and it affects both TGF-β and BMP signaling in several cellular compartments that clearly have an impact on tumor formation. Recent studies have shown that impairment of TGF-β-Smad signaling in lymphocytes can affect intestinal epithelial cell neoplasm formation (58, 114). Therefore, the results obtained with the $Apc^{1638N/wt};Tgfbr2^{IEKO}$ mouse model more specifically identify the role of TGF-β signaling deregulation in the epithelial cells during the process of intestinal tumor formation, which we propose is the most relevant model for assessing the role of TGF-β signaling deregulation in human intestinal cancer.

To investigate if loss of the growth inhibitory effect of TGF-β on the epithelial cells plays a role in the progression of the intestinal neoplasms of the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ animals, we determined the proliferation index of representative adenomas arising in the $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ and $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice. Interestingly, we observed no difference between the proliferation indices of tumors from $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ and $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ mice (data not shown, P = 0.24 as determined by the nonparametric Wilcoxon's rank sum test).

Effects of *Tgfbr2* inactivation on production of factors implicated in tumor invasion in neoplasms arising in the $Apc^{1638N/wt}$ mice

To assess the mechanism(s) affecting tumor progression that result from the cooperation of *Apc* inactivation with *Tgfbr2* deletion in the tumors arising in the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice, we derived cultures of epithelial cells from neoplasms of each genotype. We first characterized the status of *Apc* and *Tgfbr2* in these lines by assessing the cell lines for allelic imbalance of the *Apc* locus and for deletion of the $Tgfbr2^{E2flx}$ allele. A tumor cell line derived from $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice, named AVcTT, displayed *Apc* loss of heterozygosity (LOH) and loss of *Tgfbr2*, whereas a cell line derived from a tumor from the $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ mice, named ATT, displayed only *Apc* LOH (**figure 2-3A**). Both cell lines demonstrated increased Wnt pathway activity by the superTOPFLASH reporter assay; however, only the tumor cell line from the $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice demonstrated loss of TGF-β-induced phosphorylation of Smad2 and TGF-β-mediated transcription (**figure 2-3B-D**). As anticipated, the p3TP-lux luciferase reporter assay showed no basal or TGF-β inducible
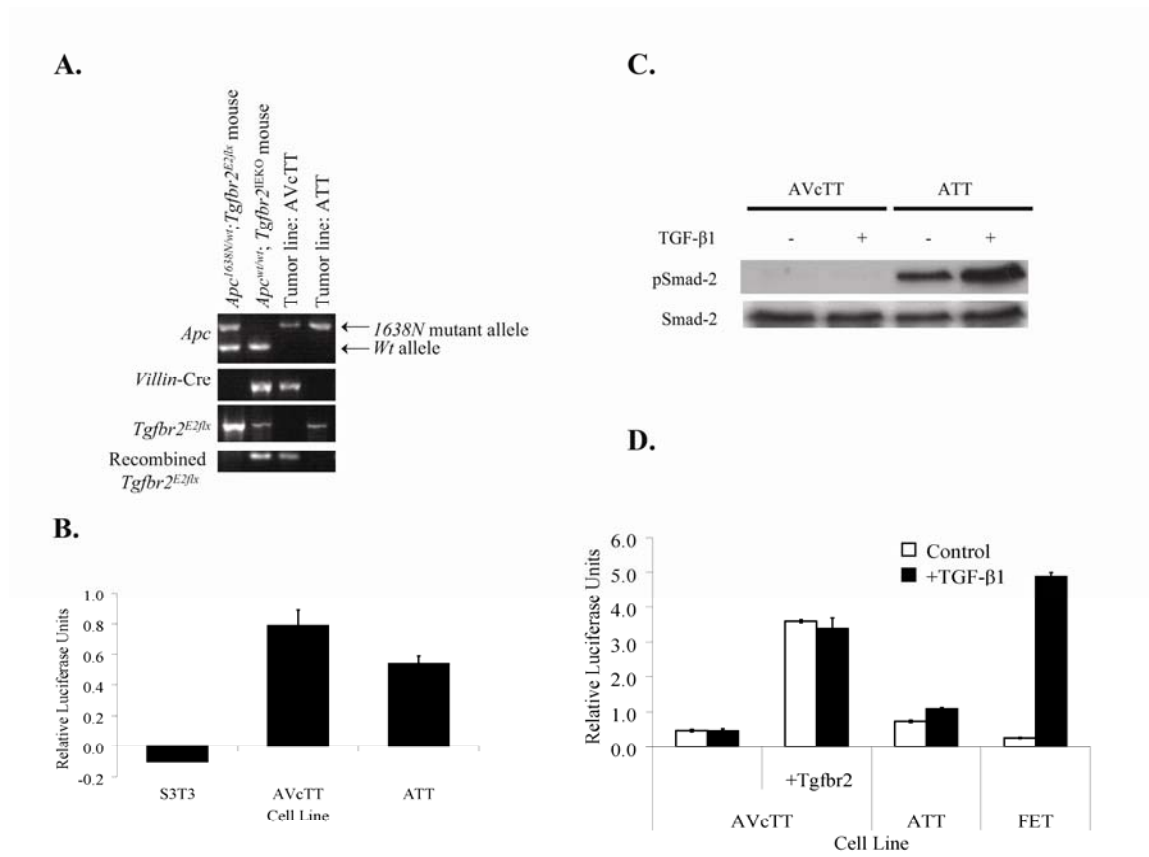
Figure 2-3. Status of Wnt and TGF-β signaling in tumor-derived epithelial cells. A. Results of PCR-based analysis indicating *Apc* LOH in tumor lines derived from *Apc^{1638N/wt}*;*Tgfbr2*^IEKO (AVcTT) and *Apc^{1638N/wt}*, *Tgfbr2^{E2flx/E2flx}* (ATT) neoplasms, and recombination of the *Tgfbr2^{E2flx}* allele in the AVcTT but not in the ATT line. Genomic DNA obtained from normal intestinal tissue was used as the reference DNA in the assays. **B.** β-catenin-mediated transcription assessed with the superTOPFLASH reporter showing an increase in both the AVcTT and ATT tumor lines. S3T3 cells were used as negative control. **C.** Analysis of Smad-2 phosphorylation by Western blot indicating loss of TGF-β responsiveness in the AVcTT but not in the ATT line. **D.** TGF-β-induced transcription evaluated with the p3TP-lux reporter indicates that TGF-β resistance can be restored in the AVcTT line by co-transfection with *Tgfbr2*. Of note, we observed similar results with the CAGA luciferase reporter. The human colorectal cancer cell line FET was used as positive control for TGF-β-induced transcription.

activity in the AVcTT cell line, but p3TP-lux reporter activity could be induced by reconstitution of the AVcTT cell line with wild type *Tgfbr2*.

We then assessed the effect of TGFBR2 inactivation on the secretion of proteins that promote tumor invasion. We determined the concentration of secreted TGF-β1 in the conditioned media from the AVcTT cells and found it to be higher than that in media from the ATT cell line (**figure 2-4A**). Moreover, we found that the concentration of TGF-β1 in tumor tissues was also higher in the tumors from the *Apc*[1638N/wt];*Tgfbr2*[IEKO] mice compared to those from the *Apc*[1638N];*Tgfbr2*[E2flx/E2flx] mice (**figure 2-4B**). These results suggest that TGF-β1 secretion is elevated in the tumors from the *Apc*[1638N/wt]; *Tgfbr2*[IEKO] mice and could have oncogenic effects by inducing angiogenesis, extracellular matrix remodeling and immune suppression.

We also evaluated the production of the matrix metalloproteases MMP-2 and MMP-9 in the cell cultures derived from *Apc*[1638N];*Tgfbr2*[IEKO] and *Apc*[1638N];*Tgfbr2*[E2flx/E2flx] tumors. We observed increased *Mmp2* mRNA expression and gelatinolytic activity in the AVcTT cell line compared to the ATT cell line, and found that this effect could be inhibited by reconstitution of the AVcTT line with *Tgfbr2* (**figure 2-5, A-B**). Assessment of these metalloproteases in tumor tissues showed that MMP-2 has a tendency to a higher enzymatic activity in the tumors derived from *Apc*[1638N];*Tgfbr2*[IEKO] as compared to those from *Apc*[1638N];*Tgfbr2*[E2flx/E2flx] mice; however, we did not find any significant difference in MMP-9 activity in these samples (**figure 2-5C**). We used *in situ* zymography to better identify the cell type involved in MMP production/activity in tumor tissues. As shown in **figure 2-6** minimal MMP activity was detected in the tumors from *Apc*[1638N];*Tgfbr2*[E2flx/E2flx] mice. Conversely, gelatinolytic activity was clearly observed in
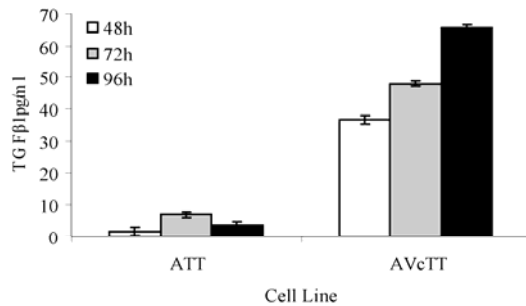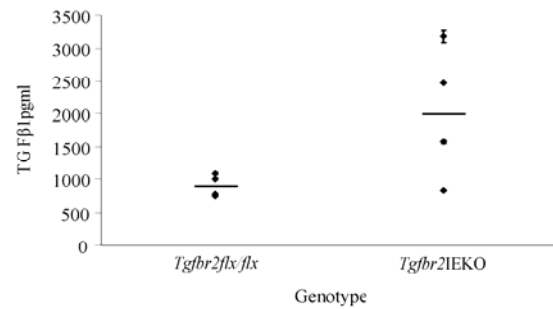
Figure 2-4. Disruption of *Tgfbr2* expression in intestinal epithelial cells alters TGF-β1 balance both *in vitro* and *in vivo*. A. The concentration of TGF-β1 in the conditioned media from AVcTT is greater than that from the ATT tumor line. **B.** The amount of TGF-β1 is higher in tumors from $Apc^{1638N/wt}$;$Tgfbr2^{IEKO}$ mice than in lesions from $Apc^{1638N/wt}$,$Tgfbr2^{E2flx/E2flx}$ animals.
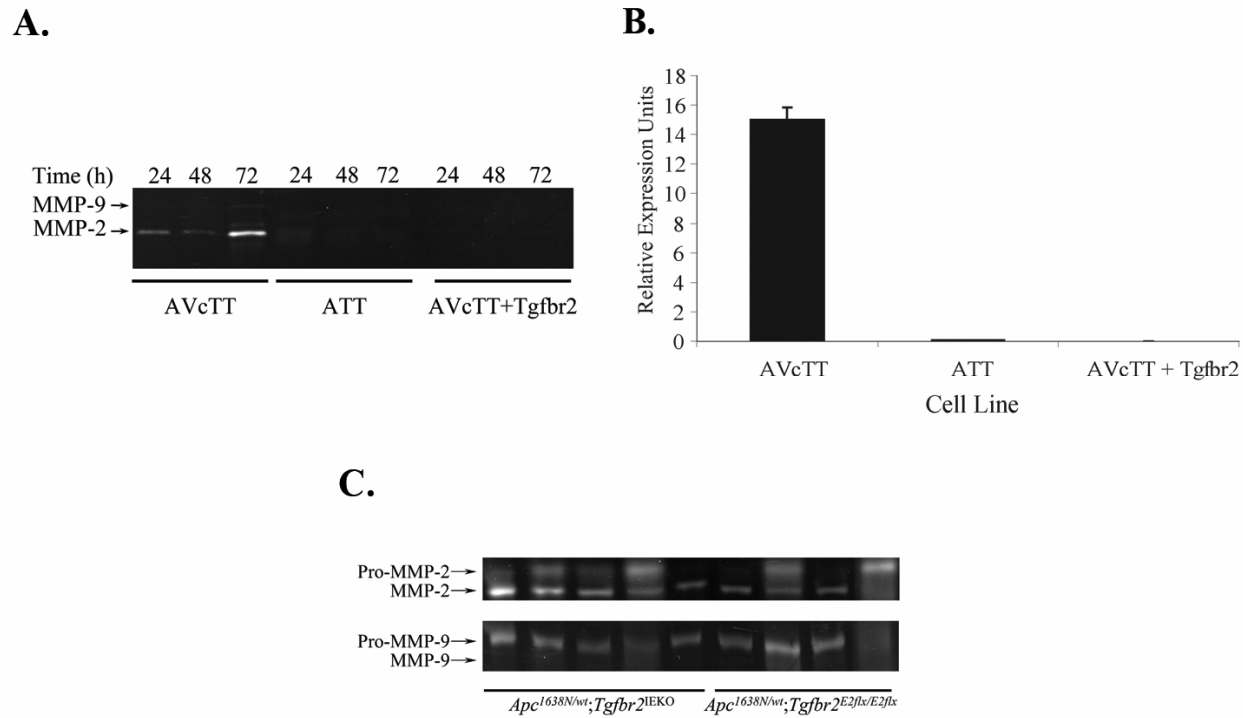
Figure 2-5. Effect of *Tgfbr2* inactivation on the expression of proteins implicated in extracellular matrix remodeling and invasion. A. MMP-2 and MMP-9 activities evaluated through zymography reveal that their enzymatic activities are higher in the AVcTT than in the ATT line. The gelatinolytic activities of these metalloproteases are significantly reduced in the AVcTT line after stable reconstitution of *Tgfbr2*. **B.** Quantitative RT-PCR analysis show that MMP-2 expression is higher in the AVcTT than in the ATT line, and that it is reduced in the AVcTT line after stable reconstitution of *Tgfbr2*. **C.** The enzymatic activity of MMP-2 is also elevated in lesions arisen in $Apc^{1638N/wt};Tgfbr2^{IEKO}$ mice. Inverted images were analyzed with Scion Image Software, and the average density for MMP-2 in $Apc^{1638N/wt};Tgfbr2^{IEKO}$ tissues was 1368.8±739.5; and for $Apc^{1638N/wt},Tgfbr2^{E2flx/E2flx}$ samples was 702.0±149.5.
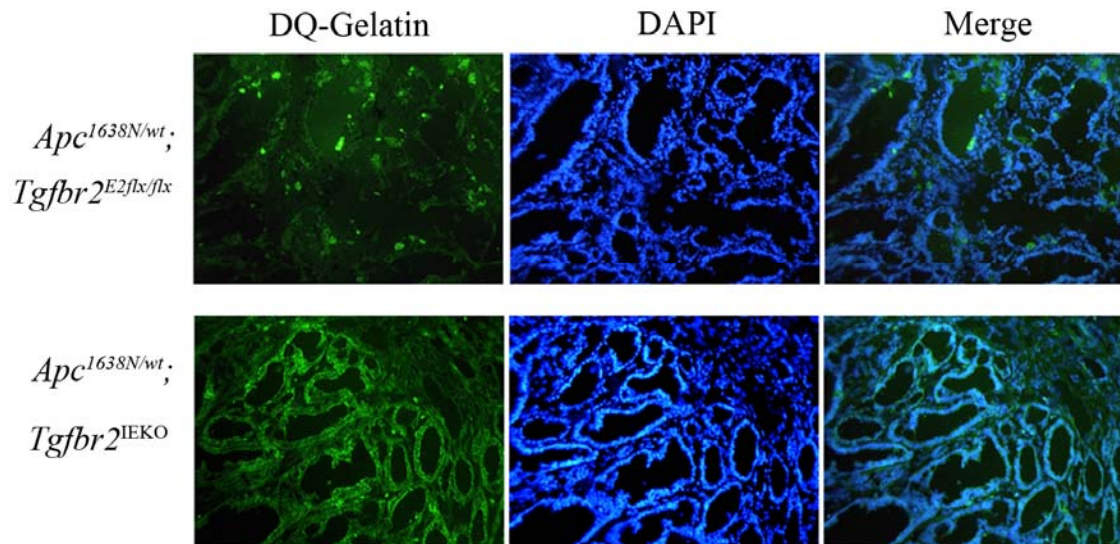
Figure 2-6. *In situ* **gelatin zymography of representative tumors from the** *Apc*$^{1638N/wt}$*;Tgfbr2*^{IEKO} **and** *Apc*$^{1638N/wt}$*;Tgfbr2*$^{E2flx/E2flx}$ **mice**. Tumors from *Apc*$^{1638N/wt}$*;Tgfbr2*^{IEKO} (n=3) show increased MMP activity than those from the *Apc*$^{1638N/wt}$*;Tgfbr2*$^{E2flx/E2flx}$ mice (n=2). Of note, sections incubated with, 10 μmmol/L 1,10-phenanthroline (a broad-spectrum MMP inhibitor) did not show any proteolytic activity (data not shown), strongly suggesting that MMPs are indeed responsible for the degradation of DQ-gelatin

the Apc^{1638N};$Tgfbr2^{IEKO}$ mice and it was restricted to the epithelial component, thus confirming our observations on the *in vitro* models. Of note, evaluation of *Mmp2* mRNA expression by quantitative RT-PCR in the primary tumors from the $Apc^{1638N/wt}$; $Tgfbr2^{IEKO}$ and the $Apc^{1638N/wt}$; $Tgfbr2^{E2flx/E2flx}$ mice did not show a consistent difference between the two groups of mice, possibly because the heterogeneity of the tumor tissue compared to the cultures of epithelial cells, or because of the effect of differences in the proportion of stromal and epithelial cells in the tumors that were analyzed.

Discussion

Our results from the *Villin*-Cre;$Tgfbr2^{E2flx/E2flx}$ ($Tgfbr2^{IEKO}$) mice demonstrate that *Tgfbr2* null intestinal epithelium *in vivo* is not highly susceptible to spontaneous tumor formation. Nevertheless, in the context of an *Apc* mutation, *Tgfbr2* inactivation promotes the progression of adenomas to adenocarcinomas. These results are consistent with *Tgfbr2* acting as a tumor suppressor gene in the intestines that primarily has effects on cells previously initiated into the polyp-carcinoma progression sequence. Furthermore, and importantly, we can conclude from this mouse model that this effect is cell autonomous and not a consequence of impaired TGF-β signaling in T-cells or stromal cells, which has been shown to effect tumor formation in mouse models (103, 114). Interestingly, although TGF-β has been shown to have potent inhibitory effects on epithelial cells *in vitro*, we do not observe any detectable difference between the $Tgfbr2^{IEKO}$ and the $Tgfbr2^{E2flx/E2flx}$ mice with regard to proliferation within the small intestine or colon. We also found no alterations in the histological appearance of the intestinal epithelium of the $Tgfbr2^{IEKO}$ mice, which is noteworthy because TGF-β has

been implicated in regulating apoptosis and differentiation as well as proliferation in the intestinal epithelium (108, 123, 124). These results demonstrate that TGF-β signaling is dispensable for the homeostasis of the intestinal epithelium and may only be physiologically required under specific circumstances, such as in response to exposure to specific inflammatory stimuli (40, 45, 125). The absence of an effect of TGF-β inactivation in the intestinal epithelium is in contrast to the result of BMP signaling disruption in the same tissue, which has been shown to lead to ectopic crypt formation and increased cell proliferation secondary to deregulated sonic hedgehog signaling (104, 126, 127). It is not clear whether the lack of a phenotypic effect is the consequence of compensation by other TGF-β ligand family members, such as activin, or whether it is because TGF-β signaling is not required for the *in vivo* regulation of intestinal epithelial proliferation, differentiation, and induction of programmed cell death.

In light of the unremarkable effect of *Tgfbr2* deletion on the basal status of the intestinal epithelium, it is perhaps not surprising that we did not observe the development of spontaneous intestinal neoplasms beyond those observed in the control mice. Our findings provide further insight into the role of TGF-β signaling on intestinal tumor formation that has been derived from other mouse models with disrupted elements in the TGF-β signaling pathway, including the *Tgfb1*^{-/-}; *Prkdc*[scid/scid], ITF-dnRII (dominant negative *Tgfbr2* transgene in the intestinal epithelium), and *Smad3*^{-/-}, mouse models. None of these models show spontaneous intestinal tumor formation without the presence of a concurrent factor, either intestinal *Helicobacter* infection or azoxymethane treatment (40, 44, 45, 125). Of interest, though, these models do not permit the precise assessment of TGF-β signaling disruption on cell autonomous events that affect intestinal tumor

formation either because they are constitutional knock-out models (e.g. $Smad3^{-/-}$ or $Tgfb1^{-/-};Prkdc^{scid/scid}$) or because they rely on dominant negative $Tgfbr2$ transgenes. The $Tgfb1^{-/-}; Prkdc^{scid/scid}$ and $Smad3^{-/-}$ require *Helicobacter* colonization to develop tumors, and it is not clear whether the bacterial infection induces tumor formation through direct effects on the intestinal epithelium or through indirect paracrine effects mediated by lymphocytes that are TGF-β resistant (58). Furthermore, effects of azoxymethane in the ITR-dnRII construct may result from attenuation of TGF-β signaling or blockade of BMP signaling rather than loss of TGF-β receptor function as occurs with the majority of *TGFBR2* mutations in human colon cancer (128).

Our prior assessment of the $Fabp^{4xat-132}$-Cre;$Tgfbr2^{E2flx/E2flx}$ mouse, the only model to date that permits the assessment of cell autonomous effects of TGF-β signaling on intestinal neoplasm formation, showed that colon neoplasms would form after exposure of the mice to azoxymethane, which can induce neoplasms through β-catenin dependent and independent pathways (41, 129, 130). Thus, to determine the effect of $Tgfbr2$ inactivation in an *in vivo* model that recapitulates the genetic alterations observed in human colon cancer, we generated the $Apc^{1638N/wt}$;$Tgfbr2^{E2flx/E2flx}$ +/- *Villin*-Cre mouse model. Our results with this model provide compelling evidence that TGFBR2 acts to suppress the progression of initiated neoplasms and that inactivation of TGF-β signaling in the context of *Apc* mutations strongly promotes the malignant transformation of adenomas to adenocarcinomas. Furthermore, in light of the increased formation of intestinal adenocarcinomas observed in the Cis-$Apc^{\Delta716}$(+/-), $Dpc4^6$(+/-) mice, our results in the $Apc^{1638N/}$;$Tgfbr2^{E2flx/E2flx}$ +/- *Villin*-Cre suggest that inactivation of Smad signaling is a central effect of TGFBR2 inactivation (46).

With regard to the relevant biological consequences that contribute to colon cancer formation as a result of *Tgfbr2* inactivation, we assessed the effects of loss of *Tgfbr2* on TGF-β1 secretion and MMP-2 and MMP-9 activity, which are candidate mechanisms that could influence tumor invasion. We assessed these mechanisms through the use of tumor epithelial cell line systems and primary tumors from the mice. We characterized the cell lines for TGF-β responsiveness and activation of the Wnt signaling pathway to accurately determine the status of these pathways in relationship to the effects on TGF-β1 secretion and MMP activity. It is of note that the cell line established from a neoplasm from an *Apc^{1638N/wt};Tgfbr2*^{flx/flx} mouse (ATT) showed a modest level of p3TP-Lux induction with TGF-β that was substantially lower than the TGF-β responsive colon cancer cell line, FET. We believe that this is secondary to saturation of the TGF-β receptor with autocrine TGF-β, but it is also possible that the TGF-β signaling pathway is attenuated in this cell line. Our studies of the cell lines and tumor tissues demonstrate that loss of TGF-β signaling leads to invasive tumors and that these neoplasms exhibit profound desmoplasia and mucinous features. These histological characteristics implicate factors that regulate extracellular matrix remodeling, and our *in vitro* studies and studies of the primary tumors have provided evidence suggesting that *Apc^{1638N/wt};Tgfbr2*^{IEKO} tumors produce considerably higher levels of TGF-β1 and MMP-2, which could account for their increased invasiveness. Furthermore, we have also established that TGF-β signaling regulates MMP-2 expression at the level of mRNA synthesis in our *in vitro* models. We have not investigated the role of loss of specific Smad and non-Smad TGF-β mediated signaling pathways in tumor formation in the *Apc^{1638N/wt}; Tgfbr2*^{IEKO} mice, but based on studies of the Cis-*Apc*^{Δ716}(+/-), *Dpc4*^6(+/-)

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mice and *Smad3*^{-/-} mice, we predict that impaired Smad signaling has a prominent role in the genesis of these tumors (42, 46).

In summary, we have shown in an *in vivo* model system that loss of *Tgfbr2* in the intestinal epithelium contributes to intestinal cancer formation by promoting the progression of adenomas initiated by *Apc* mutations. The results of these studies using the *Villin-Cre*;*Tgfbr2*$^{E2flx/E2flx}$ mice provide evidence from an *in vivo* model system that inactivation of *TGFBR2* has a pathogenic role in the formation of human colon cancers by promoting the progression of colon adenomas to invasive adenocarcinomas. These findings have significant implications regarding therapeutic strategies that target the TGF-β signaling pathway for the treatment of cancer.

CHAPTER III

TRANSFORMING GROWTH FACTOR-β SIGNALING PROMOTES RESISTANCE TO ANOIKIS IN A SUBSET OF HUMAN COLORECTAL CANCER CELL LINES

Abstract

TGF-β is a cytokine involved in the regulation of numerous cellular responses, and it is now generally accepted that the TGF-β signaling pathway is implicated in both tumor suppressing and tumor promoting processes. The relevant biological processes that TGF-β can affect that influence tumor formation include, among others, proliferation, apoptosis, and differentiation. Of particular importance to the invasive and metastatic behavior of cancer is the acquisition of resistance to anoikis, or cell death induced by inappropriate cell-cell or cell-extracellular matrix attachment, which allows anchorage-independent survival and facilitates colonization of secondary sites. In order to analyze the role of TGF-β signaling in regulating anoikis in colon cancer, we evaluated the effect of TGF-β1 on anoikis in a panel of established colorectal cancer cell lines. TGF-β1-treated HCT116+Chr3 and CBS showed a significant reduction of cell death upon loss of anchorage indicating that TGF-β protects these cell lines from anoikis. Moreover, suppression of autocrine TGF-β signaling in HCT116+Chr3 inhibited this effect. We also found that TGF-β induces the activation of the PIK3/AKT and MAPK/ERK pathways, and modulates the expression of the Bcl-2 family members Bim and Bcl-2, and that inhibition of the PIK3 and MAPK pathways attenuated the protective effect against anoikis. Thus, TGF-β may paradoxically promote the malignant behavior of a subset of

TGF-β responsive colon cancer cell lines through cell autonomous mechanisms by blocking anoikis. Several signaling cascades are involved in the TGF-β mediated effect on anoikis, and they appear to cooperate to promote cell survival after loss of anchorage.

Introduction

The Transforming Growth Factor-β (TGF-β) superfamily comprises a large number of cytokines that regulate tissue homeostasis and development due to their prominent roles in the regulation of fundamental processes such as cell proliferation, differentiation, motility and programmed cell death. TGF-β has been shown to not only play a central role in development and normal organ function, but also to affect many pathological processes such as cirrhosis, fibrotic kidney disease, and tumorigenesis (reviewed in (131, 132)).

The role of TGF-β signaling during the process of tumor development is complex and its effects are context-dependent and can be paradoxical. Results from *in vitro* and *in vivo* model systems as well as from epidemiological studies have made evident that the TGF-β signaling pathway can behave both as a tumor suppressor and as a tumor promoter pathway (27, 49). The identification of inactivating mutations in components of the TGF-β signaling pathway including *TGFBR2* and *SMAD4* has provided strong evidence that TGF-β can act as a tumor suppressor in primary human cancer (108, 109). Evidence for a tumor suppressor function of TGF-β signaling is also apparent from animal models that demonstrate an increased susceptibility to tumor development in mice in which the TGF-β signaling cascade has been inactivated or attenuated (40, 42, 43, 46, 72, 133). The tumor suppressor mechanisms of TGF-β include, among others, its ability to inhibit

cell-cycle progression as well as to induce senescence and apoptosis (131). The tumor suppressor role of TGF-β is evident in cancers arising from a variety of organ systems, but is most obvious in tumors arising from the gastrointestinal tract, including colorectal cancer, gastric cancer, and pancreatic cancer (132). The tumor promoting capability of this signaling pathway, on the other hand, has been demonstrated by the increase in invasiveness and metastatic potential of neoplasms arising in transgenic mice in which TGF-β1 or the TGF-β type I receptor are overexpressed or consitutively activated in tumor cells (49, 80, 134). Furthermore, the concept that TGF-β signaling facilitates tumor progression is supported by the observation that some tumor cell lines lose their metastatic potential in xenograft systems upon disruption of the TGF-β signaling pathway (37). It appears that TGF-β signaling may induce tumor progression due to the following: 1) its ability to promote Epithelial to Mesenchymal Transition (EMT) (37); 2) its immunosuppressant function (40), and/or 3) its ability to inhibit cell death caused by growth factor deprivation (95, 135). Furthermore, epidemiologic studies of individuals with colorectal cancer correlating alterations in the TGF-β pathway with survival or response to treatment have produced conflicting results (38, 39, 136-138). Thus, although TGF-β signaling pathway can inhibit tumor formation, it also appears capable of paradoxically promoting invasion and metastasis in established cell lines, especially in the context of breast cancer.

One of the fundamental attributes of tumor cells that dictates their potential to invade tissue and metastasize is their resistance to apoptosis triggered by inadequate cell-cell and/or cell-matrix contacts (98). This form of cell death is known as *anoikis* (from the Greek for 'homelessness', reviewed in (99, 100)). To become resistant to anoikis

represents a selective advantage for tumor cells because it confers on them the ability to survive without proper attachment and, consequently, gives them the capacity to colonize secondary sites. Indeed, tumor xenograft models have shown that anoikis-resistant cancer cell lines have increased survival in blood circulation and enhanced capacity to form metastases (101, 102, 139, 140). In light of the conflicting results of the epidemiological studies of TGF-β signaling deregulation in colorectal cancer, which suggest that the role of TGF-β in colorectal cancer behavior can be paradoxical or tumor suppressing, and the evidence from *in vitro* systems demonstrating both tumor suppressing and tumor promoting effects of TGF-β in colorectal cancer cell lines, we have assessed the effect of TGF-β on the induction of anoikis, which is a behavior predicted to be associated with the progression of advanced colorectal cancer, in a panel of colon cancer cell lines that are responsive to TGF-β mediated growth inhibition

Experimental Procedures

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 DNA topoisomerase I inhibitor Camptothecin (cat# 208925), the MEK1/2 inhibitor U0126 (cat# 662005), and the PIK3 inhibitor LY294002 (cat# 440204) were obtained from Calbiochem (San Diego, CA). TGF-β1 and IGF-I were purchased from R&D Systems (Minneapolis, MN) and Diagnostics Systems Laboratories (Webster, TX), respectively. The antibodies for p-AKT (Ser473) (cat# 9271), AKT (cat# 9272), p-ERK1/2

(Thr202/Tyr204) (cat# 9101), ERK1/2 (cat# 9102), Bcl2 (cat# 2872), Bcl-x$_L$ (cat# 2762), Bax (cat# 2772), and Bak (cat# 3792) were purchased from Cell Signaling (Beverly, MA). The antibody for Bim (cat# 38-6500) was obtained from Zymed Laboratories (South San Francisco, CA). The antibodies for actin (cat# sc-1616), goat anti-mouse IgG-HRP (cat# SC-2005), and goat anti-rabbit IgG-HRP (cat # SC-2004) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Cell culture

The colon cancer cell lines HCT116, FET, Moser, GEO and CBS were generously provided by Michael Brattain (Roswell Park Cancer Institute, Buffalo, NY). The HCT116 plus chromosome 3 cell line (designated here as HCT116+Chr3) was kindly provided by Richard Boland (Baylor University Medical Center, Dallas, TX) and John Carethers (University of California San Diego, San Diego, CA). HCT116, HCT116+Chr3, HCT116+Chr3+DN2R, FET and Moser were grown in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY), and CBS and GEO were maintained in McCoy's 5A Medium (Sigma-Aldrich), and all were supplemented with 10% FBS (HyClone, Logan, UT).

Generation of HCT116+Chr3+DN2R

An isogenic cell line with suppressed TGFBR2 activity was generated by transfecting the HCT116+Chr3 cell line with a plasmid expressing a dominant negative *TGFBR2* gene, pIRES-puro2-DN2R, which was constructed by cloning the DN2R transgene, provided by Harold Moses (Vanderbilt University Medical School, Nashville, TN) into the pIRES-

puro2 vector (Clontech, Mountain View, CA). The transfection was performed using FuGene (Roche, Indianapolis, IN) following the manufacturer's protocol. A pool of clones was selected with 1.5μg/ml puromycin (Sigma-Aldrich) and used in the subsequent experiments.

Thymidine incorporation assay

With the aim of evaluating cell proliferation by means of DNA synthesis, subconfluent cells were treated with 10ng/ml TGF-β1 in serum free conditions. 46h after starting the treatment, the cells were pulsed with 4 μCi/ml [Methyl ^{3}H]-Thymidine (Perkin Elmer, Wellesley, MA) and incubated at 37°C for 2 additional hours. The cells were fixed with 1ml 10% Trichloroacetic Acid for 30 minutes, after which they were solubilized with 300μl of 0.2N NaOH. 100μl-aliquot of the cell lysates were resuspended in 4ml of scintillation fluid and the incorporation of the radioisotope was measured with a scintillation counter. All the samples were prepared in triplicate.

p3TP-lux reporter assay

In order to evaluate TGF-β-mediated transcription, tumor-derived cells were transiently transfected with the p3TP-lux reporter (kindly provided by Joan Massagué, Memorial Sloan-Kettering Cancer Center, New York, NY) concomitantly with the pRL-TK reporter construct (Promega, Madison, WI). Subsequently, the cells were treated with 10ng/ml TGF-β1, and luciferase activity was evaluated 48h after transfection using the Dual Luciferase Reporter Assay System (Promega) with a Veritas luminometer (Turner Biosystems, Sunnyvale, CA).

Anoikis assay

For the evaluation of cell death induced by the loss of anchorage, the cells of interest were first plated in regular cell culture vessels with FBS-containing medium at a 50% confluence. 24 hours later, when the cells were properly attached to the plastic, the medium was replaced with SFM and the cells were treated or not with 10ng/ml TGF-β1. After 48 hours, the cells were detached from the vessels and resuspended in SFM. The cells were then seeded at a concentration of 1×10^6 cells/ml in Ultra Low Attachment Plates (Corning, Corning, NY), which prevent cell anchorage to the substrate, and treated or not with 10ng/ml TGF-β1 or any additional compounds to be tested. After 8 hours in suspension, the cells were harvested and cell death was evaluated by Annexin V and Propidium Iodide staining (Annexin-V-FLUOS Staining Kit, Roche) following the manufacturer's recommended protocol. Alternatively, the cells were deprived of attachment for 24 hours and the occurrence of apoptosis was analyzed by Caspase-3 activation through western blotting (protocol described below) or DNA fragmentation analysis (Cell Death Detection ELISA Kit, Roche). For the AnnexinV-PI staining, particularly clumpy cells were treated with Accutase (Innovative Cell Technologies, San Diego, CA) to improve single cell suspension quality and facilitate flow cytometry performance.

Western blotting

 For evaluation of protein expression during anoikis western blotting was used. Cells were maintained in suspension as described above, harvested, lysed with RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche) and phosphatase

inhibitor cocktails 1 and 2 (Sigma-Aldrich), and then used for SDS-PAGE. PVDF membranes (Pierce, Rockford, IL) were probed with the antibodies of interest under the conditions suggested by the maker of each antibody, and the reaction was detected by chemiluminescence.

Results

TGF-β responsiveness is restored in HCT116 upon chromosome 3 transfer

The DNA mismatch repair (MMR) deficient human colon carcinoma cell line HCT116 is resistant to TGF-β due to biallelic mutational inactivation of *TGFBR2*. An MMR proficient clone of HCT116 has been made through the transfer of chromosome 3 into this cell line, which reconstitutes not only the MMR gene *MLH1* but also *TGFBR2*, permanently restoring TGF-β responsiveness in the HCT116+Chr3 cell line (141). Our analysis revealed that in contrast to the parental HCT116 cells, the HCT116+Chr3 line shows a dramatic induction of the p3TP-lux reporter activity, and a suppression of cell proliferation upon TGF-β1 stimulation (**figure 3-1**). These results demonstrate that TGF-β sensitivity is effectively restored in HCT116 upon chromosome 3 transfer, and moreover, that this new cell line is a well defined reagent to study the effects of restoring TGF-β signaling in resistant cells, because only one functional copy of *TGFBR2* has been introduced and the reconstituted *TGFBR2* is in its native chromatin environment.
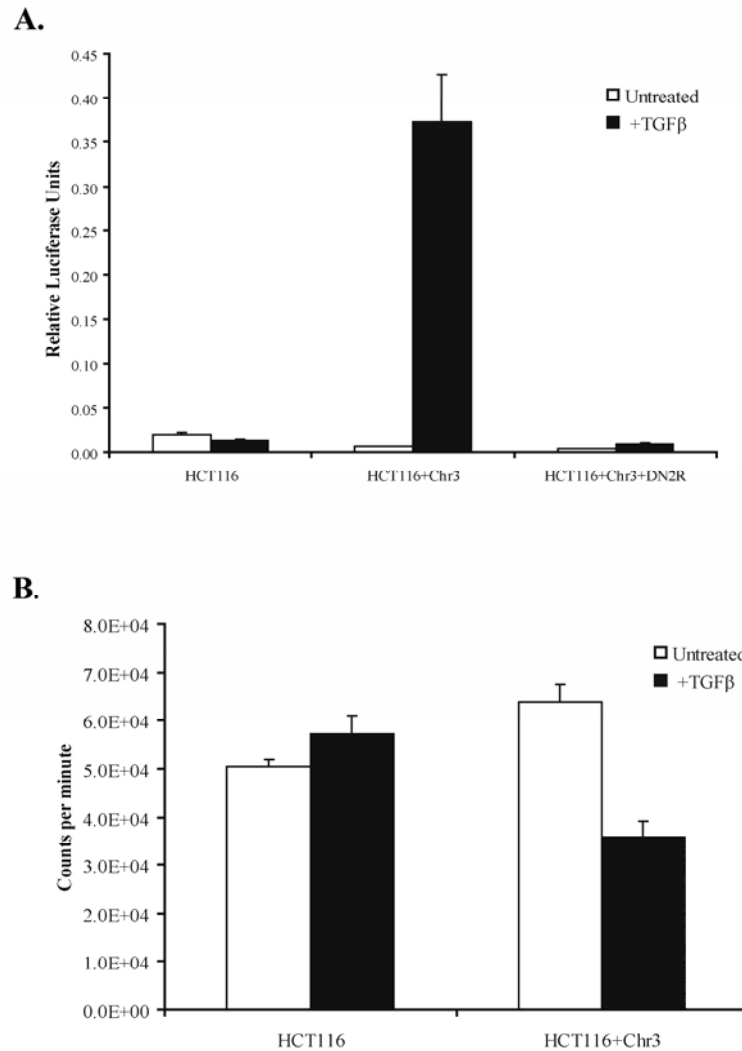
Figure 3-1. TGF-β1 induces transcriptional activity of the p3TP-lux reporter construct and growth arrest in HCT116+Chr3. A. Parental cell line HCT116, as well as HCT116+Chr3 and HCT116+Chr3+DN2R cells were transienlty transfected with the p3TP-lux and pRL-TK reporter constructs, and treated with 10ng/mL TGF-β1. **B.** DNA synthesis was evaluated in HCT116 and HCT116+Chr3 treated with 10ng/mL TGF-β1 for 48h. All experiments were performed in triplicates. The bars correspond to S.E.

TGF-β1 protects the HCT116+Chr3 cell line from anoikis

TGF-β signaling is involved in the regulation of multiple cellular responses disrupted during the process of tumor formation that can affect cancer progression. Deregulation of anoikis, for instance, contributes to the malignant behavior of many types of neoplastic cells by allowing anchorage-independent survival and facilitating colonization of secondary sites. With the aim of evaluating the effect of TGF-β1 on HCT116+Chr3 undergoing this type of cell death, the parental cell line HCT116, HCT116+Chr3, and HCT116+Chr3 transduced with a dominant negative mutant of TGFBR2 (HCT116+Chr3+DN2R) were maintained under conditions of no attachment with or without exogenous TGF-β1. After eight hours in suspension, early apoptotic cells were detected by changes in their cell membrane by Annexin-V and Propidium Iodide staining. Alternatively, the cells were collected after 24h and the extent of DNA fragmentation was determined using a Cell Death Detection ELISA assay. As shown in **figure 3-2**, autocrine TGF-β1 protects HCT116+Chr3 from undergoing anoikis as judged by the lower amount of cell death observed in untreated HCT116+Chr3 as compared to that of HCT116+Chr3+DN2R. In addition, the protective influence is further enhanced by the addition of exogenous TGF-β1.

TGF-β1 protects the colorectal cancer line CBS from undergoing anoikis

In order to determine if TGF-β also protects other TGF-β responsive colon cancer cell lines against anoikis, we analyzed the effect of TGF-β stimulation on a set of cell lines derived from human colorectal carcinomas deprived from anchorage. Furthermore, since TGF-β responsiveness was reconstituted in HCT116+Chr3 by the insertion of one copy
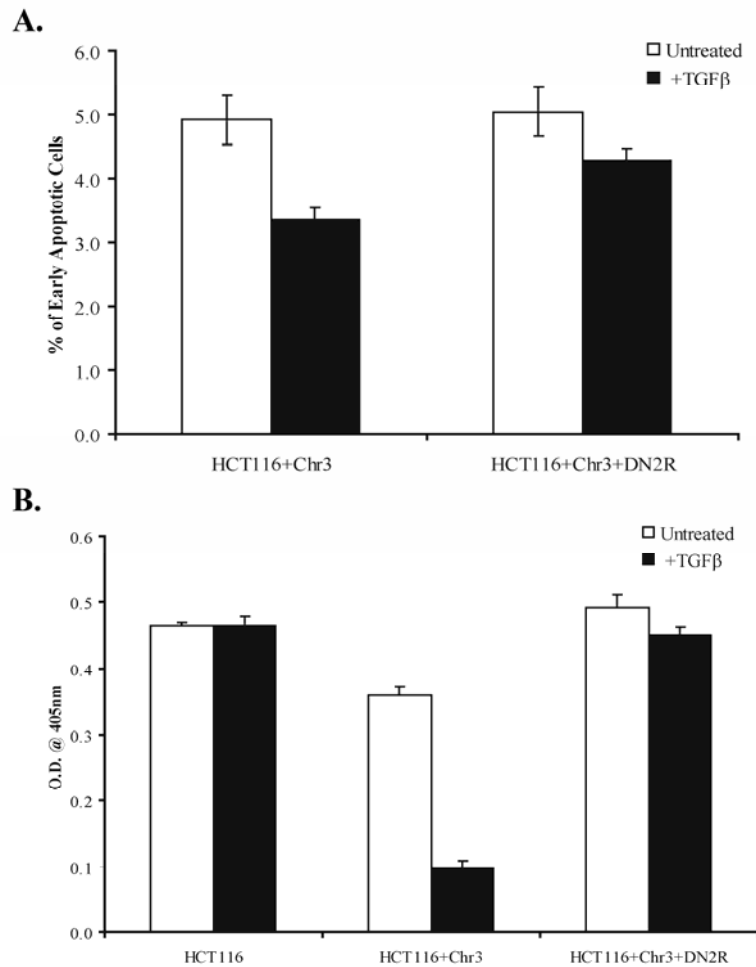
Figure 3-2. TGF-β1 protects HCT116+Chr3 cells from anoikis. The cells were treated with 10ng/mL TGF-β1 in serum free conditions in regular tissue culture flasks. After 48h, 1x10^6 cells/mL were placed in suspension in "Ultra Low Attachment Clusters" (Costar) with the same concentration of TGF-β1. **A.** 8h after, early changes in the cell membrane organization were evaluated by Annexin V and Propidium Iodide staining using flow cytometry. **B.** Alternatively, 24h later DNA fragmentation was assessed with the ELISA-based assay. The assays were run in triplicate. The bars represent S.E.

of the *TGFBR2* in its native chromatin environment, we chose to study the response to the loss of substrate in a subset of colorectal cancer cell lines naturally sensitive to TGF-β growth inhibition. Thus, we evaluated the occurrence of cell death upon the loss of anchorage in the human colorectal cancer lines GEO, Moser, FET and CBS through analysis of DNA fragmentation and Caspase-3 activation. Our analysis revealed that, even though TGF-β has been shown to cause growth arrest to all these cell lines, its effects in terms of anoikis are quite diverse. Hence, our results show that while exogenous TGF-β protects CBS from undergoing anoikis, it increases the amount of cell death in FET lacking anchorage. Further, we found that treatment with TGF-β does not have a significant effect on anoikis in GEO and Moser (**figure 3-3**).

Smad-independent pathways are activated by exogenous TGF-β1 in HCT116+Chr3 and CBS that are in an anchorage free state

Although TGF-β1 has been shown to induce cell death in several cell lineages, including some colon adenoma cell lines, there are some reports of cell lines protected by this cytokine from apoptosis triggered by different stimuli. It has been established, for instance, that TGF-β1 blocks cell death caused by growth factor deprivation to the mouse mammary gland NMuMG cell line (95), to A549 human lung carcinoma cells (94), and to activated rat hepatic stellate cells (HSC) (96).

The paradoxical effects of TGF-β in terms of apoptosis regulation emphasize the relevance of the cellular context in controlling the responses to this cytokine. Since this contextual effect is likely due to differential signaling pathway alterations, we studied whether non-Smad signaling pathways may be modulated by TGF-β1 and involved with
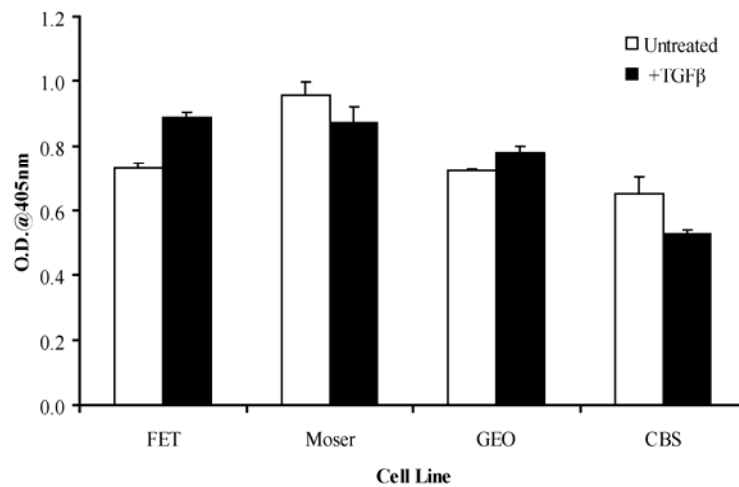
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Figure 3-3. Effect of TGF-β1 treatment on colorectal cancer cell lines deprived from anchorage. The TGF-β-responsive colon cancer cell lines FET, Moser, GEO and CBS were treated as previously described (legend of figure 2B). The amount of anoikis was assessed with the "Cell Death Assay" (Roche) after 24 hours.

the suppression of anoikis in HCT116+Chr3. We evaluated the PIK3/AKT pathway, which has been implicated in the protective effect of TGF-β1 against apoptosis (95). We observed that exogenous TGF-β1 induces AKT phosphorylation in HCT116+Chr3 kept in suspension (**figure 3-4A**). Additionally, we found that inhibition of autocrine TGF-β signaling in HCT116+Chr3, by forced expression of the DN2R mutant, dramatically reduces the basal activity of ERK1/2 in HCT116+Chr3 (**figure 3-4B**).

Next, we investigated if TGF-β-induced activation of the MAPK/ERK and PIK3/AKT pathways have a functional role in the protection against anoikis. For that purpose, we applied the pharmacological inhibitors U0126 and LY294002 to TGF-β-treated HCT116+Chr3 in order to reduce the activity of MEK1/2 and PIK3, respectively, and evaluated the amount of anoikis under such conditions. We found that blockade of these enzymes dramatically increases the amount of cell death in HCT116+Chr3 kept in suspension and, although the cells were more sensitive to blockade of the MAPK/ERK pathway alone, preventing the activity of the two pathways concurrently had an additive effect on the induction of anoikis **(figure 3-4C)**. In addition, we found that treatment of HCT116+Chr3+DN2R with LY294002 alone, or in combination with U0126, increased the amount of cell death to a greater extent than in HCT116+Chr3 (**figure 3-4C**). From these results we infer that autocrine TGF-β signaling can partially prevent cell death after loss of anchorage even in the presence of MEK1/2 and PIK3 inhibitors. Furthermore, we also found that the cells that were exposed to the pharmacological inhibitors and simultaneously treated with exogenous TGF-β1 showed a lower induction of cell death when compared to untreated cells or the cells that express the DN2R mutant. Thus, these observations implicate other mechanisms in TGF-β-mediated resistance against cell death

(**figure 3-4C**). Taken together, our results suggest that even though the activation of the PIK3/AKT and MAPK/ERK pathways is necessary for TGF-β to protect HCT116+Chr3 against anoikis, modulation of these two signaling cascades is likely not the only mechanism through which TGF-β exerts its protective effect.

We next assessed the roles of MAPK/ERK and PIK3/AKT in TGF-β-mediated protection of CBS cells against anoikis. We observed that exogenous TGF-β treatment induces the phosphorylation of ERK1/2, but not of AKT. Furthermore, with the aim of establishing if ERK1/2 activation in TGF-β-treated CBS cells is relevant for the protection against anoikis, we treated the cells with the MEK1/2 inhibitor U0126 and measured the effect effect of ERK1/2 inhibition on anoikis. This analysis showed that TGF-β-induced activation of the MAPK/ERK pathway is necessary for increased resistance against anoikis, since inhibition of these enzymes partially overcomes TGF-β's protective effect (**figure 3-5**).

Activation of TGF-β signaling correlates with increase in Bcl-2 expression

The preceding results provided evidence that MAPK/ERK1/2 and PIK3/AKT-independent mechanisms are modulated by TGF-β signaling so that HCT116+Chr3 and CBS are protected from undergoing anoikis even upon blockade of these signaling cascades.

The Bcl-2 family of proteins plays a fundamental role in the regulation of apoptosis, and several of its members have been shown to be targeted for disruption during colon cancer progression (142, 143). For that reason we decided to evaluate the expression of pro- and anti-apoptotic members of this family of proteins in both lines while undergoing anoikis.

Figure 3-4. TGF-β-induced activation of the MAPK/Erk and PIK3/AKT pathways in HCT116+Chr3 is necessary for protection against anoikis. **A.** Western blot analysis of phosphorylated Erk 1/2 (Thr202/Tyr204) and total ERK1/2 in cells treated with TGF-β1. **B.** Western blot analysis of phosphorylated AKT (S473) and total AKT in cells treated with TGF-β1. **C.** Cell death analysis for HCT116+Chr3 and HCT116+Chr3+DN2R deprived from substrate for 24h and treated with 10mM LY294002, 5mM U0126 and/or 10ng/mL TGF-β1. These results are representative of several assays. Each experiment was done in triplicate. The bars indicate S.E.

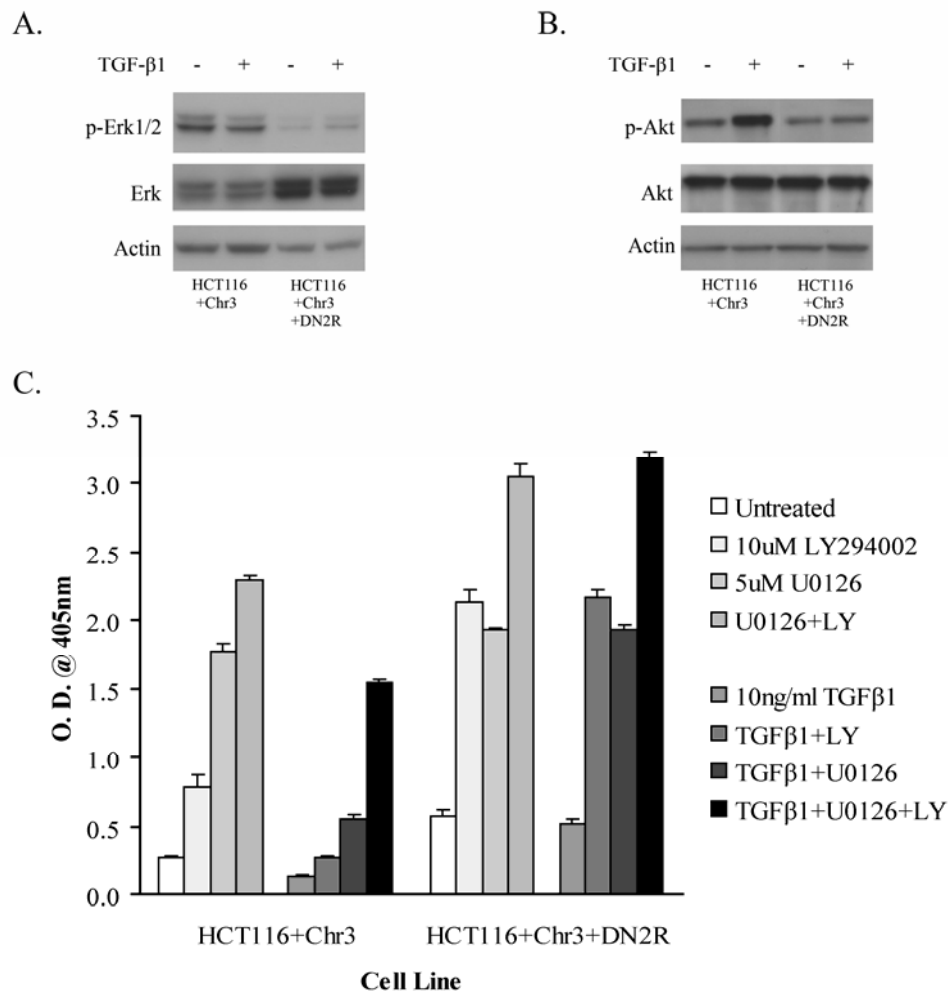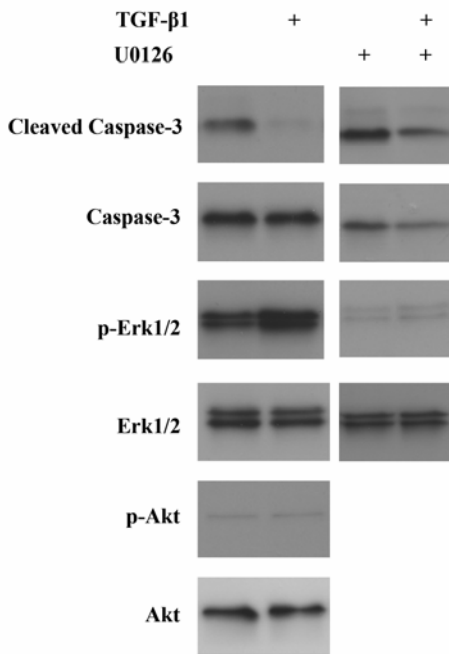**Figure 3-5. TGF-β-induced activation of the MAPK/Erk pathway in CBS is necessary for protection against anoikis.** Western blot analysis of Caspase-3, ERK1/2 and AKT and their active forms in CBS maintained in suspension for 24 hours. The cells were treated with 5uM of U0126 and/or 10ng/mL TGF-β1.

We observed that TGF-β treatment inhibits the expression of the pro-apoptotic protein Bim in HCT116+Chr3 deprived from anchorage. Importantly, we also found that TGF-β-induced downregulation of Bim expression is independent of the MAPK/ERK and PIK3/AKT pathways, because even in the presence of pharmacological inhibitors to MEK1/2 or PIK3, TGF-β potently inhibits the expression of Bim (**figure 3-6A**). We also established that TGF-β stimulation of HCT116+Chr3 is associated with an increase in the expression of anti-apoptotic Bcl-2, possibly due in part to Bim downregulation. Of note, we observed that TGF-β does not alter other Bcl-2 family members like Bcl-x_L (**figure 3-6A**), Bax or Bak (not shown). In summary, these results suggest that the modulation exerted by TGF-β signaling upon the activity of PIK3/AKT, MAPK/ERK and members of the Bcl-2 family is responsible for the persistent resistance to anoikis displayed by HCT116+Chr3 as compared to HCT116+Chr3+DN2R. Additionally, we also established that TGF-β treatment of the CBS cell line does not affect Bim expression but does cause an increase in the level of expression of total Bcl-2, presumably enhancing its antiapoptotic activity and contributing to the TGF-β-mediated protective effect (**figure 3-6B**).

Signaling network deregulation in FET, Moser and GEO and TGF-β's effect in anoikis

Our analysis of the expression of activated ERK1/2 and AKT showed that they are involved in TGF-β-mediated protection of HCT116+Chr3 and CBS. Consequently, we examined the status of these pathways in the colorectal cell lines that are not protected from anoikis by TGF-β in order to determine the specificity of TGF-β induction of these pathways in the settings of anoikis. This analysis revealed that TGF-β1 does not induce the activation

A.

B. CBS
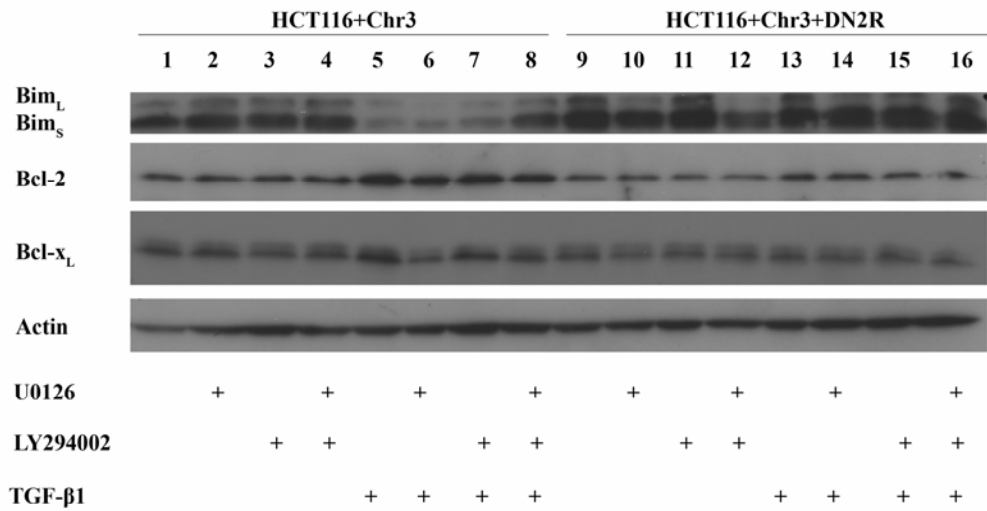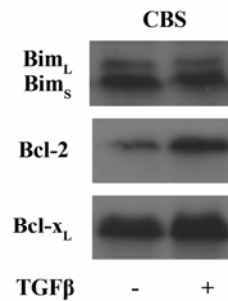
Figure 3-6. TGF-β signaling modulates the expression of Bim and Bcl-2 in HCT116+Chr3, and the expression of Bcl-2 in CBS deprived from anchorage. A. Western blot analysis of Bim_S, BimL, Bcl-2 and Bcl-x_L in HCT116+Chr3 and HCT116+Chr3+DN2R deprived from anchorage for 24h and treated with TGF-β1, the pharmacological inhibitors LY294002 and U0126. **B.** Western blot analysis of Bim_S, Bim_L, Bcl-2 and Bcl-x_L in CBS deprived from anchorage for 24h and treated with exogenous TGF-β1.

of ERK1/2 or AKT in Moser or GEO (**figure 3-7**), which is presumably associated with the lack of a protective effect. Conversely, we found that although TGF-β treatment promotes anoikis in FET, it induces the activation of ERK1/2, but not AKT, a similar effect to that seen in CBS cells, which are protected from this type of cell death by TGF-β (**figure 3-7**).

Confronted with the paradoxical results obtained with FET, in which TGF-β enhances the occurrence of anoikis while it stimulates the activation of the MAPK/ERK signaling pathway as it does in cells that are protected against cell death, we hypothesized that critical components of the signaling network required for TGF-β to exert a protective effect against anoikis were absent in FET cells. Therefore, we studied whether the induction of PIK3/AKT activity concomitant with TGF-β pathway stimulation would lead to resistance against anoikis as shown in HCT116+Chr3. For this purpose, we first treated the cells with IGF-I and found that this treatment alone reduces the amount of cell death after loss of anchorage (**figure 3-8**). Subsequently, we were able to establish that when applied simultaneously with IGF-I, TGF-β1 does not reduce the protective effect of PIK3/AKT activation induced by IGF-I, but on the contrary, it enhances the protection exerted by this cytokine, further protecting the cells from anoikis (**figure 3-8**). Moreover, we found that the enhanced protective effect exerted by the combination of TGF-β and IGF-I requires the activation of MAPK/ERK pathway, because treatment with the MEK1/2 inhibitor U0126 increases the amount of cell death to the levels observed in untreated cells.
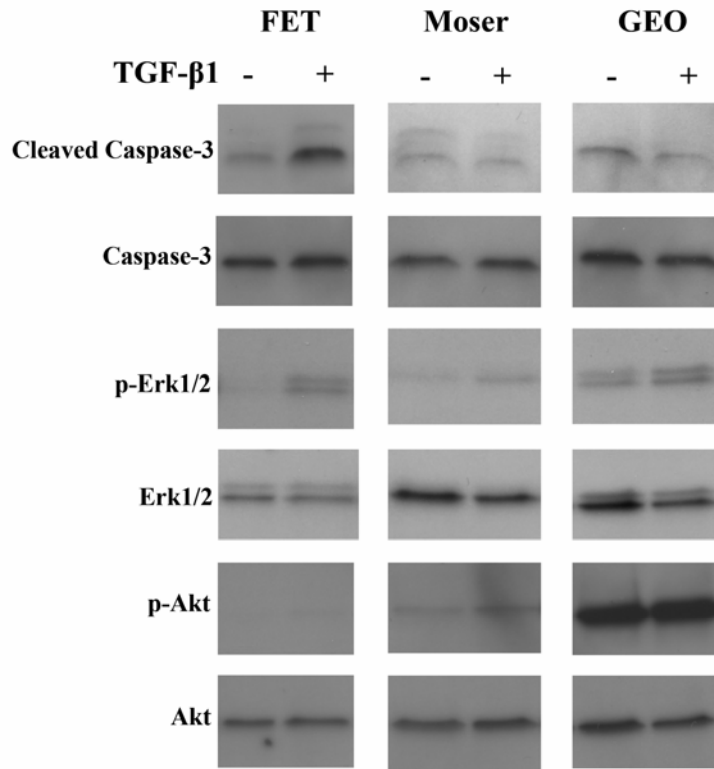
Figure 3-7. Effect of TGF-β1 treatment on Caspase-3, ERK1/2 and AKT activation in colorectal cancer cell lines deprived from anchorage. Western Blot assays for Caspase-3, ERK1/2 and AKT and their active forms in FET, Moser and GEO maintained in suspension for 24 hours with or without exogenous TGF-β1.
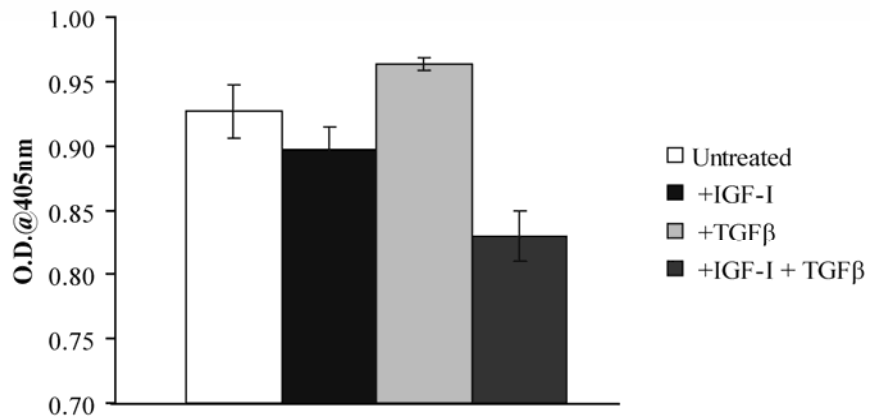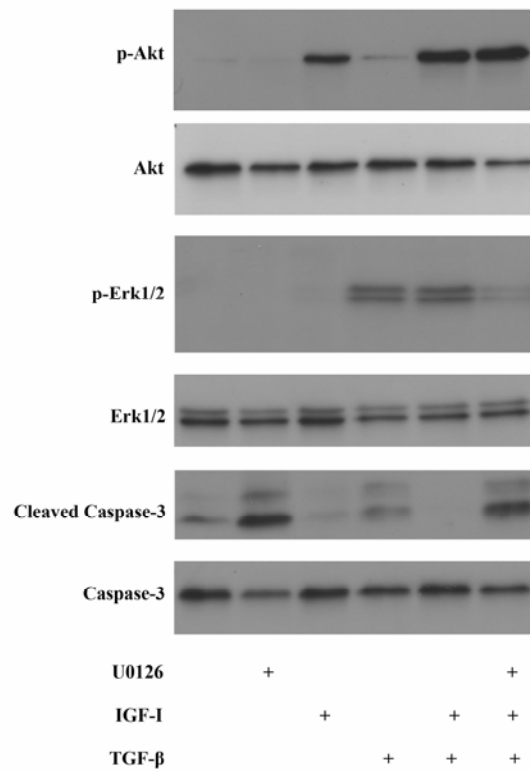
Figure 3-8. The TGF-β, MAPK/Erk and PIK3/AKT pathways cooperate to prevent anoikis in FET cells. A. FET cells were treated as described in figure 2. 100ng/mL IGF-I alone or in combination with 10ng/mL TGF-β were added while the cells were in suspension, and the amount of cell death was assessed with the ELISA-based assay after 24h. **B.** Western Blot assays for Caspase-3, AKT and ERK1/2 and their active forms in FET cells treated with IFG-I, U0216 and/or TGF-β1.

Discussion

Survival signals are conveyed to epithelial cells upon engagement of cell surface receptors with the adequate components of the extracellular matrix (ECM), or receptors of neighboring cells (98). Cell death triggered by the lack of appropriate cell-cell or cell-ECM contacts, called anoikis, is postulated to be a mechanism for maintaining tissue architecture, and in the context of cancer progression, it represents a barrier for tumor invasion and metastasis (98-100). Our studies indicate that in a subset of colorectal cancer cell lines, several of such survival signaling cascades, the MAPK/ERK, the PIK3/AKT, and the mitochondrial cell death pathways are modulated by TGF-β signaling, resulting in increased resistance against anoikis. Moreover, these findings provide evidence that the cellular context determines the effects TGF-β exerts on colorectal cancer lines, and that paradoxically, it can induce cell cycle arrest and resistance against anoikis concurrently.

Several reports have shown that tumor cells with an increased ability to survive in conditions of detachment also have an enhanced capacity to metastasize in *in vivo* systems. Tumor cells less susceptible to anoikis due to alterations in proteins involved in integrin signaling (144), cell-cell adhesion (102) or the mitochondrial cell death pathway (145) have greater metastatic potential. Our results suggest then that by increasing resistance to cell death caused by lack of or by inadequate anchorage, TGF-β signaling enhances the capacity of some colorectal cancer cells to invade and colonize secondary sites. This finding strongly supports previous data that indicate that late-stage events in colorectal cancer progression can be promoted by cell-autonomous TGF-β signaling (37).

Our results also highlight that TGF-β's effects on tumor progression are context-dependent. We were able to establish that even though TGF-β stimulation causes growth inhibition of HCT116+Chr3, CBS, Moser, GEO, and FET; it induces different responses at the level of anoikis in these colorectal cancer lines. It is very likely that the diversity in the responses is due to the different oncogenic backgrounds of these cell lines even though they were all derived from human colorectal cancers. Accordingly, we established that TGF-β inhibits cell death upon detachment in HCT116+Chr3 and CBS, and that in HCT116+Chr3 this protective effect is associated with the activation of the MAPK/ERK and the PIK3/AKT pathways, as well as with the modulation of expression of pro-apoptotic Bim and anti-apoptotic Bcl-2. In contrast, the protective effect in CBS is associated only with TGF-β-induced activation of MAPK/ERK and the upregulation of Bcl-2 expression. Also, we found that exogenous TGF-β does not have an effect on the occurrence of anoikis in Moser or GEO cells, which we predict is related to the inability of TGF-β to modulate the activity of the MAPK/ERK or PIK3/AKT pathways in these cell lines. Finally, we were able to determine that in the background of exogenous activation of PIK3/AKT, TGF-β promotes resistance against anoikis in FET cells, and that activation of the MAPK/ERK pathway was required for this protective effect.

A number of reports have shown that TGF-β signaling exerts a protective effect against apoptosis triggered by different stimuli in various cell systems, and that diverse molecular mechanisms mediate the resistance against cell death in these different cell lines. Thus, TGF-β protects microglia cells from FasL-induced death by a mechanism dependent on the activity of MEK (146). In contrast, neuronal cells are protected from apoptosis caused by Ca^{2+} overloading or growth factor deprivation by TGF-β-induced

upregulation of Bcl-2 expression (135). Furthermore, TGF-β prevents cell death upon

growth factor withdrawal to mammary epithelial cells in an AKT-dependent fashion (95).

We now provide evidence of the molecular mechanisms through which TGF-β protects

human colorectal cancer lines against cell death caused by the lack of anchorage.

The relevance of the MAPK/ERK and PIK3/AKT cascades in protection against anoikis

has been described in a wide variety of model systems. It has been reported that

oncogenic forms of RAS (147), RAF and AKT (148, 149) promote resistance against

anoikis in epithelial cells, and it has become clear that the contribution of each one of

these pathways to the protective effect depends on the cell type. Furthermore, it has been

reported that TGF-β signaling interacts with these two survival pathways, both in Smad-

dependent and Smad-independent manners, and in concert, they modulate several cell

responses of tumor cells to diverse apoptotic stimuli (reviewed in (150)).

The results of our studies also implicate the regulation of the Bcl-2 family of proteins in

TGF-β-mediated protection against anoikis. We found that the pro-apoptotic BH3-only

protein Bim, and its downstream target, the pro-survival protein Bcl-2 are modulated by

TGF-β in HCT116+Chr3 deprived from anchorage. Bim is an important regulator of

apoptosis in epithelial cells, and downregulation of its expression in MCF-10A cells

prevents the occurrence of anoikis (151, 152). Interestingly, in MCF-10A cells

upregulation of Bim expression after loss of anchorage requires the activity of the

MAPK/ERK pathway, but not of PIK3/AKT (151). In contrast, we found that in

HCT116+Chr3 maintained in suspension, upregulation of Bim expression is independent

of both MAPK/ERK and PIK3/AKT activities, since use of the pharmacological

inhibitors U0126 or LY294002 did not effect Bim expression.

Finally, Bcl-2 is aberrantly overexpressed in a number of human cancers, and the overexpression of Bcl-2 has been shown to promote resistance against apoptosis induced by different stimuli, including the lack of attachment (145). Bcl-2's survival function is neutralized by its interaction with BH3-only proteins like Bim, and by post-transcriptional modifications (153). Our results indicate that the TGF-β-induced increase in Bcl-2 expression observed in HCT116+Chr3 may be due in part to the downregulation of Bim expression, but also to regulation of Bcl-2 phosphorylation in Serine 70, which also determines its survival function (NMM and WMG unpublished data). Thus, these results suggest that protein kinases that have been associated with Bcl-2 phosphorylation such as JNK or PKC, may play a role in TGF-β-mediated protection against anoikis.

Thus, we provide evidence of the molecular mechanisms through which cell-autonomous TGF-β signaling is able to promote colon cancer progression. Furthermore, our studies highlight the relevance of the cellular context in the determination of the cell responses to TGF-β stimulation. Hence, if the TGF-β signaling pathway is to be considered for potential therapeutic strategies, it will be crucial to evaluate the oncogenic background of each neoplasm in order to determine the impact of inhibiting a pathway that can be both tumor inhibitor and tumor promoter.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Colorectal cancer is a pathological condition that results from the successive accumulation of genetic and epigenetic alterations in normal colonic mucosa. This type of cancer is the third most common cancer in both men and women in the United States, and the second most common cause of cancer-related death (1). The high incidence of this condition in the population of the developed world, in addition to the modest improvement in the 5-year relative survival rate of patients diagnosed with advanced stages of this disease in the last twenty years, demand a comprehensive investigation of the molecular basis of this type of cancer in order to identify potential targets for therapeutic intervention. Moreover, the growing appreciation of the complexity of the effects that altered oncogenes and tumor suppressor genes have on the behavior of cancer cells also creates the need to study these effects in a context that is relevant to the primary cancer under investigation. Thus, the studies described here were aimed at achieving a better understanding of the role of TGF-β signaling in intestinal cancer development through the use of *in vivo* and *in vitro* model systems in which deregulated TGF-β signaling could be studied in the context of distinct signaling network deregulation patterns. These approaches allowed us to investigate different aspects of this signaling pathway and their impact on the process of intestinal carcinogenesis.

We first generated a novel mouse model in which TGF-β signaling is impaired in the epithelial compartment of both small and large intestines. Using Cre-LoxP technology we designed the *Tgfbr2*^{IEKO} mouse model in which *Tgfbr2* is deleted exclusively in the

epithelial cells of the intestine. It is important to highlight that the $Tgfbr2^{IEKO}$ mice closely replicates a genetic alteration frequently found in at least one fourth of advanced sporadic human colorectal cancers, i.e. inactivating mutations of $TGFBR2$ (35), and it presents several advantages over other approaches that have been used to block or attenuate TGF-β signaling $in\ vivo$. The TGF-β type II receptor is a required component of the heteromeric TGF-β receptor complex, and it is a unit for which no surrogates have been found so far (131); therefore, targeting this gene for deletion ensures complete blockade of all TGF-β-induced cellular responses. This approach to achieve TGF-β signaling inactivation differs from those based on the deletion of TGF-β's downstream effectors, such as $Smad2,\ Smad3$ and $Smad4$, because the latter affect Smad-dependent signals but not those independent from that pathway. Furthermore, animal models based on $Smad4$ constitutive haploinsufficiency disturb not only TGF-β signals but also those from BMP ligands, which have been shown to be essential for normal development of the intestinal epithelium (104). Alternative strategies that have been employed to the same end have other significant limitations. Constitutive deletion of $Tgfb1$ in immunodeficient animals, for instance, impairs TGF-β signaling in epithelium, stroma and immune compartments, all of which are involved in the development of intestinal neoplasms; and the use of dominant-negative TGFBR2 transgenes may affect both TGF-β- and BMP-induced signals (128). Consequently, the specific effects of TGF-β signaling inactivation in tumor cells in such animal models are not clear. Therefore, the $Tgfbr2^{IEKO}$ mouse model is an innovative and important tool for evaluating the specific effects of TGF-β signaling pathway inactivation on intestinal biology and pathologies such as carcinogenesis.

Our studies with the *Tgfbr2*[IEKO] mouse model show that deletion of *Tgfbr2* in the epithelial cells of small intestine and colon does not appreciably alter the homeostasis of these tissues. Accordingly, *Tgfbr2*[IEKO] mice do not display changes in normal intestinal epithelial cell proliferation in comparison to *Tgfbr2*[E2flx/E2flx] mice, and the intestinal tissue architecture of these two genotypical groups is identical. Additionally, we found that abrogation of TGF-β signaling in this cellular compartment does not promote the development of spontaneous tumors even in elderly animals. These findings challenge the significance of a multitude of reports based on *in vitro* studies that stress the importance of the cytostatic effect of TGF-β on most epithelial cells (78). Our results also suggest that there are mechanisms that compensate for the lack of TGF-β activity in *in vivo* circumstances. Accordingly, at least in the absence of tumor-initiating alterations, Activin-induced activation of Smad2/3 and other non-Smad pathways may be a part of the mechanism that compensates for the deficiency of TGF-β signaling in intestinal epithelial cells *in vivo*. Thus, in the context of an intact signaling network, loss of TGF-β mediated signaling in the intestinal epithelium can be accommodated and the tissue can maintain its normal histological state and gross function.

The *Tgfbr2*[IEKO] mouse model also allowed us to evaluate the effect of inactivating TGF-β signaling in epithelial cells in which the Wnt/β-catenin pathway is aberrantly activated, a situation that recapitulates a set of conditions commonly observed in human intestinal tumors. Thus, the *Apc*[1638N/wt]; *Tgfbr2*[IEKO] mouse demonstrated that the inactivation of TGF-β signaling in the background of oncogenic activation of the Wnt/β-catenin pathway dramatically promotes intestinal tumor progression. The synergistic cooperation between these two genetic alterations is reflected in a highly significant increment in the

number of adenocarcinomas in the $Apc^{1638N/wt}$; $Tgfbr2^{IEKO}$ animals compared to the $Apc^{1638N/wt}$; $Tgfbr2^{E2flx/E2flx}$ control group. Importantly, the progression from benign lesions to invasive adenocarcinomas in these animals does not seem to be due to the lack of the growth inhibitory effect of TGF-β over epithelial tumor cells, because we found that cell proliferation in tumors with or without *Tgfbr2* is not statistically different, similarly to what we found in the normal intestinal mucosa of these mice. We established, nevertheless, that TGF-β directly regulates the expression and activity of MMP-2 and MMP-9 in tumor epithelial cells, which we predict is associated with the increased invasiveness of tumors that lack *Tgfbr2*. These results suggest that primary effects of TGF-β signaling inactivation in *Apc*-initiated neoplasms are deregulation of host-tumor interactions and extracellular matrix remodeling, and not cytostasis of tumor epithelial cells.

Matrix metalloproteinases are the principal ECM-degrading enzymes. During the formation of tumor metastasis, the proteolytic activities of these enzymes are required for the process of ECM-remodeling associated with tumor cell invasion of the stroma, intra- and extravasation into and from the circulatory system, respectively (154). Several studies have reported increased protein expression and activity of MMP-2 and MMP-9 in tumor tissue from colon cancer patients in comparison to their normal colonic mucosa (reviewed in (154)). Moreover, some of such studies have indicated that there is a positive correlation between the expression of these proteases and the Dukes' tumor stage (155, 156). These findings reinforce the concept that the activities of MMP-2 and MMP-9 are required for tumor invasion and metastasis, which according to our studies, are at least partially regulated by TGF-β signals conveyed to tumor epithelial cancer cells.

Previous studies have indicated that TGF-β controls the expression of MMP-2 and MMP-9 in a cell type-dependent fashion. It has been shown that TGF-β stimulation to different types of cancer cells induces the expression and activity of one or both of these two enzymes, and that their expression is associated with increased capacity of the tumor cells to migrate and invade in *in vitro* assays (157, 158). Conversely, it has been established that TGF-β can also have a negative impact on MMP activity by promoting the expression of tissue-specific inhibitors of these enzymes in certain cell types. Accordingly, TGF-β treatment of fibrosarcoma cells induces the expression of Tissue Inhibitor of Metalloproteinase-1 or TIMP-1, which impairs the ability of these cells to invade in *in vitro* conditions (159).

Through our animal model we were also able to establish that tumor tissues derived from *Apc*$^{1638N/wt}$; *Tgfbr2*^{IEKO} animals contain higher amounts of TGF-β1, which are likely related to the intense stromal component and mucinous cysts observed in these tumors. Furthermore, the analyses we carried out with tumor-derived epithelial cells *in vitro* suggest that reduced consumption of TGF-β1 by *Apc*$^{1638N/1638N}$;*Tgfbr2*^{IEKO} cells may be an important contributor to the overall increase in the amount of this cytokine found in the tumors. It has been established that TGF-β stimulation promotes proliferation and activation of tumor-associated fibroblasts (82). Furthermore, high levels of TGF-β1 in the tumor microenvironment have also been associated with defective immune responses against tumor cells and with increased formation of blood vessels (52, 68). These observations highlight the significance of paracrine TGF-β signaling as mediator of the interactions between tumor epithelial cells and the stromal and immune components of the tumor. Additionally, since the *Apc*$^{1638N/wt}$; *Tgfbr2*^{IEKO} mice is a conditional knockout

model, it facilitates a more accurate analysis of TGF-β-mediated interactions between epithelial and stromal tumor cells that are critical for tumor progression.

In aggregate, our data indicate that TGF-β signals conveyed to intestinal epithelial cells with abnormally activated Wnt/β-catenin pathway potently suppresses tumor progression, and that this is not entirely caused by TGF-β's growth inhibitory effect, but very likely by TGF-β-regulated host-tumor interactions and extracellular matrix remodeling.

In marked contrast, the *in vitro* studies we carried out with human colorectal cancer cell lines indicate that TGF-β signals can exert a pro-tumorigenic effect by protecting cancer cells from apoptosis triggered by inadequate cell-cell or cell-matrix interactions. Thus, we were able to establish that TGF-β stimulation in the context of MAPK/ERK and/or PIK3/AKT activation induces resistance against anoikis in a subset of human colorectal cancer cell lines, and this is also associated with modulation of the expression of Bcl-2 family members. These results provide plausible mechanisms to explain conflicting data from different epidemiological studies that have suggested that maintenance of an intact TGF-β pathway in tumor cells has pro-tumorigenic effects in colorectal cancer(38, 39), as our data indicate that these conflicting results may reflect the context dependence of the effects of TGF-β signaling inactivation on the behavior of colorectal cancer cells.

Through the use of both *in vivo* and *in vitro* model systems, the studies described here enhance our understanding of the role of TGF-β signaling in the process of intestinal carcinogenesis. Our data show that in this type of malignancy TGF-β signals paradoxically contribute to both tumor suppression and tumor promotion by virtue of their effects on tumor epithelial and stromal cells (figure 4-1). Moreover, they reiterate that the ultimate effect of TGF-β signals, or the lack of them, is determined after the
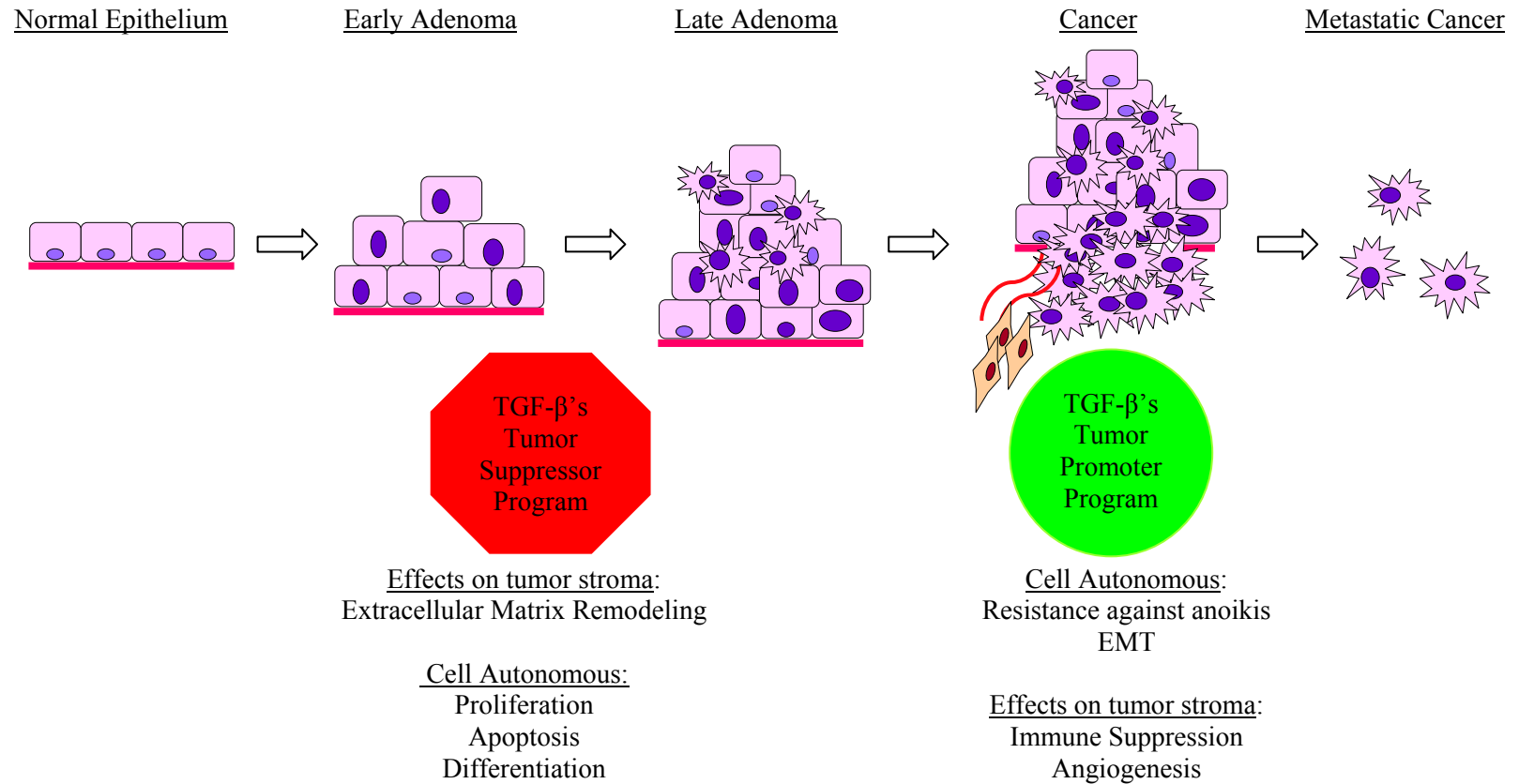
Figure 4-1. Model of the role of TGF-β signaling in intestinal cancer development

integration of multiple signals simultaneously conveyed to tumor cells. Thus, in the background of deregulated signaling networks, such as abnormally activated Wnt/β-catenin, PIK3/AKT and/or MAPK/ERK signaling pathways, TGF-β signals transmitted to tumor cells are modulated differently than in normal conditions, and consequently their effects are quite distinct from those seen in normal cells.

The paradoxical effects of TGF-β signaling in our model systems of intestinal cancer are apparent in several respects. In the murine model we found that tumor progression is suppressed by the expression of an intact *Tgfbr2* in intestinal epithelium of *Apc*$^{1638N/wt}$; *Tgfbr2*$^{E2flx/E2flx}$ mice. In this case, we determined that the ability of TGF-β to inhibit tumor progression is probably related to a cell-autonomous function of this cytokine, i.e. TGF-β's ability to downregulate the expression and activity of matrix metalloproteinases in tumor epithelial cells. However, the human colorectal cancer model systems we studied showed that TGF-β stimulation to the cell lines HCT116+Chr3 and CBS maintained *in vitro* confers on them resistance to anoikis and, presumably, it enhances their metastatic potential. These results suggest that through another cell–autonomous mechanism TGF-β signaling can promote cellular behaviors associated with tumor progression. On the other hand, we established that intestinal tumors from *Apc*$^{1638N/wt}$; *Tgfbr2*^{IEKO} mice contain high amounts of TGF-β1 that, as described previously, alters host-tumor interaction favoring tumor progression. Thus, TGF-β signaling on stromal cells may promote tumor progression in the *Apc*$^{1638N/wt}$;*Tgfbr2*^{IEKO} animal model through cell-nonautonomous mechanisms. These conflicting effects, that illustrate various cell-autonomous and -nonautonomous functions of TGF-β, underscore the relevance of the

cellular context and state on the definition of the net effect of TGF-β signals on tumor development.

An interesting point that our results raise is the observation that in tumors from the $Apc^{1638N/wt}$; $Tgfbr2^{E2flx/E2flx}$ mice, in which $Tgfbr2$ is not deleted and that presumably maintain active TGF-β signaling, TGF-β signals do not seem to exert tumor promoting functions as we anticipated from the data obtained from our human colorectal cancer systems. According to that, we expected that advanced tumors derived from epithelial cells responsive to TGF-β would progress to metastatic cancer more frequently than those that are not responsive. Our analysis from the ATT cell line described in chapter 2 demonstrate that these tumor-derived epithelial cells do not undergo spontaneous mutations that would inactivate TGF-β signaling, although this pathway might have been attenuated to a certain extent considering the modest induction of the p3TPlux reporter activity. Therefore, if these cells are representative of the tumors of this genotype, epithelial cells from these neoplasms do not acquire the oncogenic background that allows TGF-β signaling to act as a tumor promoting factor. Indeed, given that TGF-β-induced resistance against anoikis in human colorectal cancer cells is associated with aberrant activation of the PIK3/AKT and/or MAPK/ERK pathways, we evaluated AKT and ERK1/2 activation in the ATT line with and without TGF-β1 stimulation. The results of this assay indicated no TGF-β-induced activity of these pathways (not shown). Therefore, we propose that in the absence of cross-talk between TGF-β and these two signaling pathways, tumors from $Apc^{1638N/wt}$; $Tgfbr2^{E2flx/E2flx}$ do not progress to metastatic cancer. Future research with genetically engineered mice with activated PIK3/AKT and/or MAPK/ERK pathways, for instance, and bred to the $Apc^{1638N/wt}$; $Tgfbr2^{E2flx/E2flx}$

and $Apc^{1638N/wt}$; $Tgfbr2^{IEKO}$ mice could provide additional insight into this proposed model. Alternatively, it is also plausible that in *in vivo* settings, TGF-β's oncogenic effects are predominantly through its influence on host-tumor interactions, as observed in the $Apc^{1638N/wt}$; $Tgfbr2^{IEKO}$ model, and through tumor promoting cell-autonomous functions like protection against anoikis are rather infrequent.

Inactivating mutations of *TGFBR2* and *SMAD4* are frequently found in colon cancer (35), and they might be biologically equivalent. However, since TGF-β signaling can influence Smad-independent signaling cascades, it would be valuable to determine the difference between abrogating all TGF-β-mediated cellular responses and inactivating only those mediated by the canonical Smad pathway. Thus, generation of a *Smad4* intestinal conditional knockout model by crossing $Smad4^{co/co}$ mice (160), for example, with $Apc^{1638N/wt}$;*Villin*-Cre animals should facilitate the analysis of the biological differences between inactivating *Tgfbr2* or *Smad4* in the background of Wnt/β-catenin activation. The results of such studies may have some therapeutic relevance for the treatment of the two kinds of neoplasms.

TGF-β signaling certainly has an important role in the development of most human solid tumors, and as such it has been considered as a very suitable therapeutic target. However, in view of the dual nature of this cytokine the decision to enhance or to repress TGF-β signals in order to prevent or treat tumor development in patients depends on the characteristics of each specific case. For early stage tumors it could be possible to attempt restoring or enhancing TGF-β signals so as to intensify the growth inhibitory effects of this cytokine. Hence, in cases in which the defect in the pathway is due to reduced receptor expression or activation of other oncogenes, the use of small molecules

may be beneficial. *In vitro* analyses have shown that treatment with the histone deacetylase inhibitor MS-275 inhibits proliferation of breast cancer cells refractory to TGF-β-induced growth arrest, and that its effect is associated with a significant increase in TGFBR2 expression (161). Similarly, it has been demonstrated that Captopril, an angiotensin-converting enzyme inhibitor, and FTI-277, a farnesyltransferase inhibitor, promote the expression of TGFBR2 in renal and pancreatic cancer cells, respectively. Thus, these reports suggest that increasing TGFBR2 expression in cancer cells with an attenuated but functional TGF-β pathway sensitizes them to the growth inhibitory signals of this cytokine (162, 163).

In contrast, in instances in which excess of TGF-β signaling has tumor-promoting effects, like in advanced or metastatic disease, it would be desirable to inhibit the activity of this pathway. To this end, several strategies are under development and include natural and synthetic inhibitors of TGF-β (decorin and the antisense oligonucleotide AP-12009); TGF-β neutralizing antibodies (Lerdelimumab, Metelimumab, and GC-1008); soluble forms of TGFBR2 and TGFBR3 (SR2F and Betaglycan); and TGFBR1 kinase inhibitors (LY550410, SB-505124 and SD-208) (reviewed in (164)). All these approaches have promising therapeutic potential given the magnitude of all the tumor promoting activities of TGF-β signaling; however, their use in colorectal cancer has to be carefully evaluated in light of the results of our animal model and those from Wu and collaborators (165). Their work shows that abrogation of TGF-β1 and TGF-β2 expression in the weakly tumorigenic human colon cancer cell line CBS dramatically elevates tumor growth in a xenograft model. Thus, the data collected from these two *in vivo* models strongly suggest that blockade of TGF-β signaling to treat intestinal tumors that exploit the pro-

tumorigenic program of TGF-β signals would potentially promote the progression of other tumors at earlier phases of development in which TGF-β signaling is predominantly a tumor suppressing pathway.

In summary, in order to design effective cancer therapies that target the TGF-β signaling pathway, it is crucial to identify the factors that determine the balance between the opposing properties of TGF-β signals such us the ones described in this study. Hence, in order to formulate personalized therapies based on the manipulation of TGF-β signaling in tumors, it is necessary to establish the influence of other signaling cascades on TGF-β signals in a context-dependent fashion and also their mechanism of action. Therapeutic approaches designed with such underlying principles should potentially have better efficacy and less toxicity than the ones that are in use currently.

It is important then to consider therapeutic approaches based on the regulation of the specific signal transduction pathways that result aberrantly up- or down-regulated as consequence of TGF-β signaling inactivation because they could represent viable alternatives to the strategies based on direct manipulation of the TGF-β pathway. According to our findings, the expression and activity of MMP-2 and MMP-9 is up-regulated in intestinal tumor epithelial cells that lack *Tgfbr2*, therefore therapeutic routes leading to the specific inhibition of these proteases during intestinal cancer progression could be beneficial, and they may also circumvent the potential deleterious effects of complete TGF-β signaling inactivation.

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