Spring 5-6-2017

Understanding the Relationship between TGF-Beta and IGF-1R Signaling in Colorectal Cancer

Katie L. Bailey
University of Nebraska Medical Center

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Understanding the Relationship between TGF-β and IGF-1R Signaling in Colorectal Cancer

by

Katie Bailey, Ph.D.

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Department of Eppley Cancer Center
Under the Supervision of Professor Jing Wang

University of Nebraska Medical Center
Omaha, Nebraska
April, 2017

Supervisory Committee:

Jing Wang, Ph.D.           Jennifer Black, Ph.D.
Xu Luo, Ph.D.              Joyce Solheim, Ph.D.
Understanding the Relationship between TGF-β and IGF-1R Signaling in Colorectal Cancer

Katie Bailey, Ph.D.

University of Nebraska Medical Center, 2017

Advisor: Jing Wang, Ph.D.

Early in colorectal cancer development, there is loss of tumor suppressor pathways and gain of oncogenic pathways. TGFβ signaling acts as a tumor suppressor pathway early in cancer development by regulating transcription factors that are responsible for cellular growth and apoptosis. Unfortunately, some aspect of the TGFβ signaling pathway is lost in approximately 30-40% of colorectal cancer patients. Another pathway that is deregulated in 30% of human colon cancer is the oncogenic IGF-1R pathway. The dysregulation of these pathways leads to colorectal cancer progression.

The TGFβ signaling pathway activates the receptor-regulated Smads (Smad2/3) that translocate into the nucleus and regulate various target genes. Previous studies have shown that Smad2 and Smad3 can play different roles in the progression of cancer. In PDAC cells, the knock down of Smad3 led to increased proliferation, while the knock down of Smad2 decreased cellular migration. Studies from our lab have shown repression of vascular endothelial growth factor A (VEGFA) expression was TGFβ/Smad3 dependent but not Smad2. Another lab looked at the different types of proteins that interacted with Smad2 and Smad3. Insulin Receptor Substrate (IRS)-1 was found to associate with Smad3.

IRS-1 is an adaptor protein in the IGF-1R signaling pathway. When IGF-1R becomes auto phosphorylated, then IRS-1, or IRS-2, can bind and create docking sites for downstream signaling pathways involved in promoting tumorigenesis. We found that with the loss of TGFβ signaling
there was increased expression and phosphorylation of IRS-1 that was associated with increased proliferation and decreased cell death. We concluded that IRS-1 was being regulated in a TGFβ/Smad3-dependent manner. Upon knocking down Smad2, we observed decreased expression and phosphorylation of IGF-1R and the downstream markers, along with decreased proliferation and increased cell death. We demonstrated for the first time Smad2 and Smad3 have antagonist roles in the progression of colorectal cancer.

The significance of this dissertation work is the identification of the novel cross talk between TGFβ and IGF-1R signaling at the level of Smad2 and Smad3 regulation. These results give insight into new potential therapeutic targets and a better understanding of how the TGFβ and IGF-1R signaling pathways are working during the progression of colorectal cancer.
Dedication

I would like to dedicate this dissertation work to my wonderful and loving husband, who has been my rock through this whole experience.
Acknowledgement

I would like to thank my former advisor, Dr. Michael Brattain, for all the guidance and support he gave me over the 5 years I worked with him. Through his guidance I have learned how to be an independent and critical thinker. He not only taught me so much about cancer biology but also important life skills that I use everyday. I was very fortunate to have known such a brilliant person and one of the key lessons I will take away from being his student is to never stop asking questions and looking for the answers.

I would like to thank Dr. Jing Wang for taking on the role of being my advisor my last year of graduate school. I feel like the last year of your graduate career is when you need your advisor the most and she has stepped up. Thank you for helping me finish my last year.

I would like to thank everyone on my supervisory committee for all your support and guidance. I would especially like to thank Dr. Joyce Solheim. Throughout my 6 years she has been the one person I know I can turn to when I’m lost or have questions. She made my graduate experience so enjoyable and I will always remember her.

I would like to thank all the past members of Brattain lab and the present members of Wang’s lab. Everyone in both labs were extremely helpful if I ever had any questions and enjoyable to work with.

Lastly, I would like to thank my family and especially my little ray of sunshine, my son.
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>RI</td>
<td>TGFβ receptor type I</td>
</tr>
<tr>
<td>RII</td>
<td>TGFβ receptor type II</td>
</tr>
<tr>
<td>R-Smads</td>
<td>receptor-regulated Smad2/3</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Type I insulin-like growth factor receptor</td>
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<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
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<tr>
<td>GFDS</td>
<td>Growth factor and serum deprivation stress</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin embedded</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
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<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite Instability</td>
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<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor-A</td>
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<td>IPA</td>
<td>Ingenuity pathway analysis</td>
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<td>PKA</td>
<td>cAMP-dependent Protein Kinase A</td>
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<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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<td>MH1</td>
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Chapter I

Introduction
1.1 Overview of colorectal cancer

Colorectal cancer is the third leading cause of cancer related deaths in the United States (1) and is defined as either having cancer in the colon or the rectum (2). There are 4 different stages that are used to diagnose how aggressive the cancer is and to determine the appropriate action for treatment (2). The stages are: in situ, local, regional and distant. In situ is a precancerous polyp that has not invaded the wall of the colon and is 100 percent treatable by removal of the polyp. Local is cancer cells that have spread into the wall of the colon (3). When cancer is removed at this stage the 5-year survival rate is greater than 90 percent (4). Regional cancer is broken down into 2 stages, where the cancer has either spread completely through the muscle layer of the colon and not spread to nearby organs or has spread to nearby lymph nodes (3). The 5-year survival rate for regional disease is roughly between 60-80% depending on if the cancer has spread or not (4). The last stage is known as distant because the cancer has spread to another organ system that is not near the colon (3). The final stage has a 5-year survival rate of less than 10% (5). The current treatment options for individuals who are diagnosed with distant disease are chemotherapy, radiation and targeted therapy that can be used in combination or alone (2).

For colorectal cancer to progress, 3 types of events need to occur: oncogenes gain function, loss of tumor suppressor function and alterations in DNA methylation patterns (4). The Vogelgram model describes the molecules and processes involved in the transformation of normal colon epithelium into carcinoma (Fig. 1.1) (6). One process that is of importance is the loss of tumor suppressor transforming growth factor β (TGFβ) signaling. TGFβ signaling is lost in roughly 30% of patients that have colorectal carcinoma (7). A common occurrence that leads to the loss of tumor suppressive signaling is through a mutation in the TGFβ receptor type II (RII)
from microsatellite instability during the late adenoma stage of colorectal cancer that leads to carcinoma (6).

1.2 TGF-β signaling and cancer regulation

TGFβ signaling is mainly present during embryonic development and plays a key role in tissue homeostasis (7). The TGFβ ligand is a cytokine that binds to RII, causing auto phosphorylation on the receptor, which then transphosphorylates TGFβ receptor type I (RI). The phosphorylation of RI leads to the activation of this Ser/Thr kinase receptor that can now bind to and phosphorylate receptor-regulated Smads-2 and 3 on their C-terminal ends (8). The Smad proteins have two conserved regions, which are known as the N-terminal Mad homology domain-1 (MH1) and C-terminal Mad homology domain-2 (MH2) (8). Once receptor-regulated Smads are phosphorylated they can form a complex with the mediator Smad4 and accumulate in the nucleus effecting transcription of various genes that regulate many aspects within the cell (Fig. 1.2) (9).

TGFβ signaling regulates proliferation and apoptosis, and modifies the microenvironment (7). When TGFβ signaling is lost, patients develop more preneoplastic lesions and polyps that advance into colorectal carcinoma (9).

Work from our lab has shown that when TGFβ is reintroduced into colorectal cancer cells the expression and activity of various oncogenic pathways are regulated, leading to cell death and decreased metastasis (10-13). The phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT) pathway is one of the oncogenic pathways regulated by TGFβ. Previous work from our lab has shown that with the loss of TGFβ, AKT activity increases leading to cell survival (12). However, if TGFβ signaling is re-expressed this pathway can inhibit AKT phosphorylation and destabilize the X-linked inhibitor of apoptosis protein (XIAP)/Survivin (cell survival) complex, leading to cell death (14).
1.3 IGF-1R signaling pathway and role in cancer

PI3K/AKT are molecules that are activated downstream from the type I insulin-like growth factor receptor (IGF-1R) signaling pathway (15). IGF-1R is a tyrosine kinase receptor that becomes auto phosphorylated upon binding of the IGF-1 ligand to the receptor. Insulin and IGF-2 are ligands that can also bind to IGF-1R and activate the pathway but the main ligand that has the highest binding affinity is IGF-1 (15). Once IGF-1R becomes auto phosphorylated at the C-terminus this creates a docking site for insulin receptor substrate-1 and 2 (IRS-1 and 2) (Fig. 1.3) (16). IRS-1 and 2 are adaptor proteins that bind to IGF-1R through their pleckstrin homology (PH) and phospho tyrosine binding (PTB) domains located at the N terminus (Fig. 1.4) (17). Upon binding to IGF-1R, IRS-1 and IRS-2 become phosphorylated on their C-terminus, which creates docking sites and activation of downstream signals (17). IGF-1R signaling is over expressed in many types of cancers and plays a role in protein synthesis, cell survival, gene transcription, proliferation, cell motility and metastasis (18-20). IRS-1 and IRS-2 have also shown to play a role in the progression of many types of cancers, including breast and colon (21, 22). When IRS-1 and IRS-2 are over expressed in a breast cancer cell line (MCF-7) there is increased migration capability seen in the cells (23). In vivo studies have shown this same phenotype with increased tumorigenesis and metastasis when IRS-1 and IRS-2 are over expressed in the mammary gland (24). Since the IGF-1R pathway is over expressed in more than 30% of patients that have late stage colorectal cancer our lab is working on trying to identify molecular targets within the IGF-1R pathway that can be used for therapeutic purposes.

1.4 Scope of the dissertation

Even with advances in understanding late stage colorectal cancer there needs to be new treatment strategies and interventions created to help prolong the life of individuals who are diagnosed with late stage colorectal cancer. We identified a relationship between tumor
suppressive TGFβ and oncogenic IGF-1R signaling in a colorectal cancer cell line. The first part of my dissertation is focused on my research to understand this cross-talk relationship and the impact this relationship has on the progression of colorectal cancer. We discovered a novel cross talk relationship between TGF-β/Smad3/IRS-1 that affects cell survival and growth in colon cancer cells. We also identified a novel interaction between Smad-2 and IGF-1R that impacts the tumorigenesis of colorectal cancer cells. Understanding these signaling interactions in colon cancer will help find better therapeutic options for people who match the same type of profile.
**Figure 1.1 Progression model of colorectal cancer**

The Vogelgram model shows the progression of colorectal cancer disease from the normal epithelium to cancer and the associated markers that are either lost or over expressed with the advancement of disease. Figure reproduced from Walther et al. (2009) Nature Reviews doi:10.1038/nrc2645 with permission.
Figure 1.1
Figure 1.2 TGF-β signaling pathway

Diagram illustrating the steps of the TGFβ signaling pathway upon ligand binding and activation.

Figure reproduced from Lampropoulos et al. (2011) Cancer Letters doi: 10.1016/j.canlet.2011.09.041 with permission.
Figure 1.2
**Figure 1.3 IGF-1R signaling pathway**

Diagram showing some of the downstream effects once the IGF-1R pathway is activated. IRS-1 and IRS-2 can both activate the PI3K signaling pathway leading to increased cell survival, migration and proliferation. GRB2 can bind to phosphorylated IRS-1 and induce cellular differentiation and proliferation. IRS-1 can translocate into the nucleus and act as a transcriptional co-factor that helps in promoting transcription of various genes involved in cell cycle progression, like cyclin D1.
Figure 1.3
**Figure 1.4 IRS proteins**

IRS-1 and IRS-2 protein structures are shown. N-terminus contains the PH and PTB domain. The C-terminus becomes phosphorylated on tyrosine residues upon binding to IGF-1R and allows for binding of downstream effector molecules. Figure reproduced from Mardilovich et al. (2009) Cell Communication and Signaling, BioMed Central doi: 10.1186/1478-811X-7-14 with permission.
Figure 1.4

IRS-1

PH | PTB | PI3K | Grb2 | SHP2

IRS-2

PH | PTB | KRLB | PI3K | Grb2 | SHP2
Chapter II

Materials and methods
2.1 Cell lines and reagents

The human colon cancer cell lines FET, FET-DNRII, CBS, CBS-RRII and GEO were described previously (13). All cells were cultured in McCoy's SA serum-free medium (Sigma) supplemented with 10 ng/ml epidermal growth factor (EGF), 20 µg/ml insulin, and 4 µg/ml transferrin (25). When cells were subjected to growth factor deprivation stress (GFDS), they were cultured in McCoy's SA serum-free medium in the absence of EGF, insulin and transferrin. Cells were maintained at 37°C in a humidified incubator with 5% CO2. TGFβ and the TGFβ RI kinase inhibitor (Alk5i) were obtained from R&D Systems and Calbiochem respectively. Alk5i is an ATP competitive inhibitor. Colon cancer cells were treated with 5 µM of Alk5i for 2 hours followed by 1 hour incubation with TGFβ (5 ng/ml). The following antibodies were used in this study: anti-IRS-1 (1:1000), Cell Signaling #2382, anti-IRS-2 (1:1000), Cell Signaling #4502, anti-p-IRS-1Y632 (1:600), Santa Cruz sc-17196, anti-p-IRS-1/2Y612 (1:600), Santa Cruz sc-17195-R, anti-IGF-1R (1:1000), Cell Signaling #3027, anti-p-IGF-1R, Cell Signaling #3918, anti-Smad3 (1:1000), Cell Signaling #9523, anti-p-Smad3S423/425 (1:1000), Cell Signaling #9520, anti-Smad2 (1:1000), Cell Signaling #3122, anti-p-Smad2S465/467 (1:1000), Cell Signaling #3101, anti-AKT (1:1000), Cell Signaling #9272, anti-p-AKT573 (1:1000), Cell Signaling #9271, anti-p-ERK202/Y204 (1:1000), Cell Signaling #9101, anti-XIAP (1:1000), Cell Signaling #14334, anti-Cyclin D1 (1:1000), Cell Signaling #2978 and anti-Actin (1:1000), Sigma #A2066.

2.2 Transfections

2.2.1 Transformation and starter culture

To amplify the vector concentration, to have a successful transfection, we took 7 µL of the vector to 50 µL of the competent DH5α cells. The mixture was kept on ice for 30 minutes. Then we used heat shock for 45 seconds in a 42°C water bath to allow the vector to be taken up by the
competent cells. After heat shock the competent cells were kept on ice for 2 minutes. Then 500 µL of LB media were added to the competent cells and the cells were put on the shaker for 1 hour at 37°C. Once the transformation was complete, a starter culture was created using 100 µL of the transformation solution, 5 mL of LB media and 20 µL of ampicillin to select for the bacteria that contain the desired vector.

2.2.2 Stable

FET cells were infected with a retroviral shRNA vector targeting Smad3 as described previously (10). A scrambled shRNA was used as a control. Smad3 knockdown was confirmed by western blot analysis. A stable shRNA vector of Smad2 was generously given from, John A. Copland III. The stable over expressing CD8-IGF-1R vector was generously provided by Dr. Adrian Lee’s lab. To generate stable cell lines, we followed the protocol from Thermo Scientific Open Biosystems.

2.2.3 Transient transfection of siRNA

IRS-1 ON-TARGETplus Human SMARTpool siRNA IRS-1 #3667 (Thermo Scientific) was transiently transfected into colon cancer cells according to the Dharmacon siRNA transfection protocol.

2.3 Western blot analysis

Cells were lysed in TNESV buffer containing 50 mM Tris, 150 mM NaCl, 0.5% NP-40, 500 mM EDTA and 10% SDS, 5 mM Na3VO4, 50 mM NaF and 10 µg/ µL of β- glacerophosphate and 1 mM PMSF. Proteins were separated by a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked using 5% milk in TBST at room temperature for an hour. After blocking, the membrane was incubated with a primary antibody followed by incubation with a secondary antibody. After washing, enhanced chemiluminescence detection was used to visualize the proteins of interest.

2.4 Proliferation and apoptosis assays
For proliferation assays, cells were plated at 3x10³ cells per well in a 96-well plate. Five days later, cells were stained for 2 hours with 3-(4,5 Dimethylthiazol-2-yl)-2,5-
diphenyltetrazoliumbromide (MTT) (Sigma). The OD at 570 nm was read on a ELx808™
Absorbance Microplate Reader (BioTek, Winooski) after the purple precipitates were dissolved
in DMSO.

Apoptosis was detected using a DNA fragmentation ELISA assay kit (Roche) according to the
manufacturer’s protocol. Briefly, cells were seeded in 96-well plates and subjected to GFDS.
Cells were stained with MTT to determine cell numbers or lysed for ELISA assays to determine
apoptosis. Relative apoptosis was determined by dividing ELISA values by MTT values for each
sample.

2.5 RNA purification and cDNA preparation

RNA was isolated from cells using the Roche high pure RNA isolation kit according to the
manufacturer’s instructions. The amount of RNA isolated was quantified by spectrophotometry
and the samples were kept at -80°C. To create the cDNA, 1 μg of RNA was reverse transcribed
using the TaqMan reverse transcription kit according to Applied Biosystems protocol.

2.6 Quantitative real time PCR

A 1:10 dilution of the prepared cDNA and primer probes, from Applied Biosystems, for Smad3
was used. GAPDH was used as a standardizing control. Each sample was replicated 3 times using
the Stepone software version 2.0 (Applied Biosystems), according to the manufacturer’s
protocol. To calculate the fold change of expression as compared to the GAPDH control, the
comparative threshold cycle (Ct) was used.

2.7 TUNEL, Ki67 and IHC staining
Sections of formalin-fixed paraffin-embedded (FFPE) xenograft tumors were mounted on slides and examined for proliferating and apoptotic cells by Ki-67 staining and TUNEL assay, respectively, as described previously (26, 27).

IHC staining was performed on paraffin sections using the Novolink™ Min Polymer Detection System (Leica) as described previously (27, 28). Briefly, slides were subjected to antigen retrieval using Novocastra Epitope Retrieval Solutions, pH 6, followed by incubation with an anti-IRS-1 antibody (1:700, Abcam, ab52167), anti-IRS-2 (1:500, Abcam, #ab46811), anti- p-IRS-1/2Y612 (1:400, Santa Cruz, sc-17195-R) and anti- IGF-1R (1:100, Cell Signaling, #3027) overnight at 4 °C.

Slides were developed with DAB after incubation with Novolink polymer. Finally, slides were counterstained with hematoxylin. For each sample, ten randomly chosen fields were captured at 20 × magnification and quantified with Imagescope Software (Aperio Technologies, Inc.).

2.8 Statistical analysis

Statistical analyses were performed using Student’s t-test and two-way ANOVA. A P value less than 0.05 was considered significant.

2.9 In vivo xenograft studies

Animal experiments were approved by the University of Nebraska Medical Center (UNMC) Institutional Animal Care and Use Committee (IACUC #07-047-08-FC). FET control and DNRII cells (5 x 10^6) were injected into the flank of 4-6 week old male BALB/c nude mice (Harlan Laboratories) that were randomized (n=10). Tumors were measured every other day for 28 days. Tumor volumes (V) were calculated using the formula V = W2 × L × 0.5, where W represents the largest tumor diameter and L represents the next largest tumor diameter (26, 27). Upon termination of the experiments, tumors were dissected, placed in 10% neutral buffered formalin for 48hrs, and processed and embedded in paraffin for TUNEL, Ki67 and IHC analysis.
2.10 Orthotopic implantation studies

The protocol for orthotopic implantations have been described previously (14). GEO-GFP tagged cells (7x10^6) were injected into the dorsal surface of BALB/c nude mice. A xenograft was allowed to grow between 3-4 weeks. The xenograft tissue was then removed and minced into 1mm^3 cubes. Then in another BALB/c nude mouse the tissue was implanted into the subserosal layer of the cecum. Visualization of primary tumor formation and metastasis could be observed through imaging of the GFP.
CHAPTER III

TGFβ/Smad3 regulates proliferation and apoptosis through IRS-1 inhibition in colon cancer cells

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# Deceased

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The following chapter has been published in Plos One, April 2017.
3.1 Abstract

In this study, we have uncovered a novel crosstalk between TGFβ and IGF-1R signaling pathways. We show for the first time that expression and activation of IRS-1, an IGF-1R adaptor protein, is decreased by TGFβ/Smad3 signaling. Loss or attenuation of TGFβ activation leads to elevated expression and phosphorylation of IRS-1 in colon cancer cells, resulting in enhanced cell proliferation, decreased apoptosis and increased tumor growth *in vitro* and *in vivo*. Downregulation of IRS-1 expression reversed Smad3 knockdown-mediated oncogenic phenotypes, indicating that TGFβ/Smad3 signaling inhibits cell proliferation and increases apoptosis at least partially through the inhibition of IRS-1 expression and activation. Additionally, the TGFβ/Smad3/IRS-1 signaling axis regulates expression of cyclin D1 and XIAP, which may contribute to TGFβ/Smad3/IRS-1-mediated cell cycle progression and survival. Given that loss of TGFβ signaling occurs frequently in colon cancer, an important implication of our study is that IRS-1 could be a potential therapeutic target for colon cancer treatment.
3.2 Introduction

Transforming growth factor β (TGFβ) signaling regulates many important cellular functions, including differentiation, proliferation, migration and apoptosis (14). The TGFβ pathway is activated upon TGFβ ligand binding to TGFβ receptor type II (RII), which phosphorylates and activates TGFβ receptor type I (RI), leading to phosphorylation of Smad2/3 (R-Smads). R-Smads then complex with Smad4 and translocate to the nucleus to regulate expression of various target genes (29).

TGFβ signaling has been shown to inhibit cell proliferation, induce apoptosis and suppress tumorigenicity in many colon cancer cell lines (12). In addition, abrogation of TGFβ signaling increases metastasis, whereas enhanced TGFβ signaling suppresses metastasis in an orthotopic model of colon cancer (11, 14). In genetic mouse models, Smad3 or Smad4 mutation increases malignancy and invasiveness of intestinal tumors in APC<sup>min/+</sup> or APC<sup>Δ716/+</sup> mice (30, 31), pointing to a significant role of TGFβ signaling in repressing malignant progression in colon tumors. Indeed, TGFβ signaling is defective in 30% - 40% of colon cancer patients due to defects in TGFβ RII or Smads (32-34), and loss or reduction of TGFβ signaling is tightly associated with the development of metastasis in patient sample studies (35, 36).

In addition to inactivation of TGFβ signaling, cancer cells evade tumor suppressive TGFβ signaling by activation of oncogenic pathways (14, 16). An oncogenic pathway that is activated in 30% of human colon cancer is the type I Insulin Like Growth Factor Receptor (IGF-1R) signaling pathway (16, 37). IGF-1R signaling is activated through the binding of Insulin Like Growth Factor (IGF) to the receptor, leading to receptor auto phosphorylation and binding of the adaptor proteins, Insulin Receptor Substrates (IRS)-1 and 2 (16). IRS-1 and IRS-2 have been shown to play differential roles in promoting tumorigenesis (16, 38, 39). Specifically, IRS-1 is constitutively activated in a number of solid tumors including, breast, colon, hepatocellular and lung cancers.
Overexpression of IRS-1 leads to increased proliferation, mitogenesis, migration and anti-apoptotic signaling (23, 41-44). In mouse mammary gland, overexpression of IRS-1 leads to mammary hyperplasia and tumorigenesis (40). On the other hand, knockdown of IRS-1 leads to reduced cell migration and decreased tumor growth (21, 23).

One of the main downstream signaling pathways activated by phosphorylated IRS-1 is the phosphoinositide 3-kinase (PI3K)/AKT pathway (16, 45). When IRS-1 is overexpressed, the PI3K/AKT pathway becomes constitutively activated, leading to increased cell survival and tumor growth (43). It has been shown that PI3K/AKT signaling upregulates expression of x-linked inhibitor of apoptosis protein (XIAP) and inhibits cell death (12). XIAP inhibits apoptosis through direct interaction with caspases and is overexpressed in various types of cancer including colon, prostate, cervical and hepatocellular cancer, leading to chemotherapy resistance in many patients (46-48).

In addition to activation of PI3K/AKT signaling, IRS-1 also translocates to the nucleus, where it acts as a transcriptional co-factor and binds to the cyclin D1 promoter to increase its transcription (41). Cyclin D1 is an important regulator of cell cycle progression, which inactivates the retinoblastoma protein to promote cell entry into G1 phase (49). Overexpression of cyclin D1 is one of the most common abnormalities in human cancer (49). In colon cancer, overexpression of cyclin D1 is observed in 55% of patients (50). Cyclin D1 overexpression leads to increased proliferation, angiogenesis and cell survival (50), which are essential to maintain the malignant phenotype in colon cancer (51).

It has been previously reported that TGFβ induces phosphorylation of IRS-2 in non-malignant mink lung epithelial cells (Mv1Lu) and that transfection with IRS-1 or IRS-2 cDNA confers sensitivity to growth inhibition by TGFβ, suggesting that IRS proteins are involved in TGFβ-mediated growth inhibition (43, 52). Other studies have shown that IRS-1 prevents TGFβ-
induced apoptosis (43). Thus, understanding of the crosstalk between TGFβ and IGF-1R/IRS-1 signaling remains limited. In this study, we show that abrogation of TGFβ signaling increased expression and phosphorylation of IRS-1 in colon cancer cells in vitro and in a tumor xenograft model in vivo. Further studies indicate that TGFβ/Smad3 suppresses expression and phosphorylation of IRS-1. Knockdown of IRS-1 expression in Smad3 knockdown cells decreased cell proliferation and increased apoptosis under stress, reversing Smad3 knockdown-mediated oncogenic phenotypes. The underlying mechanism involves regulation of XIAP and cyclinD1 expression by the TGFβ/Smad3/IRS-1 signaling axis. Our studies identify a novel crosstalk between TGFβ and IGF-1R signaling through the adaptor protein, IRS-1, indicating that one of the mechanisms by which TGFβ elicits its tumor suppressor function is through inhibition of IRS-1 expression and activation in colon cancer cells. Therefore, IRS-1 could be a potential therapeutic target for colon cancer treatment.

3.3 Results

3.3.1 Abrogation of TGFβ signaling leads to increased expression and activation of IRS-1 in colon cancer cells in vitro and in vivo.

As reported previously, FET colon cancer cells, isolated from a well-differentiated early stage colon tumor, express autocrine TGFβ signaling and are sensitive to TGFβ-mediated growth inhibition and apoptosis (13). When injected subcutaneously into athymic nude mice in a xenograft model in vivo, FET cells formed a nodule first, which then regressed by day 12. However, abrogation of TGFβ signaling in FET cells by expression of dominant negative RII (DNRII) led to sustained tumor growth (Fig. 1A) (13). Thus, TGFβ signaling appears to suppress tumorigenesis in colon cancer cells. Ki67 and TUNEL staining were performed to determine the proliferative and apoptotic indices within tumors respectively, which showed an increased number of proliferative cells and fewer apoptotic cells in DNRII tumors than in control tumors.
(Fig. 1B, left panels). Quantification of percentage of positively stained cells revealed an approximately 3-fold increase in proliferative cells and an almost 75% decrease in apoptotic cells in DNRII tumors compared with control tumors (Fig. 1B, right panels). These results indicate that the promoting effect of inactivation of TGFβ signaling on tumor growth is a combined result of increased proliferation and decreased apoptosis.

It has been shown that IGF-1R signaling is important for proliferation of colon cancer cells and that IRS-1 is one of the main adaptor proteins required for IGF-1R signaling (24, 53). To determine the mechanisms underlying the suppressive effect of TGFβ signaling on growth of colon cancer cells in the xenograft studies, we determined the expression and phosphorylation of IRS-1 in xenograft tumors by immunohistochemistry (IHC) staining. DNRII tumors showed more intense staining for IRS-1 and more IRS-1 positive cells than control tumors (Fig. 1C, left panels). Quantification of the data revealed that the average intensity of IRS-1 staining and the percentage of IRS-1 positive cells were approximately 71% and 62% higher in DNRII tumors than in control tumors, respectively (Fig. 1C, right panels). Activation of IRS-1, as reflected by its phosphorylation, was also determined by examining the levels of IRS-1 phosphorylated at tyrosine 612 (Y612). Quantification demonstrated that the average intensity of p-IRS-1-Y612 staining and the percentage of p-IRS-1-Y612 positive cells were approximately 48% and 46% higher in DNRII tumors than in control tumors, respectively (Fig. 1C). Taken together, these results indicated that abrogation of TGFβ signaling led to increased expression and activation of IRS-1 in colon cancer cells in vivo, suggesting that TGFβ may regulate IRS-1 expression and activation.

3.3.2 TGFβ signaling inhibits expression and phosphorylation of IRS-1 in colon cancer cells.

To explore the regulation of IRS-1 by TGFβ signaling, expression and phosphorylation of IRS-1 were determined in FET control and DNRII cells. Consistent with in vivo findings, IRS-1
expression and phosphorylation were upregulated in DNRII cells compared with control cells (Fig. 2A).

As described previously, exogenous expression of TGFβ RII in CBS colon cancer cells (CBS-RII) enhanced TGFβ signaling, resulting in reduced proliferation, increased apoptosis and suppressed metastatic potential in an orthotopic model in vivo (11). We therefore examined expression and activation of IRS-1 in CBS control and CBS-RII cells. The results show that IRS-1 expression and phosphorylation are decreased in CBS-RII cells compared with control cells (Fig. 2B), indicating that enhancing TGFβ signaling by TGFβ RII upregulation suppresses the expression and activation of IRS-1 in colon cancer cells.

To directly demonstrate whether TGFβ regulates expression and activation of IRS-1, CBS-RII cells were treated with TGFβ in the presence or absence of a TGFβ RI kinase inhibitor. As expected, phosphorylation of Smad2 was increased upon TGFβ treatment and addition of the TGFβ RI inhibitor prevented TGFβ-induced Smad2 phosphorylation (Fig. 2C). In addition, TGFβ treatment decreased the expression and phosphorylation of IRS-1 in CBS-RII cells and the RI inhibitor prevented the inhibitory effect of TGFβ (Fig. 2C). These results demonstrate that TGFβ suppresses expression and activation of IRS-1 in colon cancer cells.

3.3.3 TGFβ/Smad3 elicits its function through the inhibition of IRS-1 expression/activation

Since Smad3 is an important effector in TGFβ signaling, we next determined whether downregulation of Smad3 would affect IRS-1 expression and activation in colon cancer cells. Smad3 was knocked down in FET cells using shRNA (designated FET/S3KD, Fig. 3A). A scrambled shRNA was used as a control. As expected, Smad3 knockdown reduced TGFβ signaling as reflected in decreased overall levels of Smad3 phosphorylation (Fig. 3A). Notably, expression of IRS-1 was markedly increased in Smad3 KD cells (Fig. 3A), accompanied by increased levels of phosphorylation at both Y632 and Y612 (Fig. 3A). These results were consistent with those
obtained in FET/DNRII cells (Fig. 2A). Of note, IRS-1 mRNA expression was not affected by Smad3 knockdown (Fig. 3B), suggesting that Smad3 regulates IRS-1 expression primarily at the post-transcriptional level. Together the data indicate that attenuation of TGFβ signaling by inactivation of TGFβ RII or decreasing Smad3 expression results in increased IRS-1 expression and activation.

As expected, knockdown of Smad3 expression also resulted in increased proliferation and decreased apoptosis in the context of growth factors and serum deprivation stress (Fig. 3C and 3D). To determine whether IRS-1 plays a role in the pro-proliferative and anti-apoptotic effects of Smad deficiency, expression of IRS-1 was knocked down in FET/S3KD and control cells using a pool of siRNA-targeting IRS-1. Levels of total and phosphorylated IRS-1 were markedly reduced by IRS-1 siRNAs in FET/S3KD and control cells (Fig. 3E). Scrambled or IRS-1 siRNA treated FET control and FET/S3KD cells were subjected to growth factor and serum deprivation stress (GFDS) and effects on proliferation and apoptosis were determined. IRS-1 knockdown significantly reduced cell proliferation and increased stress-induced apoptosis in FET/S3KD cells while having little effect in control cells (Fig. 3F and 3G). The failure to affect these cellular processes in control cells likely reflects the low basal levels of p-IRS-1 and the involvement of other growth regulatory pathways. Taken together, our studies demonstrate that TGFβ/Smad3 inhibits cell proliferation and induces apoptosis through down-regulation of IRS-1 expression/activation in colon cancer cells.

To determine the mechanism by which the TGFβ/Smad3/IRS-1 axis regulates cell proliferation and survival, expression of important effectors in cell cycle progression and apoptosis were examined. Expression of cyclin D1 and XIAP was markedly increased in DNRII and Smad3 KD cells (Fig. 4A and 4B). IRS-1 siRNAs decreased the expression of these molecules in Smad3 KD cells, consistent with effects on cell proliferation and survival, but had little effect in
control cells (Fig. 4C). These results indicate that TGFβ/Smad3 suppresses cyclin D1 and XIAP expression through inhibition of IRS-1 expression/activation, suggesting that cyclin D1 and XIAP may be involved in the TGFβ/Smad3/IRS-1 signaling axis regulating cell proliferation and survival.

3.4 Discussion

In this study, we have uncovered a novel crosstalk between TGF-β and IGF-1R signaling pathways through the adaptor protein, IRS-1, in colon cancer cells. In a xenograft tumor model, abrogation of TGFβ signaling led to increased colon cancer cell proliferation, decreased apoptosis and enhanced tumor growth in vivo, associated with elevated expression and activation of IRS-1. These results indicate that TGFβ signaling regulates IRS-1 expression and activation and that IRS-1 may be a target of TGFβ-mediated tumor suppressor function. Further studies showed that TGFβ signaling suppresses expression and phosphorylation of IRS-1 in colon cancer cells. Knockdown of Smad3 attenuated TGFβ signaling and increased IRS-1 expression and activation, leading to increased cell proliferation and reduced apoptosis under stress. Additional studies indicated that knockdown of IRS-1 expression prevented the effects of Smad3-deficiency on cell proliferation and apoptosis, indicating that TGFβ/Smad3 mediates tumor suppressor function through the inhibition of IRS-1 expression and activation.

Mechanistically, the TGFβ/Smad3/IRS-1 axis regulates expression of cyclin D1 and XIAP, which play important roles in cell proliferation and survival, respectively. Taken together, our study suggests a novel model of crosstalk between TGFβ/Smad3 signaling and IRS-1 in colon cancer cells (Fig. 5). In this model, TGFβ/Smad3 inhibits IRS-1 expression/activation, which leads to suppression of XIAP and cyclin D1 expression. Consequently, TGFβ signaling elicits its tumor suppressor function through inhibiting survival, proliferation and tumorigenesis. Given that the TGFβ signaling pathway is lost in approximately 30% of colon cancer patients and that IRS-1 has
been implicated in the progression of various cancers including colon cancer (7, 21, 22, 54), our study suggests that IRS-1 could be a potential therapeutic target in colon cancer management.

It has been reported that IRS-1 expression is increased in colon adenomas compared with normal colon epithelium (22). Tumor suppressor microRNA, miR-145, has been shown to downregulate IRS-1 expression in the colon (55-57). In addition, miR-128 suppresses cell growth and metastasis by targeting IRS-1 in colon cancer (58). Furthermore, miR-126 regulates cell proliferation, migration and invasion of colon cancer cells by targeting IRS-1 (54). In our study, we show, for the first time, that TGFβ signaling inhibits IRS-1 expression and activation in colon cancer cells in vitro and in vivo and that upregulated IRS-1 contributes to attenuation of TGFβ signaling-mediated tumor suppressive phenotypes. The frequent loss of TGFβ signaling in colon cancer may account for increased IRS-1 expression. Therefore, our study uncovers another mechanism by which IRS-1 expression is regulated in colon cancer.

Our findings further show that TGFβ/Smad3 inhibits IRS-1 protein expression (Fig. 2 and 3A) but has little effect on IRS-1 mRNA levels (Fig. 3B), indicating that TGFβ/Smad3 signaling regulates IRS-1 expression at the post-transcriptional level. Since many miRNAs have been shown to regulate IRS-1 expression (54-58), it is possible that TGFβ/Smad3 signaling may mediate expression of those miRNAs. In addition, it has been shown that degradation of IRS-1 protein is regulated by ubiquitination. For example, Cbl-b is an E3 ubiquitin ligase that has been reported to specifically promote the degradation of IRS-1 protein (59). Further studies are needed to investigate whether TGFβ/Smad3 regulates Cbl-b expression and/or activity in colon cancer cells and whether Cbl-b is responsible for suppression of IRS-1 expression by TGFβ/Smad3 signaling.

Overexpression of IRS-1 has been associated with inhibition of cell death and increased proliferation in different cell types (42, 43, 60). One of the main downstream pathways
activated by IRS-1 is the PI3K/AKT pathway (16, 45). Additional mediators of IRS-1-mediated function have been reported, including ERK signaling and Sox9 expression (54, 61). Here we show that IRS-1 regulates expression of cyclin D1 and XIAP in colon cancer cells. Given their function in regulating cell cycle and survival, effects on cyclinD1 and XIAP may contribute to IRS-1 mediated enhancement of cell proliferation and inhibition of apoptosis in colon cancer cells.

In summary, we have uncovered a novel crosstalk between TGFβ and IGF-1R signaling. This study shows, for the first time, that expression and phosphorylation of IRS-1, an IGF-1R adaptor protein, is decreased by TGFβ/Smad3 signaling. Loss or attenuation of TGFβ signaling leads to elevated expression and activation of IRS-1, resulting in enhanced cell proliferation, decreased apoptosis and increased tumor growth in vitro and in vivo. Down-regulation of IRS-1 expression reversed attenuated TGFβ signaling-mediated phenotypes in vitro. Since loss of TGFβ signaling is frequent in colon cancer, our study points to IRS-1 as a potential therapeutic target for colon cancer management.

3.5 Figures
Figure 3.1 Abrogation of TGFβ signaling led to increased expression and activation of IRS-1 in colon cancer cells in vivo. A, Xenograft tumor growth curves of FET control and DNRII cells are shown. n=10. B, Representative images of Ki67 and TUNEL staining are shown (left). Percentage of positive staining cells was determined (right). C, Representative images of IRS-1 and pIRS1Y612 staining in xenograft tumors are shown (left). Quantification of staining intensity and percentage of positive cells was performed (right). Scale bars, 5 µm. The data are presented as the mean ± SD. *P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 3.1

A

![Graph showing tumor volume over days with control and DNRII groups.]

B

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Figure 3.2 TGFβ inhibits expression and phosphorylation of IRS-1 in colon cancer cells. A, Expression and phosphorylation of IRS-1 at both Y632 and Y612 were higher in FET-DNRII cells than in control cells, as shown by western blot analysis. B, Expression and phosphorylation of IRS-1 at both Y632 and Y612 were lower in CBS-RII cells than in the control cells, as shown by western blot analysis. C, TGFβ treatment of CBS-RII cells decreased expression and phosphorylation of IRS-1, an effect that was prevented by the addition of TGFβ RI kinase inhibitor.
Figure 3.2

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Figure 3.3 Knockdown of Smad3 led to increased expression and phosphorylation of IRS-1, which contributed to Smad3 knockdown-induced increased proliferation and decreased apoptosis under stress. A, knockdown of Smad3 in FET cells attenuated TGFβ signaling and increased IRS-1 expression and phosphorylation. B, knockdown of Smad3 in FET cells had little effect on IRS-1 mRNA expression. C, MTT assays showed that cell proliferation was increased in Smad3 knockdown cells compared with control cells. D, DNA fragmentation assays showed protection from apoptosis in Smad3 knockdown cells compared with control cells under GFDS. E, A siRNA pool targeting IRS-1 was transfected into FET-Smad3 knockdown and control cells. Expression and phosphorylation of IRS-1 were markedly reduced in siRNA-transfected cells compared with scrambled siRNA-transfected control cells. F, MTT assays showed that knockdown of IRS-1 expression partially prevented Smad3 knockdown-mediated increased proliferation of FET cells. G, DNA fragmentation assays showed that knockdown of IRS-1 expression abrogated Smad3 knockdown-mediated reduction of apoptosis under GFDS. For F and G, two-way ANOVA was used to compare the difference between Cont Scr and S3KD Scr and Student’s t-test was used to compare the difference between S3KD Scr and siIRS-1. The data are presented as the mean ± SD of three replicates. *P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 3.3

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B

Field Change in IRS-1 and p-IRS-1 Expression

Control | S3 KD
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0.0      | 1.54   

C

Absorbance

Control | S3 KD
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0.0      | 2.00   

D

Field Change of Amplitude

Control | S3 KD
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0.0      | 1.54   

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F

Absorbance

Scir | siIRS1 | Scir | siIRS1
-----|--------|-----|--------
0.0  | 0.37   | 0.0 | 0.37   

G

Field Change of Amplitude

Scir | siIRS1 | Scir | siIRS1
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0.0  | 1.54   | 0.0 | 1.54   

Figure 3.4 The TGFβ/Smad3/IRS-1 signaling axis regulates expression of cyclin D1 and XIAP.

A&B, Expression of cyclin D1 and XIAP was higher in FET-DNRII (A) and Smad3 knockdown (B) cells. C, Knockdown of IRS-1 led to reduced expression of cyclin D1 and XIAP in Smad3 knockdown cells.
### Figure 3.4

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Figure 3.5 A proposed model of crosstalk between TGFβ signaling and IRS-1 in the regulation of XIAP and cyclin D1 expression, cell survival, proliferation and tumorigenesis in colon cancer cells.
Chapter IV

Understanding the Relationship between Smad2 and IGF-1R
4. Understanding the Relationship between Smad2 and IGF-1R

4.1 Summary/Abstract

In this study, we have discovered a relationship between Smad2 and the IGF-1R signaling pathway in a colorectal cancer cell line. With the loss of Smad2 there was decreased expression and activation of IGF-1R and the downstream effector molecules. We also observed decreased proliferation and increased apoptosis both in vitro and in vivo. Over expressing IGF-1R reversed the Smad2 knock down tumor suppressive effects in vitro. This study shows for the first time Smad2 could be having a cross talk relationship with IGF-1R in a colorectal cancer cell line.
4.2 Introduction

TGFβ is a signaling pathway that plays a role in tumor suppression early on in cancer development. However, as the disease progresses the TGFβ pathway acts as a tumor promoter by inducing epithelial to mesenchymal (EMT) transition (62). Receptor-regulated Smads are activated by TGFβ, translocate to the nucleus and act as transcription factors to either repress or activate certain target genes (63). In colorectal cancer progression, microsatellite instability (MSI) is a hallmark feature that occurs with the progression of the disease. When MSI occurs, more than 80% of patients develop a mutation in the tumor suppressor TGFβ signaling pathway that leads to further progression of colorectal cancer (63). Previous studies have shown that if Smad2 or Smad3 is mutated in colorectal cancer there is progression of the disease, suggesting Smad2 and Smad3 act as tumor suppressors in colorectal cancer (63).

Previous work from our lab has shown that tumor suppressive TGF-β signaling represses vascular endothelial growth factor A (VEGFA) expression in a Smad3-dependent manner but not Smad2 (10). In other types of cancers Smad2 and Smad3 play different roles in cancer progression, but this has not been identified in colorectal cancer. When Smad2 was knocked down in HaCaT keratinocyte cells, there was no effect on TGFβ-induced cell cycle arrest. However, when Smad3 was knocked down there was abrogation of the TGFβ-induced growth arrest as well as inhibition of c-MYC (64). In a pancreatic ductal adenocarcinoma (PDAC) cell line, PANC-1, Smad3 knock down increased proliferation (59). These studies indicate Smad3 plays a role in tumor suppression through inhibiting cell cycle progression. When Smad2 was knocked down in PANC-1 cells there was decreased migration (59). This study identified Smad2 and Smad3 having antagonistic roles in cancer progression of PANC-1 cells.

In our study, we utilized a colon cancer cell line that still has functional tumor suppressive TGFβ signaling and is IGF-1R dependent, FET (53). IGF-1R is a known oncogenic pathway that is
upregulated in many types of cancers (16). This pathway plays a role in anti-apoptotic signaling and cell cycle progression (65). Our lab has previously shown there is a cross talk relationship between TGFβ and IGF-1R signaling and we wanted to determine what roles Smad2 and Smad3 played in this cross talk. We determined that Smad3 played a role in the tumor suppressive TGFβ signaling by controlling the expression of IRS-1 and downregulating cyclin D1 and XIAP. However, knockdown of Smad2 decreased expression and phosphorylation of IGF-1R and IRS-1. From this study, we hypothesize that Smad2 plays a role in promoting proliferation and inhibiting apoptosis in an IGF-1R dependent manner. The mechanism to which this novel Smad2/ IGF-1R cross talk is occurring still needs to be identified.

4.3 Results

4.3.1 Successful knock down of Smad2 in FET cell line

Knock down of Smad2 in the FET colorectal cancer cell line at the protein showed there was a 99.8% knock down as compared to the control. This observation was also seen at the RNA level (Fig. 4.1). There was no change in Smad3 expression (Fig. 4.1).

4.3.2 Decreased phosphorylation of IGF-1R and downstream markers when Smad2 is knocked down

We previously determined that Smad3 played a tumor suppressive role in colorectal cancer by regulating IRS-1 expression at the post-transcriptional level. To determine if Smad3 and Smad2 had similar roles in colorectal cancer we knocked down Smad2. We observed decreased expression of phosphorylated levels of IGF-1R and decreased expression and phosphorylation of IRS-1 in the Smad2 knock down cell line (Fig. 4.2). We looked at downstream targets of the IGF-1R pathway and observed decreased expression of both phosphorylated AKT and ERK (Fig. 4.2). We performed QPCR analysis on IRS-1 and determined there were no significant differences seen at the mRNA level (Fig. 4.2B), this finding indicates that the
expression differences between the control and Smad2 knock down cell line we are observing are due to the protein stability difference between these cell lines.

4.3.3 Decreased proliferation and increased cell death when Smad2 is knocked down

As compared to the control, the Smad2 knock down cell lines showed decreased proliferation and increased cell death (Fig. 4.3A and B). We also over expressed IGF-1R in the Smad2 knock down cell line to see if we could reverse the phenotype we are observing when Smad2 is knocked down. We observed increased proliferation and decreased cell death when IGF-1R was over expressed in the Smad2 knock down cell line (Fig. 4.3A and B).

4.3.4 Knock down of Smad2 in vivo shows decreased proliferation and increased apoptosis

We performed a small xenograft study using the FET control and FET Smad2 knock down cell lines. After 8 days, the xenografts were removed from the mice. The tumor volumes in the Smad2 knock down mice were significantly smaller than the control (Fig 4.4A). A visual representation of the xenografts also confirmed this finding (Fig. 4.4B). We did H&E staining of the xenograft tissue and observed more necrotic tissue in the Smad2 knock down sample as compared to the control sample (Fig. 4.5). We stained for Smad2 expression and observed decreased expression of Smad2 in the knock down sample as compared to the control (Fig. 4.6A). Quantification of the percentage of Smad2 positive cells showed a decrease in the Smad2 knock down sample (Fig. 4.6B). Ki-67 and TUNEL staining showed a decrease in the number of proliferative cells (75% reduction) in the Smad2 knock down tumors and increased number of apoptotic cells (85% increase), respectively (Fig. 4.7 and 4.8), confirming the results we were observing in vitro.

4.4 Discussion

TGFβ signaling acts as a tumor suppressor early in cancer progression and the main proteins responsible for signaling are the R-Smads. Smad2 and Smad3 are highly conserved and
share 84% sequence homology (66), because of this homology they do have redundant roles in response to TGFβ (8). However, Smad2 and Smad3 have also shown to have non-redundant roles when they are knocked out in mice (8). Smad3 has shown to play more of a dominant role in tumor suppression than Smad2 (8). Pull down studies, along with mass spectrometry, identified several proteins that were specific to just Smad2 and Smad3, proving that these proteins can have different roles (8).

*In vitro* analysis of Smad2 and Smad3 knock down in MDA-MB-231 cells showed different phenotypes. When Smad3 was knocked down, the cells proliferated at a similar rate as the control cells. However, when Smad2 was knocked down the proliferation rate was reduced as well as the migratory ability of the cells (66). We show for the first time in colorectal cancer cells that when Smad2 is knocked down *in vitro* and *in vivo* there is decreased proliferation and increased apoptosis. We also looked at the IGF-1R signaling pathway since our lab has previously shown there is a cross talk relationship between TGFβ and IGF-1R (12). We observed decreased expression and phosphorylation of IGF-1R and the downstream markers affected by this pathway. We determined the change in expression is at the post-transcriptional level. These results indicate Smad2 could have an oncogenic role in the progression of colorectal cancer and may be a possible target for therapeutic treatment for individuals who have enhanced IGF-1R signaling and functional Smad2. Further studies need to be done to determine if this is a true cross talk between IGF-1R and Smad2 or just an effect from knocking down Smad2 that leads to a reduced tumorigenic phenotype within this colorectal cancer cell line.

### 4.5 Figures
Figure 4.1 Knock down of Smad2 in FET cell line

Western blot showing Smad2 and Smad3 expression in the control and Smad2 knock down cell lines (left panel). Quantification of mRNA expression of Smad2 between control and Smad2 knock down cell lines (right panel). The data are presented as the mean ± SD of three replications. *** P < 0.001.
Figure 4.2 Decreased expression and activation of the IGF-1R signaling pathway

A) Knock down of Smad2 showing decreased activation of IGF-1R and downstream effector molecules as depicted through western blot analysis. B) comparison of IRS-1 mRNA levels between FET control and S3KD cell lines showed no significant difference.
Figure 4.2

(A) Western blot analysis showing the expression levels of various proteins in control and S2KD conditions. The proteins analyzed include Smad-2, pSmad2, IRS-1, p-IRS-1Y632, IGF-1R, p-IGF-1RY1135, AKT, p-AKT(S473), and p-ERK(T202/Y204).

(B) Bar graph showing the fold change in IRS-1 mRNA expression between control and S2KD conditions. The data indicates a significant reduction in IRS-1 expression in the S2KD condition compared to control. The graph is labeled with NS (not significant).
Figure 4.3 Decreased proliferation and increased cell death when Smad2 is knocked down

A) MTT assays show decreased proliferation when Smad2 is knocked down but this phenotype can be reversed upon over expression of IGF-1R. B) DNA fragmentation assays showed there was an increase in cell death upon knock down of Smad2 and no significant difference in cell death when IGF-1R is over expressed. The data are presented as the mean ± SD of three replications. *P <0.05, P** <0.01, P*** <0.001.
Figure 4.3

A

B

Absorbance

Fold Change of Apoptosis

Control  S2KDSH1  S2KDSH1 CD8a-GF-1R

Control  S2KDSH1  S2KDSH1 CD8a-GF-1R

*** NS

** NS
Figure 4.4 Decreased tumor growth in FET Smad2 knock down xenografts as compared to the control

A) Xenograft tumor growth curves of FET control and S2KD cells are shown. n=10. B)
Representative images showing tumor volume for FET control and S2KD. The data are presented as the mean ± SD. *P <0.05, P** <0.01.
Figure 4.4

A. 
- Psr Control
- S2 KD

B.
Control

S2 KD
Figure 4.5 H&E of FET control and Smad2 knock down xenograft tissue

Representative image showing H&E stained xenograft tumors for FET control and S2KD.
Figure 4.5

Control

S2 KD

T

N

N

N
Figure 4.6 IHC analysis of Smad2 expression between control and Smad2 knock down xenograft tissue

Representative image of Smad2 staining in xenograft tumors for FET control and S2KD (left). Quantification of percentage positive was performed (right). The data are presented as the mean ± SD. ***P < 0.001.
Figure 4.6

Negative Cont  Smad2

Control

S2 KD

% of Smad2 Positive Cells

Control  S2 KD
Figure 4.7 Decreased Ki-67 staining in the Smad2 knock down xenograft tissue as compared to the control

Representative image of Ki-67 is shown (left). Quantification of percentage positive was performed (right). The data are presented as the mean ± SD. ***P <0.001.
Figure 4.7

Control

Ki-67

S2 KD

% of Ki-67 positive cells

Control

S2 KD

***
Figure 4.8 Increased TUNEL staining in the Smad2 knock down xenograft tissue as compared to the control

Representative image of TUNEL staining is shown (left). Quantification of percentage positive cells was performed (right). The data are presented as the mean ± SD. ****P < 0.0001.
Figure 4.8

Comparison of control and S2 KD conditions for TUNEL assay.
Chapter V

Determining if IRS-1 and/or IRS-2 could be used as therapeutic targets in metastatic CRC
5.1 Summary/Abstract

IRS-1 and IRS-2 are adaptor proteins that are downstream of the IGF-1R pathway and activate downstream molecules involved in promoting tumorigenesis. In this study, we created inducible systems to determine the effect IRS-1 and IRS-2 have on promoting colorectal cancer progression. We observed decreased expression of downstream signaling molecules and cellular proliferation upon knock down of IRS-1 and IRS-2.
5.2 Introduction

Through various studies, IRS-1 and IRS-2 have been shown to have non-redundant roles. IRS-1 plays a role in recruiting proteins involved in mitogenic signaling and IRS-2 has a role in cellular motility (16). Besides having roles in proliferation and motility, IRS-1 and IRS-2 can activate downstream pathways involved in cell survival, protein synthesis and growth, and glucose metabolism (16).

IRS-1 and IRS-2 have both been shown to play a role in the progression of cancer. When either IRS-1 or IRS-2 are over expressed in MCF-7 or MDA-MB-231 breast cancer cell lines, there is enhanced migration capability of the cells with a lower dose of growth factor being used (23). IRS-1 and IRS-2 have also shown to cause mammary hyperplasia and metastasis in a mammary epithelial cell line, MCF-10A (24). Specifically, IRS-1 has been shown to be over expressed in many types of cancers (67). In colorectal cancer, when IRS-1 expression was compared between primary and metastatic colorectal cancer there was increased expression in the metastatic tissue as compared to the primary tumor (22), implicating IRS-1 in the progression of colorectal cancer.

Our lab went on to further understand the roles of IRS-1 and IRS-2 in the progression of colorectal cancer. Utilizing Oncomine data we looked at the expression of IRS-1 and IRS-2 in colorectal adenoma versus colorectal carcinoma and found that there was increased expression of both IRS-1 (fold change 2.79) and IRS-2 (fold change 1.55) in colorectal carcinoma as compared to colorectal adenoma (Fig. 5.1). We used Ingenuity Pathway Analysis (IPA) to determine the molecular networks associated with IRS-1 and IRS-2 in colorectal cancer metastasis (data not shown). We identified different networks specific to just IRS-1 or IRS-2, which led us to the hypothesis that IRS-1 and IRS-2 are promoting colorectal cancer through differential mechanisms. We developed an inducible system to test this hypothesis and to
eventually determine if IRS-1 and IRS-2 could be used as therapeutic targets for metastatic colorectal cancer.

5.3 Results

5.3.1 Expression of IRS-1 and IRS-2 in colorectal cancer cell lines

We looked at the basal expression levels of IRS-1 and IRS-2 in FET, CBS and GEO colon cancer cell lines. CBS and GEO are tumorigenic and have 50-60% metastatic potential. We observed increased expression of IRS-1 and IRS-2 in GEO and CBS as compared to the less tumorigenic cell line FET (Fig. 5.2). Indicating that as the disease progresses there is increased expression of these adaptor proteins.

5.3.2 Treatment with MK-0646 shows reduced expression of IRS-1 and IRS-2

To validate that the expression of IRS-1 and IRS-2 can be affected by the inhibition of IGF-1R, we used a small molecule inhibitor known as MK-0646. MK-0646 binds to the IGF-1 receptor causing internalization and degradation of the receptor (68). In vivo analysis showed the inhibitor was successful at reducing IGF-1R expression (Fig. 5.3). We probed for IRS-1 and IRS-2 and observed decreased expression in the treated sample as compared to the control (Fig. 5.3), confirming that IRS-1 and IRS-2 expression is affected by the regulation of IGF-1R expression and signaling.

5.3.3 IRS-1 and IRS-2 are upregulated in colon cancer metastasis

An orthotopic implantation study, using the human colon cancer cell line GEO, was performed by a lab member to recapitulate colon cancer metastasis in vivo (Fig. 5.4A). Using the tissue from the primary tumor and liver mets, we probed for IRS-1 and IRS-2 to compare the expression levels between these two types of tumors. We observed increased expression of both IRS-1 and IRS-2 in the liver mets as compared to the primary tumor (Fig. 5.4B). We also performed IHC staining for IRS-1 with the primary and liver met tissue sections and observed
increased expression of IRS-1 in the liver met sample as compared to the primary tumor (Fig. 5.4C), confirming the findings we obtained through western blot analysis.

5.3.4 Inducible knock down of IRS-1 leads to decreased activation of downstream markers and reduced proliferation

To determine what role IRS-1 plays in colorectal cancer metastasis we created an inducible system to knock down IRS-1 when doxycycline (Dox) was present (Fig. 5.5). The inducible system allows us to manipulate when IRS-1 will be knocked down for the cell lines to be used in vivo. We transfected three different IRS-1 shRNAs into the colon cancer cell line, GEO. We also transfected a non-targeting vector into the GEO cells for a control. We added Dox to the cells for 3 days and then checked the expression of IRS-1. We observed decreased expression of IRS-1 when Dox was present and there was no effect of the non-targeting vector or when there was no Dox present in the cells transfected with IRS-1 shRNA (Fig. 5.6A). There was also no effect on the expression of IRS-2, showing that the shRNA vector was specific for IRS-1 (Fig 5.6A). We probed for downstream targets that are activated by IRS-1 and observed decreased phosphorylation of Erk and AKT (Fig. 5.6A). We also observed decreased proliferation when IRS-1 was knocked down (Fig. 5.6B).

5.3.5 Creating an IRS-2 inducible system and decreased proliferation when IRS-2 is knocked down

To create an inducible system for IRS-2, we had to excise the shRNA fragment for IRS-2 from the GIPZ vector and ligate the fragment into a Lentiviral vector. We performed this experiment and then added Dox to the GEO cells for five days. We observed decreased expression of IRS-2 as compared to the non-targeting vector and there was no effect on the expression of IRS-1 (Fig. 5.7A). We also observed decreased proliferation when IRS-2 was knocked down (Fig. 5.7B).
5.4 Discussion

IRS-1 and IRS-2 have increased expression in breast, hepatocellular, lung, medulloblastoma, mesothelioma, ovarian, pancreatic, prostate, renal and colorectal cancers (22, 40). When either IRS-1 or IRS-2 is over expressed in the mammary gland these epithelial cells develop mammary hyperplasia, tumorigenesis and metastasis (24). Besides acting as adaptor proteins at the cell membrane, IRS-1 and IRS-2 can translocate to the nucleus and increase transcription of genes involved in promoting tumorigenesis (41, 69). These features make IRS-1 and IRS-2 desirable targets for therapeutic treatment of cancer. In colorectal cancer, we observed increased expression of both IRS-1 and IRS-2 in liver metastasis compared to the primary tumor in vivo. When IRS-1 and/or IRS-2 were knocked down, there was decreased proliferation and decreased expression of downstream signaling molecules. Further studies need to be performed to determine if IRS-1 and IRS-2 influence cell survival and if either of these adaptor proteins could be potential therapeutic targets for metastatic colorectal cancer patients with upregulated expression of IRS-1 and/or IRS-2.

5.5 Figures
Figure 5.1 Increased mRNA expression of IRS-1 and IRS-2 in colorectal carcinoma vs colorectal adenoma

Oncomine analysis showing increased expression of IRS-1 and IRS-2 with the progression of colorectal cancer. Fold change for IRS-1 is 2.79 and for IRS-2 is 1.55.
Figure 5.1
**Figure 5.2 Protein expression of IRS-1 and IRS-2 in FET, CBS and GEO**

Western blot analysis showing expression of IRS-1 and IRS-2 in three colorectal cancer cell lines, FET, CBS and GEO.
Figure 5.2

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<th>FET</th>
<th>CBS</th>
<th>GEO</th>
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<tr>
<td>β- actin</td>
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Figure 5.3 Decreased expression of IGF-1R, IRS-1 and IRS-2 upon treatment with MK-0646

Western blot analysis showing GEO colon cancer cells treated with MK-0646 and the expression of IGF-1R, IRS-1 and IRS-2 between the control and treated cells.
Figure 5.3
Figure 5.4 Increased expression of IRS-1 and IRS-2 in liver metastasis as compared to the primary tumor

A) Visual representation of orthotopic implantation method. GEO colon cancer cells tagged with GFP for visualization of tumor cells showing the primary tumor and liver metastasis. B) Western blot analysis of IRS-1 and IRS-2 expression in the primary and liver metastasis samples. C) IHC of IRS-1 expression in the primary and liver metastasis samples compared to the normal tissue of the colon and liver.
Figure 5.4

A. Whole body | Open abdomen
Primary tumor | Liver Metastasis

B. Primary | Liver Mets
IRS-1 | IRS-2 | β-actin

C. Normal area | Tumor area | Negative control
Primary | Metastasis

10X

A.

B.

C.
Figure 5.5 Diagram illustrating how to create an inducible system

Diagram showing the order of steps to do a transfection with an inducible lentiviral IRS shRNA vector.
Figure 5.5

TRIPZ Inducible Lentiviral shRNA Vector

+ Dox

Knockdown of IRS

Transcription of shRNA

Inducible GEO shIRs

- Dox

GEO or GEO-GFP

Collect supernatant and add to GEO or GEO-GFP cells

Antibiotic Selection

IRS Expression

No transcription
Figure 5.6 Inducible knock down of IRS-1 leads to decreased expression of downstream markers and decreased proliferation

A) Western blot analysis showing a successful knockdown of IRS-1 without affecting the expression of IRS-2. We also observed decreased expression of downstream markers, p-Erk and p-AKT. B) MTT assay showing decreased proliferation when IRS-1 is knocked down. The data are presented as the mean ± SD of three replicates. *P < 0.05, *** P < 0.001.
Figure 5.6

A.

-Dox  +Dox

IRS-1
IRS-2
p-Erk-1/2
Thr202/Tyr204
p-AKT
Thr308
Actin

B.

Absorbance

IR519 Dox  IR519 +Dox  IR519 Dox  IR519 +Dox
Figure 5.7 Knock down of IRS-2 leads to decreased proliferation

A) Western blot analysis showing knock down of IRS-2 and there was no change in IRS-1 expression. B) MTT assay showing reduced proliferation when IRS-2 is knocked down. The data are presented as the mean ± SD of three replicates. *** P < 0.001.

This was a single blot that was rearranged.
Figure 5.7

A.

<table>
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<th>Actin</th>
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</tr>
<tr>
<td>IRS2sh1 + Dox</td>
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B.

Range: 0-2

Absorbance

IRS2SH1 - Dox

IRS2SH1 + Dox

** ***
Chapter VI

Summary and Future Directions
6.1 Central theme

The central theme of this dissertation is the novel cross talk between TGFβ and IGF-1R at the level of Smad2 and Smad3. Both TGFβ and IGF-1R pathways play a role in the progression of colorectal cancer. By identifying how TGFβ and IGF-1R signaling impacts the progression of colorectal cancer we have better insight into how to treat patients.

6.2 Current standing

Currently we know that there is a cross talk relationship at the level of TGF-β/Smad3/IRS1. We identified IRS-1 regulation by Smad3 at the post transcriptional level and concluded that Smad3 is working as a tumor suppressor in colorectal cancer. With the knock down of Smad2 there was decreased proliferation and increased cell death, along with decreased activity of IGF-1R. From these two different studies, we concluded that the R-Smads are playing antagonistic roles in the progression of colorectal cancer through the IGF-1R pathway.

6.3 Future directions

6.3.1 Determining metastatic potential with Smad3 knock down

The next step is to study the Smad3 knock down effect in vivo. We need to determine if Smad3 knock down can affect the growth and metastasis in a more advanced colon cancer cell line, like GEO. For therapeutic purposes, it would be of importance to determine if the in vitro observation of reduced IRS-1 expression along with repression of XIAP and cyclin D1 also occurs in vivo when Smad3 is knocked down. This study would determine if IRS-1 could be a potential therapeutic target for individuals that have lost functional TGFβ signaling.

6.3.2 Understanding the linkage between Smad2 and IGF-1R

The next step would be to determine if there is a cross talk between Smad2 and IGF-1R in vitro for potential therapeutic purposes, it is of importance to know whether our initial Smad2
knock down observation of decreased IGF-1R signaling, decreased proliferation and increased cell death is not just a cell line specific event, TGFβ-dependent effect, or an off-target effect. It would also be important to determine the in vivo effect on primary tumor growth as well as liver and lung metastasis. Our in vivo observation of decreased tumor size, growth and cell survival indicates Smad2 could be promoting tumorigenesis in this colorectal cancer cell line and give better insight into how to treat patients that have functional TGFβ signaling.

6.3.3 Determining therapeutic potential for IRS-1 and IRS-2

To move forward with the inducible IRS-1 and IRS-2, some functional studies still need to be done in vitro. It is important to determine if IRS-1 and/or IRS-2 can affect cell survival and proliferation before moving to in vivo analysis. The next step would be to determine if IRS-1 and/or IRS-2 are required for metastasis or the maintenance of metastasis using the inducible system in vivo. This will identify if IRS-1 and/or IRS-2 can be used as therapeutic targets for individuals with late stage colorectal cancer.

6.4 Final Conclusions

Overall, the work of this dissertation was to try to better understand the progression of colorectal cancer and find therapeutic targets to treat the disease. From this dissertation, we can conclude that molecular pathways are very complex and don’t work in just one way. Receptor-regulated Smads that should be working in a tumor suppressive manner can have antagonistic roles and one R-Smad can lead to the progression of colorectal cancer. These facts need to be kept in mind when patients are being treated for colorectal cancer so a therapeutic drug doesn’t cause more harm than good. By identifying new cross talk relationships between signaling pathways we can better treat patients and give them a more individualized treatment plan that matches their same genomic profile.
Chapter VII

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23. de Blaquiere GE, May FE, Westley BR. Increased expression of both insulin receptor substrates 1 and 2 confers increased sensitivity to IGF-1 stimulated cell migration. Endocr Relat Cancer. 2009 Jun;16(2):635-47.


