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LGR5 ACTIVATES TGFβ SIGNALING AND SUPPRESSES METASTASIS IN COLON CANCER

By

Xiaolin Zhou

A DISSERTATION

Presented to the Faculty of

The Graduate College in the University of Nebraska

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

Department of Eppley Cancer Institute

Under the Supervision of Professor Jing Wang

University of Nebraska Medical Center

Omaha, Nebraska

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LGR5 ACTIVATES TGFβ SIGNALING AND SUPPRESSES METASTASIS IN COLON CANCER

Xiaolin Zhou, Ph.D.

University of Nebraska Medical Center, 2015

Advisor: Jing (Jenny) Wang

Metastasis is the major cause of death in colorectal cancer patients, mainly due to the ineffectiveness of current therapies once metastases begin to form. Further insight into the biology of colorectal cancer metastasis is therefore essential in order to gain a greater understanding of this process and ultimately to develop better cancer therapies to prevent or target metastasis. LGR5 is leucine-rich repeat containing G protein-coupled receptor (GPCR) and was discovered as a marker for proliferating adult stem cells in small intestine. LGR5 and its homologs LGR4 and LGR6 are receptors of R-spondins (RSPOs), which are secreted agonists of canonical Wnt signaling. Upon binding RSPOs, they form a physical complex with Wnt receptor Frizzled and its co-receptor LRP6 to regulate Wnt canonical signaling. Here we show that RSPO-LGR5 activates TGFβ signaling in colon cancer cells, enhancing TGFβ-mediated growth inhibition and stress-induced apoptosis. Knockdown of LGR5 attenuates TGFβ signaling, increases cell proliferation, survival and metastasis. Mechanistically, with the presence of RSPOs, LGR5 forms and enhances complex formation with TGFβ receptors to activate downstream signaling. Our study underlies a novel crosstalk between Wnt and TGFβ signaling in colon cancer cells. Since TGFβ signaling is defective in most late stage colon cancer and its restoration has been

shown to suppress colon cancer tumorigenicity, our findings would provide an alternative mechanism to activate TGFβ signaling and suppresses colon cancer tumorigenicity and metastasis.

DEDICATION

This dissertation is dedicated to my dear husband, Yinbo Zhang, who has been a constant source of support, encouragement and love during all the challenges in graduate school.

I am deeply grateful for having you in my life!

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As a joint M.D-Ph.D student of Shanghai Jiaotong University (SJTU) and University of Nebraska Medical Center (UNMC), I am very fortunate to have performed my Ph.D phase in the Cancer Research Graduate Program in UNMC. There are many people to thank for their part in this milestone achievement. Firstly, I would like to thank my supervisor, Dr. Jing (Jenny) Wang, for providing me a nice research environment in the lab and her mentorship has enlightened me a lot in moving my research project forward. I am grateful for all the suggestions and opportunities she offered me and I will remember my time in the lab very fondly.

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Thirdly, I would also like to recognize all the people who have given their time to help with my project, teach me techniques and trouble shoot my failed experiments. I'd like to thank Dr. Liying Geng for her contribution to my work by helping me identify the complex formation between LGR5 and TGFβ receptors. She was so passionate on this

part of work that enlightened me to overcome the most difficult time in this project. The project won't be completed without the help of Liying and I am so grateful for having such a productive colleague and friend in my lab. Dr. Andy Chaudhuri, a former lab member, who has trained me to obtain the orthotopic transplantation technique and other animal work. Dr. Geoffrey Talmon, a pathologist in UNMC hospital, who contributed a lot in choosing the appropriate human patient samples for my study and provided many valuable advices on the identification of pathological structures in each sample. Dr. Sanming Wang and Dr. Yeong Kim, two great bioinformatics specialists who have contributed a lot to the work on mining the RNA sequencing data from the TCGA platform for me. Without all those wonderful people's expertise, I would not have accomplished as much.

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my gratitude because it is so boundless. Thanks for everything he has done for me over the years being around with me listening to my problems and providing perspective. He is my best friends, my joy and my cheerleader. We have gone through the whole amazing journal together, so it only seems right that I dedicate this dissertation to him.

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ABBREVIATION

ACVR1B	Activin A receptor, type IB
ACVR2A	Activin A receptor, type IIA
APC	Adenomatous polyposis coli
ASCL2	Achaete-scute family bHLH transcription factor 2
BAX	BCL2-associated X protein
BCA	Bicinchoninic acid
CIN	Chromosomal instability
CK1	Casein kinase 1
CMV	Cytomegalovirus
CNV	DNA copy number variation
CRC	Colorectal cancer
CTNNB1	Catenin (cadherin-associated protein), beta 1 (ß-catenin)
DAPI	4', 6-diamidino-2-phenylindole
DCC	Deleted in colorectal carcinoma gene, netrin 1 receptor
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
FKBP12	FK506 binding protein 1A

Fz	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFDS	Growth factor depreviation stress
GFP	Green fluorescence protein
GPCR	G protein coupled receptor
GSK3	Glycogen synthase kinase 3
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
IF	Immunofluorescence
IHC	Immunohistochemistry
IP	Immunoprecipitation
KD	Knockdown
KRAS	Kirsten rat sarcoma viral oncogene homolog
LEF	Lymphoid enhancer binding factor
LGR4/5/6	Leucine rich repeat containing G protein coupled receptor 4/5/6
LRP6	Low density lipoprotein receptor-related protein 6
MAPK	Mitogen-activated protein kinase
microRNA	Micro ribonucleic acid
MMR	Mismatch repair
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OD	Optical density
PARP	Poly-ADP-ribose polymerase
PBS	Phosphate buffer saline
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
POD	Anti-DNA-peroxidase
RIPA	Radio-immunoprecipitation assay
RNF43	Ring finger protein 43
RSPO	R-spondin
RT-PCR	Reverse transcription and polymerase chain reaction
SBE	Smad binding element
SDS	Sodium dodecyl sulfate
SEER	Surveillance epidemiology and end results
SEM	Standard error of the mean
SF medium	Serum-free medium
SM medium	Serum-free McCoy's 5A medium
TCF	Transcription factor
TCGA	The cancer genome atlas
TGFBR1	Transforming growth factor, beta receptor I
TGFBR2	Transforming growth factor, beta receptor II
TGFβ	Transforming growth factor, beta
TLE	Transducin-like enhancer
TMA	Tissue micro-array

TNM	Tumor-node-metastasis
TP53	Tumor protein p53
TUNEL	Terminal nucleotidyl transferase-mediated nick end labeling
VEGF	Vascular endothelial growth factor
ZNRF3	Zinc and ring finger 3

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CHAPTER 1

Introduction

1.1 Overview of Colorectal Cancer Disease

1.1.1 Colorectal Cancer Facts and Statistics

New Cases and Death Rate: Colorectal (colon and rectum) cancer is expected to be the third leading cause of cancer related death in both men and women based on 2007-2011 cases and deaths record from the USA National Cancer Institute. In the year 2014, it is estimated that there will be 136,830 new cases of colorectal cancer and an estimated 50,310 people will die of this disease **(Figure1.1)**.

Risks Factors: Hereditary and medical factors that increase risk to colorectal cancer include a personal or family history of colorectal cancer or polyps, a personal history of chronic inflammatory bowel disease (e.g., ulcerative colitis or Crohn disease), certain inherited genetic conditions (e.g., Lynch syndrome) and type 2 diabetes.

Stage of Colorectal Cancer: As many other cancer types, colorectal cancer disease is treated and managed according to their stages. Currently, the most commonly used staging system is the TNM (Tumor, Node, Metastasis) staging system **(Figure 1.2)**, which reflects the depth and the potential spread of the tumor. The anatomic TNM staging system of colon cancer has greatly benefited the management of the disease, however, oncologists are still facing the challenge of cancer recurrence risks and patients response to intervention therapies. At this time, efforts are being made to identify the genes and proteins that are associated with cancer initiation and/or recurrence. One advancement is the development of multi-gene assays that provide prognostic and predictive information to aid in decisions regarding adjuvant therapy in patients with colon cancer (Benson and Hamilton 2011).

Survival Rate: Localized cancer is referred to as stage I cancer. If it has spread to a different part of the body, the stage is regional or distant. The earlier colon and rectum cancer is caught, the better chance a person has of surviving five years after being diagnosed. The overall 5-year survival rate for colorectal cancer is 64.7%. However, cancer stage has a strong influence on the length of survival. At the time of diagnosis, there are about 40% of patients presenting with localized tumors, 36% of patients have the disease spread to regional lymph nodes and 20% of them have distal metastasis. The 5-year survival rate for patients with localized and regional tumor is 89.8% and 70.5%, respectively. While it drops dramatically to 12.9% when the tumor has metastasized to other organs **(Figure 1.3)**.

Early Detection and Treatment: At the age of 50, men and women who are at average risk are encouraged to begin screening for colorectal cancer. Screening can detect cancer at an early stage and also allow for the removal of colorectal polyps that might become cancerous. Surgery is the most common treatment for all stages of cancer. Even if the surgery removes all the cancer that can be seen at the time of the operation, some patients may be given chemotherapy or radiation therapy after surgery (i.e., adjuvant therapy) to kill any cancer cells that are remaining in the body. Presently, researchers have discovered more about the gene and protein profiles in cells that cause cancer and newer drugs that specifically target the cancer cells are being developed (i.e., targeted therapy). For example, some monoclonal antibodies and inhibitors have been used in combination with the other treatments to attack the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and some kinases that prompt cancer cells to grow. However, as the outcome of patients with metastasis or with later stage of tumor is still

poor, more efforts are being made to elucidate better prognostic biomarkers and prediction biomarkers to improve the management and to assess total outcome after certain treatments in colorectal cancer patients.

1.1.2 Colon Cancer Progression

Cancer progression involves all of the changes that transform cells from normal to cancerous. The transformation of colon epithelium cells from normal to life-threatening metastatic cancer cells can be dissected into the following key steps: adenoma initiation, carcinoma initiation and metastatic cell initiation. In this section, I will introduce the progression process of colorectal cancer on both the morphological level and the molecular level.

1.1.2a Morphological Progression: Most colorectal cancers progress through a series of morphological changes **(Figure 1.4)**. In the first histological signs, one or more crypts will show accumulation of excess cells at the epithelial surface. The cells in these aberrant crypt foci may appear normal (i.e., hyperplasia), or show some abnormality (i.e., dysplasia). The accumulated cells will grow out from the epithelial surface to form a polyp or an early adenoma, but all cells are growing within the epithelial mucosa at this stage. However, as an early adenoma continues to grow, it will obtain more intracellular disorders to become a later adenoma or an invasive carcinoma. At these stages, cells will display more histological abnormality; tumors are tending to invade and infiltrate local structures and, eventually, spread from the site of its origin to adjacent and/or distant sites in the body, i.e., metastasis.

Metastasis is widely accepted as a multi-step process that involves the following steps

(**Figure 1.5**):

Local Invasion and Intravasation: Cancer cells invade into the local normal tissue and/or migrate into the nearby lymph vessels or blood vessels.

***Survive in Circulation System*:** Cancer cells start to circulate in the lymphatic and/or the blood circulation system. They need to survive against the harsh conditions and escape from the surveillance of the immune system to reach other parts of the body.

Arrest and Extravasation: Cancer cells arrest in capillaries at a distant location. They then invade the walls of the capillary vessels and migrate into the surrounding tissue.

Tumorigenicity and Proliferation: Cancer cells start amplification at the secondary site to form micrometastases.

Angiogenesis: Micrometastases stimulate the growth of new blood vessels to obtain the oxygen and nutrients necessary for continued tumor growth to form macrometastases.

However, only a very small population of cancer cells is capable of going through all the processes and form metastasis at the secondary site, counting on the malignant properties of individual cells. Thus, to study metastasis, the aforementioned properties and phenotype changes are widely used in research field as critical indicators to determine a metastatic cell.

1.1.2b Molecular Progression: It is widely accepted that particular molecular changes often associate with morphological stage and play important roles in driving progression. Studies from the past few decades have revealed that the following three genetic and/or genomic alterations contribute to the most molecular changes during colorectal cancer progression.

The Chromosomal Instability (CIN): A type of chromosomal abnormality, such that either whole or part of the chromosome are deleted or duplicated, leading to the DNA copy number variation (CNV), which results in a series of genetic changes that involve the activation of oncogenes such as *KRAS* and inactivation of tumor suppressor gene such as *TP53, DCC/SMAD4,* and *APC*. Synergistically, these gene alterations will also accelerate the alteration rate of the genome stability. CIN accounts for around 85% of colorectal cancer cases.

Inherited Mismatch Repair (MMR) Deficiency: Inherited mutations in the DNA MMR system cause microsatellite instability (MSI), which causes alterations of genes such as transforming growth factor receptor II (TGFBR2) and BAX.

Epigenetic hypermethylation: Silencing of MMR or other DNA repair system by hypermethylation on their corresponding functional gene's promoter also results in an increased MSI and somatic mutation rate.

The aforementioned events will directly result in a substantial amount of genetic/genomic changes (i.e., DNA copy number variation, somatic mutation and mRNA expression changes) during the process of cancer progression. However, only few of altered genes are playing the 'drivers' role, companied with enormous passenger genes' alterations. A rich history of investigations has uncovered several critical genes and pathways important in the initiation and progression of colorectal cancer, which has been summarized in **Figure 1.4**. Briefly, the initiation of adenoma is mostly driven by the mutation of *APC* and/or *CTNNB1*, the two regulatory gene of the canonical Wnt signaling pathway. Mutation of *RAS* is contributed to the transformation of an early adenoma to an

intermediate adenoma. Mutation of *DCC, SMAD2* and *SMAD4* result in a deregulation of Wnt and TGFβ signaling pathways and drive the tumor to grow into late stage adenoma. The initiation of carcinoma usually occurs in accompany with the homozygous mutation of *TP53* and *TGFBR2* mutation. These genes function as tumor suppressors that inhibit cell division and introduce cell apoptosis under stress. Tumor cells, which escape from the surveillance of these proteins, will consequently transform into cancerous stages.

In terms of metastasis, the following three theories have been widely accepted to explain the origin and rising of metastatic cells.

Cancer Stem Cell Theory: Recently, several concepts regarding the origins of cancer and metastasis have converged. Special interest has focused on the possibility that normal tissue specific stem cells and cancer stem cells also known as cancer initiating cells play fundamental roles in the malignant process. The cancer stem cell theory proposed that cancer stem cells, mostly rising from normal somatic stem cells, are origin of cancer (Barker, Ridgway et al. 2009). These cells may be numerically rare in primary tumor but nonetheless give rise to the distal metastasis and account for the poor response to intervention therapies in cancer patients (Dean 2005, Huang and Wicha 2008). Several *in vitro* and *in vivo* studies have proved such cells are present in colorectal cancer and other solid tumors. The normal stem cell surface markers, CD133, CD44 and EpCAM, as well as other stem cell-associated proteins, nestin, Bmi1 and Msi-1 can be used to identify the colon cancer stem cell population. (Ricci-Vitiani, Fabrizi et al. 2009).

Multi-Progression Theory: Alternatively, the multi-progression theory proposed that metastatic cells could arise at different stages from different origin of cells. Broadly,

further accumulations of the genetic changes in cancer cells are required to drive the progression to metastatic stages. However, the genetic mutation rate and progression speed present an enormous variation among patients (Campbell, Yachida et al. 2010). This theory emphasizes that the ongoing clonal transformation/'evolutions' among cells in both the primary tumor and secondary lesion are the causative reason of metastases, but the accomplishment of the transformation/'evolution' happens in a stochastic manner depending on when the cancer cells could acquire the mutations on the metastatic drivers gene. Comparative analysis between metastatic and non-metastatic cells revealed that differential expression of certain genes is associated with the metastatic potential of cancer cells (Hernandez, Smith et al. 2000, Ji, Greening et al. 2013). Don X. Nguyen et. al., reviewed several studies in characterizing some molecules that drive the progression on each step of metastasis **(Figure 1.6)** (Nguyen, Bos et al. 2009). Previous studies from Dr. Brattain's and Dr. Wang's lab also showed that *PIK3CA* mutations (Guo, Rajput et al. 2007), genes altered in TGFβ signaling pathway (Wang, Han et al. 1996, Wang, Yang et al. 2008, Liu, Rajput et al. 2011) and loss of microRNA-192 expression (Geng, Chaudhuri et al. 2014) play critical roles in colon cancer metastasis.

Cancer-Stromal Crosstalk Theory: Efforts also have been made in studying the cancer-stromal crosstalk and genetic changes in stromal tissue in cancer progression for the past two decades. Multiple reports have demonstrated that cancer cells can produce a range of growth factors (e.g. bFGF, VEGF, PDGF, and TGFβ) that modulate their surrounding stromal cells to activate critical signals that drive proliferation, angiogenesis, and motility while suppressing cell death (Mueller and Fusenig 2004). Increasing evidence has also indicated that the stromal cells themselves may also obtain mutations from environmental

stimulation or inherited genetic or genomic events to promote cancer to metastasize (Tlsty and Hein 2001).

As a summary of this section, the morphological change of colorectal cancer progression is always accompanied with certain amount of molecular deregulations, which may be playing the driver's role in promoting the progression process. Several theories have been applied to identify the malignant cells that carry the most altered and critical molecular changes during the disease progression. Therefore, a comprehensive understanding of colorectal cancer progression models on both morphological and molecular levels is very important for studying colon cancer, which ultimately will shed lights on the development of the intervention therapies of the disease.

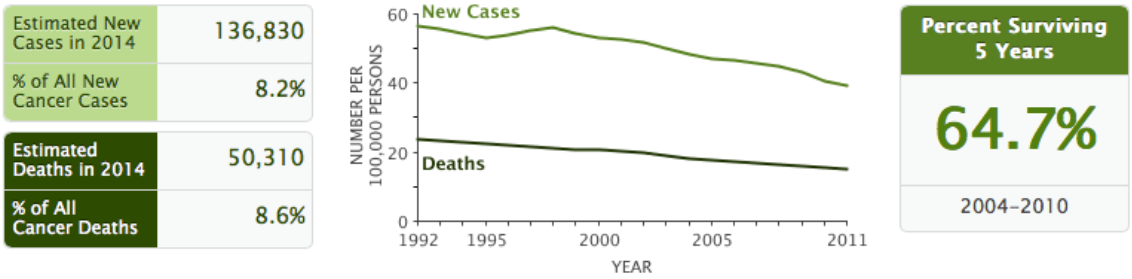


Figure 1.1 New Cases and Deaths of CRC. The number of new cases of colon and rectum cancer was 43.7 per 100,000 men and women per year, accounting for 8.2% of all new cancer cases. The number of deaths was 15.9 per 100,000 men and women per year, counting for 8.6% of all cancer deaths. Approximately 4.7 percent of men and women will be diagnosed with colon and rectum cancer at some point during their lifetime, based on 2009-2011 data. And the five-year survival rate is about 64.7%, based on 2004-2010 data.

Figure and data were obtained from SEER Stat Fact Sheets, 2015, http://seer.cancer.gov/statfacts/html/colorect.html

Primary Tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria[a]
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the pericolorectal tissues
T4a	Tumor penetrates to the surface of the visceral peritoneum[b]
T4b	Tumor directly invades or is adherent to other organs or structures[b,c]

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in one regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in four or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in seven or more regional lymph nodes

Distant Metastasis (M)

M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site (eg, liver, lung, ovary, nonregional node)
M1b	Metastases in more than one organ/site or the peritoneum

Stage	T	N	M	Dukes*	MAC*
0	Tis	N0	M0	-	-
I	T1	N0	M0	A	A
	T2	N0	M0	A	B1
IIA	T3	N0	M0	B	B2
IIB	T4a	N0	M0	B	B2
IIC	T4b	N0	M0	B	B3
IIIA	T1-T2	N1/N1c	M0	C	C1
	T1	N2a	M0	C	C1
IIIB	T3-T4a	N1/N1c	M0	C	C2
	T2-T3	N2a	M0	C	C1/C2
	T1-T2	N2b	M0	C	C1
IIIC	T4a	N2a	M0	C	C2
	T3-T4a	N2b	M0	C	C2
	T4b	N1-N2	M0	C	C3
IVA	Any T	Any N	M1a	-	-
IVB	Any T	Any N	M1b	-	-

Note: cTNM is the clinical classification, pTNM is the pathologic classification. The y prefix is used for those cancers that are classified after neoadjuvant pretreatment (e.g., ypTNM). Patients who have a complete pathologic response are ypT0N0cM0 that may be similar to Stage Group 0 or I. The r prefix is to be used for those cancers that have recurred after a disease-free interval (rTNM).

*Dukes B is a composite of better (T3 N0 M0) and worse (T4 N0 M0) prognostic groups, as is Dukes C (Any TN1 M0 and Any T N2 M0). MAC is the modified Astler-Coller classification.

Figure 1.2 Definition for T, N, M. (Left Panel) and Anatomic Staging of CRC (Right Panel)

Used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois. The original source for this material is the AJCC Cancer Staging Manual, Seventh Edition (2010) published by Springer-Verlag New York, www.springer.com.

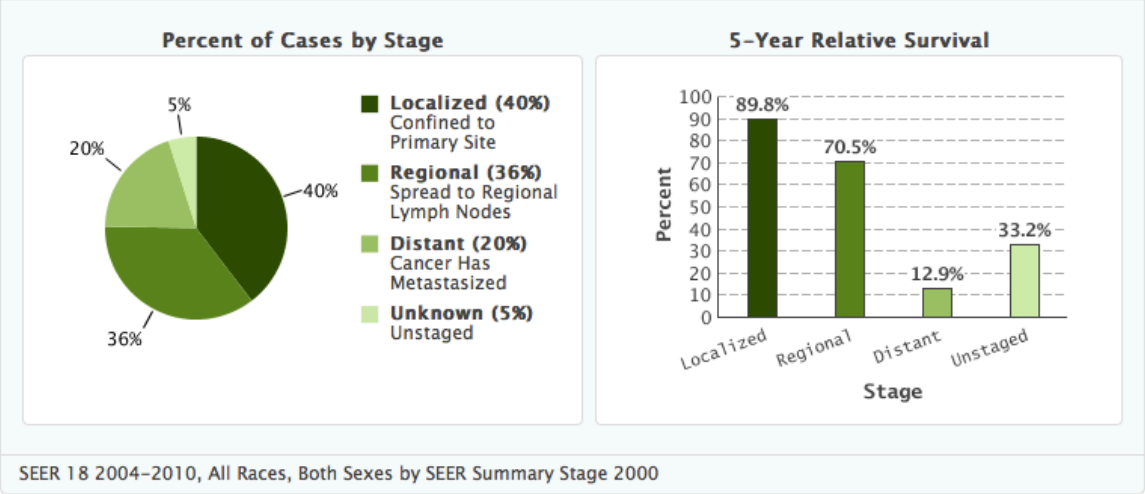


Figure 1.3 Percent of Cases and 5-Year Relative Survival by Stage at Diagnosis. At the time of diagnosis, there are about 40% of patients presenting with localized tumors, 36% of patients have the disease spread to regional lymph nodes and 20% of them have distal metastasis. The 5-year survival rate for patients with localized and regional tumor is 89.8% and 70.5%, respectively. While it drops dramatically to 12.9% when the tumor has metastasized to other organs.

Figure and data were obtained from SEER Stat Fact Sheets, 2015. http://seer.cancer.gov/statfacts/html/colorect.html

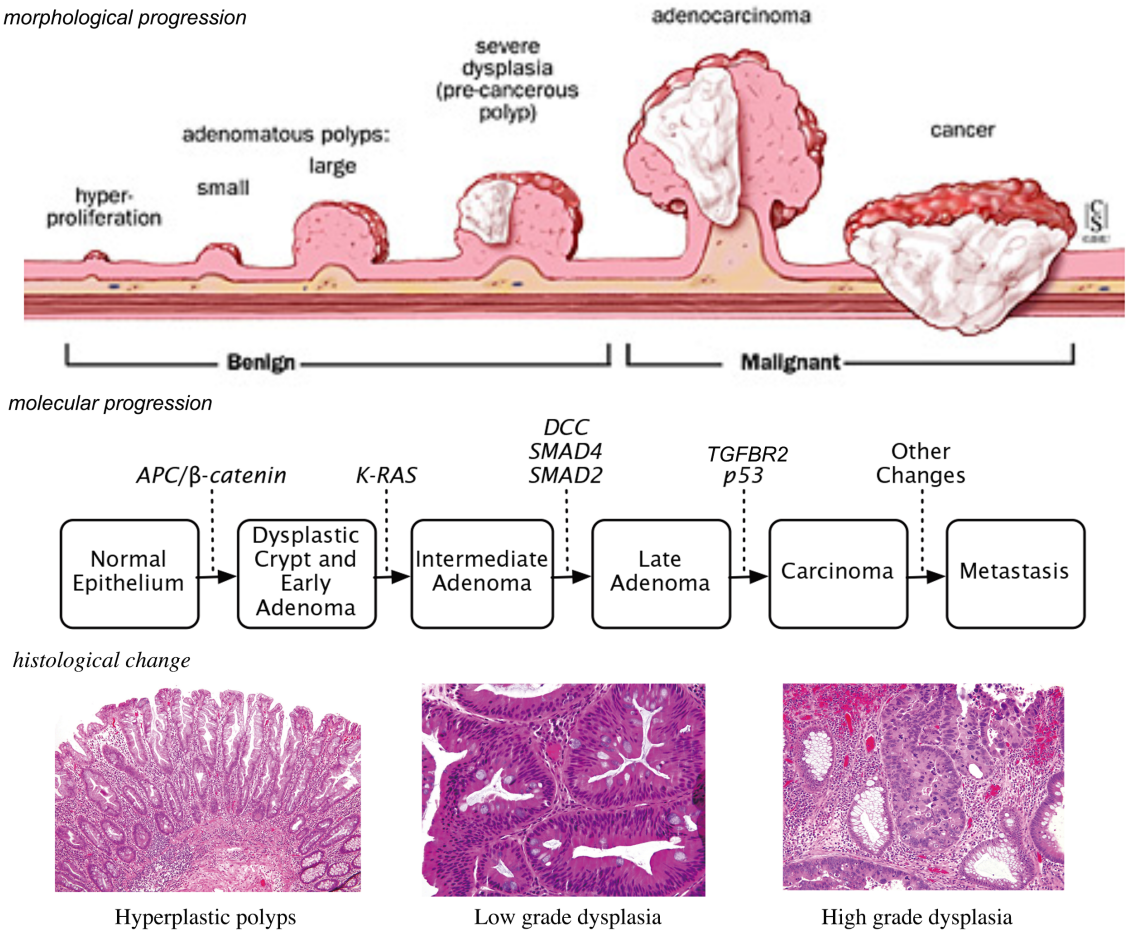


Figure 1.4 Classic Molecular Progression of Colon Cancer. Particular genetic changes often associated with the morphological stage, suggesting that the genetic changes play an important role in driving progression. Approximately 50–85% of colorectal cancers follow this pathway. *(Frank 2007) (Fleming, Ravula et al. 2012)*

Figure is rewrote from Figure 3.2 of Frank 2007, H&E stainings were obtained from Ravula et al. 2012 with permission.

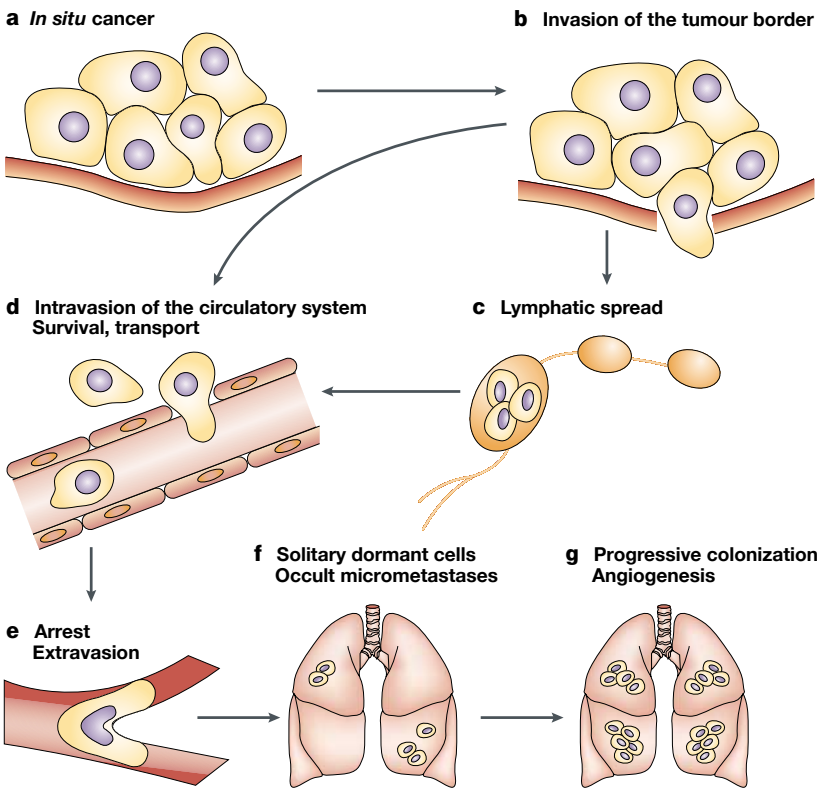


Figure 1.5 Multi-step Processes of Cancer Metastasis. The process begins with **a.** an *in situ* cancer. **b.** Invasion requires destruction of proteins in the matrix and stroma, EMT changes and increased motility of cancer cells. **c.** Metastatic cells can enter via the lymphatics, or **d.** directly through the circulation. **e.** Metastatic cells survive and arrest in the secondary site, and extravasation of the circulatory system follows. **f.** Metastatic colonization of the secondary site progresses through single cells to micrometastases and **g.** progressively grows into macrometastases. *(Steeg 2003)*

Figure was obtained from Figure 1 of Steeg 2003 with permission.

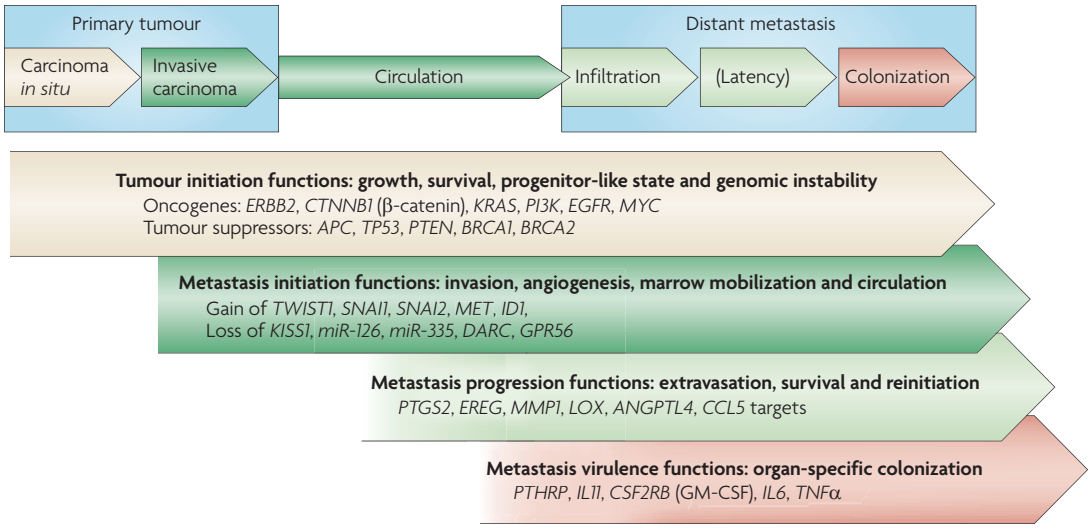


Figure 1.6 Metastasis-associated Genes. Functionally classified genes are associated with different steps of colon cancer development (Nguyen, Bos et al. 2009)

Figure was obtained from Figure 1 of Nguyen, Bos et al. 2009 with permission.

1.2 Altered Signaling Pathways in CRC

Combined with the classic genetic alteration models that are described in the last section, a comprehensive molecular study in colorectal cancer from The Cancer Genome Atlas (TCGA) Network in 2012 has also enriched people's understanding of how some well-defined pathways are deregulated (Cancer Genome Atlas 2012). The group has conducted an integrated analysis of mutations, DNA copy number alterations, promoter methylation status and messenger RNA and microRNA expression. From their analysis, they found that the Wnt signaling pathway was altered in 93% of all tumors, including biallelic inactivation of *APC* or activating mutations of *CTNNB1* in 80% of cases, which indicates that Wnt signaling alterations are prerequisites of tumor initiation. The TCGA group also found the TGFβ signaling pathway is universally deregulated with genomic alterations in *TGFBR1, TGFBR2, ACVR2A, ACVR1B, SMAD2, SMAD3* and *SMAD4* in 27% of the non-hypermutated and 87% of the hypermutated tumors. Genetic alterations in the PI3K and RAS-MAPK pathways are also common in CRC. The p53 pathway was found altered in 59% of non-hypermutated cases.

Therefore, according to this TCGA's analysis, together with a history of studies in CRC, activation of the Wnt signaling pathway at the onset of tumorgenisis and inactivation of the TGFβ signaling pathway in later progression stages are nearly ubiquitous events in CRC. In this dissertation, I will demonstrate the novel crosstalk between TGFβ signaling pathway and a Wnt target gene, LGR5.

1.2.1 Canonical Wnt Signaling Pathway

The canonical Wnt signaling pathway has been well characterized from two decades of studies. As was illustrated in **Figure 1.7,** in the absence of Wnt ligands, β-catenin is constantly degraded by the destruction complex, which is composed of the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 sequentially phosphorylate the N-terminal region of β-catenin, resulting in the recruitment of β-Trcp, an E3 ubiquitin ligase subunit, and subsequent β-catenin ubiquitination and proteasomal degradation. This continual distruction of β-catenin prevents the accumulation of this protein and translocation to nucleus. In this case, Wnt target genes are repressed by TCF-TLE/Groucho and histone deacetylases (HDAC). With the presence of Wnt ligands, the seven-transmembrane receptor Frizzled (Fz) and its co-receptor, low-density lipoprotein receptor related protein 6 (LRP6) or its close relative LRP5 will activate and form a likely Wnt-Fz-LRP6 complex. The complex formation will recruit the scaffolding protein Dishevelled (Dvl) to phosphorylate LRP6 and sequentially activate the destruction complex. These events lead to inhibition of Axin-mediated β-catenin phosphorylation and thereby to the stabilization of β-catenin. Accumulated cytoplasmic β-catenin will travel to the nucleus to replace the repressors from TCF/LEF and activate Wnt target gene expression (MacDonald, Tamai et al. 2009).

1.2.2 Roles of LGR5 and RSPOs in Colorectal Cancer

LGR5 (GPR49) is a leucine-rich repeat containing G protein-coupled receptor (GPCR) and one of the LGR family members (Hsu, Liang et al. 1998, McDonald, Wang et al. 1998). As most of the other GPCRs, the structure of LGR5 and its homologues, LGR4

and LGR6, contain an N-terminal extracellular domain (i.e., ectodomain), a seven-transmembrane domain and a C-terminal intracellular domain **(Figure 1.8)**.

LGR5 and its homologs LGR4 and LGR6 are receptors of R-spondins (RSPOs) (Carmon, Gong et al. 2011, de Lau, Barker et al. 2011, Glinka, Dolde et al. 2011), which are secreted agonists of canonical Wnt signaling (Binnerts, Kim et al. 2007, Kim, Wagle et al. 2008). Peng and de Lau et al., first reported the crystal structure of a Fu1-Fu2 fragment of RSPO1 and its complex with the ligand-binding ectodomain of LGR5 (Peng, de Lau et al. 2013). And this short RSPO1 Fu1-Fu2 fragment suffices for full activity of the downstream signaling of LGR5. A similar complex structure of RSPO1 Fu1-Fu2 with the ectodomain of LGR4 was reported by Wang et al., indicating a common machanism of signaling transduction of LGR4, LGR5 and LGR6. (Wang, Huang et al. 2013)

Unlike other GPCRs, LGR4, LGR5 and LGR6 are not coupled to either G proteins or β-arrestin when they are stimulated by RSPOs (Carmon, Gong et al. 2011). Instead, upon binding RSPOs, they form a physical complex with Wnt receptor Frizzled and its co-receptor LRP6 to regulate Wnt canonical signaling (de Lau, Barker et al. 2011). LGR5 itself is also a target gene of the Wnt signaling pathway (Haegebarth and Clevers 2009), indicating a feedback loop between the receptor and the Wnt signaling pathway **(Figure 1.9)**. In the beginning, people believed RSPO-LGR5 acted as an enhancer of Wnt signaling pathway (Carmon, Gong et al. 2011, Glinka, Dolde et al. 2011). However, recent evidence has shown that RSPO-LGR5 axis is a negative feedback factor of Wnt signaling. RSPO-LGR5 can form a supercomplex with LRP6-Fz. Though this can transiently increase Wnt signaling activation, eventually it will lead to an enhanced internalization and degradation of the receptors (Carmon, Lin et al. 2012, Snyder,

Rochelle et al. 2013). The role of LGR5 in regulating Wnt signaling becomes more dynamic with the identification of the complex formation between RSPO-LGR5 and two Wnt antagonists, ZNRF3 and its homolog RNF43. (Chen, Chen et al. 2013, Peng, de Lau et al. 2013, Xie, Zamponi et al. 2013). The authors believe that RSPO-LGR5 can competitively bind with these Wnt antagonists to regulate Wnt signaling activity. As a summary of all the findings described previously, LGR5 acts as a negative regulator when Wnt signaling is over-activated, while it neutralizes the Wnt antagonists when they are redundant. Therefore, other than playing a single role as either an enhancer or an inhibitor, LGR5 is more like a buffering molecule whose role is to prevent the Wnt signaling pathway from getting over-activated or –under-activated.

The physiological role of LGR5 during normal intestinal development and tumor initiation has been studied for the past decade. Early in 2004, Morita et al. has reported that LGR5 null mice suffer a neonatal death and the lethality is associated with ankyloglossia and gastrointestinal distension (Morita, Mazerbourg et al. 2004), indicating an important functional role of LGR5 in the gastrointestinal development. The possible underlying mechanism was first revealed by Garcia and Ghiani et al. in 2009 that LGR5 deficiency leads to premature Paneth cell differentiation associated with the activation of the Wnt signaling pathway. These results supported a possible negative feedback role of LGR5 in regulating the over-activated Wnt signaling pathway in normal intestinal development (Garcia, Ghiani et al. 2009).

Nowadays, the concepts regarding normal stem cells and cancer stem cells have converged. The relationship between LGR5 and intestinal cancer was first revealed by Clevers and Barker' group, who have successfully identified the molecule as a marker for

proliferating adult stem cells in the small intestine in 2007 (Barker, van Es et al. 2007). The group also observed that conditional deletion of *APC* in LGR5-positive stem cells but not in LGR5-negative cells led to a rapid growth of large adenomas in mouse small intestine (Barker, Ridgway et al. 2009), suggesting that intestinal tumors arise from LGR5-positive stem cells.

However, whether LGR5-positive stem cells are the only origin of colon tumor is still under debate. A recent study showed that combination of *APC* loss and *KRAS* mutation can also initiate tumors from LGR5-negative villus cells (Schwitalla, Fingerle et al. 2013). Taken together with the colon cancer metastasis theories described in the previous section, many cancer researchers believe malignant cells can also arise from the non-stem cells lineage. Several clinical studies have evaluated the relationship between expressions of certain stem cell markers with the most clinically relevant features of colorectal cancer. These results suggest that, despite the increased expression of stem cell markers, including EpCAM, CD166, CD44s, ASCL2 and LGR5, it is the overall decreased expression of these markers that is linked to a more aggressive tumor phenotype and poorer prognosis (Lugli, Iezzi et al. 2010, de Sousa, Colak et al. 2011). This studies indicate that those stem cell markers might play a suppressor role in cancer malignancy or cancer cells that rising from the other lineages of epithelial cells, other than stem cell lineage are responsible for the malignancy of the disease.

As for LGR5, conflicting reports have been published regarding the functional role of LGR5 in colon cancer tumorigenesis. Some studies reported that RSPO-LGR5 signaling has a tumor suppressive phenotype in colon cancer (Walker, Zhang et al. 2011, Wu, Qiu et al. 2014), whereas others showed that LGR5 promotes tumorigenicity (Tsuji, Kawasaki

et al. 2014). The role of LGR5 in colon cancer tumorigenesis remains ambiguous, which is likely due to the dual role of RSPO-LGR5 axis in regulating Wnt signaling pathway at the onset of the disease. As Wnt signaling is widely and coercively activated after tumor initiation, it is yet to be determined whether LGR5 still continuous contributes to the later stages of colon cancer progression and metastasis.

1.2.3 TGFβ Signaling Pathway

The transforming growth factor-beta (TGFβ) signaling pathway is a key player in cancer biology, and its deregulation can result in tumor development. TGFβ comprises a group of multifunctional polypeptides that regulate cellular processes through binding to three major TGFβ receptors, type I (RI), type II (RII) and type III (RIII) (Hu, Datto et al. 1998). As indicated in **Figure 1.10**, upon binding TGFβ, RII recruits and activates RI, which then phosphorylates and activates Smad2 and Smad3. Activated Smad2 and/or Smad3 form complexes with Smad4 and translocate to the nucleus, where they will further recruit transcriptional coactivators, corepressors and chromatin remodeling factors to regulate gene expression (Massague, Seoane et al. 2005). The expression of each target gene is dependent on transcription partner cofactors whose expressions are highly restricted by cells type and their environmental condition.

However, TGFβ activation can also shift to its non-canonical pathway when the signaling transduction components in the canonical pathway are impaired. The non-canonical TGFβ signaling includes various branches of MAP kinase (MAPK) pathways, Rho-like GTPase signaling pathways and phosphatidylinositol-3-kinase (PI3K)/AKT pathways (Zhang 2009). The non-canonical pathways could mediate the epithelial-mesenchymal

transition (EMT) phenotypes or compete with the canonical effects of TGFβ. Consequently, the output of a TGFβ response is highly contextual throughout development, across different tissues and also in cancer.

TGFβ is an autocrine-negative growth factor, as evidenced by stimulation of growth of several cell lines treated with TGFβ-neutralizing antibody (Arteaga, Coffey et al. 1990, Hafez, Infante et al. 1990, Wu, Theodorescu et al. 1992, Wu, Sun et al. 1993, Wang, Han et al. 1996, Hu, Datto et al. 1998). Defective TGFβ signaling occurs in 30-50% of colon cancer patients and in most of the hyper-mutated tumor type. (Markowitz, Wang et al. 1995, Grady, Myeroff et al. 1999, Salovaara, Roth et al. 2002, Cancer Genome Atlas 2012). We and others have demonstrated experimentally that TGFβ mediates tumor suppressor activity in a variety of cancers including colon cancer, and that loss of TGFβ signaling leads to malignancy (Wu, Theodorescu et al. 1992, Wu, Sun et al. 1993, Sun, Wu et al. 1994, Ye, Foster et al. 1999, Wang, Sergina et al. 2004, Veenendaal, Kranenburg et al. 2008). For example, transfecting TGFβ1 antisense into the TGFβ responsive FET colon cancer cells led to robust malignant progression (Wu, Theodorescu et al. 1992, Wu, Sun et al. 1993); Inactivation of TGFβ RII led to increased tumorigenicity in athymic mice (Ye, Foster et al. 1999). In contrast, we demonstrated that restoration of TGFβ signaling by ectopic expression of TGFβ receptors (RI or RII) resulted in reduced tumorigenicity (Sun, Wu et al. 1994, Wang, Han et al. 1996). Taken together, these studies indicate that TGFβ signaling plays an essential role in deterring tumor progression in human colon carcinoma cells. Studies of human tumor samples indicate that activity of the TGFβ signaling pathway is reduced in metastatic colon

tumors as compared to primary tumors by measuring phospho-Smad2 in a tissue array study (Veenendaal, Kranenburg et al. 2008). In addition, another tissue array study of 310 colon carcinomas show that lower expression of TGFβ1 and loss of nuclear Smad4 expression are associated with presence of lymph node metastasis (Bacman, Merkel et al. 2007). These studies indicate that loss or reduction of TGFβ signaling in human colon cancer is associated with development of metastasis, and that our studies of TGFβ as a metastasis suppressor are relevant to human colon cancer.

Traditionally, TGFβ's tumor suppressive activity has been attributed to its ability to inhibit cell cycle progression. However, its role in apoptosis as a mechanism of tumor suppression is under-explored. Our preliminary studies indicate that abrogation of TGFβ signaling enables increased survival under GFDS in colon cancer cells. In addition, we have shown that loss of TGFβ signaling is associated with increased metastasis, whereas enhanced TGFβ signaling suppresses metastasis in an orthotopic model of colon cancer. These results suggest that endogenous TGFβ acts as an intrinsic fail-safe mechanism in the early stages of carcinogenesis by promoting cell death under stress conditions to prevent malignant progression, whereas abrogation of TGFβ signaling leads to activation of oncogenic signals that protect tumor cells from apoptosis. However, it is not clear how loss of TGFβ signaling leads to resistance to stress-induced apoptosis. Answering these questions will provide evidence for new TGFβ tumor suppressor functions that could be mimicked by novel treatments.

In contrast to its tumor suppressor function in colon cancer, TGFβ functions as a tumor promoter in other types of cancer including breast cancer. Many studies have shown that

TGFβ promotes metastasis in breast cancer (Wakefield and Roberts 2002). However, it has been reported that knockout of type II TGFβ receptor in mammary epithelia induced tumor formation and promoted metastasis, indicating that TGFβ suppresses metastasis in breast cancer (Forrester, Chytil et al. 2005, Yang, Huang et al. 2008)(17;18). The contradicting role of TGFβ in metastasis of colon and breast cancer suggests that different mechanisms are involved. The metastasis promoting activity of TGFβ in breast cancer has been associated with increased motility and invasion, and with induction of an epithelial to mesenchymal transition (EMT) (Wakefield and Roberts 2002), which confers resistance to the apoptotic effects of TGFβ (Valdes, Alvarez et al. 2002).

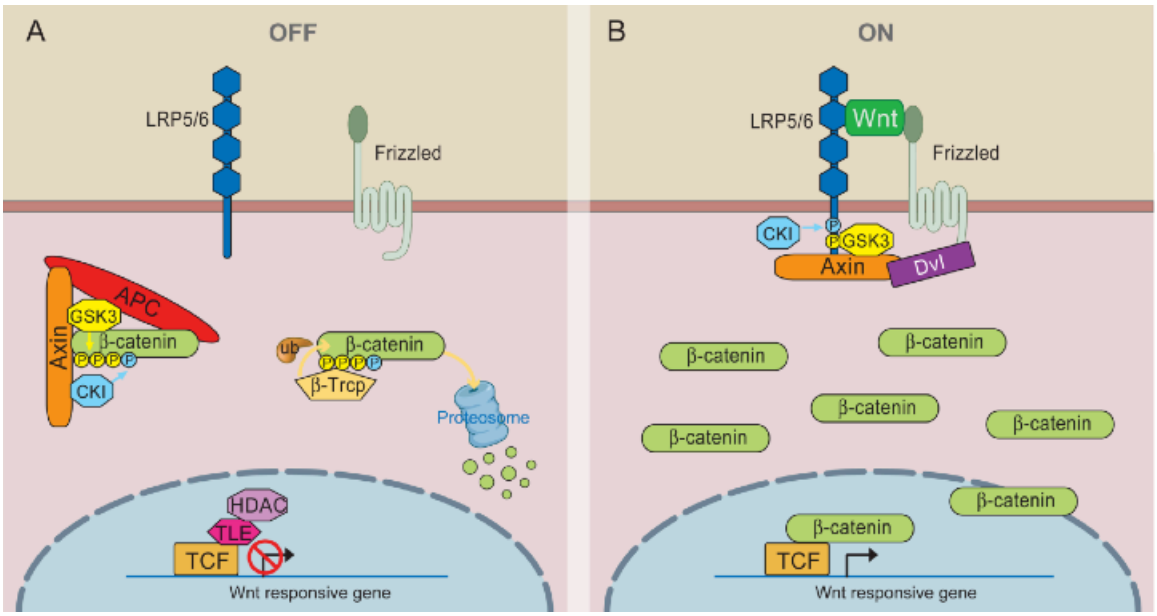


Figure 1.7 Canonical Wnt Signaling Pathway. **A.** In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, APC, GSK3 and CK1, and is phosphorylated by CK1 (blue) and subsequently by GSK3 (yellow). Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-Trcp, which targets β- catenin for proteosomal degradation. Wnt target genes are repressed by TCF-TLE/Groucho and HDAC. **B.** In the presence of Wnt ligand, a receptor complex forms between Fz and LRP5/6. Dvl recruitment by Fz leads to LRP5/6 phosphorylation and Axin recruitment. This disrupts Axin-mediated phosphorylation/degradation of β-catenin, allowing β-catenin to accumulate in the nucleus where it serves as a co-activator for TCF to activate Wnt responsive genes. *(MacDonald, Tamai et al. 2009)*

Figure obtained from MacDonald, Tamai et al. 2009 with permission.

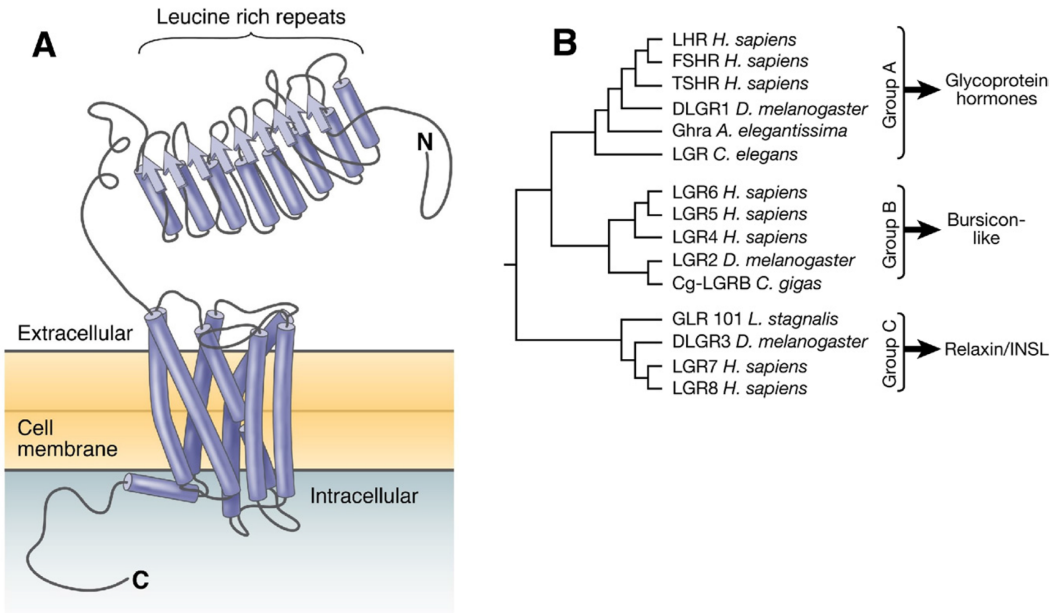


Figure 1.8 LGR5/GPR49 is a G-protein–coupled Receptor Related to the Glycoprotein Hormone Receptors. A. Predicted structure of LGR5, containing a large extracellular domain with multiple leucine-rich repeats that mediate ligand interaction, a 7TM domain, and a C-terminal intracellular domain for signal transduction. **B.** Phylogenetic relationship between LGR5 and related family members. The ligands for LGR4, LGR5, and LGR6 have not been identified. *(Barker and Clevers 2010)*

Figure obtained from Gastroenterology, Volume 138, Issue 5, 2010, 1681 – 1696 with permission

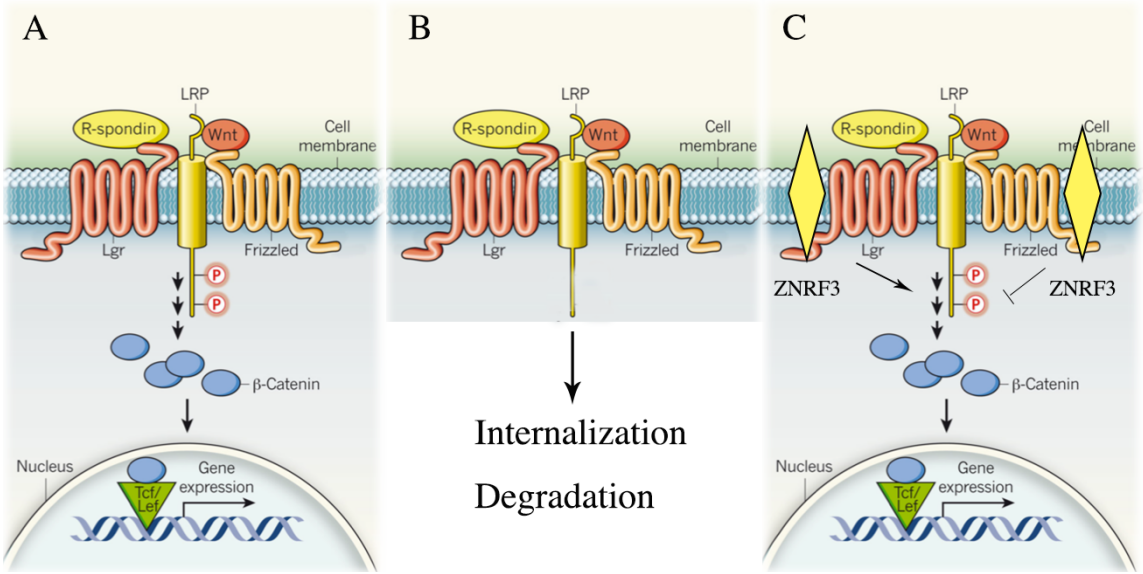


Figure 1.9 RSPO-LGR5 Regulation of Wnt Signaling Pathway. A. After binding with Wnt-Fz-LRP, RSPO-LGR5 transiently amplify the Wnt signaling and then **B.** bring the whole complex into cytoplasm for degradation. **C.** RSPO-LGR5 can competitively bind to the Wnt antagonist ZNRF3 to prevent its inhibition of Wnt signaling.

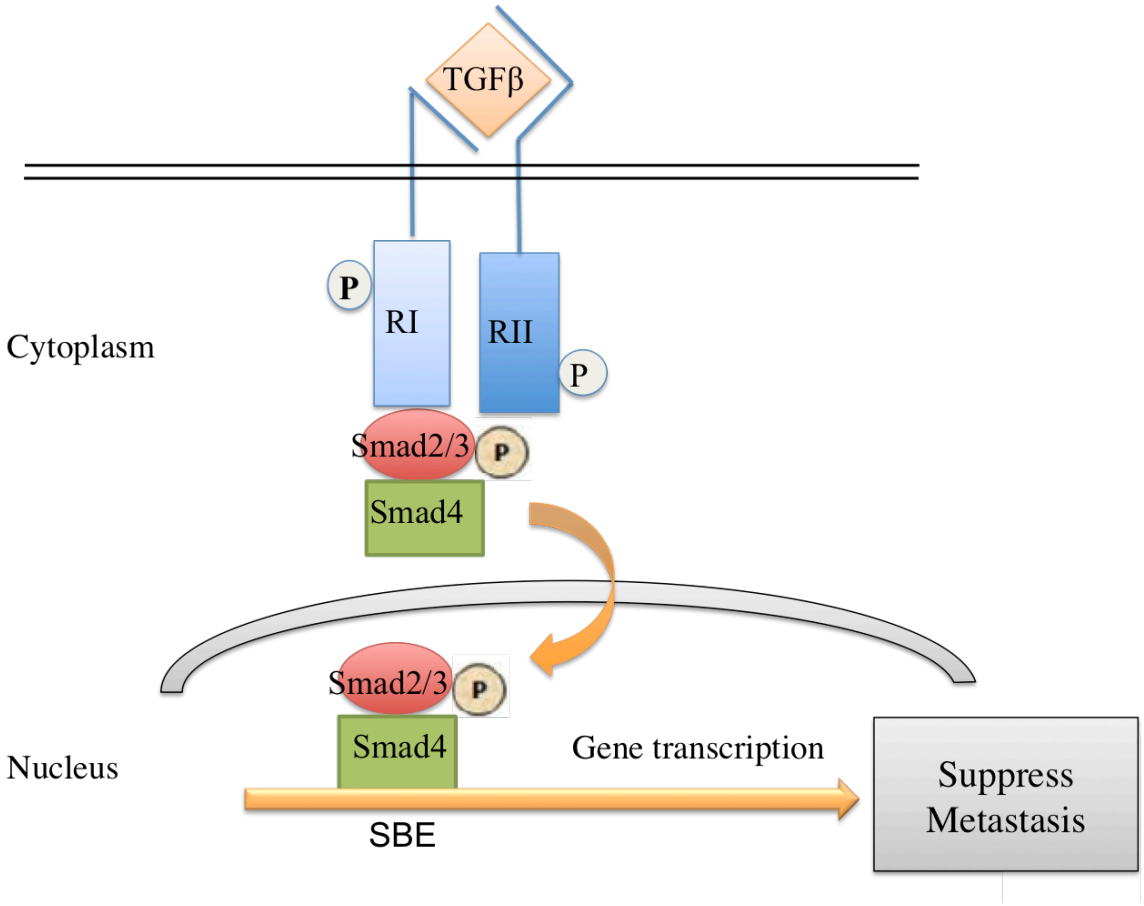


Figure 1.10 Canonical TGFβ Signaling Pathway. TGFβ ligands dimerizes and binds to RII and subsequently recruits RI, which is activated by the phosphorylation of the GS domain in the cytoplasmic region. Activated RI further activates smad2/3, which will form complex with Smad4, translocate to nucleus and bind to the smad binding elements (SBE) on DNA to facilitate target gene transcription.

1.3 Study Strategies of Colorectal Cancer Metastasis

The aforementioned introductions have demonstrated the process of cancer progression morphologically and molecularly. The goal of the studies in this dissertation is to reveal the role of RSPO-LGR5 axis in colorectal cancer metastasis. Although different theories have conflicting views on the origins or the rising routes of metastatic cells, our objective is to characterize RSPO-LGR5's function in regulating malignant properties of colon cancer cells and the underlying mechanisms by which altered critical signaling pathways drive the metastatic process.

We manipulated the expression of LGR5 by using shRNA interference to knock down its expression and/or using retroviral infection to increase its expression in colon cancer cell lines. Cell malignant phenotypes were characterized *in vitro* and *in vivo*. For *in vitro* studies, we characterize the cells' proliferation, apoptosis and colony/tumor formation capability. For *in vivo* studies, we used an orthotopic transplantation technique to determine the metastatic capability of the cells in animal models. Alterations of certain genetic molecules were determined by Western Blot, RT-PCR and immunohistochemistry (IHC) staining. Cell signaling activity was measured by luciferase reporter assay or activation and/ or expression of the signaling effectors or target genes. Protein-protein interactions were determined by immuneprecipitation (IP), immunofluorescence and confocal microscopy.

Paraffin embedded human colon cancer specimen were collected from the UNMC tissue bank as tissue micro array (TMA) blocks, correlations between interested molecules were examined by IHC staining. In silico analysis were perform on the Oncomine, Nexus Copy Number and the cancer genome atlas (TCGA) platform.

CHAPTER 2

Materials and Methods

** The Institutional Biosafety Committee (IBC) has approved the protocol of all experiments involving hazardous biological materials in the study.*

** The Institutional Animal Care and Use Committee (IACUC) has approved the protocol of all the animal experiments in the study.*

** The Institutional Review Board (IRB) has approved the protocol of all the experiments involving human subjects in the study.*

2.1 Cell Lines and Cell Culture

The human colon carcinoma cell lines TENN, RKO, HCT116, HCT116b, CBS and FET cells were isolated from human colon cancer patients and established in tissue culture as described previously(Brattain, Brattain et al. 1981). Cells were maintained at 37°C in a humidified incubator with 5% CO_2 and cultured in SM medium (Serum-free McCoy's 5A medium; Sigma) supplemented with 10 ng/mL of epidermal growth factor (EGF), 20 mg/mL of insulin, and 4 mg/mL of transferrin. When cells were under growth factor deprivation stress (GFDS), they were cultured in SM medium in the absence of growth factor or serum supplements for the indicated time without changing medium in between.

2.2 Plasmids

Two LGR5 shRNAs were subcloned into FSIPPW lentiviral vector under the H1 promoter. The two pLKO.1 lentiviral TGFBR1 shRNA constructs were gifts from Dr. Luzhe Sun (University of Texas Health Science Center, TX, USA). Full-length human TGFBR2 cDNA was amplified from FET cell and subcloned into the pMX retroviral vector. The N-terminal HA-tagged full-length human LGR5 retroviral plasmid was a gift from Dr. Keith Johnson (University of Nebraska Medical Center, NE, USA).

2.3 Transfection and Stable Cells Establishment

Transfections were performed using Lipo-fectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The lentiviral constructs were co-transfected with two lentiviral packaging plasmids pMD2.G and psPAX2 (gifts from Dr. Didier Trono, Addgene plasmid #12259 and #12260) into the 293TN cells. The retroviral constructs were co-transfected with pVSV.G (a gift from Tannishtha Reya, Addgene Plasmid #14888) into 293GP cells. Viruses were harvested at 48h and used to infect cells. Stable CBS/FET-LGR5 shRNA knockdown cells, HCT116-TGFBR1 knockdown cells and HCT116-TGFBR2 overexpression cells were established under the selection of 2ng/ml puromycin for five days. HCT116-LGR5/HA overexpression cells were stably generated after one-week selection with 600ng/ml G418. Stable pools were then confirmed by western blotting

2.4 Antibodies and Reagents

LGR5 monoclonal antibody (EPR3065Y) (#ab75850) and phospho-smad2 (Ser465/467) (#AB3849) was purchased from Abcam and EMD Millipore respectively. Ki67 (#550609) was obtained from BD Biosciences. Cleaved PARP (#5625), smad2/3 (#8685), smad4 (#9515), TGFBR1 (#3712), TGFBR2 (#11888), LRP6 (#2560), phospho-LRP6 (Ser1490) (#2588) and beta-actin (#4970) antibodies were obtained from Cell Signaling Technology. Recombinant human TGFβ1 and RSPO1-4 were purchased from R&D Systems. TGFβ-receptor type I kinase inhibitor (SB525334), salinomycin and tharpsigargin were purchased from Sigma-Aldrich.

The Cell Death Detection ELISA plus kit was from Roche Life Science. ApopTag Plus

Peroxidase In Situ Apoptosis Detection kit (TUNEL Assay) was from Millipore. The IHC staining kit was from DAKO North America. Hematoxylin and Eosin were obtained from Sigma-Aldrich.

2.5 RT-PCR

Total RNA was isolated from cell lines or tumor samples using TRIZOL reagent (Invitrogen). A total of 2μg of total RNA was reverse-transcribed to cDNA with MLV-reverse transcriptase (Promega). β-actin was used as endogenous control.

2.6 Western-Blot Analysis

Cells were washed three times with cold phosphate buffer saline (PBS) then lysed in RIPA buffer (EMD, Millipore) containing 50 mmol/L NaF, 1 mmol/L Na3VO4, 25 μg/mL β-glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche Life Science) for 30 min on ice. For complete lysis, sonication was used to disrupt the cell membranes and shear DNA. Cell debris was removed by centrifuging the lysate at 1,4000 rpm for 20 min at 4 °C. Protein concentration of the supernatant was measured by the BCA protein assay according to the manufacture's instruction (Pierce Biotechnology). After denaturing in 4xSDS sample buffer (12% SDS, 25% Glycerol, 150mM Tis-HCl, 0.05% Bromophenol blue, 6% β-mercaptoethanol), protein lysate was resolved by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Thermo Scientific). The membrane was blocked with 5% non-fat dry milk in PBST (50mM Tris, pH 7.5, 150mM NaCl, 0.01% Tween 20) for one hour at room temperature and then incubated with primary antibodies for overnight at 4 °C. After three times washing with PBST, the membrane was incubated with species-specific horseradish peroxidase-conjugated

secondary antibody for 1 hour at room temperature. Super Signal West Pico or Femto Chemiluminescent System (Thermo Scientific) was used for protein detection.

2.7 Immunoprecipitation (IP)

Cells were lysed and protein concentration was measured as was described previously. Immunoprecipitation (IP) was performed with 1mg protein aliquots using magnetic beads (PureProteome Protein G or Protein A Magnetic Beads, Millipore). The 1mg protein sample was mixed with the primary antibody according to the dilution described in manufacture instruction and incubated overnight at 4°C. The following day, 50μL suspended beads per IP reaction were aliquot into 1.5 mL microcentrifuge tubes followed by three times washing with PBST. The IP samples containing the primary antibody were incubated with the washed magnetic beads for 30 minutes at room temperature with continuous mixing to generate the IP immune complex. Following pelleting of the beads, the IP supernatant fraction was removed to a new tube and stored for further analysis. The beads were washed three times with 500μL of PBST. After the final wash, the pelleted magnetic beads were re-suspended in 60uL of 1×SDS sample buffer. The samples were denatured at 95°C for 10min. The beads were pelleted and the supernatant were transferred to a new tube. Proteins in the complex were then detected by Western blotting technique.

2.8 Immunofluorescence (IF) Microscopy

Immunofluorescence microscopy was performed to visualize the localization of the proteins involving the complex formation. Cells were grown on poly-D-lysine–coated glass cover slips suitable for immunofluorescence microscopy. Following drug treatment,

cells were washed with IF buffer (1% BSA in PBS) and fixed with 4% w/v formaldehyde for 20 min at room temperature. Following a 1 min wash with PBS, cells were permeabilized with 0.3% Triton X-100 in IF buffer for 15 min. After further washing, the cells were blocked with 10% goat serum in IF buffer for 1h at room temperature. Cells were then processed with primary antibodies overnight at 4°C, and then washed three times for 1min with IF buffer. Secondary antibodies (Invitrogen) conjugated with Alexa Fluor 488 and Alexa Fluor 594 was used for staining as appropriate and incubated with the cells in the dark. Following washing and DAPI treatment, the cell staining was visualized with a Zeiss 710 confocal laser-scanning microscope equipped with 4 lasers: a Blue Diode 405nm; an Argon Laser 458/477/488 514nm; a DPSS 561nm and a HeNe 633nm. The image-processing software such as Zeiss and ImageJ were used for the quantitation of the fluorescence and analysis of colocalization, etc

2.9 MTT Assay

MTT assay was used to quantify the number of viable cells. Briefly, 50% v/v MTT reagent (2% w/v 3-[4,5-Dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) was added to each culture well followed by incubation at 37 °C for 2 hours. The medium was removed and cells were dissolved in dimethyl sulfoxide (DMSO) followed by a gentle shaking for 10 minutes. 200µL dissolvent were then transferred to 96-well plates and the absorbance was read at a 570 nm wavelength under the microplate reader (Bio-Rad). All samples were measured in triplicates.

2.10 Colony Formation and Soft Agarose Assay.

Colony formation assay was used to measure the cell proliferation capability and

anchorage-dependent growth. To perform the assay, cells were seeded in six-well plates at 500 cells per well density and colony number was measured by MTT staining after 3 weeks. Soft agar assay were performed to test the anchorage-independent growth of the cells and also to determine the tumorogenesity of the cells *in vitro*. To perform the experiment, 1ml SF medium contained 0.8% soft agar (BD Biosciences) was poured in six-well plates as a base layer. Cells were then suspended in SF medium containing 0.4% low melting point agarose (Thermo Scientific) and seeded upon the base layer at a density of 3000 cells per well. Plates were maintained at 37°C in a humidified incubator. Colonies were stained by 1% w/v iodonitrotetrazolium violet (Sigma-Aldrich) after 3 weeks and the numbers of colonies were counted under a microscope. All experiments were conducted in triplicates.

2.11 Cell Apoptosis Assay

Cell apoptosis was measured by DNA fragmentation assay (cell death ELISA assay). Cells were seeded in 96-well plates and allowed to grow to 80% confluence. The cells were then changed to SM medium for GFDS culture for 2-5 days as was indicated in each of the experiments. Apoptosis measurement was then performed using a cell death ELISA kit (Roche Life Science) as described in the manufacturer's protocol. DNA fragments were measured on the quantitative “sandwich enzyme immunoassay” principle. Briefly, anti-DNA-peroxidase (POD) reacted with the DNA fragments in the cell lysate. Histone contained DNA fragments were then captured by the anti-histone antibody coated on the ELISA plates. With the presence of HRP substrate (ABTS, 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt substrate), the DNA fragments-POD complexes were detected under the absorbance reader at a 405 nm

wavelength. The viable cell number was measured by MTT staining to normalize the assay. The ratio of OD 405 / OD 570 is directly proportional to the number of the apoptotic cells in the culture. All experiments were conducted in triplicates.

2.12 Luciferase Reporter Assay

Luciferase reporter assay was used to determine the Wnt or TGFβ signaling activity in different cell lines or with different ligand treatment. To test Wnt activity, a TOPFlash plasmid (EMD Millipore) that contains two sets of three copies of the wild-type TCF binding regions before the luciferase expression gene were transiently transfected to the cells. Respectively, an SBE4-Luc (Addgene plasmid # 16495) plasmid that contains four copies of smads-binding elements (SBE4) before the luciferase expression gene were transfected into the cells for the detection of TGFβ activity. The pRL-CMV (Promega) plasmid that constitutively expresses the renilla gene was used as an internal control. To perform the experiment, 8000 cells per well were seeded in 96-well the day before transfection. TOPFlash or SBE4-Luc was co-transfected with the pRL-CMV and cells were maintained in culture for 2-3 days as was indicated in the experiments. Luciferase activity measurements were performed using the Dual Luciferase Assay System (Promega). The luminescent signals of firefly and renilla were read under the BioTek Synergy MX Reader according to the manufacture instruction. All experiments were conducted in triplicates.

2.13 *In Vivo* Orthotopic Transplantation

Orthotopic mouse model of colon cancer has been well established to represent the human colon cancer progression and metastasis. Two techniques have been widely used

to establish the model. One technique involves injection of a colon cancer cell suspension into the cecal wall. The other technique involves transplantation of a piece of subcutaneous tumor onto the cecum. Both techniques are similar and require mouse anesthesia and laparotomy for exposure of the cecum. In our study, we have used the later technique due to the reason that the subcutaneous tumor can introduce a more heterogeneous population of cancer cells that has been established in xenograft model, which better represents the nature property of primary tumors in human colon cancer disease.

To start with, the control and LGR5 knockdown CBS cells were stably transfected with GFP construct. Exponentially growing GFP-labeled cells ($5x10^6$) were inoculated subcutaneously onto the dorsal surfaces of the athymic nude mice (Harlan Laboratories). Once xenografts were established (~ 5 weeks post-inoculation), mice were terminated and tumors were excised into $1mm^3$ pieces. For implantation procedures, animals were anesthetized with isoflurane inhalation. A 1cm laparotomy was done for the exteriorization of the cecum and ascending colon of the animal. The serosa was disrupted at two locations of the cecum. Two pieces of xenograft were positioned and sub-serosally implanted using an 8-0 nylon suture at the disrupted serosal locations. The bowel was then returned to the peritoneal cavity and the abdomen was closed with 5-0 vicryl sutures. Buprenex was delivered to the animal three times a day at the dose of 0.1mg/kg for three days post-surgery. Fluorescence imaging was performed weekly to follow tumor growth and progression (LightTools). Around seventy days post-implantation, animals were euthanized. Organs were explanted, imaged, and immediately placed in buffered 10%

formalin. Tissues were then processed and embedded in paraffin. Slides were cut for H&E and IHC staining.

2.14 Hematoxylin and Eosin (H&E) and Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded tissue blocks of primary tumor, liver and lung were made as was described previously and sectioned for further analysis. Distal metastasis to liver and lung were evaluated from the H&E staining of liver and lung tissue; Local invasiveness and lymph tubular metastases were also evaluated from the H&E staining of primary tumor. IHC staining of TUNEL, Ki67 and pSmad2 expression were performed in the primary tumor sections. TUNEL assays were performed using ApopTag Peroxidase In Situ Oligo Ligation Apoptosis Detection Kit (Millipore). The sources of primary antibodies used for IHC are: Ki67 (BD Pharmingen) and pSmad2 (Cell Signaling Technology). Tissue slides were subjected to antigen retrieval using Novocastra Epitope Retrieval Solutions, pH 6 (Leica), followed by incubation with primary antibodies at 4°C overnight. A biotin goat anti-mouse and -rabbit secondary antibody (BD Pharmingen) was used for Ki67 and pSmad2, respectively, followed by incubation with streptavidin-horseradish-peroxidase (BD Pharmingen). Apoptosis and proliferation were determined quantitatively by counting the number of positively stained cells for TUNEL and Ki67 at ×20 magnification. Staining density of pSmad2 was quantified with Imagescope Software (Leica Biosystems). 4-6 animals were analyzed for each cell type. Ten histologically fields were randomly selected from each section for analysis.

2.15 *Ex Vivo* Intestinal Crypt Culture

In recent years, protocols for growing organoids containing crypts and villus from adult

mouse intestinal epithelium in matrigel have been widely developed. As the crypts directly isolated from mouse can be maintained in culture for years, this model has proven to serve as a powerful system to investigate regulatory and pathological mechanisms of the intestinal epithelium on both cellular level and molecular level.

In our study, intestinal crypts from wide-type and $Apc^{min/+}$ mouse were isolated according to the methods described in previous publications (Barker, Ridgway et al. 2009, Sato, Vries et al. 2009). Briefly, crypts were released from mouse small intestine by incubation for 30 min at 4 °C in PBS containing 2 mM EDTA. Followed by a few steps of wash and centrifugation, isolated crypts were counted and pelleted. A total of 500 to 1000 crypts were mixed with 50 ml of Matrigel (BD Bioscience) and plated in 24-well plates. After polymerization of Matrigel, 500μl of DMEM/ F12 medium containing N2 supplement, B27 supplement (Gibco), 1 μM N-acetyl cysteine, 10 mM Hepes, glutamine, penicillin/streptomycin (Sigma-Aldrich), 100 ng/mL mNoggin, 50 ng/mL mEGF, and 500 ng/mL mRSPO1 (R&D Systems) were added to each well. Culture medium containing the growth factors was changed every 4 days. For passaging the culture, organoids were removed from Matrigel and mechanically dissociated into single-crypt domains, and then transferred to fresh Matrigel. Passage was performed every 1–2 weeks with 1:2 and 1:5 split ratios for wide-type and $Apc^{min/-}$ organoids, respectively. Intestinal crypts from $Apc^{min/+}$ were initially cultured without mRSPO1. Under these conditions, only organoids with endogenously activated β-catenin/ TCF pathway survived beyond 7 days.

2.16 Conditional Medium

To produce conditional medium containing active RSPO1 ligands, the expression vector inserted with the RSPO1 recombinant cDNA was stably introduced into HEK293 cells. To harvest the conditional medium, 1×10^6 cells were plated in 10 mL culture medium in the 10 cm tissue culture dishes or T-75 flasks and grown for 4 days (approximately to confluency). Take off the medium and sterile filter to collect the first batch of medium, then add 10 mL fresh culture medium and culture for another 3 days to collect the second batch of medium. Mix the first and the second batch with 1:1 ratio as working medium.

2.17 Statistical Analyses.

The data represent the mean ± SEM. Statistical comparisons were performed using Student's t-test. Unless otherwise indicated. A P-value < 0.05 was considered to be statistically significant.

CHAPTER 3

LGR5 Inhibit Colon Cancer Metastasis Through the Activation of TGFβ Signaling Pathway

3.1 LGR5 expression inversely correlates with colon cancer tumor stages.

As introduced in the previous chapters, several studies have shown that expression of LGR5 decreases during colon cancer progression (de Sousa, Colak et al. 2011) and that re-expression of LGR5 or its ligands RSPOs suppresses tumor formation (de Sousa, Colak et al. 2011, Walker, Zhang et al. 2011, Wu, Qiu et al. 2014). To confirm these findings, we have assessed copy number variation (CNV) of LGR5 from the Cancer Genome Atlas (TCGA) on 445 cases of colon primary tumors. The data suggest that CNV loss of LGR5 is enriched in late stage colon cancer stage III patients and with high lymph node spread and/or distant metastasis ($P < 0.05$) **(Table 3.1)**. More importantly, LGR5 CNV loss correlates with poor survival probability of colon cancer patients ($P = 0.08$) **(Figure 3.1A)**.

Although the loss of LGR5 DNA copy number associates with late stage colon cancer and the event is enriched in patients with poor survival, it is not known whether expression of LGR5 shows the same correlation. We therefore determined expression of LGR5 in primary tumors of different cancer type and different tumor stages. A comparison of the LGR5 mRNA expression between normal colon, colon adenomas and carcinomas was performed in the Oncomine® Research Edition platform. The analysis showed that although LGR5 mRNA expression is higher in adenomas and carcinomas than in normal controls, its expression decreases in carcinomas when compared to adenomas. **(Figure 3.1B).** These data suggest that LGR5 expression may increase during the onset of colon tumorigenesis and decrease thereafter during tumor progression. We therefore hypothesized that LGR5 expression inversely correlates with advanced stages in human colon tumors. To test this hypothesis, we examined LGR5 protein expression in

tissue samples from normal colon and colon cancer patients at stage I, II or IV. IHC staining results revealed that expression of LGR5 in adenocarcinomas of stage I and II was statistically higher than that in normal colonic epithelial cells (**Figure 3.1C).** However, there was a significant decrease in LGR5 expression in stage IV tumors as compared to those of stages I or II. The percentage of LGR5 positive cells and the intensity of the staining in individual tissue samples are shown in **Figure 3.1D&E**. These results indicate that LGR5 expression increases during the onset of colon tumorigenesis and decrease thereafter during tumor progression.

Additionally, LGR5 is differentially expressed in colon cancer cell lines. Examination of LGR5 protein and mRNA expression in human colon cancer cell lines showed that highly metastatic cell lines such HCT116, RKO and TENN cells express lower amounts of LGR5 than moderately, weakly or non-metastatic CBS, HCT116b and FET cells respectively at both protein and mRNA levels (**Figure 3.1F**), indicating an inverse correlation between LGR5 expression and metastatic potential.

enrichment analysis of LGR5 CNV loss in primary tumor with different lymph node metastasis and TNM stages of the individuals			
clinical characteristic	No. in Factor Group	No. in Selected Samples	enrichment P-Value
Lymph node metastasis			
N0	9	211	1.00
N1	7	73	0.19
N2	10	64	0.01
TNM stage			
Stage I	3	70	0.88
Stage II	2	37	0.75
Stage IIA	3	120	0.98
Stage IIB	1	10	0.50
Stage IIIA	2	14	0.22
Stage IIIB	4	50	0.47
Stage IIIC	6	36	0.03
Stage IVA	2	17	0.23
Stage IVB	2	2	0.07

Table 3.1 Enrichment Analysis of LGR5 CNV Loss in Primary Tumor with Different Lymph Node Metastasis and TNM Stages of the Individuals. Patients' information and DNA copy number of total 466 cases of colon cancer was obtained from TCGA database. The DNA copy number variation (CNV) and enrichment analysis were generated on Nexus Copy Number software, BioDiscovery. LGR5 CNV loss is enriched in patients with N2 lymph node metastasis ($p = 0.01$), and in stage IIIC patients ($p = 0.03$).

Figure 3.1A LGR5 CNV Inversely Correlates with Patients Survival Rate. K-M analysis for LGR5 CNV loss in colon cancer patients was performed on Nexus Copy Number software, BioDiscovery. LGR5 CNV loss (green line) has less survival capability than LGR5 CNV normal patients ($p = 0.08$).

Figure 3.1

A

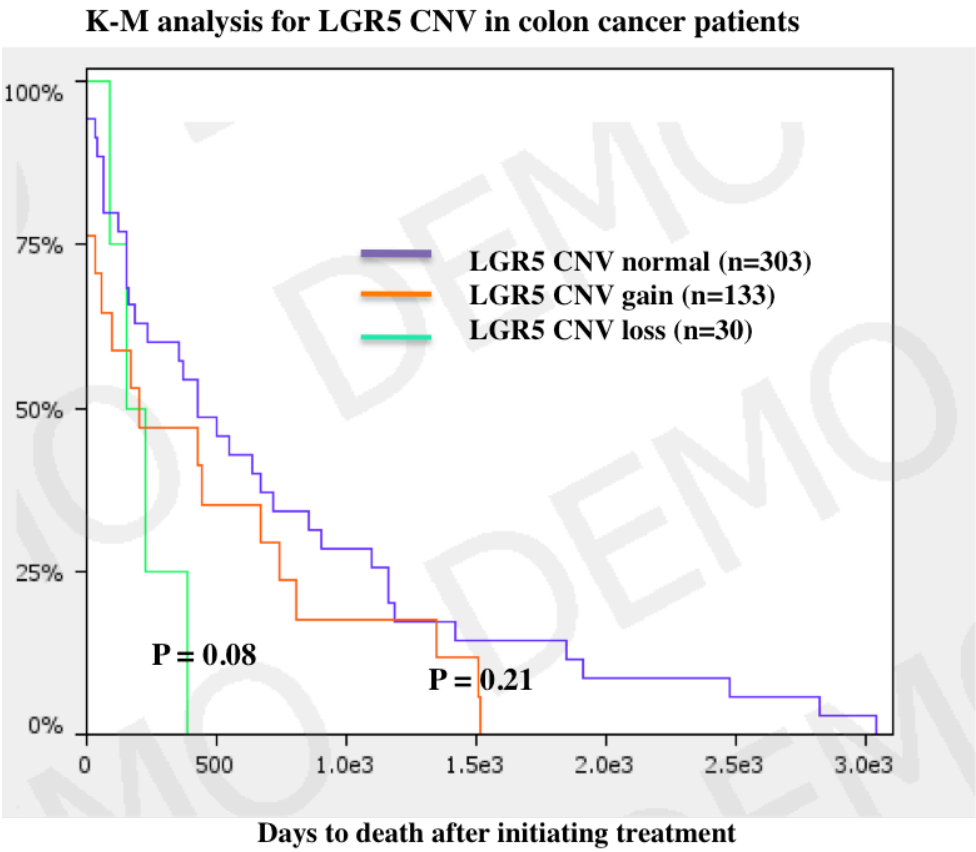
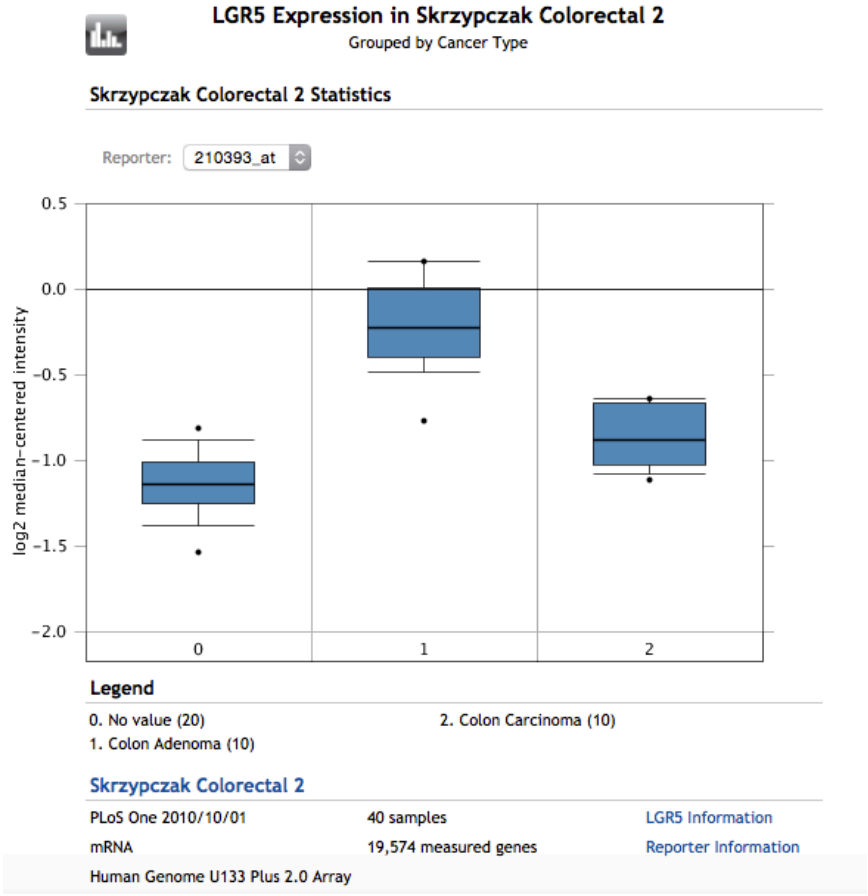

K-M analysis for LGR5 CNV in colon cancer patients
100%
75%
50%
25%
0%
0
500
1.0e3
1.5e3
2.0e3
2.5e3
3.0e3
LGR5 CNV normal (n=303)
LGR5 CNV gain (n=133)
LGR5 CNV loss (n=30)
P = 0.08
P = 0.21
Days to death after initiating treatment

Figure 3.1B LGR5 Expression Inversely Correlates with Tumor Grade. Comparison analysis of LGR5 mRNA expression between normal to adenoma, and to carcinoma was performed on Oncomine data analysis platform based on the Skrzypczak Colorectal 2 dataset. LGR5 mRNA expression increases in adenoma by 30 fold comparing to normal and drop to 8 fold in carcinoma.

Figure 3.1

B


LGR5 Expression in Skrzypczak Colorectal 2
Grouped by Cancer Type
Skrzypczak Colorectal 2 Statistics
Reporter: 210393_at
log2 median-centered intensity
0.5
0.0
−0.5
−1.0
−1.5
−2.0
0
1
2
Legend
0. No value (20)
1. Colon Adenoma (10)
2. Colon Carcinoma (10)
Skrzypczak Colorectal 2
PLoS One 2010/10/01
40 samples
LGR5 Information
mRNA
19,574 measured genes
Reporter Information
Human Genome U133 Plus 2.0 Array

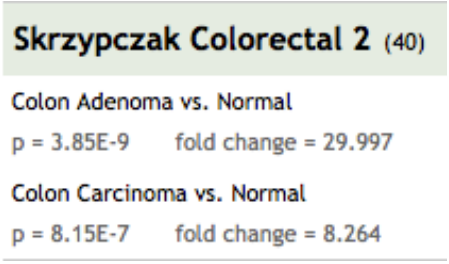
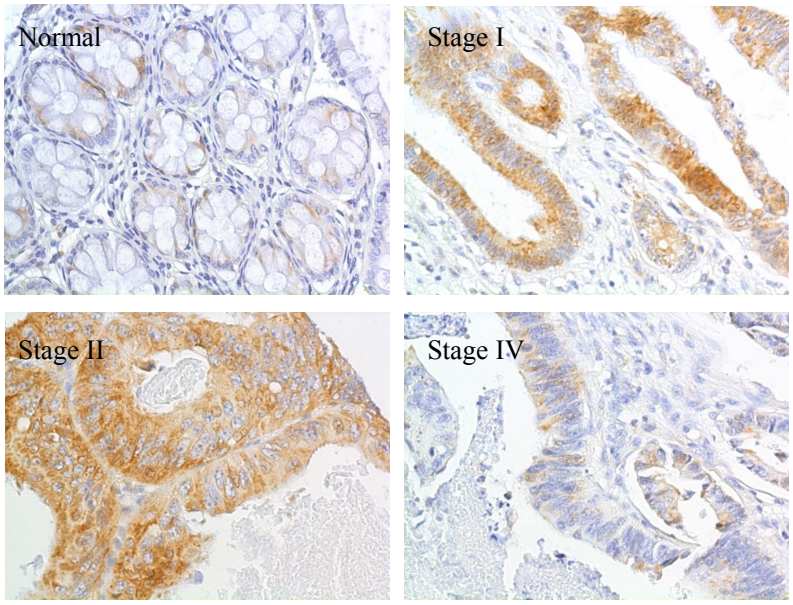

Skrzypczak Colorectal 2 (40)
Colon Adenoma vs. Normal
p = 3.85E-9 fold change = 29.997
Colon Carcinoma vs. Normal
p = 8.15E-7 fold change = 8.264

Figure 3.1 C-E LGR5 Expression Inversely Correlates with Cancer Stages. C. Immunohistochemistry staining with LGR5 antibody on different stages of primary colon cancer sections. **D.** Plots of percentage of LGR5 positive cells in each of the individual sections **E.** Plots of intensity of LGR5 staining in each of the individual sections. Error bars indicate SEM of all samples. Statistical significance was determined by Student's t-test. (* $P < 0.05$, ** $P < 0.001$, $n = 9$ in stage I, 10 in normal, stage II and IV)

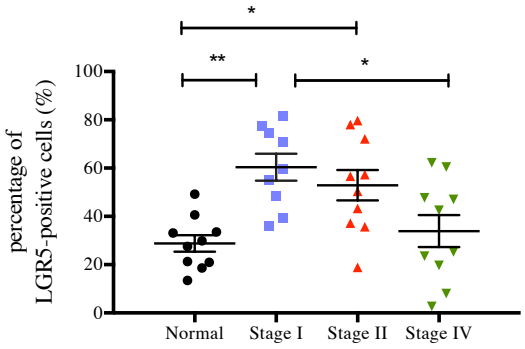
(These experiments were performed by Dr. Liying Geng)

Figure 3.1

C



D



E

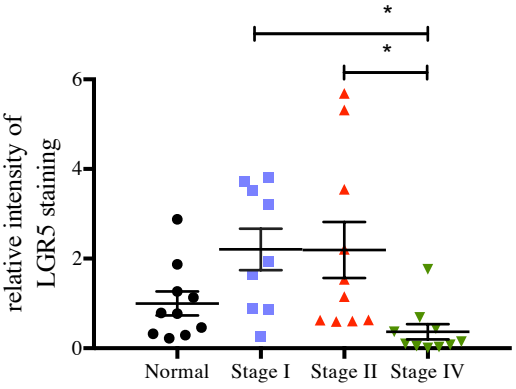
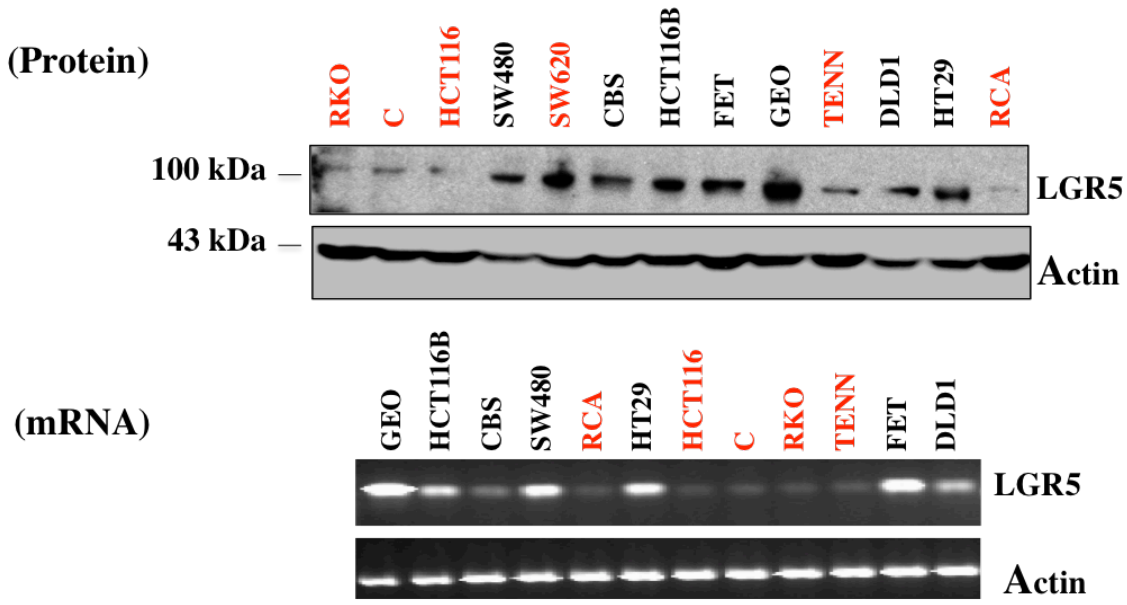
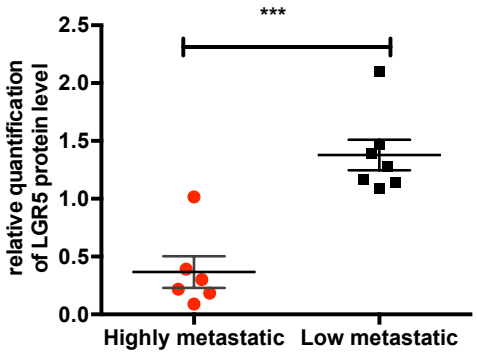


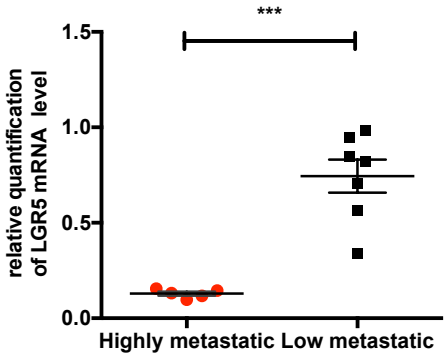
Figure 3.1F LGR5 Expression Inversely Correlates with Metastatic Potential in Colon Cancer Cell Lines. Top panel shows protein expression of LGR5 in different colon cell lines by Western blotting. Middle panel shows mRNA level of LGR5 in colon cancer cell lines by RT-PCR. Cells were grouped into highly metastatic (red) and low or non-metastatic (black). Bottom panel shows relative expression of LGR5 in each of the cell line and the comparison between highly metastatic versus low metastatic group. Quantification was performed by imageJ. Error bars indicate SEM of all samples. Statistical significance was determined by Student's t-test. (*** $P < 0.001$, $n = 6$ of highly metastatic protein samples; 5 of highly metastatic mRNA samples; 7 of low metastatic protein and mRNA samples)

Figure 3.1

F


(Protein)
RKO
C
HCT116
SW480
SW620
CBS
HCT116B
FET
GEO
TENN
DLD1
HT29
RCA
100 kDa
43 kDa
LGR5
Actin
(mRNA)
GEO
HCT116B
CBS
SW480
RCA
HT29
HCT116
C
RKO
TENN
FET
DLD1
LGR5
Actin


***
relative quantification of LGR5 protein level
2.5
2.0
1.5
1.0
0.5
0.0
Highly metastatic
Low metastatic


***
relative quantification of LGR5 mRNA level
1.5
1.0
0.5
0.0
Highly metastatic
Low metastatic

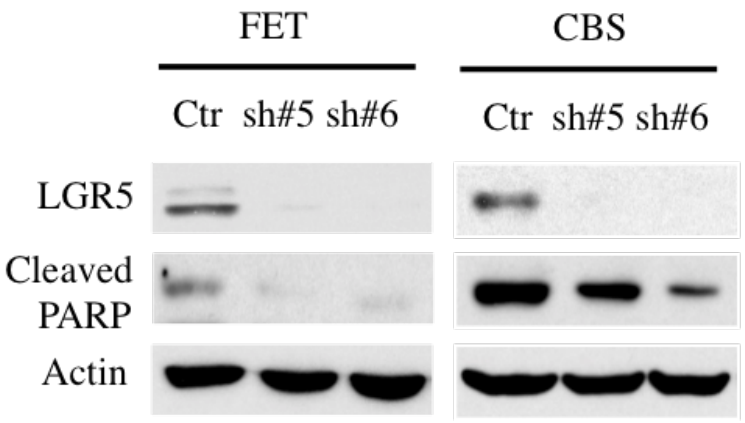
3.2 LGR5 Inhibits Colon Cancer Malignant Phenotypes *in vitro*.

Results from last section promoted us to explore whether LGR5 might play a suppressive role in colon cancer progression and metastasis. Accordingly, LGR5 expression was knocked down in FET and CBS cells using shRNAs. Expression of LGR5 was reduced by more than 90% with each of the two independent shRNAs (#5 and #6) **(Figure 3.2A)**. Since aberrant survival capacity of tumor cells is an important determinant of metastatic potential, we first evaluated the ability of colon cancer cells to withstand stress-induced apoptosis *in vitro*. Under growth factor and nutrient deprivation stress (GFDS), LGR5 knockdown cells showed increased resistance to GFDS-induced apoptosis as reflected by decreased PARP cleavage **(Figure 3.2A)** and reduced apoptosis in DNA fragmentation assays **(Figure 3.2B)**, indicating that LGR5 mediates stress-induced apoptosis. In addition, colony formation assays in adhesion and in soft agar indicated that knockdown of LGR5 expression led to almost 2 fold of increase in colony forming capacity when plated at sparse densities **(Figure 3.2C)** and 2.5 – 4.5 fold of increase in anchorage-independent growth **(Figure 3.2D).** These results demonstrate that knockdown of LGR5 expression increases cell survival and clonogenecity *in vitro*.

Figure 3.2A&B LGR5 Contributes to GFDS-induced Apoptosis. Two LGR5 shRNAs and their corresponding scramble control were introduced in FET cells and CBS cells **(A)**. Western blot analysis of poly ADP ribose polymerase (PARP) was performed in FET and CBS cells under GFDS for 48 hours. DNA fragmentation assays were performed **(B)**. Error bars indicate SEM of three replications. Statistical significance was determined by Student's t-test. *** $P < 0.001$

Figure 3.2

A


FET
CBS
Ctr sh#5 sh#6
Ctr sh#5 sh#6
LGR5
Cleaved PARP
Actin

B

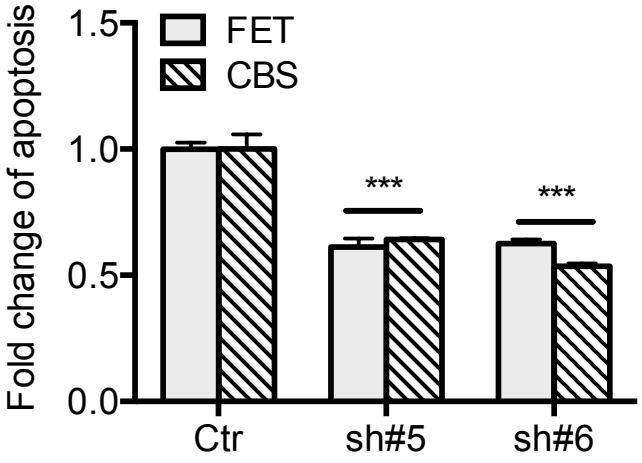
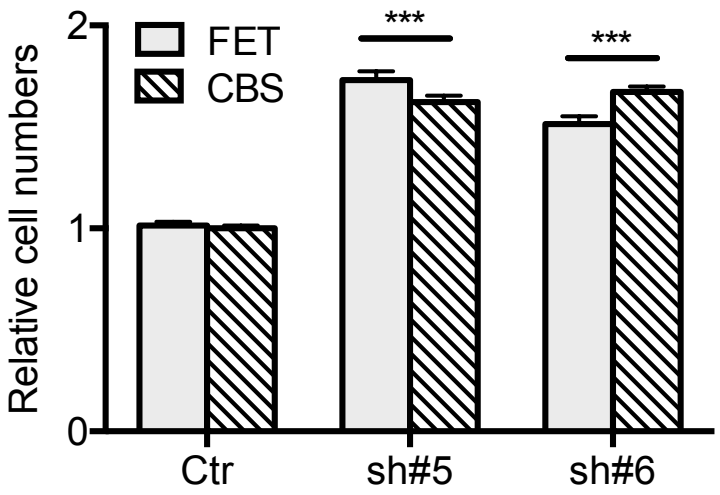

Fold change of apoptosis
1.5
1.0
0.5
0.0
FET
CBS
***
***
Ctr
sh#5
sh#6

Figure 3.2C&D LGR5 Affects Cancer Cell Tumorigenicity *in vitro*. Knock down of LGR5 increases cell colony formation as shown in **(C)**. Knock down cells as well as their control cells were plated at 500/well in 6-well plate and stained with MTT at day 14. Plates were scanned and cells were dissolved in DMSO and read at OD 570 nm. Knock down of LGR5 also increases cell anchorage-independent growth **(D)**. 3000/well of cells were seeded in 0.4% soft agar with a lower layer of 0.8% soft agar and stained with 0.01% iodonitrotetrazolium violet at Day 25. A-F. Error bars indicate SEM of three replications. Statistical significance was determined by Student's t-test. ** $P < 0.01$, *** $P < 0.001$

Figure 3.2

C


Relative cell numbers
2
1
0
FET
CBS
***
***
Ctr
sh#5
sh#6

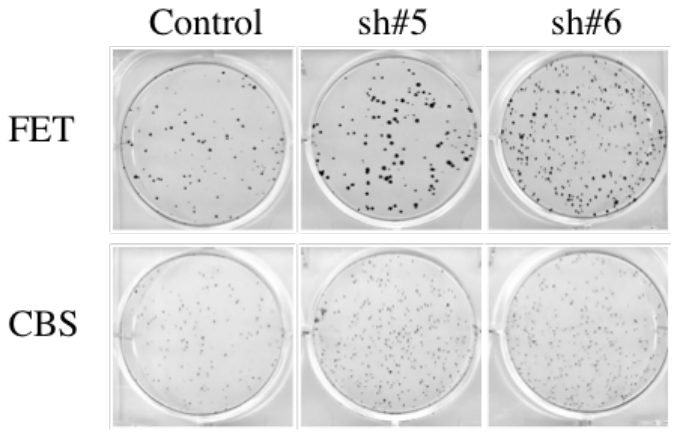
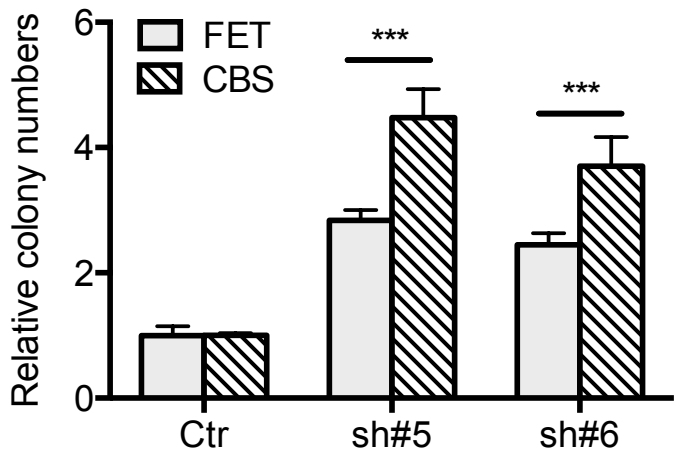
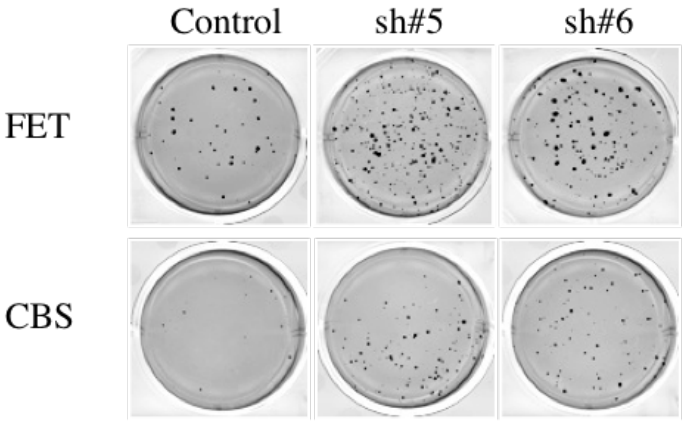

Control
sh#5
sh#6
FET
CBS

Figure 3.2

D


Relative colony numbers
6
4
2
0
FET
CBS
***
***
Ctr
sh#5
sh#6


Control
sh#5
sh#6
FET
CBS

3.3 LGR5 Inhibits Colon Cancer Metastasis *in vivo*.

We next examined the role of LGR5 in colon cancer metastasis using an orthotopic mouse model that recapitulates human colon cancer metastasis to the liver and lungs (Guo, Rajput et al. 2007, Jiang, Liu et al. 2011). We used CBS cells with LGR5 knockdown for this study since CBS cells are moderately metastatic *in vivo* (Simms, Rajput et al. 2012). Mice implanted with CBS control or LGR5 knockdown cells (LGR5 KD: combined results of two shRNAs) showed 100% primary tumor growth. Although knockdown of LGR5 expression resulted in a 23% increase in primary tumor weight **(Figure 3.3D)**, it also significantly increased the incidence of liver and/or lung metastasis from 29% to 78% **(Table 3.3).** Fluorescence imaging of explanted liver or lungs showed increased tumor burden of metastases in mice implanted with LGR5 KD cells **(Figure 3.3A)**. To confirm GFP imaging results, RNA was extracted from lungs and liver of each mouse and semi-quantitative RT-PCR was performed using a human specific GAPDH primer. The level of human GAPDH mRNA expression in each sample represents the amount of human RNA, which is a reflection of tumor burden in the lungs and livers of the mice. RT-PCR results showed that the human specific GAPDH mRNA level was much higher in LGR5 KD group than in the control group **(Figure 3.3B&C)**. These results indicate LGR5 inhibits incidence and tumor burden of colon cancer metastasis *in vivo*.

To determine whether LGR5-mediated cell proliferation and apoptosis were associated with its regulation of metastatic potential *in vivo*, TUNEL and Ki67 assays were performed. TUNEL staining of primary tumors showed that there were significantly fewer apoptotic cells in the tumors of LGR5 KD cells than in those of control cells (8.5%

vs. 29.2%, **Figure 3.3F**). Meanwhile Ki67 staining showed that tumors of LGR5 KD cells also had higher percentage of proliferative cells than those of control cells (95.7% vs. 65.8%, **Figure 3.3E**). These results indicate that the inhibitory effect of LGR5 on metastasis was a result of its combined effect on suppression of cell proliferation and survival of tumor cells *in vivo*.

Table 3.3 Summary of Liver and Lung Metastasis of Control and LGR5 Knockdown Cells

Cell Lines	Liver Metastasis	Lung Metastasis	Liver or Lung Metastasis	
CBS Control	6/24 (25%)	7/24 (29%)	7/24 (29%)	** P < 0.01
CBS LGR5 KD	18/26 (69.2%)	17/26 (65.4%)	22/26 (84.6%)	Power > 0.85

Percentage of metastasis to either liver or lung was calculated. Statistical significance was determined by Fisher Exact test. ** P < 0.01. Power > 80%

Figure 3.3A-D Reduction of LGR5 Increases Colon Cancer Metastasis. A. Green fluorescent protein (GFP) images of liver and lung metastasis are shown in the upper panel. Representatives of H&E staining of liver and lung were shown in the lower panel. Tumors were indicated by arrows. **B&C.** Number of metastasis cases was determined by semi-quantification of human specific GAPDH in liver and lung. Summary of metastasis cases were calculated in Table 3.3. **D.** Primary tumor weight of individual animal was plotted in the upper panel. Error bars indicate SEM of samples in each group. The expression of LGR5 was determined by IHC staining as indicated in the lower panel.

Figure 3.3

A.

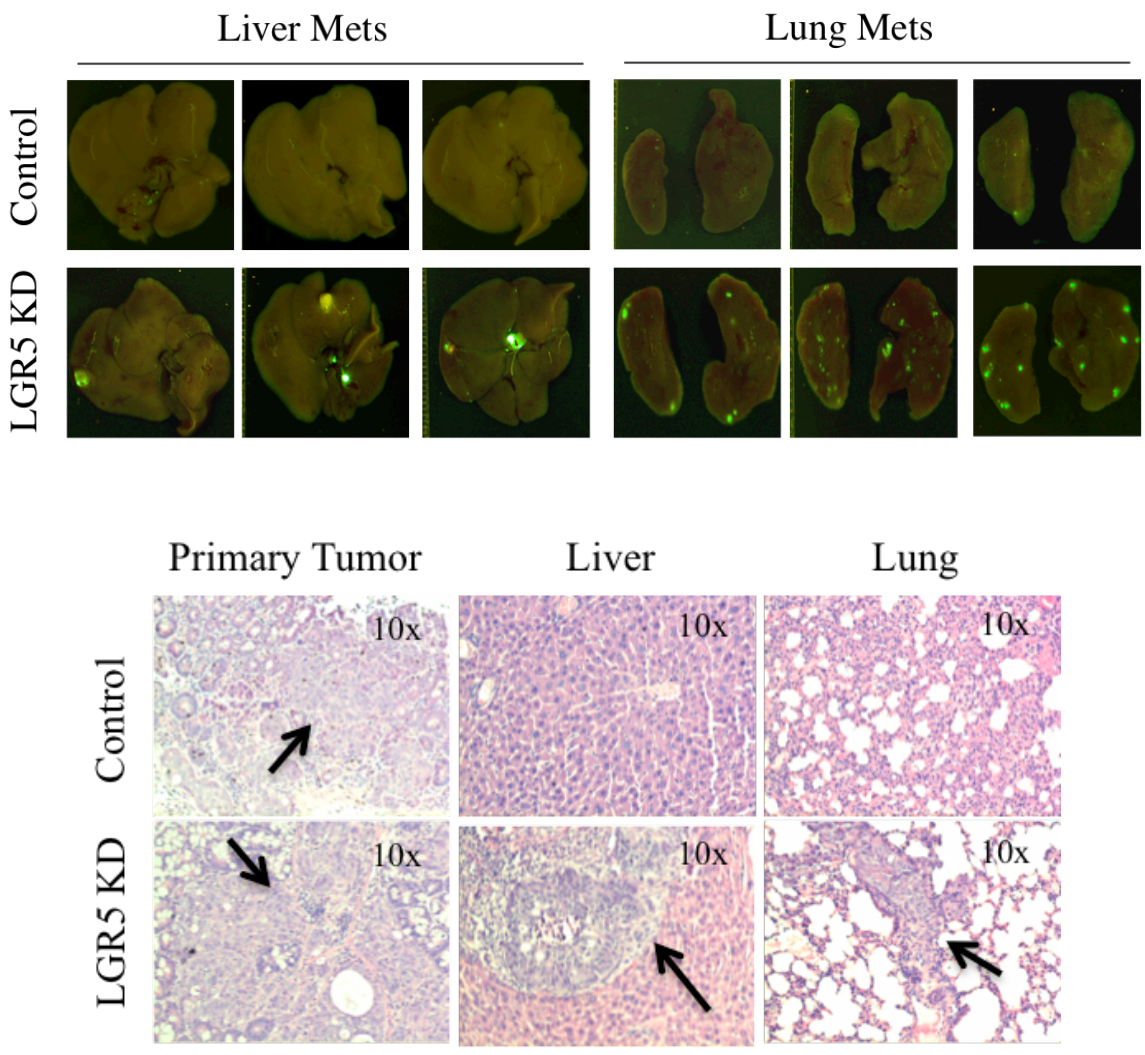
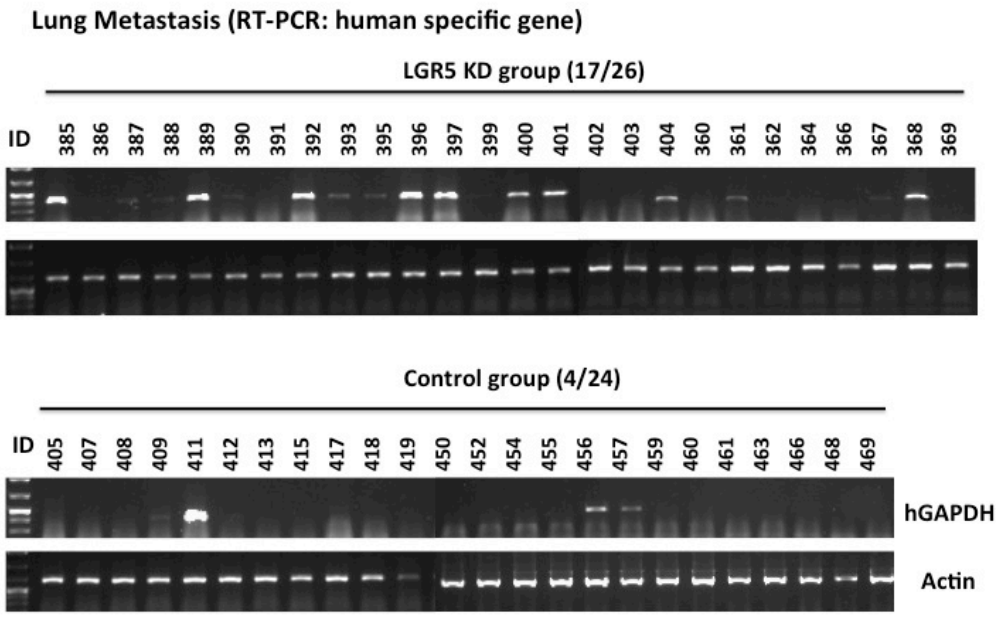

Liver Mets
Lung Mets
Control
LGR5 KD
Primary Tumor
Liver
Lung
10x
10x
10x
10x
10x
10x

Figure 3.3

B


Lung Metastasis (RT-PCR: human specific gene)
LGR5 KD group (17/26)
ID 385 386 387 388 389 390 391 392 393 395 396 397 399 400 401 402 403 404 360 361 362 364 366 367 368 369
Control group (4/24)
ID 405 407 408 409 411 412 413 415 417 418 419 450 452 454 455 456 457 459 460 461 463 466 468 469
hGAPDH
Actin

C

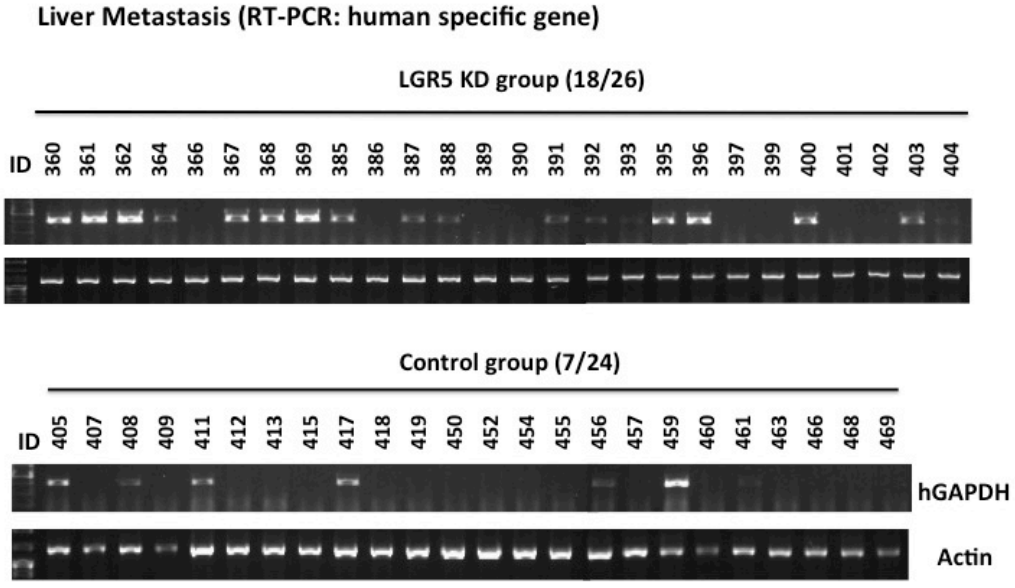
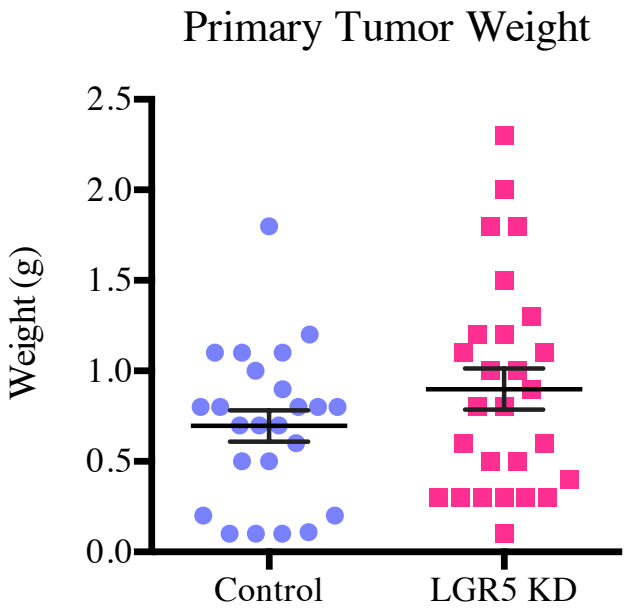

Liver Metastasis (RT-PCR: human specific gene)
LGR5 KD group (18/26)
ID 360 361 362 364 366 367 368 369 385 386 387 388 389 390 391 392 393 395 396 397 399 400 401 402 403 404
Control group (7/24)
ID 405 407 408 409 411 412 413 415 417 418 419 450 452 454 455 456 457 459 460 461 463 466 468 469
hGAPDH
Actin

Figure 3.3

D



	Control	LGR5 KD
Mean	0.70	0.90
Std. Error of Mean	0.087	0.114

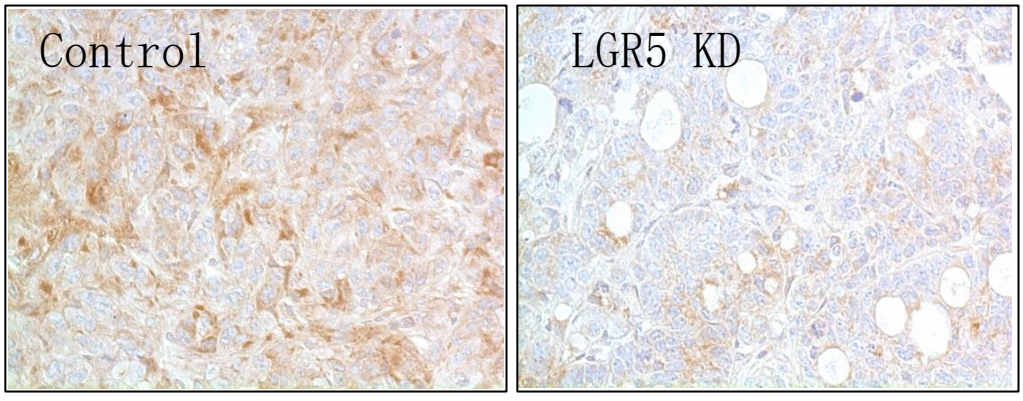
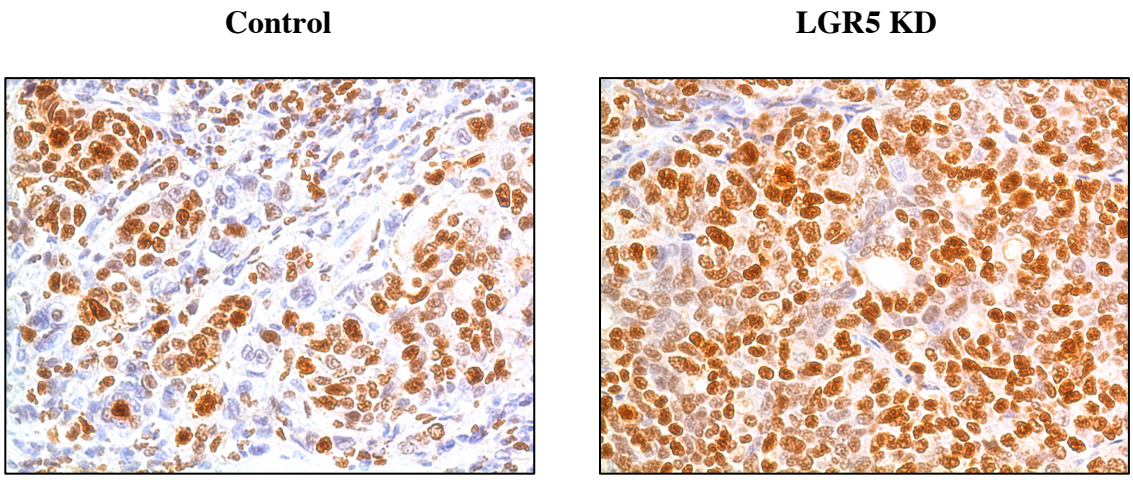


Figure 3.3E&F. LGR5 Regulates Cell Proliferation and Apoptosis *in vivo*. Images of Ki67 (E, ×20 magnification) and TUNEL (F, ×20 magnification) staining of primary tumors are shown in the upper panels. The images are representative of multiple fields of tumor sections from six tumors per group. Numbers of positive Ki67 and TUNEL staining cells were determined under the Imagescope software. The data are presented as the mean ± SEM. Statistical significance was determined by Student's t-test. *** $P < 0.001$

Figure 3.3

E


Control
LGR5 KD

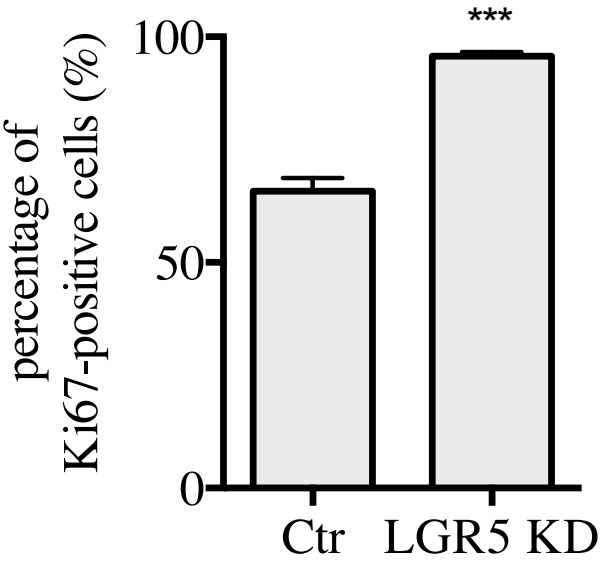
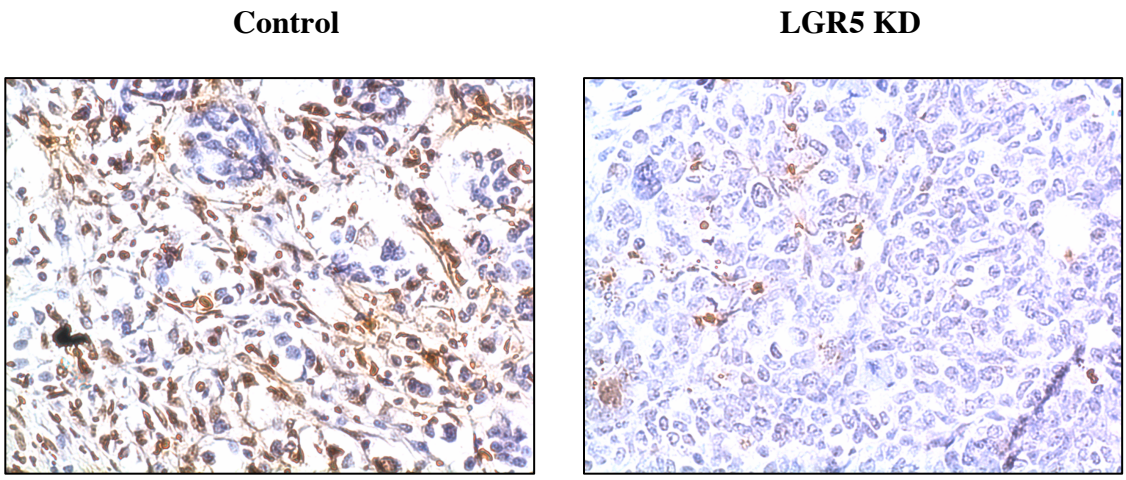
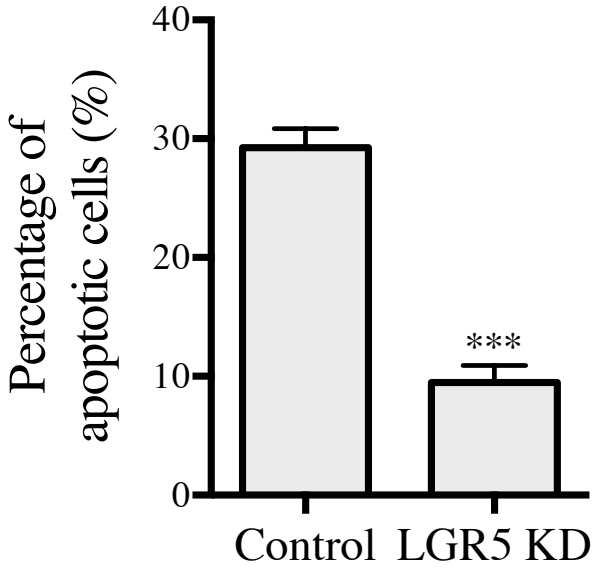

***
100
50
0
percentage of
Ki67-positive cells (%)
Ctr
LGR5 KD

Figure 3.3

F


Control
LGR5 KD


Percentage of apoptotic cells (%)
40
30
20
10
0
***
Control
LGR5 KD

3.4 LGR5 activates TGFβ signaling in colon cancer cells.

To determine the mechanisms of LGR5 function, we investigated the effect of LGR5 on markers related to cell cycle and apoptosis. We found that LGR5 can regulate cyclin D1, p21, p27 and pAKT **(Figure 3.4H)**, which all have been reported to be regulated by TGFβ signaling pathway (Datto, Li et al. 1995, Ko, Sheng et al. 1995). These findings promote us to look into the relationship between LGR5 and TGFβ signaling pathway.

We used a luciferase plasmid containing multiple Smad binding elements (pSBE4-Luc) as a surrogate to determine TGFβ promoter activity. HCT116 cells express undetectable TGFβ RII due to mutations caused by microsatellite instability, and re-expression of RII in those cells (HCT116-RII) restores their responsiveness to TGFβ (Markowitz, Wang et al. 1995, Wang, Sun et al. 1995). Since HCT116 cells express low levels of LGR5 (**Figure 3.1C**), LGR5 expression vector was transfected into those cells. Ectopic expression of LGR5 activated TGFβ signaling, as reflected by the luciferase reporter activity, in a dose-dependent manner in both HCT116 and HCT116-RII cells, with higher activation in HCT116-RII cells (**Figure 3.4A upper panel**). Expression of p21, a TGFβ target gene, was also induced by LGR5 (**Figure 3.4A lower panel, Figure 3.4H**), confirming the activation of the pathway. Exposure of cells to RSPO1 further increased LGR5-mediated activation of TGFβ promoter, whereas treatment with a RSPO1 neutralizing antibody almost completely blocked LGR5 effect (**Figure 3.4B**). The human RSPO1 neutralizing antibody is antigen affinity-purified and displays less than 1% cross-reactivity with other human RSPOs (R&D Systems). Therefore, these results indicate that RSPO1 is primarily responsible for LGR5-mediated TGFβ activation. In the following studies, RSPO1 was used to activate LGR5 function.

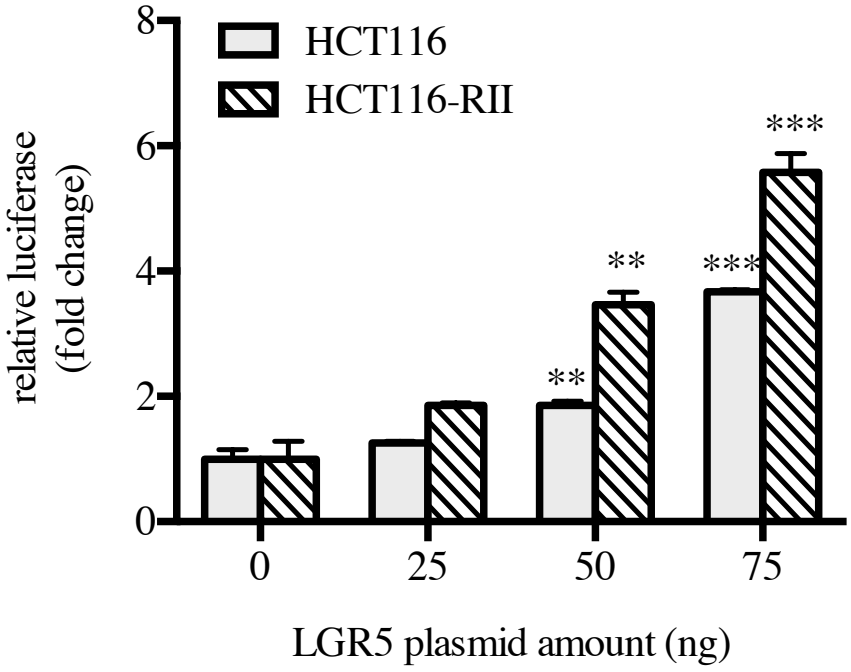
Complementarily, LGR5 expression was knocked down by two different shRNAs in FET and CBS cells (**Figure 3.2A**), which display high levels of endogenous TGFβ activity. This resulted in decreased TGFβ promoter activity (**Figure 3.4C**). In addition, treatment of FET and CBS cells with RSPO1 increased TGFβ promoter activity to a similar level as that with TGFβ. Treatment with TGFβ and RSPO1 simultaneously induced a much higher increase in TGFβ promoter activity than that of either one alone (**Figure 3.4D)**. A time course study showed that RSPO1 and TGFβ activate Smad2 with similar dynamics in FET cells (**Figure 3.4F**). These studies indicate that RSPO-LGR5 activates the canonical TGFβ pathway in colon cancer cells.

Since LGR5 activated TGFβ promoter in the absence of TGFβ RII **(Figure 3.4A**), we next determined whether TGFβ RI is required. A potent and selective inhibitor of TGFβ RI kinase**,** SB525334, was used in the following studies. The luciferase reporter assays showed that RI kinase inhibitor blocked the activation of TGFβ promoter by RSPO1 treatment in FET and CBS cells (**Figure 3.4D**) and by LGR5 expression in HCT116 cells (**Figure 3.4E**), indicating that the kinase activity of TGFβ RI is essential for RSPO1-LGR5-mediated TGFβ activation. To further determine which Smads play a role in this activation, expression of Smad2, Smad3 and Smad4 was knocked down individually by shRNAs in FET cells (**Figure 3.4G, upper panel**). Knockdown of Smad2, Smad3 or Smad4 attenuated RSPO1-LGR5-mediated activation of TGFβ signaling as reflected by reduction in fold change of luciferase reporter activity induced by RSPO1 treatment (**Figure 3.4G, lower panel**). These results indicate that Smad2, Smad3 and Smad4 are important in RSPO1-LGR5-mediated TGFβ activation.

Figure 3.4A-C LGR5 Induces TGFβ Signaling Pathway. A. LGR5 induces TGFβ signaling pathway in HCT116 cells with or without TGFβRII. Upper panel, TGFβ reporter assay in HCT116 and HCT116RII cells transfected with different dose of LGR5 expression plasmid (0, 25, 50 and 100ng). ** P <0.01, *** P < 0.001 versus 0 ng group. Lower panel, RT-PCR of p21 in control and LGR5 overexpression HCT116 cells. **B.** TGFβ reporter assay in HCT116 control and LGR5 overexpression cells with 500ng/ml RSPO1 and RSPO1 neutralizing antibody treatment for 48 hours. C. TGFβ reporter assay in FET and CBS LGR5 knock down cells (by scramble control, shRNA#5 and #6). *** P < 0.001 versus control group. **A-C**. Error bars indicate the SEM of three replications. Statistical significance was determined by ANOVA test.

Figure 3.4

A


HCT116
HCT116-RII
relative luciferase
(fold change)
8
6
4
2
0
**
**
***
***
0
25
50
75
LGR5 plasmid amount (ng)

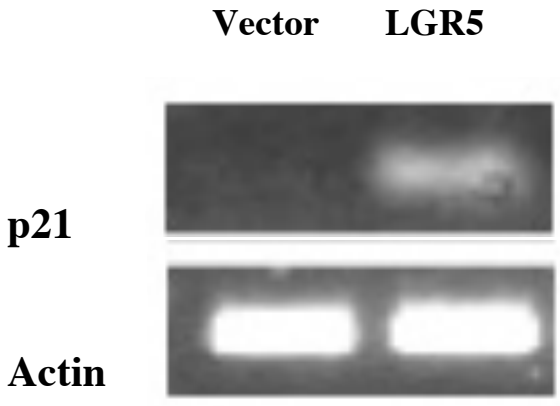
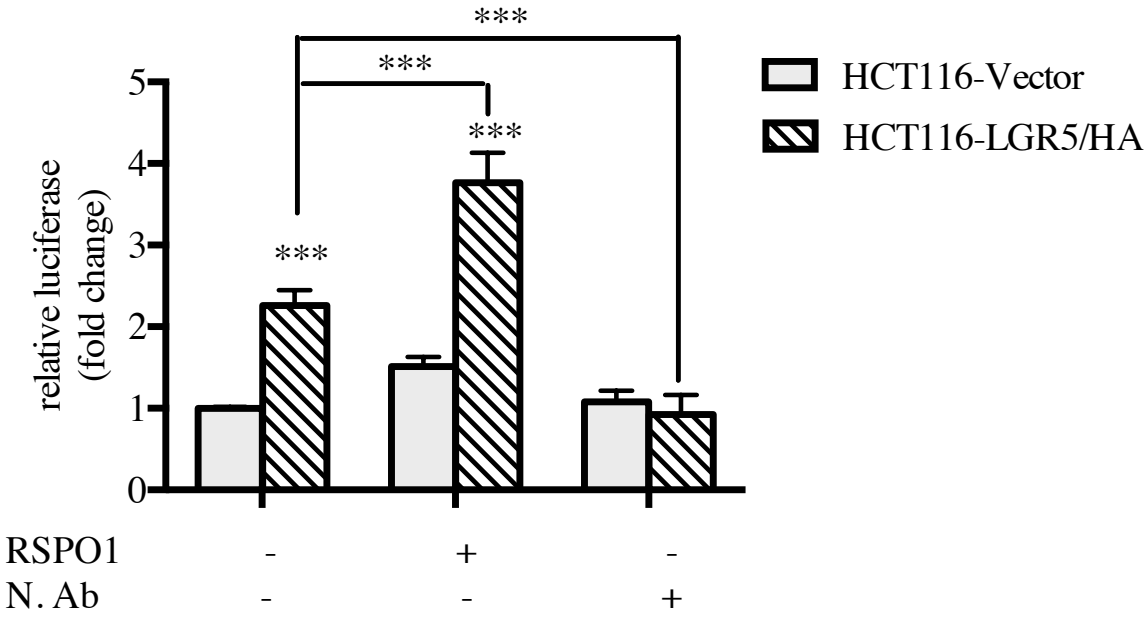

Vector
LGR5
p21
Actin

Figure 3.4

B


***
***
***
***
5
4
3
2
1
0
relative luciferase
(fold change)
HCT116-Vector
HCT116-LGR5/HA
RSPO1 - + -
N. Ab - - +

C

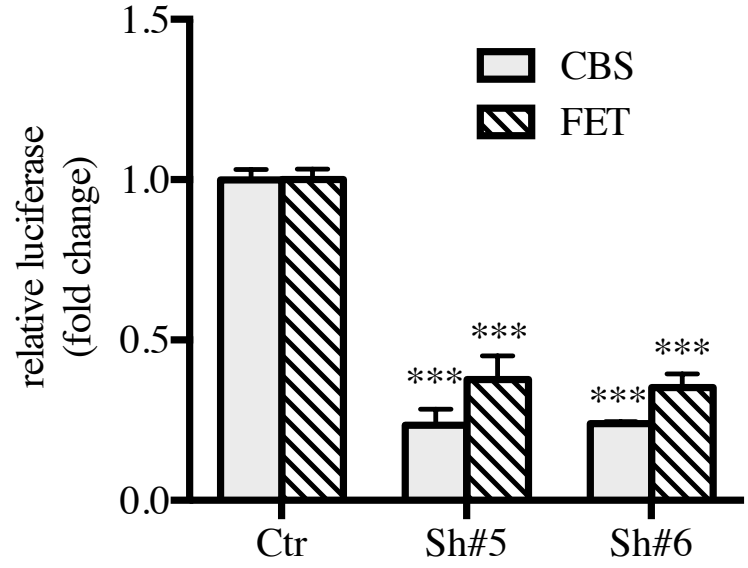
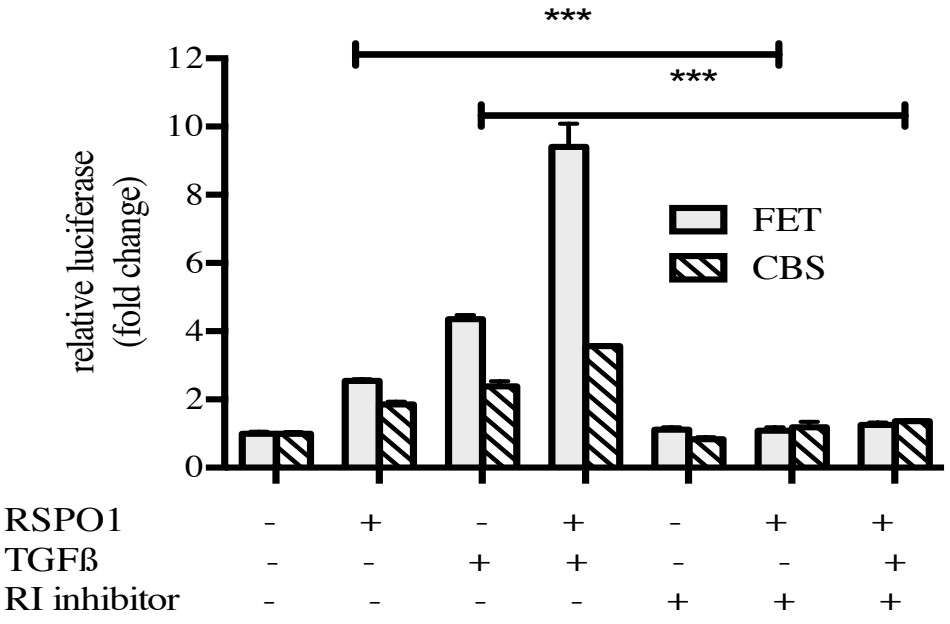

1.5
1.0
0.5
0.0
relative luciferase
(fold change)
CBS
FET
***
***
***
***
Ctr
Sh#5
Sh#6

Figure 3.4D-G LGR5-induced TGFβ Signaling Activation is Mediated by TGFβRI Kinase and Smads Activation. D. TGFβ reporter assay in FET and CBS cells following 500ng/ml RSPO1, 4ng/ml TGFβ and/or 200nM TGFβRI kinase inhibitor (SB525334) treatment for 48 hours. **E.** TGFβ reporter assay in HCT116 vector control and LGR5/HA overexpression cells following 200nM SB525334 treatment for 48 hours. **F.** Western Blot of pSmad2 with 200ng/ml RSPO1 or 4ng/ml TGFβ treatment by time course. **G.** Smad 2,3 and 4 shRNA were stably introduced into FET cells. Expression of Smad 2,3 and 4 were determined by Western blotting (upper panel). TGFβ signaling activity in those cells was measured by reporter assay (lower panel). **D&E**. Error bars indicate the SEM of three replications. Statistical significance was determined by Student's t-test, *** $P < 0.001$.

Figure 3.4

D


***
***
12
10
8
6
4
2
0
relative luciferase
(fold change)
FET
CBS
RSPO1 - + - + - + +
TGFß - - + + - - +
RI inhibitor - - - - + + +

E

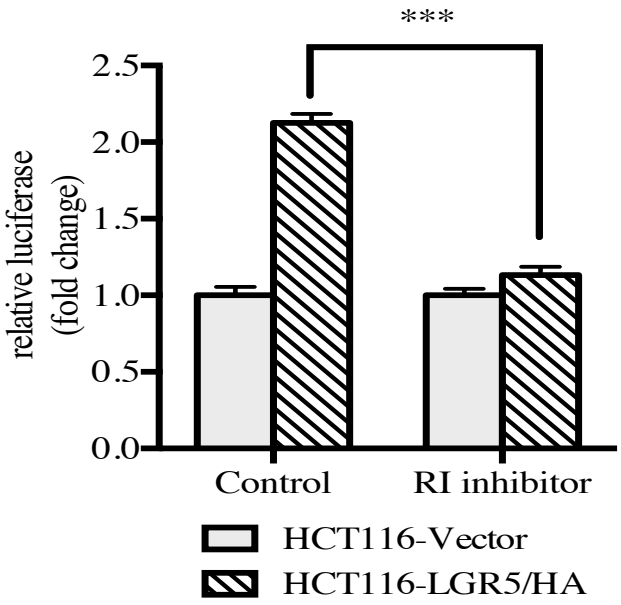
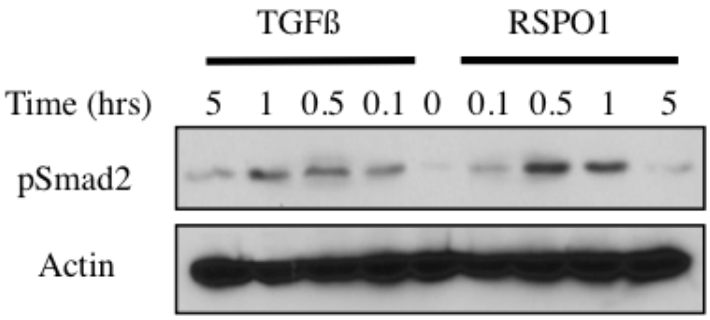

***
2.5
2.0
1.5
1.0
0.5
0.0
relative luciferase
(fold change)
Control
RI inhibitor
HCT116-Vector
HCT116-LGR5/HA

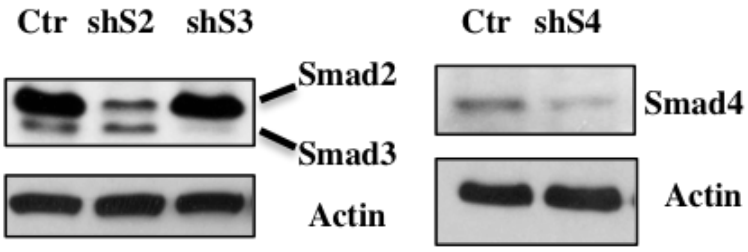
Figure 3.4

F



(This experiment was performed by Dr. Liying Geng)

G



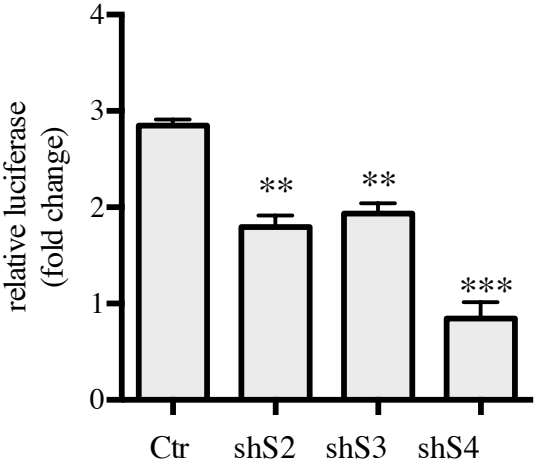
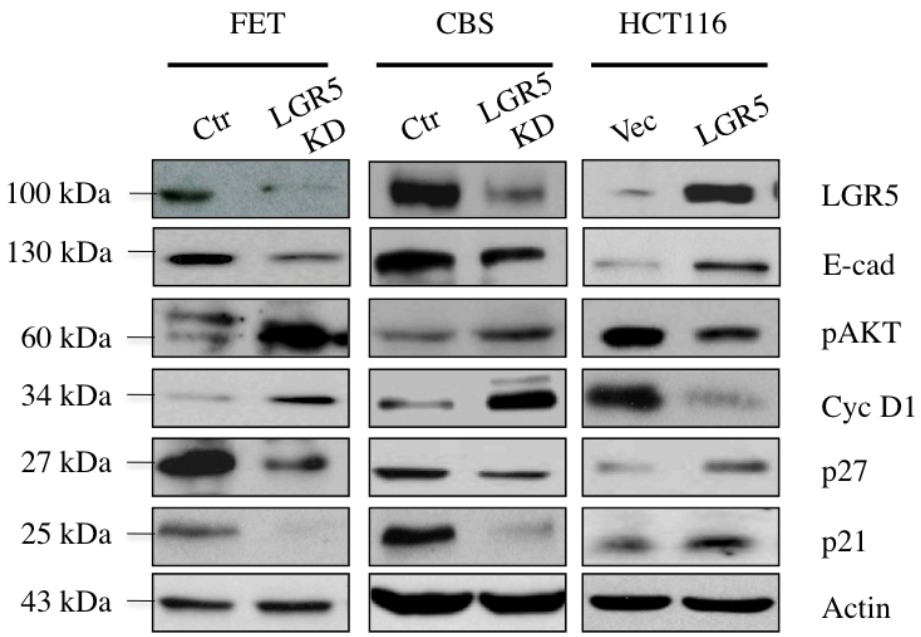


Figure 3.4H LGR5 Regulates TGFβ Signaling Target Genes. pAKT, CyclinD1, p27 and p21 expression were determined by Western blotting in LGR5 expression-modulated cells. Left panel, FET scramble control versus LGR5 knockdown cells; middle panel, CBS scramble control versus LGR5 knockdown cells; right panel, HCT116 vector control versus LGR5/HA overexpression cells.

Figure 3.4

H


FET
CBS
HCT116
Ctr
LGR5 KD
Ctr
LGR5 KD
Vec
LGR5
100 kDa
130 kDa
60 kDa
34 kDa
27 kDa
25 kDa
43 kDa
LGR5
E-cad
pAKT
Cyc D1
p27
p21
Actin

3.5 RSPO1-LGR5 Regulates Cellular Functions of Colon Cancer Cells Through TGFβ Signaling Pathway.

When HA-tagged LGR5 (LGR5-HA) was introduced into HCT116 and CBS cells that show low and intermediate levels of endogenous LGR5 respectively **(Figure 3.1C**). LGR5 expression was significantly increased **(Figure 3.5A)** in these two cell lines. LGR5-expressing cells became more sensitive to GFDS-induced apoptosis than the control cells as reflected by increased caspase-9 cleavage **(Figure 3.5A)** and induced apoptosis in DNA fragmentation assays **(Figure 3.5B)**. Furthermore, overexpression of LGR5 reduced clonogenecity in colony formation **(Figure 3.5C&E)** and soft agarose **(Figure 3.5D&F)** assays. Of note, expression of non-tagged LGR5 showed similar effect as HA-tagged LGR5 in HCT116 cells **(Data not shown)**, indicating that HA tag does not affect LGR5 function.

To determine whether LGR5 functions through the activation of TGFβ signaling, the RI kinase inhibitor**,** SB525334**,** was used in the studies to block the downstream activity. As shown in **Figure 3.5B,C&D**, the RI kinase inhibitor almost completely reversed the effect of LGR5 on cell survival and clonogenecity in HCT116 cells. Furthermore, when FET cells were treated with RSPO1, it increased GFDS-induced apoptosis and reduced clonogenecity in soft agarose to the similar degree as TGFβ **(Figure 3.5H)**. When the RI kinase inhibitor was used, it almost completely reversed RSPO1 effects. To show the specificity of the RI kinase inhibitor, RI expression was knocked down by an shRNA **(Figure 3.5I, upper panel).** Reduction in RI expression abrogated TGFβ or RSPO1-mediated induction of apoptosis **(Figure 3.5I lower panel)** and inhibition of colony formation in soft agarose **(Figure 3.5J)**. Taken together, these data indicate that RSPO1-

LGR5 increases stress-induced apoptosis and inhibits clonogenecity through the activation of the TGFβ signaling pathway.

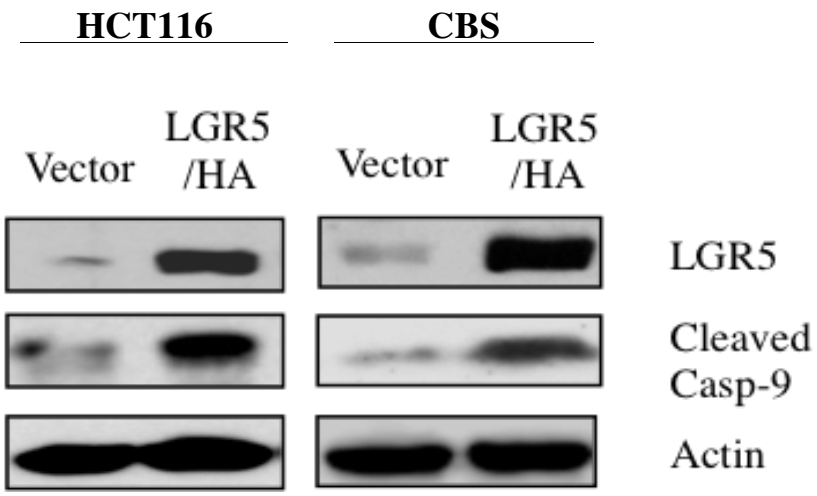
TGFβ has been shown to suppress metastasis of colon cancer cells in an orthotopic model (Barker, van Es et al. 2007, Snippert, Haegebarth et al. 2010, Barker, Rookmaaker et al. 2012). We have shown in this study that knockdown of LGR5 increased liver and lung metastasis of colon cancer cells *in vivo* **(Figure. 3.3)**. To determine whether LGR5 mediates TGFβ signaling *in vivo*, Smad2 phosphorylation was determined in the primary tumors of LGR5 KD and control cells. IHC analyses using an anti-phospho-Smad2 antibody showed that nuclear Smad2 phosphorylation was significantly reduced in the primary tumors of LGR5 KD cells as compared to those of the control cells **(Figure 3.5F, upper panel)**. Quantification of staining intensity of nuclear pSmad2 indicated that TGFβ signaling was inhibited in the tumors of LGR5 KD cells **(Figure 3.5F, lower panel)**. These results suggest that LGR5 may suppress colon cancer metastasis through the regulation of TGFβ signaling pathway. Similar effects were observed in *ex vivo* organoids culture. RSPO1 or TGFβ could inhibit the growth of $Apc^{min/+}$ organoids (**Figure 3.5G**) and activate pSmad2 and pLRP6 (**Figure 3.5H**).

Results in this chapter indicate that expression of LGR5 increases early in colon cancer development but decreases during colon cancer progression. *in vitro* and *in vivo* results in colon cancer cells further demonstrate that LGR5, associating with TGFβ signaling, plays an important role in colon cancer progression. In addition, the stage-dependent decrease of LGR5 expression suggests that LGR5 expression could be used as a potential biomarker to predict metastasis in colon cancer.

Figure 3.5A&B RI Kinase Inhibitor Abrogates LGR5-induced Apoptosis. HA-tagged LGR5 expression vector were stably introduced in HCT116 cells and CBS cells **(A)**. Western blot analysis of cleaved caspase 9 was performed after GFDS in HCT116 cell for 96 hours and in CBS cells for 48 hours. 200nM RI kinase inhibitor was applied one hour before GFDS and maintained in culture for 96 hours in HCT116 cells. DNA fragmentation assays **(B)** were performed as was described in material and methods. Error bars indicate SEM of three replications. Statistical significance was determined by Student's t-test. *** $P < 0.001$

Figure 3.5

A


HCT116
CBS
Vector
LGR5 /HA
Vector
LGR5 /HA
LGR5
Cleaved Casp-9
Actin

B

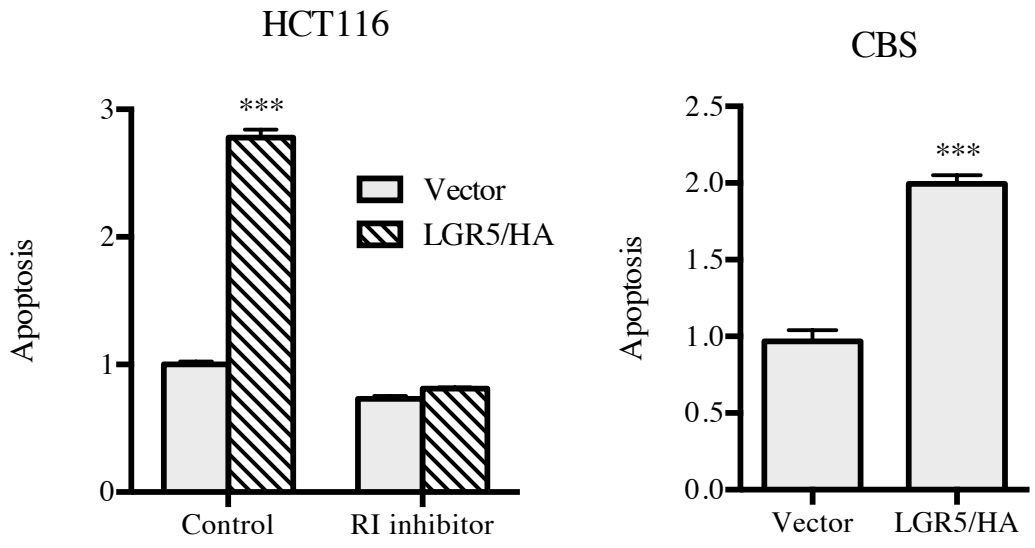

HCT116
CBS
Apoptosis
***
Vector
LGR5/HA
Control
RI inhibitor
Apoptosis
***
Vector
LGR5/HA

Figure 3.5C-E RI Kinase Inhibitor Abrogates LGR5-induced Clonogenecity Suppression. C and E. LGR5 overexpression cells as well as their control cells were plated at 500/well in 6-well plate, 200nM RI inhibitor was applied in HCT116 cells and maintained in culture for two weeks. Cells were stained by MTT on day 14, dissolved in DMSO and read at OD 570 nm. **D and F.** 3000/well of cells were seeded in 0.4% soft agar. 200nM RI inhibitor was applied in HCT116 cells and maintained in culture for 3 weeks. Cells were stained with 0.01% crystal violet on day 25. **C-F.** Error bars indicate SEM of three replications. Statistical significance was determined by Student's t-test. ** $P < 0.01$, *** $P < 0.001$

Figure 3.5

C

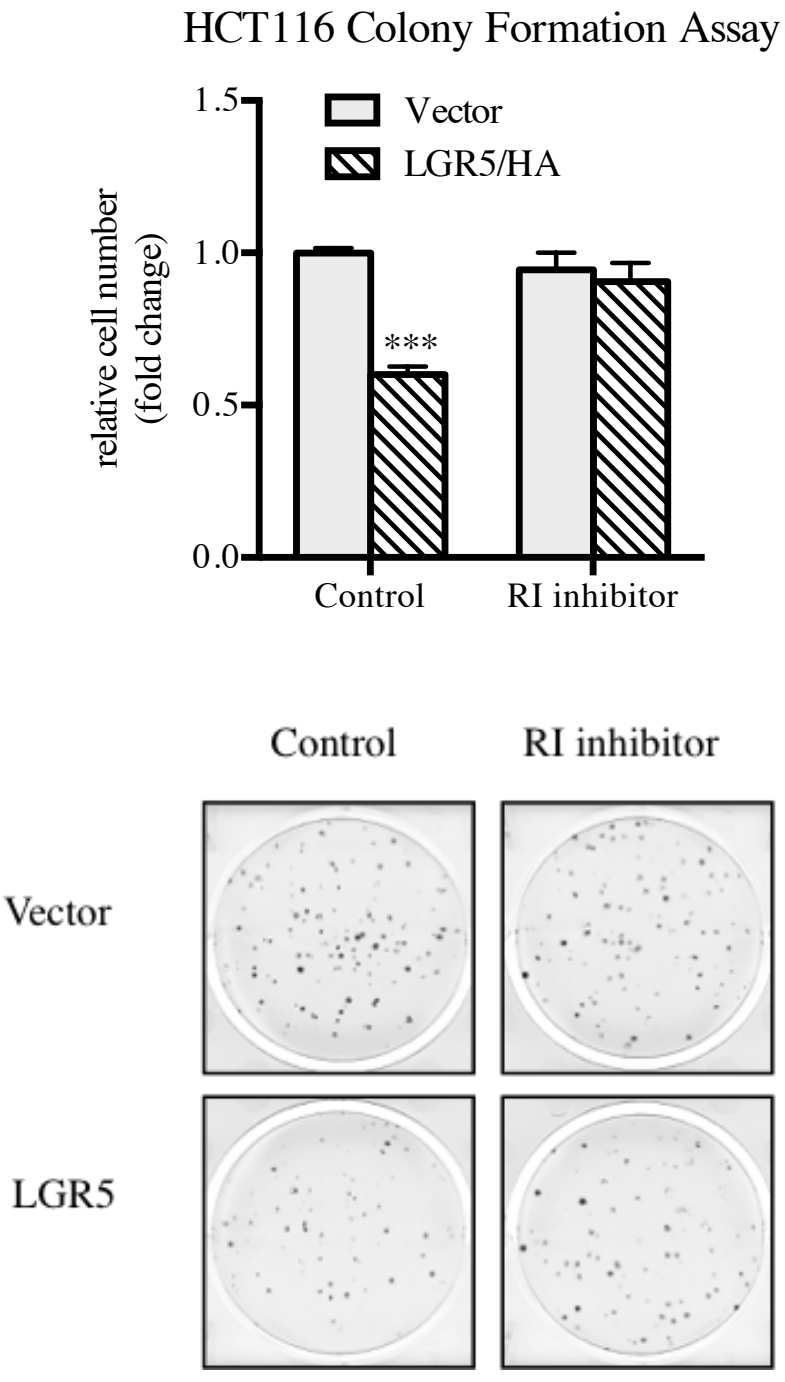

HCT116 Colony Formation Assay
relative cell number (fold change)
1.5
1.0
0.5
0.0
Vector
LGR5/HA
***
Control
RI inhibitor
Control
RI inhibitor
Vector
LGR5

Figure 3.5

D

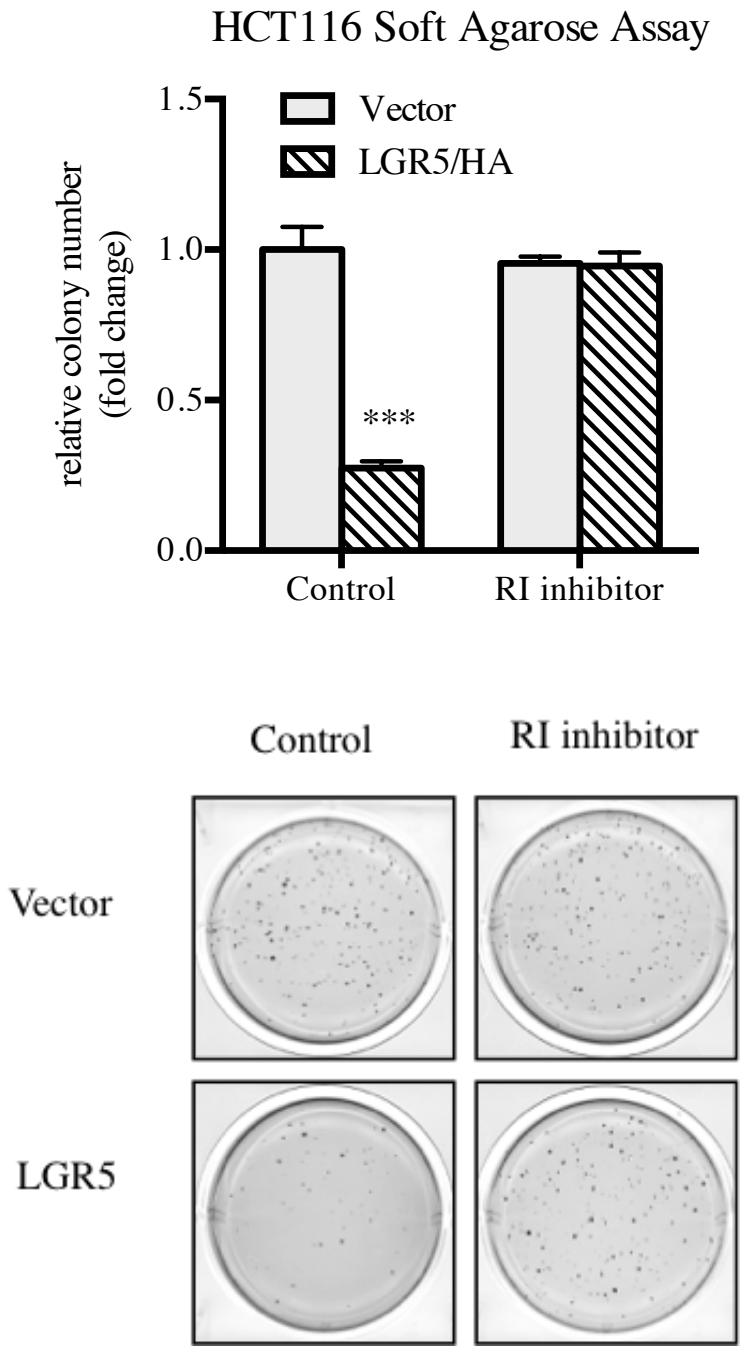
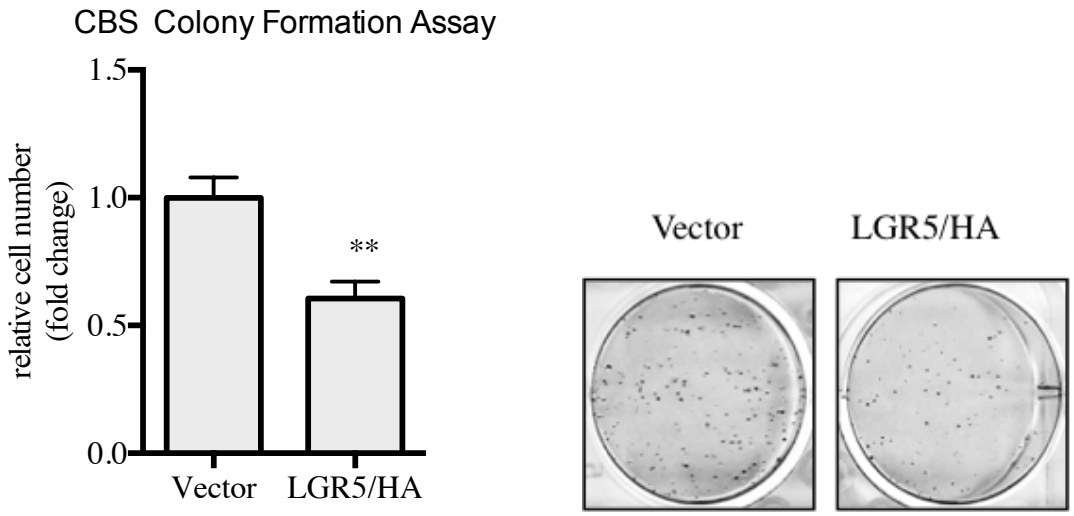

HCT116 Soft Agarose Assay
relative colony number (fold change)
1.5
1.0
0.5
0.0
Vector
LGR5/HA
***
Control
RI inhibitor
Control
RI inhibitor
Vector
LGR5

Figure 3.5

E


CBS Colony Formation Assay
relative cell number (fold change)
1.5
1.0
0.5
0.0
**
Vector
LGR5/HA
Vector
LGR5/HA

F

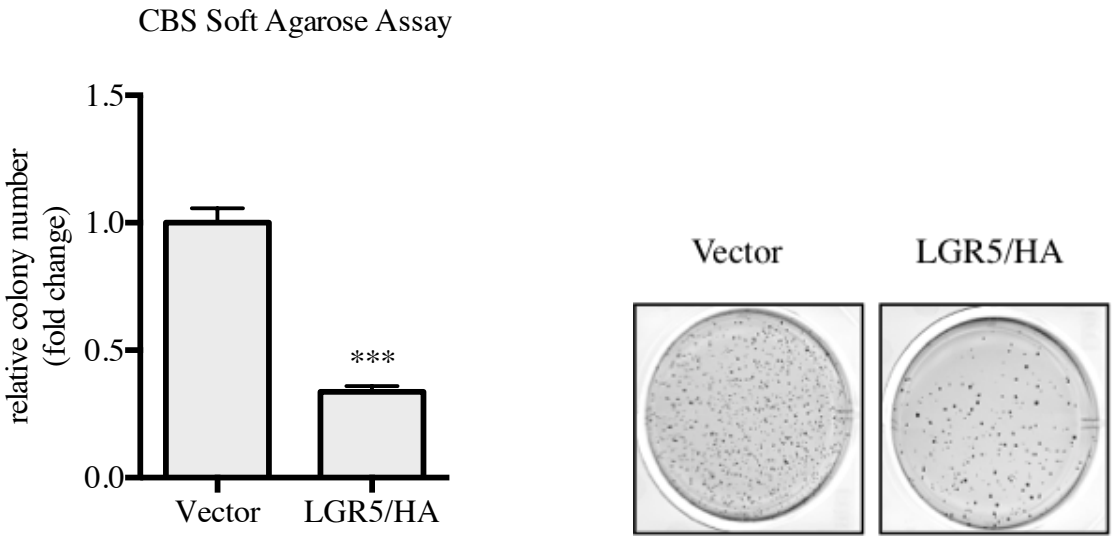

CBS Soft Agarose Assay
relative colony number (fold change)
1.5
1.0
0.5
0.0
***
Vector
LGR5/HA
Vector
LGR5/HA

Figure 3.5G&H. RI Kinase Inhibitor Attenuates RSPO1-induced Apoptosis and Clonogenecity Suppression in FET Cells. G. Cells were pretreated with 200nM RI kinase inhibitor or DMSO one hour before starting GFDS. 500ng/ml RSPO1 or 5ng/ml TGFβ were applied in followed by DNA fragmentation assay after 16 hours GFDS. **H.** 3000/well of cells were seeded in 0.4% soft agarose. 200nM RI kinase inhibitor, 500ng/ml RSPO1 or 5ng/ml TGFβ were applied and maintained in culture for 3 weeks. Cells were stained with 1% w/v iodonitrotetrazolium violet at Day 25. **G&H.** Error bars indicate SEM of three replications. Statistical significance was determined by Student's t-test. ** $P < 0.01$, *** $P < 0.001$

Figure 3.5

G

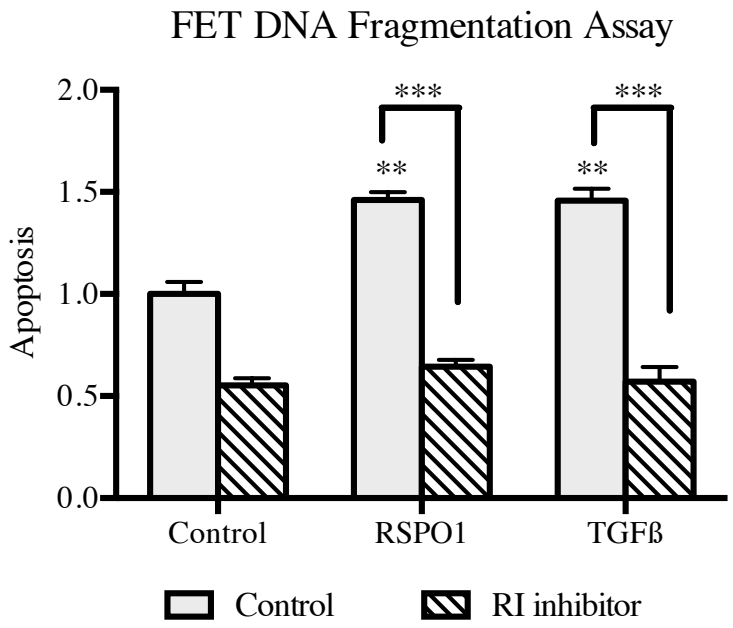

FET DNA Fragmentation Assay
Apoptosis
2.0
1.5
1.0
0.5
0.0
***
***
**
**
Control
RSPO1
TGFß
Control
RI inhibitor

Figure 3.5

H

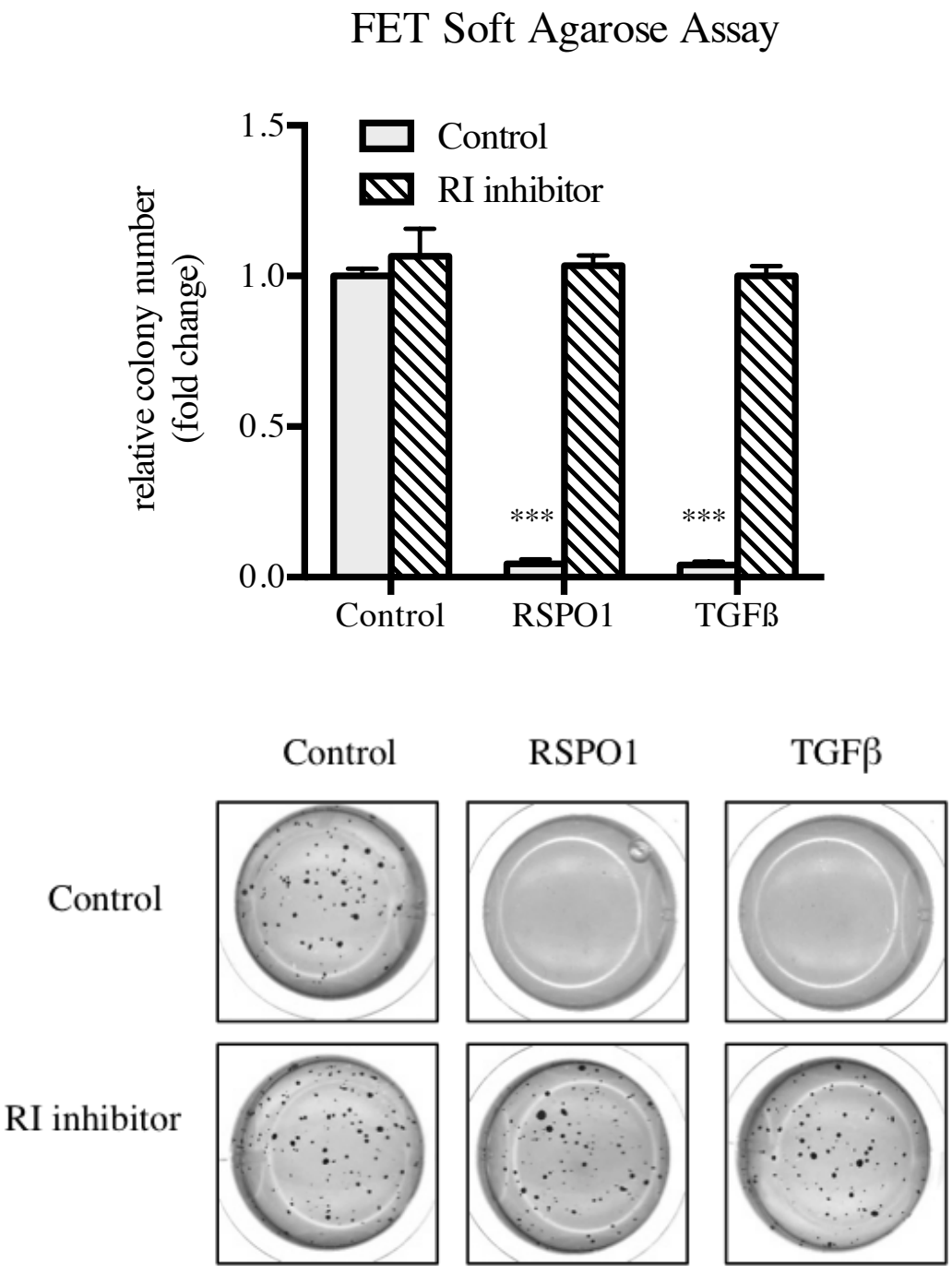
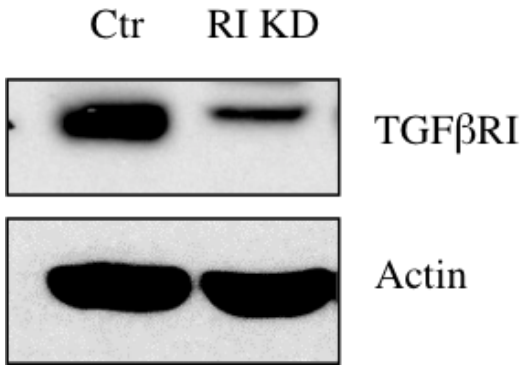

FET Soft Agarose Assay
relative colony number (fold change)
1.5
1.0
0.5
0.0
Control
RI inhibitor
***
***
Control
RSPO1
TGFß
Control
RSPO1
TGFβ
Control
RI inhibitor

Figure 3.5 I&J. RSPO1-induced Cell Apoptosis and Clonogenecity Suppression Associate with TGFβ RI. I. FET cells were stably transfected with TGFβ RI shRNA. The expression of RI was determined by Western blotting (upper panel). DNA fragmentation assay was performed after 16 hours GFDS (lower panel). **J.** 3000/well of cells were seeded in 0.4% soft agarose and were stained with 1% iodonitrotetrazolium violet at Day 25. **I&J.** Error bars indicate SEM of three replications. Statistical significance was determined by Student's t-test. *** $P < 0.001$

Figure 3.5

I


Ctr
RI KD
TGFβRI
Actin

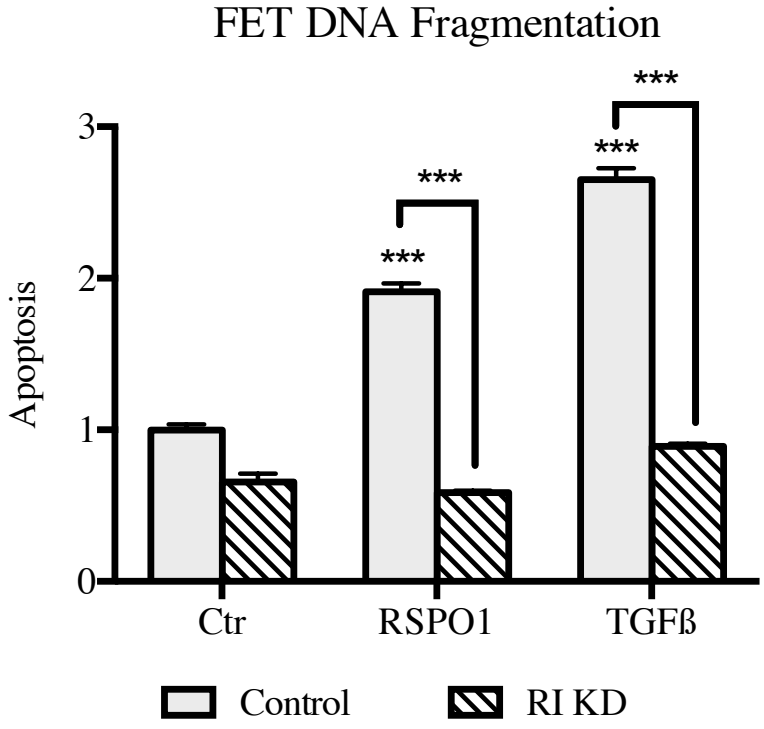

FET DNA Fragmentation
Apoptosis
3
2
1
0
***
***
***
***
Ctr
RSPO1
TGFß
Control
RI KD

Figure 3.5

J

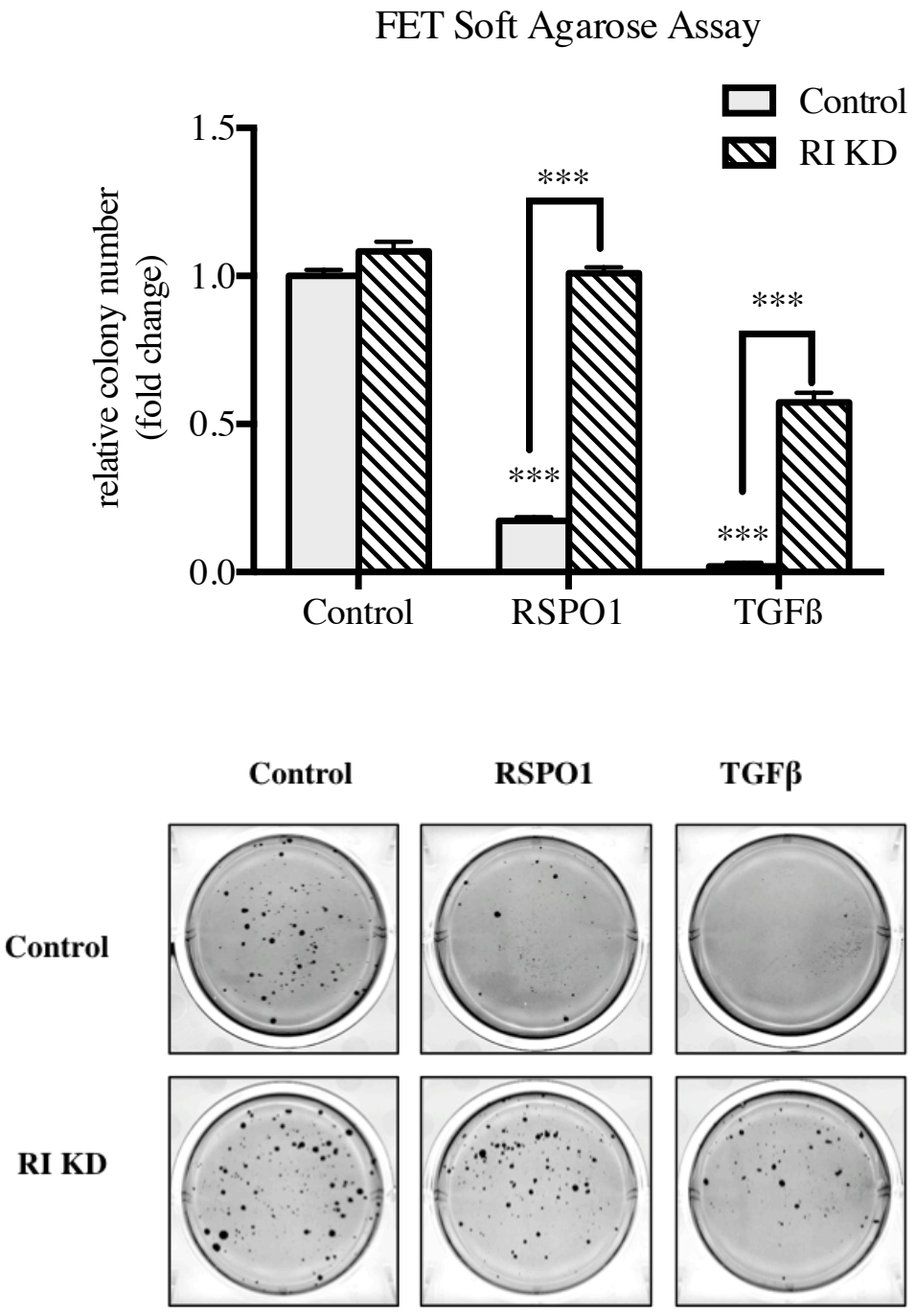

FET Soft Agarose Assay
Control
RI KD
relative colony number
(fold change)
1.5
1.0
0.5
0.0
***
***
***
Control
RSPO1
TGFß
Control
RSPO1
TGFβ
Control
RI KD

Figure 3.5F. Knockdown of LGR5 Associates with Reduced TGFβ Signaling *in vivo*. Images of pSmad2 staining in primary tumors are shown in the upper panel. The images are representative of multiple fields of tumor sections from each group. Intensity of positive and strong positive staining cells was determined under the Imagescope software. Relative intensity was calculated as percentage of strong positive to all positive fields in each section. The data are normalized as fold change and presented as the mean ± SEM. n = 7 in control group; 4 in LGR5 KD group. Statistical significance was determined by Student's t-test. ** $P < 0.01$

Figure 3.5

F

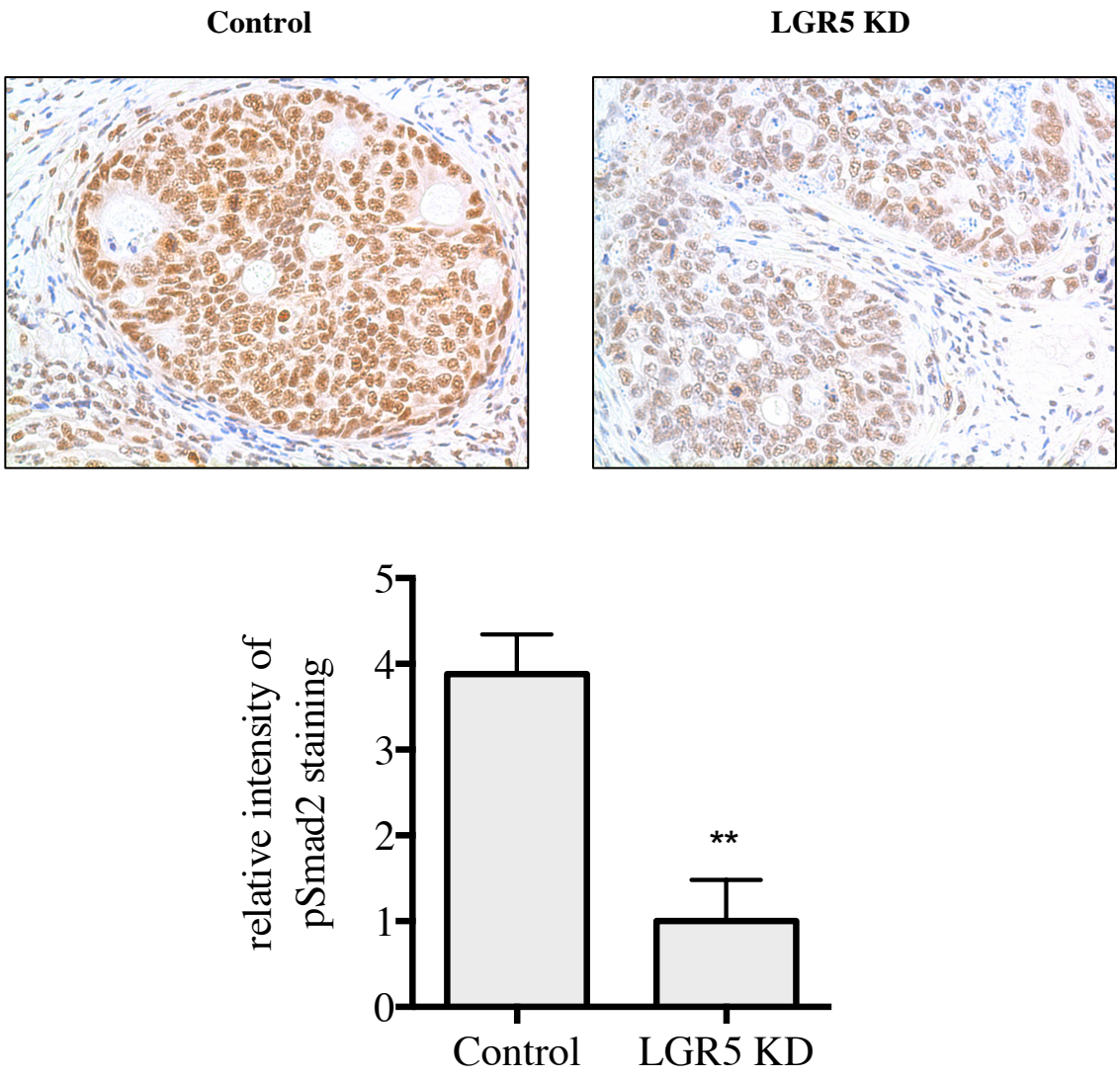
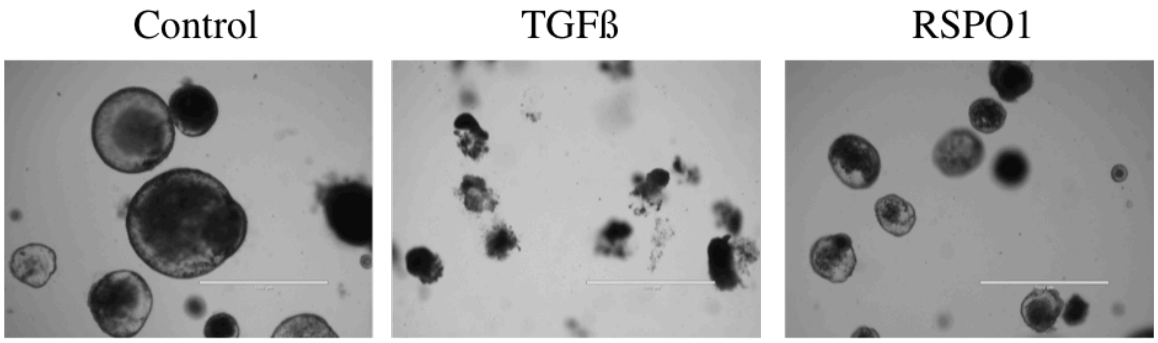

Control
LGR5 KD
relative intensity of pSmad2 staining
5
4
3
2
1
0
**
Control
LGR5 KD

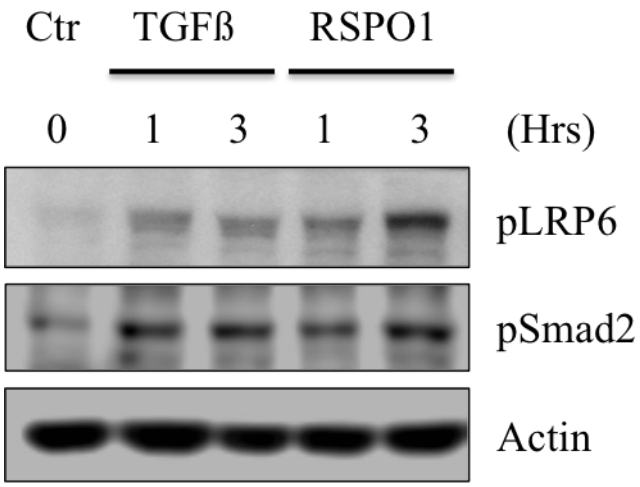
Figure 3.5G&H. RSPO1 Activates TGFβ Signaling *ex vivo*. G. 500ng/ml RSPO1 or 4ng/ml TGFβ was applied to the $APC^{min/+}$ organoids for 2 days. Bright field images were taken under 4 x magnifications. Scale bar indicates 1000 μm. **H.** Lysate of $APC^{min/+}$ organoids was collected after treating with 500ng/ml RSPO or 4ng/ml TGFβ for 1 hour and 3 hours. Expression of pLRP6 and pSmad2 were detected by western blot.

Figure 3.5

G.


Control
TGFß
RSPO1

H.


Ctr
TGFß
RSPO1
0
1
3
1
3
(Hrs)
pLRP6
pSmad2
Actin

CHAPTER 4

LGR5 Associates with TGFβ Receptors and Mediates Downstream TGFβ Signaling Pathway

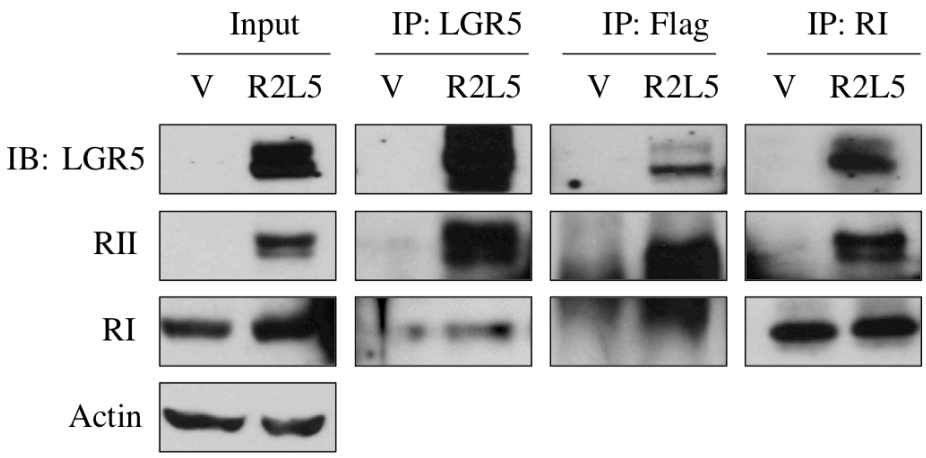
4.1 LGR5 Forms Complex with TGFβ Receptors.

TGFβ has been shown to bind TGFβ RII, which then associates with RI to transduce downstream signaling (Massague, Seoane et al. 2005). To determine the mechanism by which RSPO1-LGR5 activates TGFβ signaling, we investigated whether LGR5 forms complexes with TGFβ RI and/or RII. To facilitate co-immunoprecipitation (co-IP) assays and immunofluorescence staining to detect complex formation and co-localization, we used HCT116 cells that express abundant TGFβ RI, no TGFβ RII and little LGR5 to construct a stable cell line expressing Flag-tagged TGFβ RII and HA-tagged LGR5 (HCT116/RII/LGR5). As shown in **Figure 4.1**, RII/LGR5 cells expressed increased amount of RII and LGR5 as compared to the vector control cells. Endogenous RI was abundantly expressed in those cells. Co-IP results showed that LGR5, RI and RII all could be pulled-down together by targeting any of them, indicating the three receptors may form a super complex in colon cancer cells.

Figure 4.1 LGR5 Forms Complex with TGFβ Receptors. HCT116 cells were stably transfected with Flag-tagged TGFβRII and LGR5 (HCT116/RII/LGR5). From left to right panel: Detection of LGR5, RII and RI expression in control and overexpression cells by anti-LGR5, RI and RII antibody; Immunoprecipitation (IP) of LGR5 with LGR5 antibody followed by immunoblotting (IB) with anti-RII and -RI antibodies; IP of RII with Flag antibody followed by IB with anti-LGR5 and -RI antibodies; IP of RI with RI antibody followed by IB with anti-LGR5 and -RII antibodies.

(These experiments were performed by Dr. Liying Geng)

Figure 4.1


Input
IP: LGR5
IP: Flag
IP: RI
V
R2L5
V
R2L5
V
R2L5
V
R2L5
IB: LGR5
RII
RI
Actin

4.2 RSPO-LGR5 Mediates TGFβ Signaling by Enhancing the Complex Formation with TGFβ Receptors.

We then determined whether RSPO1 or TGFβ treatment would induce complex formation between LGR5 and TGFβ receptors in RII/LGR5 cells. Treatment of HCT116/RII/LGR5 cells with RSPO1 or TGFβ showed an increased Smad2 phosphorylation **(Figure 4.2A)**. Protein lysates from the control, RSPO1 or TGFβ-treated cells were immunoprecipitated with anti-LGR5, anti-RI or anti-RII antibody followed by western blot analysis of expression of LGR5, RI and RII in the immunoprecipitated complexes. Co-IP results showed that RSPO1 or TGFβ increased the amount of RI and RII, LGR5 and RII, LGR5 and RI associated with LGR5, RI or RII respectively (**Figure 4.2A**). These results indicated that treatment with RSPO1 or TGFβ induced the complex formation between LGR5 and RI/RII.

To further confirm the formation of the LGR5/RI/RII complex, immunofluorescence microscopy was used to determine whether LGR5 co-localized with TGFβ RI and RII. HCT116/LGR5/RII cells treated with RSPO1 or TGFβ were labeled with anti-HA (for LGR5), anti-RI or anti-Flag (for RII) antibody followed by incubation with a second antibody conjugated with Alexa Fluor 488 or 594. As shown in **Figure 4.2B-D**, treatment with RSPO1 or TGFβ increased co-localization of LGR5/RI, RII/LGR5 and RII/RI. These results indicate that LGR5 is a novel component of TGFβ receptor signaling complex and that RSPO-LGR5 activates TGFβ signaling through increasing this complex formation.

We showed earlier that ectopic expression of LGR5 activated TGFβ promoter activity in

HCT116 cells in the absence of TGFβ RII **(Figure 3.4A)**. To determine whether LGR5 could form the complex with RI in the absence of RII, we treated HCT116 cells stably expressing LGR5 (HCT116/LGR5) with RSPO1. As a result, Smad2 phosphorylation was induced **(Figure 4.2E)**. Protein lysates from the control or RSPO1-treated cells were immunoprecipitated with anti-LGR5 or anti-RI antibody followed by western blot analysis of expression of LGR5 and RI in the immunoprecipitated complexes. Co-IP results showed that RSPO1 increased the amount of RI associated with LGR5 **(Figure 4.2E)**. These results indicated that treatment with RSPO1 induced the complex formation between LGR5 and RI in the absence of RII.

To demonstrate the clinical relevance of the activation of the TGFβ pathway by RSPO/LGR5, we determined the correlation of LGR5 expression with Smad2 phosphorylation in the tissue samples of MSI patients. Since TGFβ RII is widely inactivated in MSI patients (Markowitz, Wang et al. 1995), the effect of TGFβ RII on TGFβ activation as reflected by Smad2 phosphorylation is minimized in these samples. IHC analysis showed a positive correlation between LGR5 and pSmad2 expression in MSI samples **(Figure 4.2F)**. These results indicated that LGR5-mediates the activation of TGFβ signaling in MSI patients.

Figure 4.2A-D RSPO-LGR5 Mediates TGFβ Signaling by Enhancing the Complex Formation with TGFβ Receptors. HCT116/RII/LGR5 cells were treated with 500ng/ml RSPO1 (R) or 4ng/ml TGFβ (T) at 40% confluence for 15 minutes. **A.** IP and IB were performed as was describe in **Figure 4.1**. LRP6 were detected by anti-LRP6 antibody. The activation of TGFβ signaling was determined by pSmad2. **B.** Confocal imaging of LGR5 (green) colocalization with TGFβRII (red) in control (a), under TGFβ treatment (b) and RSPO1 treatment (c). **C.** Confocal imaging of LGR5 (green) colocalization with TGFβRI (red) in control (d), under TGFβ treatment (e) and RSPO1 treatment (f). **D**. Confocal imaging of TGFβRII (green) colocalization with TGFβRI (red) in control (g), under TGFβ treatment (h) and RSPO1 treatment (i) B-D lower panel. Quantifications of colocalizaion was determined by Person's coefficient analysis.

(These experiments were performed by Dr. Liying Geng)

Figure 4.2

A

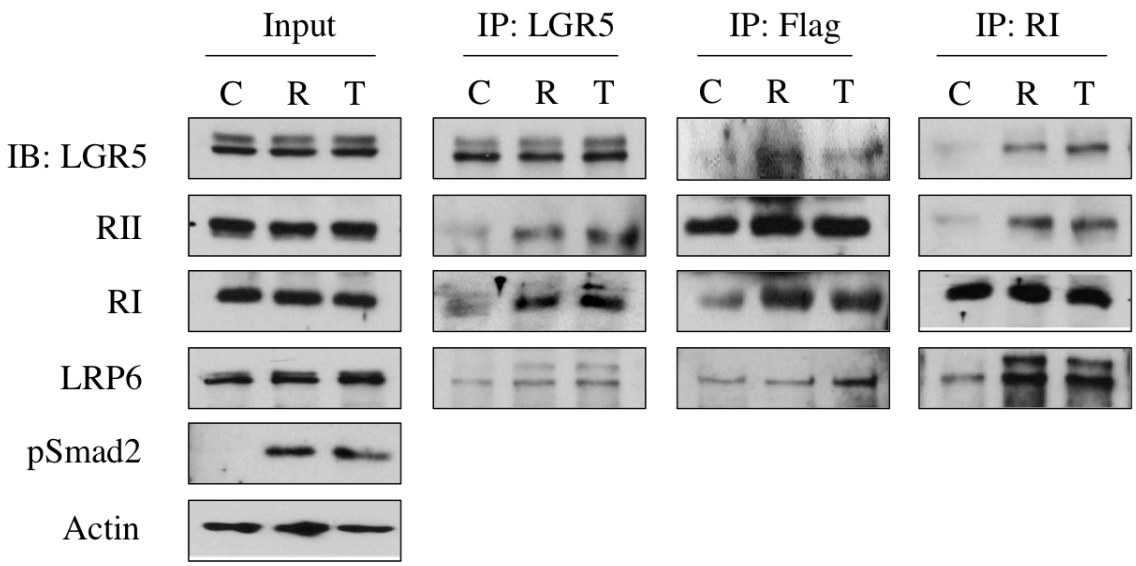
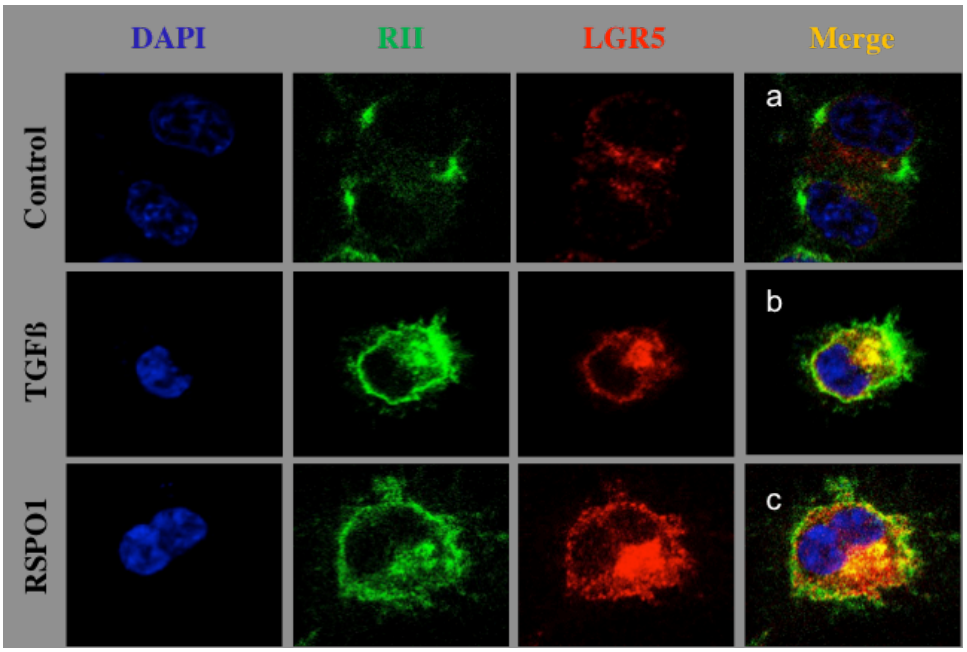

Input
IP: LGR5
IP: Flag
IP: RI
C R T
C R T
C R T
C R T
IB: LGR5
RII
RI
LRP6
pSmad2
Actin

Figure 4.2

B


DAPI
RII
LGR5
Merge
Control
TGFß
RSPO1
a
b
c

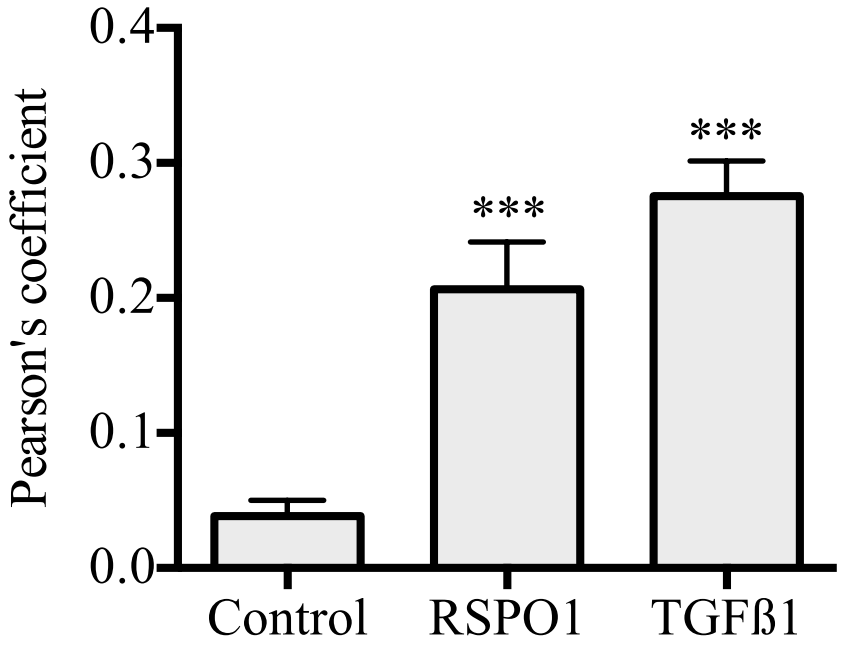
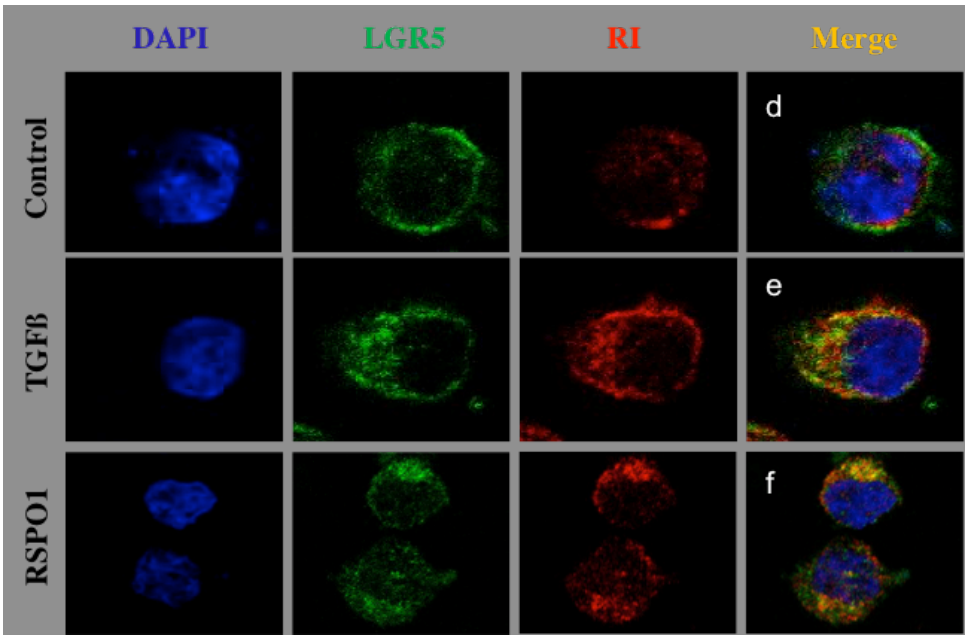

Pearson's coefficient
0.4
0.3
0.2
0.1
0.0
***
***
Control
RSPO1
TGFß1

Figure 4.2

C


DAPI
LGR5
RI
Merge
Control
TGFß
RSPO1
d
e
f

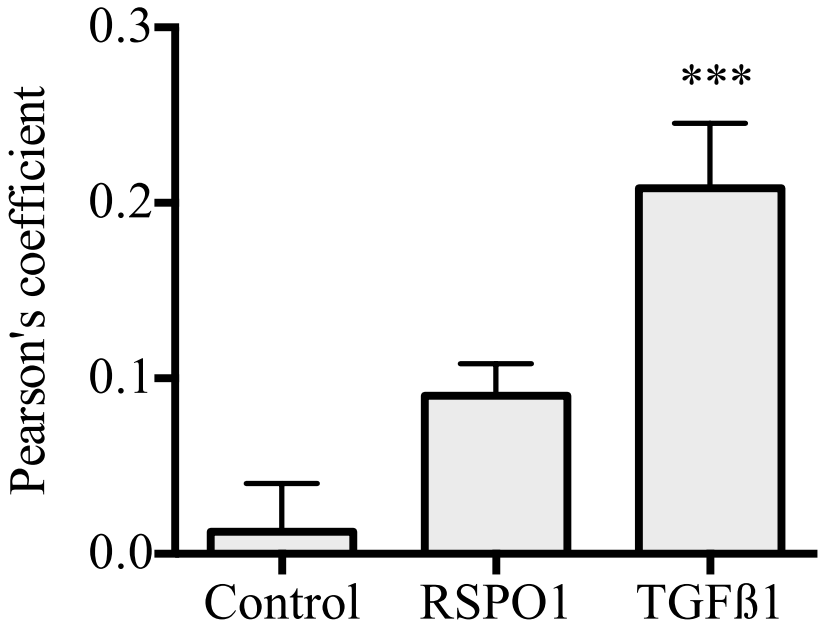
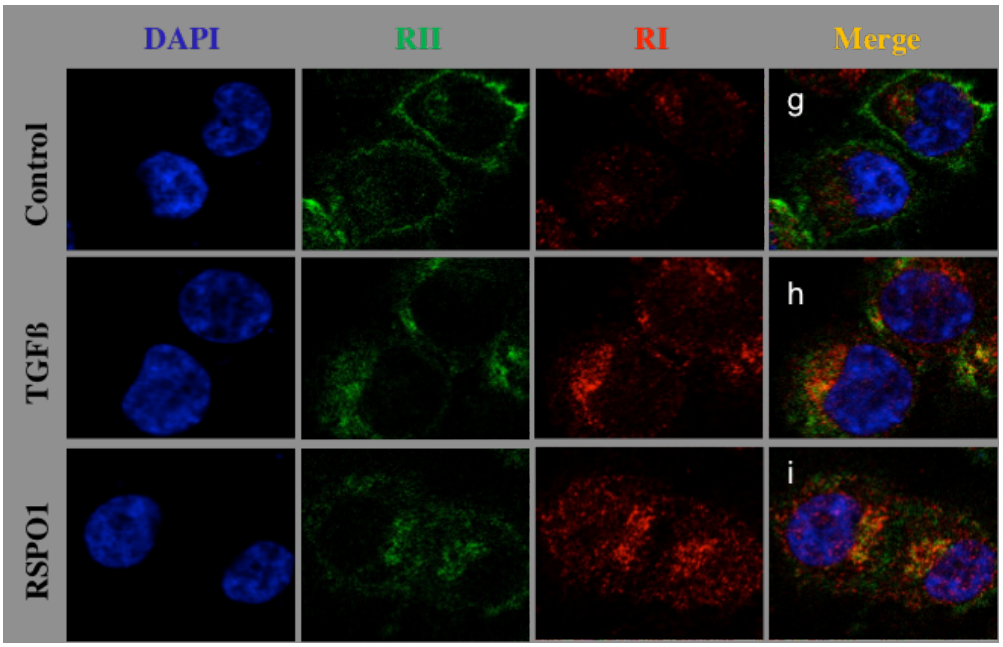

Pearson's coefficient
0.3
0.2
0.1
0.0
***
Control
RSPO1
TGFß1

Figure 4.2

D


DAPI
RII
RI
Merge
Control
TGFß
RSPO1
g
h
i

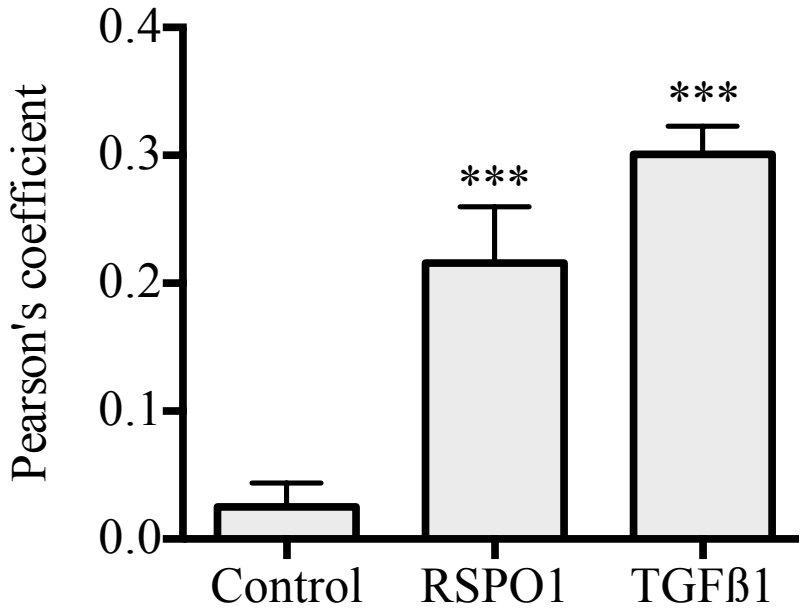

Pearson's coefficient
0.4
0.3
0.2
0.1
0.0
***
***
Control
RSPO1
TGFß1

Figure 4.2E RSPO-LGR5 Mediates TGFβ Signaling Independent of TGFβ RII. HCT116 cells were stably expressed with LGR5 and treated with RSPO1 under the condition described previously. **E.** Left panel: expression of LGR5, TGFβ RI and pSmad2 after RSPO1 treatment. Right upper panel: IP of LGR5 followed by IB with TGFβ RI. Right lower panel: IP of TGFβ RI followed by IB with LGR5.

(These experiments were performed by Dr. Liying Geng)

Figure 4.2

E

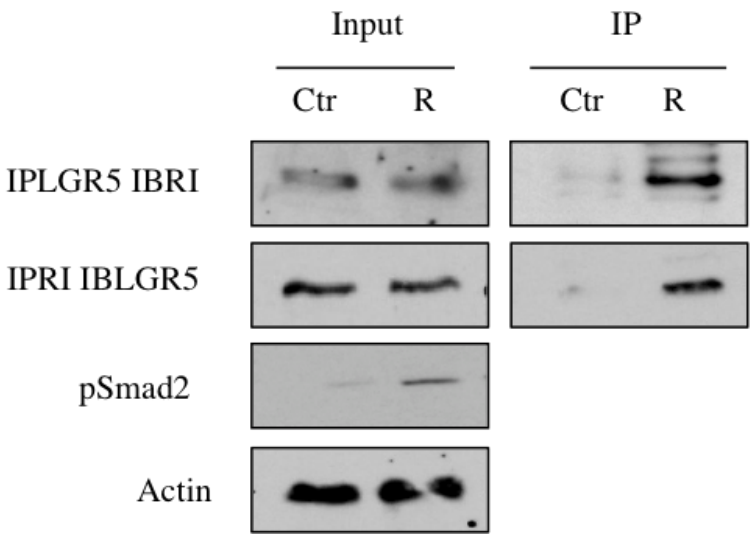
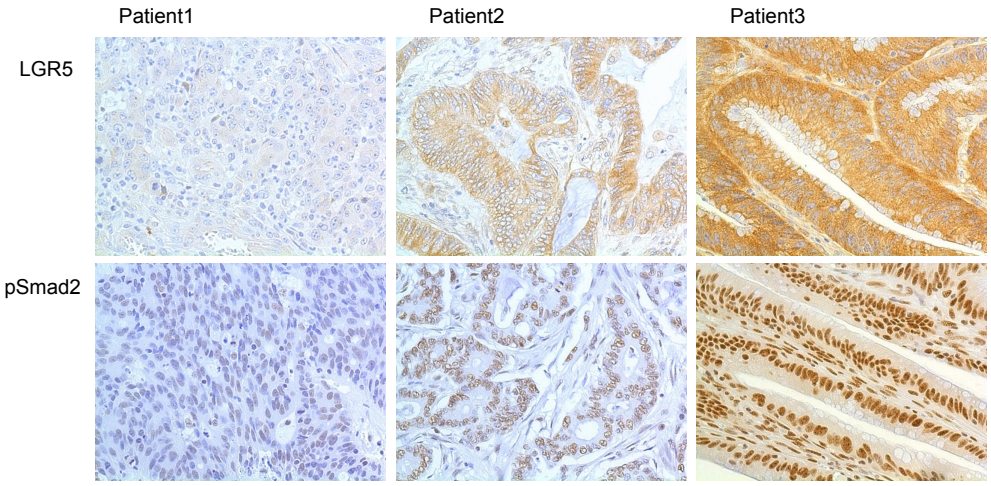


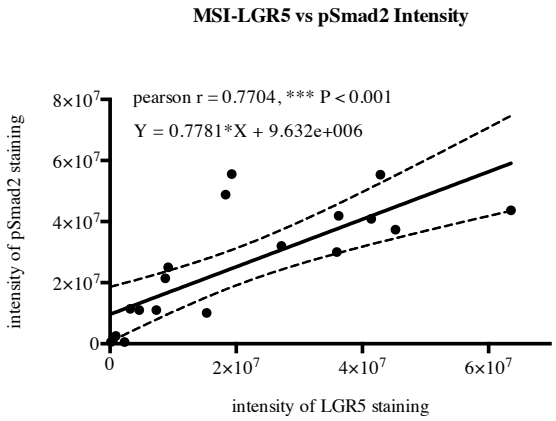
Figure 4.2F. LGR5 Expression Positively Correlates with TGFβ Activity in MSI Colon Cancer Patients. 19 MSI colon cancer patient samples were obtained from the UNMC tissue bank, followed by pSmad2 and LGR5 IHC staining. The upper panel showed representatives of the staining (× 20 magnification). The lower panel showed the correlation of LGR5 and pSmad2 staining in terms of intensity (Left) or percentage of positive cells (Right). The values are means of staining intensity or percentage of positive cells from multiple fields of each sample section. The correlation was established through Pearson's test ($r = 0.7704$ in intensity correlation, 0.8906 in percentage correlation; *** $p < 0.001$; $n = 19$). The equation was generated by lineage regression analysis. Dash lines indicate 95% confidence intervals of the slope.

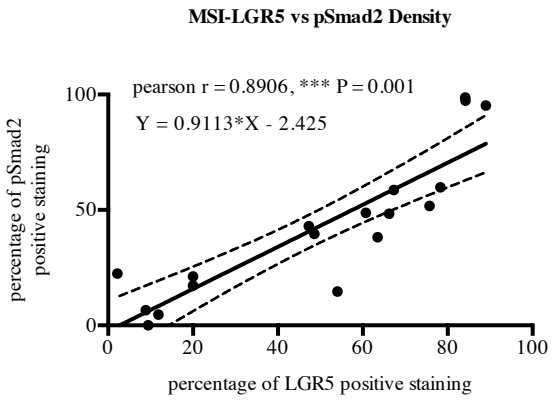
(These experiments were performed by Dr. Liying Geng)

Figure 4.2

F


Patient1
Patient2
Patient3
LGR5
pSmad2


MSI-LGR5 vs pSmad2 Intensity
pearson r = 0.7704, *** P < 0.001
Y = 0.7781*X + 9.632e+006
intensity of pSmad2 staining
intensity of LGR5 staining
8×10^7
6×10^7
4×10^7
2×10^7
0
0
2×10^7
4×10^7
6×10^7


MSI-LGR5 vs pSmad2 Density
pearson r = 0.8906, *** P = 0.001
Y = 0.9113*X - 2.425
percentage of pSmad2 positive staining
percentage of LGR5 positive staining
100
50
0
0
20
40
60
80
100

CHAPTER 5

Discussion and Future Direction

TGFβ signaling functions as a tumor suppressor and is defective in most of late stage colon cancer cases (Cancer Genome Atlas 2012). We have shown in this study that RSPO-LGR5 activates TGFβ signaling and inhibits oncogenic phenotypes of colon cancer cells. More importantly, knockdown of LGR5 expression suppressed TGFβ signaling *in vivo* and increased colon cancer metastasis in an orthotopic model. RSPO-LGR5-mediated activation of TGFβ signaling is dependent upon the RI kinase activity and expression of Smad2, Smad3 and Smad4. Mechanistically, LGR5 forms complexes with TGFβ RI and RII. Our studies have identified LGR5 as a novel signaling component of the TGFβ signaling pathway.

LGR4, LGR5 and LGR6 are close relatives of the receptors for follicle stimulating hormone, luteinizing hormone and thyroid-stimulating hormone (Barker and Clevers 2010). LGR6 marks multipotent stem cells in the epidermis (Snippert, Haegebarth et al. 2010). Both LGR4 and LGR5 are co-expressed in the stem cells of intestine, colon, stomach and hair follicle (Barker, van Es et al. 2007, Jaks, Barker et al. 2008, Barker, Huch et al. 2010). We found that LGR5 is differentially expressed in colon cancer cell lines (**Figure 3.1F**) whereas LGR4 is ubiquitously expressed at similar levels and LGR6 is absent from those cells (**Figure S1**). Unlike other GPCRs, LGR4, LGR5 and LGR6 are not coupled to either G proteins or β-arrestin when stimulated by RSPOs (Carmon, Gong et al. 2011). Instead, we show in this study that upon binding RSPOs, LGR5 forms a physical complex with TGFβ receptors RI and RII to amplify TGFβ signaling. Similarly, LGR4 and LGR6 also activate TGFβ signaling in colon cancer cells (**Figure S2**), indicating that it is a common function of this family. Since LGR4 is universally expressed and LGR5 suppression correlates with metastatic potential in colon cancer

cells, it is possible that LGR5 is a critical suppressor of colon cancer progression.

RSPO-LGR5 has been shown to modulate Wnt canonical signaling in many different cell types including colon cancer cells (Geng, Chaudhuri et al. 2014, Tsuji, Kawasaki et al. 2014, Wu, Qiu et al. 2014). Therefore, RSPO-LGR5-mediated Wnt activation seems to be cell context-dependent. In our studies, we found that RSPO-LGR5 can induce phosphorylation of LRP6 in most of the cancer cells, but none of them showed any significant change under Wnt reporter assay (data not shown), likely due to the ubiquitous and saturated Wnt activation in these cells. Nevertheless, RSPO-LGR5 activates TGFβ signaling in all cell lines including those with mutated *APC* or β-catenin (i.e. HCT116 and FET, respectively) and in those with the wild type *APC* and β-catenin (i.e. RKO, **Figure S3**), suggesting that RSPO-LGR5 mediates TGFβ activation regardless of Wnt activation status. We show here that LGR5 forms complexes with TGFβ receptors. It is yet to be investigated what the determining factors are for LGR5 to bind either or both of the Wnt or TGFβ receptors.

LGR5 activates TGFβ signaling in HCT116 cells that do not express TGFβ RII (**Figure 3.4A**) and treatment with RSPO increases TGFβ pathway activation in these cells (**Figure 3.4B**). These results suggest that RSPO-LGR5 could activate TGFβ signaling independent of RII. Knockdown of LGR5 in FET cells that express both RII and LGR5 reduces basal level of TGFβ activity and fold of TGFβ activation induced by RSPO (**Figure 3.4C**), suggesting that TGFβ-RII mediated TGFβ activation is independent of LGR5. In addition, treatment of FET and CBS cells with both TGFβ and RSPO induces much higher TGFβ activation than either treatment alone (**Figure 3.4D**). RI kinase inhibitor almost completely blocked TGFβ activation in all the treatments (**Figure 3.4D**),

indicating RSPO-LGR5 functions through TGFβRI. Furthermore, either TGFβ or RSPO induces complex formation of RI/RII/LGR5 and phosphorylation of Smad2 (**Figure 4.2**). Based on these results, we propose an updated model of TGFβ signaling (**Figure 5**): TGFβ and RSPO bind TGFβ RII and LGR5 respectively. Activated RII or LGR5 then form complexes with TGFβ RI and activate RI, which leads to phosphorylation and activation of Smads downstream. In the presence of RII, TGFβ-RII activates TGFβ signaling through RII binding with RI. Likewise, in the presence of LGR5, RSPO-LGR5 mediates TGFβ signaling through LGR5 association with RI. When both RII and LGR5 are present, RII and LGR5 both associates with RI and TGFβ signaling is further amplified. Therefore, TGFβ-RII and RSPO-LGR5 transduce TGFβ signaling independently as well as collaborately through the downstream RI-Smad pathway.

Our studies have significant implications for colon cancer development. According to our data, there are at least two ways to activate TGFβ signaling, TGFβ binding to RII and RSPO binding to LGR5. When both are present, TGFβ effect is greatly enhanced. Absence of either one will decrease TGFβ effect, but not be able to completely evade this tumor suppressor mechanism in colon cancer. Therefore, loss or reduction of both RII and LGR5 expression would significantly enhance the capacity of cancer cells to escape TGFβ tumor suppressor function, leading to cancer progression, poor prognosis and/or survival of colon cancer patients. Although loss of RII or decrease of LGR5 expression has been reported in colon cancer studies, decreased expression of both simultaneously has not been examined. Further studies of RII and LGR5 expression in human patient samples would be needed to investigate whether they could be used as potential biomarkers in colon cancer. Our results will also guide future studies towards the

application of RSPOs in treating colon cancers with elevated LGR5 expression. In addition, this study establishes a novel connection between an adult intestinal stem cell marker and a well- established tumor suppressor pathway in colon cancer.

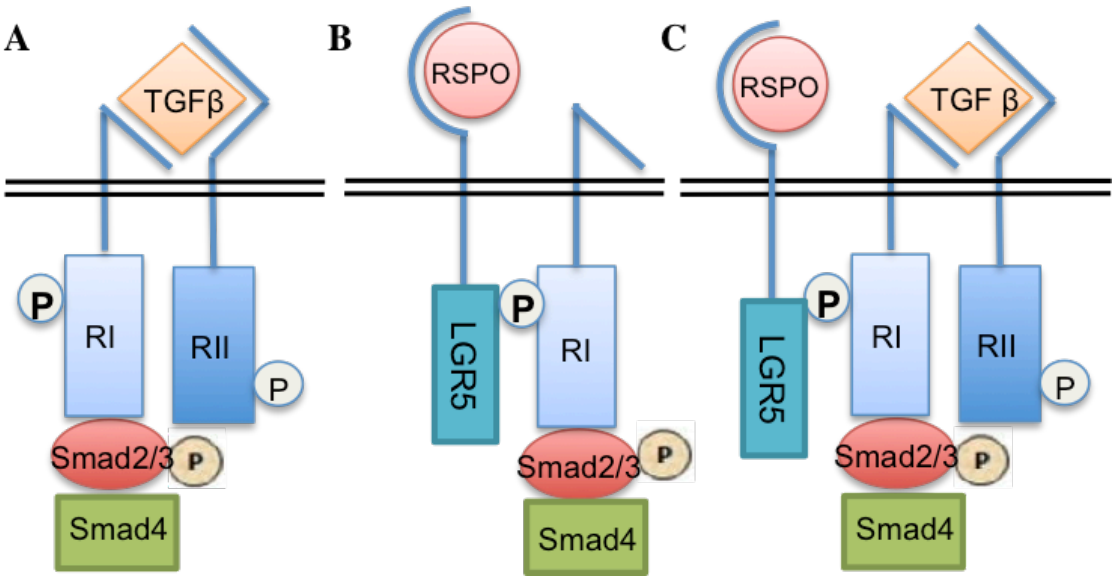


Figure 5. TGFβ Signaling Pathway Activation Models. Without the presence of RII, RSPO-LGR5 can directly activate RI to facilitate downstream Smads activation (**B**). With the presence of RII, RI activation can go through the canonical TGFβ-RII pathway (**A**) or through both RSPO-LGR5 and TGFβ-RII activation (**C**).

Supplementary Figures:

S1

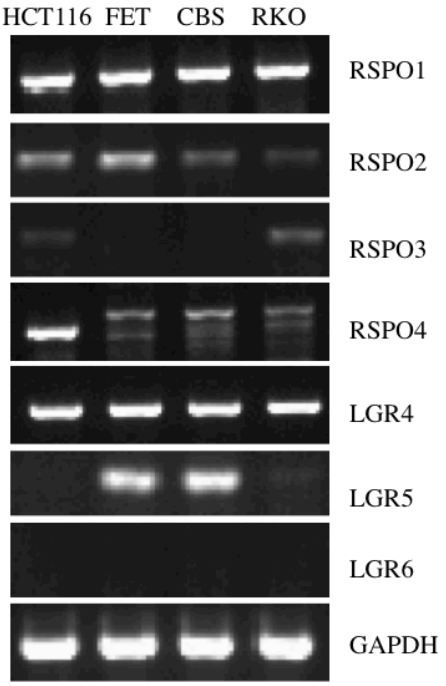


Figure S1. RT-PCR Result of RSPO1-4, LGR4, LGR5 and LGR6 in Different Colon Cancer Cell Lines.

S2

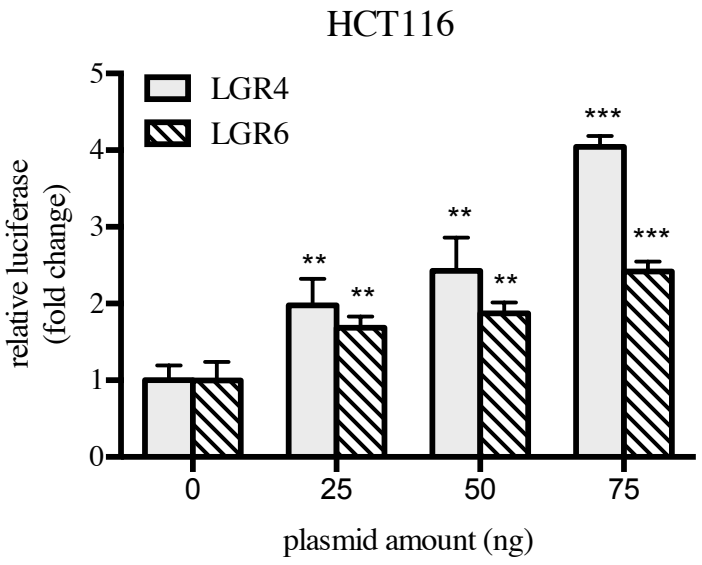


Figure S2. LGR4 and LGR6 Activate TGFβ Signaling Pathway in HCT116 Cells. TGFβ reporter assay in HCT116 cells transfected with different dose of LGR4 and LGR6 expression plasmid (0, 25, 50 and 75ng). The data are presented as the mean ± SEM. Statistical significance was determined by Student's t-test. ** P <0.01, *** P < 0.001 comparing to 0 ng group.

S3.

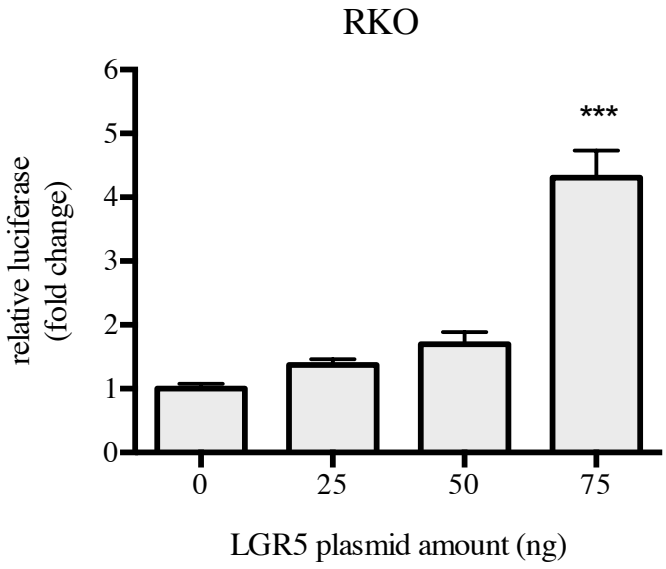


Figure S3. LGR5 Activates TGFβ Signaling Pathway in RKO Cells. TGFβ reporter assay in RKO cells transfected with different dose of LGR5 expression plasmid (0, 25, 50 and 75ng). The data are presented as the mean ± SEM. Statistical significance was determined by Student's t-test. *** $P < 0.001$ comparing to 0 ng group.

CHAPTER 6

References

Arteaga, C. L., R. J. Coffey, Jr., T. C. Dugger, C. M. McCutchen, H. L. Moses and R. M. Lyons (1990). "Growth stimulation of human breast cancer cells with anti-transforming growth factor beta antibodies: evidence for negative autocrine regulation by transforming growth factor beta." Cell Growth Differ **1**(8): 367-374.

Bacman, D., S. Merkel, R. Croner, T. Papadopoulos, W. Brueckl and A. Dimmler (2007). "TGF-beta receptor 2 downregulation in tumour-associated stroma worsens prognosis and high-grade tumours show more tumour-associated macrophages and lower TGF-beta1 expression in colon carcinoma: a retrospective study." BMC Cancer **7**: 156.

Barker, N. and H. Clevers (2010). "Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells." Gastroenterology **138**(5): 1681-1696.

Barker, N., M. Huch, P. Kujala, M. van de Wetering, H. J. Snippert, J. H. van Es, T. Sato, D. E. Stange, H. Begthel, M. van den Born, E. Danenberg, S. van den Brink, J. Korving, A. Abo, P. J. Peters, N. Wright, R. Poulsom and H. Clevers (2010). "Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro." Cell Stem Cell **6**(1): 25-36.

Barker, N., R. A. Ridgway, J. H. van Es, M. van de Wetering, H. Begthel, M. van den Born, E. Danenberg, A. R. Clarke, O. J. Sansom and H. Clevers (2009). "Crypt stem cells as the cells-of-origin of intestinal cancer." Nature **457**(7229): 608-611.

Barker, N., M. B. Rookmaaker, P. Kujala, A. Ng, M. Leushacke, H. Snippert, M. van de Wetering, S. Tan, J. H. Van Es, M. Huch, R. Poulsom, M. C. Verhaar, P. J. Peters and H. Clevers (2012). "Lgr5(+ve) stem/progenitor cells contribute to nephron formation during kidney development." Cell Rep **2**(3): 540-552.

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

Benson, A. B., 3rd and S. R. Hamilton (2011). "Path toward prognostication and prediction: an evolving matrix." J Clin Oncol **29**(35): 4599-4601.

Binnerts, M. E., K. A. Kim, J. M. Bright, S. M. Patel, K. Tran, M. Zhou, J. M. Leung, Y. Liu, W. E. Lomas, 3rd, M. Dixon, S. A. Hazell, M. Wagle, W. S. Nie, N. Tomasevic, J. Williams, X. Zhan, M. D. Levy, W. D. Funk and A. Abo (2007). "R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6." Proc Natl Acad Sci U S A **104**(37): 14700-14705.

Brattain, M. G., D. E. Brattain, W. D. Fine, F. M. Khaled, M. E. Marks, P. M. Kimball, L. A. Arcolano and B. H. Danbury (1981). "Initiation and characterization of cultures of human colonic carcinoma with different biological characteristics utilizing feeder layers of confluent fibroblasts." Oncodev Biol Med **2**(5): 355-366.

Campbell, P. J., S. Yachida, L. J. Mudie, P. J. Stephens, E. D. Pleasance, L. A. Stebbings, L. A. Morsberger, C. Latimer, S. McLaren, M. L. Lin, D. J. McBride, I. Varela, S. A. Nik-Zainal, C. Leroy, M. Jia, A. Menzies, A. P. Butler, J. W. Teague, C. A. Griffin, J. Burton, H. Swerdlow, M. A. Quail, M. R. Stratton, C. Iacobuzio-Donahue and P. A. Futreal (2010). "The patterns and dynamics of genomic instability in metastatic pancreatic cancer." Nature **467**(7319): 1109-1113.

Cancer Genome Atlas, N. (2012). "Comprehensive molecular characterization of human colon and rectal cancer." Nature **487**(7407): 330-337.

Carmon, K. S., X. Gong, Q. Lin, A. Thomas and Q. Liu (2011). "R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling." Proc Natl Acad Sci U S A **108**(28): 11452-11457.

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

Chen, P. H., X. Chen, Z. Lin, D. Fang and X. He (2013). "The structural basis of R-spondin recognition by LGR5 and RNF43." Genes Dev **27**(12): 1345-1350.

Datto, M. B., Y. Li, J. F. Panus, D. J. Howe, Y. Xiong and X. F. Wang (1995). "Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism." Proc Natl Acad Sci U S A **92**(12): 5545-5549.

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

de Sousa, E. M. F., S. Colak, J. Buikhuisen, J. Koster, K. Cameron, J. H. de Jong, J. B. Tuynman, P. R. Prasetyanti, E. Fessler, S. P. van den Bergh, H. Rodermond, E. Dekker, C. M. van der Loos, S. T. Pals, M. J. van de Vijver, R. Versteeg, D. J. Richel, L. Vermeulen and J. P. Medema (2011). "Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients." Cell Stem Cell **9**(5): 476-485.

Dean, M. (2005). "Cancer stem cells: Implications for cancer causation and therapy resistance." Discov Med **5**(27): 278-282.

Fleming, M., S. Ravula, S. F. Tatishchev and H. L. Wang (2012). "Colorectal carcinoma: Pathologic aspects." J Gastrointest Oncol **3**(3): 153-173.

Forrester, E., A. Chytil, B. Bierie, M. Aakre, A. E. Gorska, A. R. Sharif-Afshar, W. J. Muller and H. L. Moses (2005). "Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis." Cancer Res **65**(6): 2296-2302.

Frank, S. A. (2007). Dynamics of Cancer. Dynamics of Cancer: Incidence, Inheritance, and Evolution. Princeton (NJ).

Garcia, M. I., M. Ghiani, A. Lefort, F. Libert, S. Strollo and G. Vassart (2009). "LGR5 deficiency deregulates Wnt signaling and leads to precocious Paneth cell differentiation in the fetal intestine." Dev Biol **331**(1): 58-67.

Geng, L., A. Chaudhuri, G. Talmon, J. L. Wisecarver, C. Are, M. Brattain and J. Wang (2014). "MicroRNA-192 suppresses liver metastasis of colon cancer." Oncogene **33**(46): 5332-5340.

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

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

Guo, X. N., A. Rajput, R. Rose, J. Hauser, A. Beko, K. Kuropatwinski, C. LeVea, R. M. Hoffman, M. G. Brattain and J. Wang (2007). "Mutant PIK3CA-bearing colon cancer

cells display increased metastasis in an orthotopic model." Cancer Res **67**(12): 5851-5858.

Haegebarth, A. and H. Clevers (2009). "Wnt signaling, lgr5, and stem cells in the intestine and skin." Am J Pathol **174**(3): 715-721.

Hafez, M. M., D. Infante, S. Winawer and E. Friedman (1990). "Transforming growth factor beta 1 acts as an autocrine-negative growth regulator in colon enterocytic differentiation but not in goblet cell maturation." Cell Growth Differ **1**(12): 617-626.

Hernandez, A., F. Smith, Q. Wang, X. Wang and B. M. Evers (2000). "Assessment of differential gene expression patterns in human colon cancers." Ann Surg **232**(4): 576-585.

Hsu, S. Y., S. G. Liang and A. J. Hsueh (1998). "Characterization of two LGR genes homologous to gonadotropin and thyrotropin receptors with extracellular leucine-rich repeats and a G protein-coupled, seven-transmembrane region." Mol Endocrinol **12**(12): 1830-1845.

Hu, P. P., M. B. Datto and X. F. Wang (1998). "Molecular mechanisms of transforming growth factor-beta signaling." Endocr Rev **19**(3): 349-363.

Huang, E. H. and M. S. Wicha (2008). "Colon cancer stem cells: implications for prevention and therapy." Trends Mol Med **14**(11): 503-509.

Jaks, V., N. Barker, M. Kasper, J. H. van Es, H. J. Snippert, H. Clevers and R. Toftgard (2008). "Lgr5 marks cycling, yet long-lived, hair follicle stem cells." Nat Genet **40**(11): 1291-1299.

Ji, H., D. W. Greening, T. W. Barnes, J. W. Lim, B. J. Tauro, A. Rai, R. Xu, C. Adda, S. Mathivanan, W. Zhao, Y. Xue, T. Xu, H. J. Zhu and R. J. Simpson (2013). "Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components." Proteomics **13**(10-11): 1672-1686.

Jiang, Y., X. Q. Liu, A. Rajput, L. Geng, M. Ongchin, Q. Zeng, G. S. Taylor and J. Wang (2011). "Phosphatase PRL-3 is a direct regulatory target of TGFbeta in colon cancer metastasis." Cancer Res **71**(1): 234-244.

Kim, K. A., M. Wagle, K. Tran, X. Zhan, M. A. Dixon, S. Liu, D. Gros, W. Korver, S. Yonkovich, N. Tomasevic, M. Binnerts and A. Abo (2008). "R-Spondin family members regulate the Wnt pathway by a common mechanism." Mol Biol Cell **19**(6): 2588-2596.

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

Liu, X. Q., A. Rajput, L. Geng, M. Ongchin, A. Chaudhuri and J. Wang (2011). "Restoration of transforming growth factor-beta receptor II expression in colon cancer

cells with microsatellite instability increases metastatic potential in vivo." J Biol Chem **286**(18): 16082-16090.

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

MacDonald, B. T., K. Tamai and X. He (2009). "Wnt/beta-catenin signaling: components, mechanisms, and diseases." Dev Cell **17**(1): 9-26.

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

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

McDonald, T., R. Wang, W. Bailey, G. Xie, F. Chen, C. T. Caskey and Q. Liu (1998). "Identification and cloning of an orphan G protein-coupled receptor of the glycoprotein hormone receptor subfamily." Biochem Biophys Res Commun **247**(2): 266-270.

Morita, H., S. Mazerbourg, D. M. Bouley, C. W. Luo, K. Kawamura, Y. Kuwabara, H. Baribault, H. Tian and A. J. Hsueh (2004). "Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension." Mol Cell Biol **24**(22): 9736-9743.

Mueller, M. M. and N. E. Fusenig (2004). "Friends or foes - bipolar effects of the tumour stroma in cancer." Nat Rev Cancer **4**(11): 839-849.

Nguyen, D. X., P. D. Bos and J. Massague (2009). "Metastasis: from dissemination to organ-specific colonization." Nat Rev Cancer **9**(4): 274-284.

Peng, W. C., W. de Lau, F. Forneris, J. C. Granneman, M. Huch, H. Clevers and P. Gros (2013). "Structure of stem cell growth factor R-spondin 1 in complex with the ectodomain of its receptor LGR5." Cell Rep **3**(6): 1885-1892.

Peng, W. C., W. de Lau, P. K. Madoori, F. Forneris, J. C. Granneman, H. Clevers and P. Gros (2013). "Structures of Wnt-antagonist ZNRF3 and its complex with R-spondin 1 and implications for signaling." PLoS One **8**(12): e83110.

Ricci-Vitiani, L., E. Fabrizi, E. Palio and R. De Maria (2009). "Colon cancer stem cells." J Mol Med (Berl) **87**(11): 1097-1104.

Salovaara, R., S. Roth, A. Loukola, V. Launonen, P. Sistonen, E. Avizienyte, P. Kristo, H. Jarvinen, S. Souchelnytskyi, M. Sarlomo-Rikala and L. A. Aaltonen (2002). "Frequent loss of SMAD4/DPC4 protein in colorectal cancers." Gut **51**(1): 56-59.

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

Schwitalla, S., A. A. Fingerle, P. Cammareri, T. Nebelsiek, S. I. Goktuna, P. K. Ziegler, O. Canli, J. Heijmans, D. J. Huels, G. Moreaux, R. A. Rupec, M. Gerhard, R. Schmid, N. Barker, H. Clevers, R. Lang, J. Neumann, T. Kirchner, M. M. Taketo, G. R. van den Brink, O. J. Sansom, M. C. Arkan and F. R. Greten (2013). "Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties." Cell **152**(1-2): 25-38.

Simms, N. A., A. Rajput, E. A. Sharratt, M. Ongchin, C. A. Teggart, J. Wang and M. G. Brattain (2012). "Transforming growth factor-beta suppresses metastasis in a subset of human colon carcinoma cells." BMC Cancer **12**: 221.

Snippert, H. J., A. Haegebarth, M. Kasper, V. Jaks, J. H. van Es, N. Barker, M. van de Wetering, M. van den Born, H. Begthel, R. G. Vries, D. E. Stange, R. Toftgard and H. Clevers (2010). "Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin." Science **327**(5971): 1385-1389.

Snyder, J. C., L. K. Rochelle, H. K. Lyerly, M. G. Caron and L. S. Barak (2013). "Constitutive internalization of the leucine-rich G protein-coupled receptor-5 (LGR5) to the trans-Golgi network." J Biol Chem **288**(15): 10286-10297.

Steeg, P. S. (2003). "Metastasis suppressors alter the signal transduction of cancer cells." Nat Rev Cancer **3**(1): 55-63.

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

Tlsty, T. D. and P. W. Hein (2001). "Know thy neighbor: stromal cells can contribute oncogenic signals." Curr Opin Genet Dev **11**(1): 54-59.

Tsuji, S., Y. Kawasaki, S. Furukawa, K. Taniue, T. Hayashi, M. Okuno, M. Hiyoshi, J. Kitayama and T. Akiyama (2014). "The miR-363-GATA6-Lgr5 pathway is critical for colorectal tumourigenesis." Nat Commun **5**: 3150.

Valdes, F., A. M. Alvarez, A. Locascio, S. Vega, B. Herrera, M. Fernandez, M. Benito, M. A. Nieto and I. Fabregat (2002). "The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes." Mol Cancer Res **1**(1): 68-78.

Veenendaal, L. M., O. Kranenburg, N. Smakman, A. Klomp, I. H. Borel Rinkes and P. J. van Diest (2008). "Differential Notch and TGFbeta signaling in primary colorectal tumors and their corresponding metastases." Cell Oncol **30**(1): 1-11.

Wakefield, L. M. and A. B. Roberts (2002). "TGF-beta signaling: positive and negative effects on tumorigenesis." Curr Opin Genet Dev **12**(1): 22-29.

Walker, F., H. H. Zhang, A. Odorizzi and A. W. Burgess (2011). "LGR5 is a negative regulator of tumourigenicity, antagonizes Wnt signalling and regulates cell adhesion in colorectal cancer cell lines." PLoS One **6**(7): e22733.

Wang, D., B. Huang, S. Zhang, X. Yu, W. Wu and X. Wang (2013). "Structural basis for R-spondin recognition by LGR4/5/6 receptors." Genes Dev **27**(12): 1339-1344.

Wang, J., W. Han, E. Zborowska, J. Liang, X. Wang, J. K. Willson, L. Sun and M. G. Brattain (1996). "Reduced expression of transforming growth factor beta type I receptor contributes to the malignancy of human colon carcinoma cells." J Biol Chem **271**(29): 17366-17371.

Wang, J., N. Sergina, T. C. Ko, J. Gong and M. G. Brattain (2004). "Autocrine and exogenous transforming growth factor beta control cell cycle inhibition through pathways with different sensitivity." J Biol Chem **279**(38): 40237-40244.

Wang, J., L. Sun, L. Myeroff, X. Wang, L. E. Gentry, J. Yang, J. Liang, E. Zborowska, S. Markowitz, J. K. Willson and et al. (1995). "Demonstration that mutation of the type II transforming growth factor beta receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells." J Biol Chem **270**(37): 22044-22049.

Wang, J., L. Yang, J. Yang, K. Kuropatwinski, W. Wang, X. Q. Liu, J. Hauser and M. G. Brattain (2008). "Transforming growth factor beta induces apoptosis through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells." Cancer Res **68**(9): 3152-3160.

Wu, C., S. Qiu, L. Lu, J. Zou, W. F. Li, O. Wang, H. Zhao, H. Wang, J. Tang, L. Chen, T. Xu, Z. Sun, W. Liao, G. Luo and X. Lu (2014). "RSPO2-LGR5 signaling has tumour-suppressive activity in colorectal cancer." Nat Commun **5**: 3149.

Wu, S. P., L. Z. Sun, J. K. Willson, L. Humphrey, R. Kerbel and M. G. Brattain (1993). "Repression of autocrine transforming growth factor beta 1 and beta 2 in quiescent CBS colon carcinoma cells leads to progression of tumorigenic properties." Cell Growth Differ **4**(2): 115-123.

Wu, S. P., D. Theodorescu, R. S. Kerbel, J. K. Willson, K. M. Mulder, L. E. Humphrey and M. G. Brattain (1992). "TGF-beta 1 is an autocrine-negative growth regulator of human colon carcinoma FET cells in vivo as revealed by transfection of an antisense expression vector." J Cell Biol **116**(1): 187-196.

Xie, Y., R. Zamponi, O. Charlat, M. Ramones, S. Swalley, X. Jiang, D. Rivera, W. Tschantz, B. Lu, L. Quinn, C. Dimitri, J. Parker, D. Jeffery, S. K. Wilcox, M. Watrobka, P. LeMotte, B. Granda, J. A. Porter, V. E. Myer, A. Loew and F. Cong (2013). "Interaction with both ZNRF3 and LGR4 is required for the signalling activity of R-spondin." EMBO Rep **14**(12): 1120-1126.

Yang, L., J. Huang, X. Ren, A. E. Gorska, A. Chytil, M. Aakre, D. P. Carbone, L. M. Matrisian, A. Richmond, P. C. Lin and H. L. Moses (2008). "Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis." Cancer Cell **13**(1): 23-35.

Ye, S. C., J. M. Foster, W. Li, J. Liang, E. Zborowska, S. Venkateswarlu, J. Gong, M. G. Brattain and J. K. Willson (1999). "Contextual effects of transforming growth factor beta on the tumorigenicity of human colon carcinoma cells." Cancer Res **59**(18): 4725-4731.

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