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KRÜPPEL-LIKE FACTOR 4 REGULATION IN INTESTINAL EPITHELIAL DIFFERENTIATION, PROLIFERATION AND POLARITY

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KRÜPPEL-LIKE FACTOR 4 REGULATION IN INTESTINAL EPITHELIAL DIFFERENTIATION, PROLIFERATION AND POLARITY

by

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Dedication

Dedicated to my families who have been lovingly supportive to me in all my endeavors.

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KRÜPPEL-LIKE FACTOR 4 REGULATION IN INTESTINAL EPITHELIAL DIFFERENTIATION, PROLIFERATION AND POLARITY

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The zinc finger protein and transcription factor, KLF4, plays a vital role in the regulation of lineage differentiation during development, as well as the maintenance of epithelial homeostasis in the intestine. KLF4 is predominantly expressed in the epithelial cells that are fully differentiated in normal intestine. In colorectal cancer, KLF4 was identified as a tumor suppressor; it is also a stem cell factor that along with other factors can induce pluripotent stem cells. KLF4 knockout mice demonstrate decrease in goblet cell number in the colon; conditional KLF4 ablation from the intestinal epithelium leads to changes in epithelial homeostasis. This dissertation defines the role of KLF4 in normal intestinal cells and in colon cancer cells, as well as the mechanism by which KLF4 regulates intestinal homeostasis and represses tumorigenesis. I demonstrate the role of KLF4 in maintaining numbers, positions and polarity of intestinal epithelial cells, changes of which result in morphology alterations. I further delineate the mechanisms of KLF4 regulation in the intestinal stem cells and colorectal cancers, which mainly focuses

on its target Bmi1, a Polycomb group protein, and an intestinal stem cell marker. Human colorectal cancers tissue array staining showed that Bmi1 levels are significantly increased in the tumor tissues from colon cancer patients compared with adjacent normal tissues; and expression of Bmi1 is significantly associated with nuclear β-catenin. Bmi1 knockdown by lentivirus-mediated Bmi1-shRNA repressed growth of different colon cancer cell lines, inhibited xenograft tumor growth, and increased differentiation of colon cancer cells in mouse xenograft. Dominant negative TCF (dnTCF), which blocks Wnt signaling, only marginally inhibits Bmi1 expression. However, the expression of Bmi1 was directly inhibited by KLF4 in LS174T colon cancer cells, and its function in H2A ubiquitination is inhibited by KLF4. In addition, using a three-dimensional (3D) culture system for colon cancer cell lines, I demonstrate that KLF4 is important for cell polarity and position; also it is essential for epithelial crypt-cyst structure formation *in vitro*. This research provides novel insights into the mechanisms under which KLF4 functions and regulate the intestinal stem cells and colorectal cancers, and will prove useful in development of targeting therapeutics of colon cancer, as well as understanding of tumor initiation in the background of stem cell regulation.

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List of Abbreviations

AB	Alcian Blue
AML	acute myeloid leukemia
APC	adenomatous polyposis coli
Ascl2	achaete scute-like-2
Bmi1	B lymphoma Mo-MLV insertion region 1
CBC	crypt base columnar
DCAMKL-1	doublecortin and Ca^{2+}/calmodulin-dependent kinase-like-1
ChIP	chromatin immunoprecipitation
CKIα	casein kinase Iα
dnTCF	dominant-negative TCF
CRD	cysteine-rich domain
CTOS	colon tissue originated spheroids
Dox	doxycycline
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ESC	embryonic stem cell
H&E	haematoxylin and eosin
H3K27	H3 Lys27
IHC	immunohistochemistry
ING	inhibitor of growth
iPS	induced pluripotent stem
KLF4	Krüppel-like factor 4
LEF-1	lymphoid enhancer factor-1

Lgr5	leucine-rich-repeat-containing G-protein-coupled receptor
LRC	label retaining cells
mTERT	mouse telomerase reverse transcriptase
NT	neurotensin
Olfm4	olfactomedin-4
PAS	periodic acid-Schiff
PcG	polycomb group
PCP	planar cell polarity
PHD	plant homeodomain
PP2A	protein phosphatase 2A
PRC	Polycomb repressive complex
Prom1	prominin1
Shh	sonic hedgehog
WT	wild-type
3D	three-dimensional

OUTLINE

Colorectal cancer is the second most commonly diagnosed cancer and the second leading cause of cancer-related death in the United States. Among many gene mutations associated with colorectal cancer, mutation of APC (adenomatous polyposis coli) or β-catenin, which activates Wnt signaling, represents the initiation step of colon tumorigenesis. Wnt signaling is known to play multiple roles in early development and formation of human cancers. Chapters 1 and 2 will present an overview of the roles of Wnt signaling in regulating intestinal epithelial homeostasis and in colon cancer, and focus on the known and unknown about the role of Wnt signaling in normal intestine and colon cancer, as well as stem cell regulation involved in these processes. Based on the previous finding about a crosstalk between Wnt and KLF4, this dissertation focuses on KLF4 and its role in manipulating intestinal homeostasis.

KLF4 is the zinc finger transcription factor that crosstalks with Wnt signaling pathway and that plays a fundamental role determining the fate of differentiation, proliferation and cell polarity in the intestine. Chapter 3 will provide a review on KLF4 and its role in stem cells of normal intestine and of colon cancer. Chapter 4 will demonstrate experimental studies delineating the mechanisms of KLF4 regulation in intestinal epithelial cell morphology and polarity.

Bmi1 is a Polycomb group protein important for stem cell self-renewal and proliferation through histone modification, chromatin remodeling and gene silencing. The role of Bmi1 in histone regulation in cancer cell epigenetics will be reviewed in Chapter 5. And in the following Chapter 6, results from colon cancer studies will demonstrate mechanisms on how Bmi1 is manipulated by Wnt and inhibited by KLF4, and how they cooperatively determine cell fate in the process of colorectal tumorigenesis.

CHAPTER 1: WNT SIGNALING IN NORMAL INTESTINE AND COLON CANCER *

WNT SIGNALING PATHWAY

Overexpression of the Wnt-1 protein was found to be due to the Murine Mammary Tumor Virus and to lead to formation of mammary tumors (Nusse and Varmus, 1982). Wnts are secreted glycoproteins. There are nineteen Wnt genes found in human and mouse genomes. The functions of different Wnt proteins may be similar or vary. Some Wnts activate the canonical Wnt pathway, whereas others turn on the non-canonical pathway, including the planar cell polarity (PCP) pathway, and/or the Ca^{2+} pathway (Clevers, 2006; Logan and Nusse, 2004). Wnt signaling is one of the major signaling pathways that controls cell proliferation and differentiation, and it also regulates the homeostasis of many tissues (Czyz and Wobus, 2001; Malhotra and Kincade, 2009; Wodarz and Nusse, 1998). Deregulation of Wnt signaling plays a vital role in tumorigenesis (Giles et al., 2003; Kinzler and Vogelstein, 1996).

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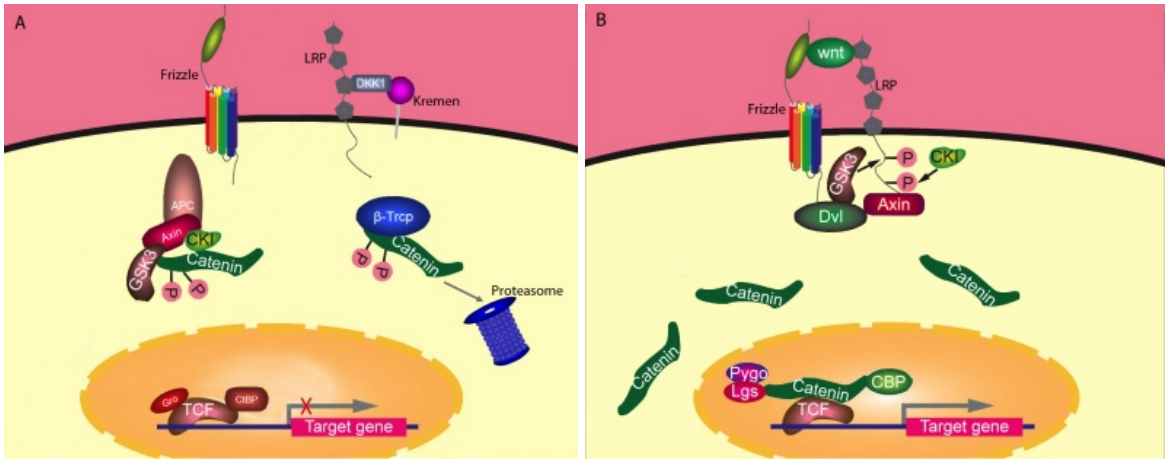

Figure 1: Wnt signaling pathway. (A) In the absence of Wnt, β-catenin is phosphorylated by the Axin destruction complex consisting of Axin, APC, GSK3β and CKIα. Phosphorylated β-catenin is recognized by β-Trcp for ubiquitination and proteasome degradation. In the nucleus, TCF recruits co-repressors such as CtBP and Groucho (Gro) to inhibit transcription. (B) Wnt binds to the receptor Frizzled and the co-receptor LRP5/6. Wnt stimulates phosphorylation of LRP5/6 and recruitment of Axin to LRP5/6, leading to release of β-catenin from the destruction complex. Stablized β-catenin enters the nucleus, binds to TCF, and recruits co-activators and P300/CBP to activate target gene expression (Yu, 2010)(Published and copy right belongs to Nova Science Publishers, Inc., New York, ISBN: 978-1-61761-206-0).

The canonical pathway is also called the Wnt/β-catenin pathway. Canonical Wnt signaling regulates β-catenin, which is a multifunctional protein depending on cellular localization. When on the cell membrane, it binds E-cadherin and regulates cell adhesion, whereas when in the nucleus, it binds TCF/LEF and regulates gene transcription. The level of cytoplasmic β-catenin is dependent on the Axin degradation complex (Fig. 1A). Axin is a scaffold protein; it interacts with APC, GSK-3β, Casein Kinase Iα (CKIα) and β-catenin through different domains. In the absence of Wnt, β-catenin is sequentially phosphorylated by CKIα and GSK-3β within the Axin complex (Amit et al., 2002; Liu et al., 2002). Phosphorylated β-catenin is recognized by β-Trcp, which is a component of E3 ubiquitin ligase complex, for ubiquitination (Hart et al., 1999; Jiang and Struhl, 1998;

Liu et al., 1999; Spencer et al., 1999; Winston et al., 1999). Poly-ubiquitinated β-catenin is then degraded by the proteasome (Fig. 1A). β-catenin phosphorylation is also regulated by Protein phosphatase 2A (PP2A). The specificity of PP2A-mediated β-catenin dephosphorylation is regulated by PR55α, a regulatory subunit of PP2A (Zhang et al., 2009). Whether β-catenin ubiquitination is regulated by a deubiquitinase is currently unknown.

In the presence of Wnt, Wnt binds to the receptor Frizzled (Fz) (Bhanot et al., 1996; Yang-Snyder et al., 1996) as well as the co-receptor Lrp5/6 (low-density-lipoprotein receptor-related proteins 5 and 6) (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000) (Fig. 1B). Fz receptors are proteins that contain seven trans-membrane repeat and an extra-cellular N-terminal cysteine-rich domain (CRD) and an intra-cellular tail domain (Dann et al., 2001; Hsieh et al., 1999). Binding of Wnt ligand to the receptor leads to receptor structural changes and results in recruitment of Disheveled to Fz through its intra-cellular domain (Axelrod et al., 1998; Rothbacher et al., 2000; Umbhauer et al., 2000; Wong et al., 2003). Fz can also activate signaling through heterotrimeric G proteins (Katanaev et al., 2005; Liu et al., 2005). Lrp5/6 are single transmembrane proteins; binding of Wnt triggers phosphorylation of Lrp5/6, providing a docking site for Axin (Mao et al., 2001), releasing β-catenin from the destruction complex (Tamai et al., 2004) (Fig. 1B).

Stabilized β-catenin translocates into the nucleus. In the nucleus, β-catenin interacts with the TCF family of transcription factors including TCF-1, lymphoid enhancer factor-1 (LEF-1), TCF-3, and TCF-4 (Brunner et al., 1997; van de Wetering et al., 1997). In the absence of β-catenin, TCF/LEF binds co-repressors such as CtBP

(Brannon et al., 1999), HDAC1 (Billin et al., 2000; Kioussi et al., 2002), and Groucho/TLE (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998) and repress transcription (Fig. 1A). Upon Wnt signaling activation, β-catenin binds TCF/LEF and recruits its co-activators and subsequently activates downstream genes (Fig. 1B). The binding is through its N- and C-terminal transactivation domains. The C-terminus of β-catenin harbors a transactivation domain (Cox et al., 1999; Hecht et al., 2000; van de Wetering et al., 1997). This transactivation domain recruits p300/CBP to activate Wnt signaling (Hecht et al., 2000; Takemaru and Moon, 2000). p300 and CBP acetylate nearby histones as paralogous transcriptional co-activators so as to loosen chromatin structure and to facilitate recruitment of other transcription factors (Goldman et al., 1997; Ogryzko et al., 1996). The N-terminal transactivation domain of β-catenin directly interacts with BCL9/Legless, and then the co-activator Pygopus is in turn recruited to the complex (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002) (Fig. 1B). Pygopus contains a plant homeodomain (PHD). The PHD domain of ING (inhibitor of growth) can interact with histone H3 that has the tri-methylation mark. This domain is also able to regulate epigenetic target gene modifications (Soliman and Riabowol, 2007).

WNT SIGNALING IN THE INTESTINE

The intestine is a tube-like organ. On top of the luminal surface of the gut, there is a continuous sheet of the epithelial cells (Fig. 2A). Villi are exclusive existing in the intestine. The epithelial sheet of the small intestine folds into finger-like protrusions and extends into the lumen, making them villi structures. At the opposite direction inwardly, the epithelial sheet invaginates to form the crypts of Lieberkühn (Crosnier et al., 2006).

The colonic epithelium has no villi, but consists entirely of crypts. Intestinal stem cells at the bottom of the crypts give rise to transit-amplifying cells that are capable of differentiating into different types of cells that are component of the small intestine, including enterocytes, goblet cells, Paneth cells and enteroendocrine cells (Fig. 2B) (Crosnier et al., 2006; Marshman et al., 2002). As the most abundant cell type, enterocytes play a primary role in absorptive functions of the intestine. Paneth cells are located at the bottom of crypts. They are responsible for lysozyme releasing as well as producing other anti-microbial molecules. Goblet cells are located along the crypt-villus axis and play a major function in secreting substances such as mucins to protect the luminal surface. Enteroendocrine cells are also located throughout the crypt-villus axis. They secrete hormones in the intestine and they are positive for neurotensin (NT). Three types of differentiated cells migrate upward along the crypt-villi axis except Paneth cells. At the top of villi, they are shed into the lumen after 3-5 days (Marshman et al., 2002).

Wnt signaling is the major player in regulating cell fate along the crypt-villus axis (Batlle et al., 2002; Korinek et al., 1998; Reya and Clevers, 2005). Since TCF4-deficient mice lack proliferating cells in the intestinal crypts (Korinek et al., 1998), Wnt signaling is known to be essential for self-renewal of intestine stem cells, and the bottom of the crypt is thought to be a stem cell niche in which Wnt plays a vital role controlling the differentiation, migration and proliferation of the epithelial stem cells (Pardal et al., 2003).

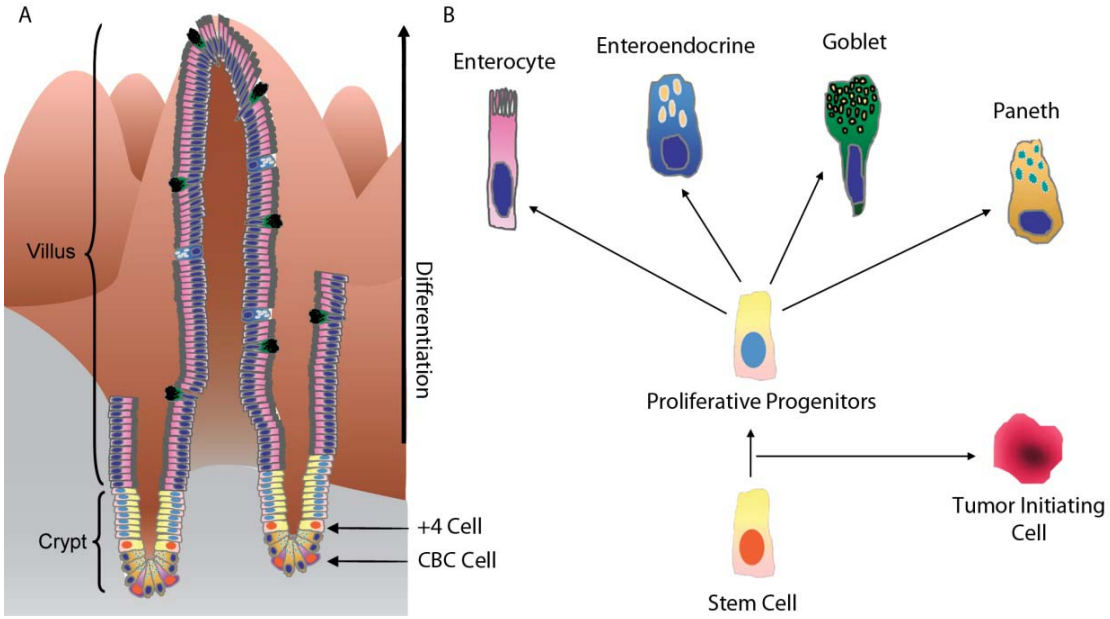

Figure 2: Wnt signaling in the intestine. (A) Structure of the intestine. The epithelium of the small intestine is composed of the crypt and villus. Stem cells (+4 and CBCs) are located at the bottom of crypts. Self-renewal and differentiation of the stem cells are regulated by Wnt signaling. (B) Lineage of intestinal stem cells. Intestinal stem cells give rise to transit-amplifying cells, which are capable of differentiating into different types of cells that are component of the intestine including enterocytes, Paneth cell, goblet cells, and enteroendocrine cell. Tumor initiating cells likely originate from stem cells or transit-amplifying cells containing APC or β-catenin mutations (Yu, 2010) (Published and copy right belongs to Nova Science Publishers, Inc., New York, ISBN: 978-1-61761-206-0).

Overexpression of a Wnt inhibitor – Dkk in the intestine results in loss of crypt and defect in differentiation of secretory cell lineages (Pinto et al., 2003). As a tumor suppressor, APC is sequestering β-catenin and functions in inhibiting Wnt signaling in colorectal cancer. Deletion of APC results in activated Wnt signaling in the mouse intestine and lengthening of the crypts (Sansom et al., 2004). As a well-established target of Wnt, Myc was tested to be crucial in mediating early stages of neoplasia after APC loss in the murine small intestine. MYC deletion in $APC^{-/-}$ mice rescues the consequences

of APC depletion in the intestine by recovering the cell proliferation and migration (Sansom et al., 2007). Myc heterozygosity was also reported to be able to attenuate phenotypes due to APC deficiency(Athineos and Sansom, 2010). In addition, crypt cell proliferation can be enhanced by expression of R-spondin1 as the Wnt agonist (Kim et al., 2005). By inducing Wnt/β-catenin signaling and promoting intestinal stem cell regeneration, R-spondin1 was also found to play a protective role against environmental disturbance on cellular homeostasis of intestinal epithelium (Bhanja et al., 2009). These data further suggest that Wnt signaling plays essential roles in maintaining the stem cell compartment and regulating cell proliferation and differentiation in the intestine.

WNT SIGNALING IN COLON CANCER

The potential importance of Wnt signaling linked to colon cancer was found by the discovery that FAP patients inherit one mutational alle in the *APC* gene (Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991; Powell et al., 1992). In addition, APC mutations have been found in more than 80% of sporadic colon cancer. These APC mutants contain C-terminal truncations. Since the Axin-binding domain and part of the β-catenin-binding domains are missing, these APC mutants are unable to regulate β-catenin phosphorylation, ubiquitination or degradation (Yang et al., 2006). This leads to β-catenin accumulation and constitutively activated Wnt signaling (Korinek et al., 1997; Morin et al., 1997). The $APC^{min/+}$ mouse model provided clear evidence showing the role of mutant APC in initiation of intestinal tumors (Moser et al., 1990; Su et al., 1992). Moreover, conditional APC gene deletion in the adult mouse intestine results in altered proliferation and differentiation patterns, which is a “crypt progenitor-like” phenotype

(Andreu et al., 2005; Sansom et al., 2004), and which eventually leads to the formation of tumors (Shibata et al., 1997).

Mutations in the β-catenin (*CTNNB1*) (Morin et al., 1997; Munemitsu et al., 1996) or Axin2 gene (Liu et al., 2000) are frequently found in sporadic colorectal tumors with wild-type *APC*. Mutations of the β-catenin gene prevent β-catenin phosphorylation by CKIα or GSK-3β, and prevent β-Trcp-mediated ubiquitination/degradation (Liu et al., 2002). These mutations result in chronic activation of Wnt signaling that ultimately lead to colon cancer. As an example, deletion of β-catenin N-terminus in the mouse intestinal epithelium leads to formation of adenomatous polyps during short period of time (Harada et al., 1999). Moreover, the colitis-associated colorectal carcinoma model in mice showed that use of 1,2-dimethylhydrazine and dextran sulfate sodium led to dysplastic lesions and colorectal cancer, which was indicated by enhanced β-catenin expression and nuclear localization (Wang et al., 2004b).

APC and β-catenin play important roles in the initiating step of colonic tumorigenesis (Kinzler and Vogelstein, 1996). In addition, down-regulation of other tumor suppressor genes such as Krüppel like factor 4 (KLF4) may also contribute to the development of colon cancer. KLF4 interacts with β-catenin to repress Wnt signaling and inhibit tumor growth (Zhang et al., 2006). $KLF^{+/-}/APC^{Min/+}$ mice developed, on average, 59% more intestinal adenomas than $Apc^{Min/+}$ mice (Ghaleb et al., 2007). The mechanism of KLF4-mediated inhibition of Wnt/ β-catenin is through competing with p300/CBP for the transactivation domain of β-catenin (Evans et al., 2010). In addition, it is important to further delineate the mechanisms of Wnt signaling crosstalk with other signaling

pathways, such as Notch, PTEN/Akt, BMP and Hedgehog, and to define their roles in colorectal tumorigenesis and other cancer types.

CHAPTER 2: WNT SIGNALING IN NORMAL INTESTINAL STEM CELLS AND COLON CANCER STEM CELLS *

Wnt signaling regulates the self-renewal and differentiation of adult stem cells. Recently, cancer stem cells have emerged as an exciting new concept in cancer research. However, many challenging questions have also arisen. What is the definition of a cancer stem cell? Where do such cells originate and what are the markers for these cells? What is the genetic and epigenetic machinery that regulate the homeostasis of this cell population? What is the mechanism underlying the process of cancer stem cell-involved tumorigenesis? This chapter will discuss the development and trends in the field of cancer stem cell research, with emphasis on colon cancer stem cells. Since Wnt signaling is involved in both intestinal stem cells and colon cancers, the discussion will include the mechanisms of Wnt signal transduction pathway both in normal intestinal stem cells and in colon cancer stem cells, as well as challenges and opportunities in this field of research.

WNT SIGNALING IN INTESTINAL STEM CELLS

Intestinal stem cells are located in the bottom of crypts (Fig. 2A). Hierarchies of stem cell models suggest that there are 4 to 6 stem cells per crypt, which decide the ultimate pattern of the entire crypt (van der Flier and Clevers, 2009). Due to the lack of specific markers, the position of these stem cells located in the crypts is still unclear. Currently, there are two models for intestinal stem cells: the +4 label retaining cells

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(LRC) model and the crypt base columnar cells (CBC) model (Scoville et al., 2008; van der Flier and Clevers, 2009).

Since the relative "quiescent" status is thought to be one of the characters of stem cells (Tumbar et al., 2004), DNA label retention is used as a marker for "stemness" (Kiel et al., 2007). The +4 LRC model is based on the results of DNA label retention experiments. Long-term BrdU and ^{3}H-thymidine label-retaining cells are located at the +4 position, which is just above the crypt base columnar (CBC) cells and Paneth cells (Marshman et al., 2002). These cells have the quiescent or slow cycling character (Marshman et al., 2002; Potten et al., 2002). Additional evidence suggests that these +4 cells indeed have stem cell activity (Bjerknes and Cheng, 1981). The neural RNA-binding protein Musashi-1, which has been proposed as a stem cell marker of human colon epithelium, is expressed in the +4 cells as well as CBC cells (Imai et al., 2001; Nishimura et al., 2003). Several other stem cell markers such as Sox4 (Van der Flier et al., 2007), phosphorylated PTEN, phosphorylated AKT and AKT-phosphorylated β-catenin (S552) (He et al., 2004), are also localized in the +4 cells. S552-phosphorylated β-catenin is associated with its nuclear localization. These observations support the notion that the +4 cells are potential intestinal stem cells. However, the label retention property of the stem cells does not address the functional characteristics of these cells. Thus, functional evidence is lacking to fully support the +4 LRC model. In addition, there are about 16 cells around the +4 position; the exact position of +4 LRCs in the intestine is not well defined (Scoville et al., 2008; van der Flier and Clevers, 2009).

By analyzing Wnt target genes, Lgr5 (or Gpr49) was identified as a novel intestinal stem cell marker. Lgr5 (leucine-rich-repeat-containing G-protein-coupled

receptor 5) encodes an orphan G-protein-coupled receptor with a leucine-rich extracellular domain (Hsu et al., 1998). Lgr5 was identified as a Wnt target gene in a micro-array study (van de Wetering et al., 2002). *In situ* hybridization and Lgr5-EGFP knock-in studies suggest that Lgr5 is expressed the crypt base columnar (CBC) cells which are intermingled with the Paneth cells (Barker et al., 2007). Lineage-tracing experiments with an inducible Cre knock-in allele and the Rosa26-lacZ reporter strain demonstrated that these CBC cells are capable of maintaining epithelial self-renewal over long periods of time and generate all of the major differentiated cell types, thus strongly suggesting that the CBCs are intestinal stem cells and the Lgr5 is a marker for these cells (Barker et al., 2008; Barker et al., 2007). When $Lgr5^{+}$ cells were isolated from the crypt and sorted with the GFP marker, a single $Lgr5^{+}$ cell was shown to generate a crypt-villus structure (Sato et al., 2009). Using the same lineage tracing system, the authors also found $Lgr5^{+ve}$ stem cells to be self-renewing and responsible for the long-term renewal of the gastric units *in vitro* (Barker et al.). These data not only support that Lgr5 is a marker for intestinal stem cells, but also indicate the general mechanism of stem cell regulation in the gastrointestinal epithelia tissues. The current barrier to use Lgr5 as a stem cell marker is the lack of a specific antibody that recognizes the extracellular domain of Lgr5.

Following the discovery of Lgr5, the stem cell transcription profile was analyzed using sorted Lgr5 stem cells. Among the genes tested by *in situ* hybridization, olfactomedin-4 (Olfm4) and achaete scute-like-2 (Ascl2) have been identified as novel stem cell markers that are expressed in Lgr5 stem cells (van der Flier et al., 2009a; van der Flier et al., 2009b). Olfm4 is a secreted protein with unknown function. An Olfm4 family member, ONT1, acts as a BMP antagonist in Xenopus (Inomata et al., 2008). Ascl2 is a basic helix-loop-helix transcription factor. Expression of Ascl2 induces ectopic

crypts on the villi; deletion of Ascl2 gene in the small intestine results in loss of Lgr5 stem cells, suggesting that Ascl2 plays a key role in regulating intestinal stem cells (van der Flier et al., 2009b). As Lgr5, Ascl2 is a Wnt target gene, while Olfm4 is not, implying that these cells are regulated by multiple signaling pathways (van der Flier et al., 2009a; van der Flier et al., 2009b).

Using similar lineage-tracing methods described above (Barker et al., 2008; Barker et al., 2007), Bmi1 was identified as another intestinal stem cell marker (Sangiorgi and Capecchi, 2008). Bmi1 belongs to the Polycomb group (PcG) gene family, which functions in gene silencing through chromatin modifications. Bmi1 was initially identified as an oncogene that regulates cell proliferation and transformation (Haupt et al., 1991; Jacobs et al., 1999b). Later it was found to play an important role in hematopoiesis and development of the nervous systems (van der Lugt et al., 1994). Bmi1 is also crucial for self-renewal of stem cells and cancer initiation (Kang et al., 2007; Lessard and Sauvageau, 2003; Valk-Lingbeek et al., 2004). The role of Bmi1 in controlling cell proliferation and self-renewal might be through its function as a Polycomb group (PcG) protein, which facilitates histone modification and regulates gene silencing (Rajasekhar and Begemann, 2007; Wang et al., 2004a; Wei et al., 2006). Although it was originally reported that Bmi1 is expressed in the +4 cells in the intestine, Van de Flier et al. reported that Bmi1 expression is enriched in Lgr5 cells, suggesting that these two stem cell markers may be expressed in overlapping cells in the intestine (van der Flier et al., 2009a). Again, the exact position of +4 LRCs requires further refinement.

Prominin1 (Prom1 or CD133) is a five-transmembrane-domain-containing glycoprotein. It has been proposed as a marker for cancer stem cells, including colon

cancer stem cells (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). To determine if Prom1 is a marker for normal intestinal stem cells, lineage-tracing experiments were performed by two groups (Snippert et al., 2009; Zhu et al., 2009). Zhu et al. reported that expression of Prom1 overlaps with Lgr5 expression in the small intestine; YFP-labeled Prom1 cells remained present at 60 days and gave rise to four differentiated cell types (Zhu et al., 2009). However, Snippert et al. demonstrated that Prom1 expression is not restricted to the CBCs, but rather is also detected in transit-amplifying progenitor cells (Snippert et al., 2009). Snippert et al. further demonstrated the stem cell tracing should be analyzed quantitatively. Most of the Prom1-YFP tracing is initiated in the transit-amplifying cells, which can only last for a few days, whereas about 10% of tracing is initiated in the stem cells, which can last 60 days (Snippert et al., 2009). In contrast, the frequency of long-term tracing in Lgr5 cells is much higher (Barker et al., 2007). This also raises a concern of the stem cell tracing technique: it can only determine if a gene is expressed in the stem cells but it cannot give a definitive answer as to whether this gene is a stem cell-specific gene.

Recently, doublecortin and Ca^{2+}/calmodulin-dependent kinase-like-1 (DCAMKL-1) was identified as a putative intestinal stem cell marker, which specifically marks the quiescent cells in the lower two-thirds of the intestinal crypt epithelium and occasionally marks CBCs (May et al., 2009). DCAMKL-1 is a microtubule-associated kinase expressed in post-mitotic neurons (Lin et al., 2000). Using Gene Ontology (GO) term enrichment analysis, DCAMKL-1 was found to be expressed in comparison with gastric epithelial progenitor and whole stomach libraries (Giannakis et al., 2006). It was later found to be expressed in gastric stem cells (Giannakis et al., 2008). Most frequently, DCAMKL-1 was found at or near position +4; much less frequently, it was localized in

CBC cells (May et al., 2009). Using conjugated anti-DCAMKL-1 antibody, which labels the extracellular C-terminus of the protein, intestinal epithelial stem cells from mouse were labeled and isolated by FACS. Sorted cells were grown in suspension culture for further analysis. It was demonstrated that the resulting spheroids in suspension culture have proliferation capacity and are positive for stem cell markers and progenitor cell markers (May et al., 2009). It has been proposed that DCAMKL-1 marks the quiescent stem cells and Lgr5 marks the cycling stem cells in the intestine. However, another group demonstrated only 21% of the DCAMKL-1 cells were localized in the crypts, while about 50% were localized on villi and 29% were localized in the crypt-villus junction. This group found that these cells also express Cox-1 and Cox-2, markers for tuft cells. Thus, DCAMKL-1 cells are probably tuft cells rather than stem cells (Gerbe et al., 2009). Tuft cells are also called brush cells; the function of these cells is currently not known. It will be interesting to investigate the lineage of tuft cells in the intestine. Despite the interesting properties of DCAMKL-1 cells in *in vitro* cultures, the role of DCAMKL-1 in stem cells has yet to be determined.

WNT SIGNALING IN COLON CANCER STEM CELLS

Since Wnt signaling plays essential roles in both normal intestinal stem cells and colon cancers, it was hypothesized that colon cancer is initiated from intestinal stem cells or progenitor cells (van de Wetering et al., 2002) (Fig. 2B). Lgr5 is a Wnt target gene as well as a stem cell marker; deletion of APC genes in Lgr5 cells leads to rapid transformation of these cells (Barker et al., 2009). In contrast, deletion of APC in non-stem cells fails to induce transformation (Barker et al., 2009). In these Wnt signaling-induced adenomas, the expression of Lgr5-EGFP was restricted to a small population of

cells, suggesting that stem cells or progenitor cells are maintained in these tumors, thus strongly supporting the cancer stem cell concept (Barker et al., 2009).

Like many other types of stem cells, cancer stem cells are basically defined by the ability to self-renew and the capability of generating differentiated cells. In addition, two other characteristics of cancer stem cells differ from other stem cells: 1) the potential of transformation toward adenomas, i.e., the ability to give rise to heterogeneous progeny of cells, and to maintain diverse and specialized progression (Dalerba et al., 2007a), and 2) the ability to 'escape' from normal cell cycle regulation and enter into the tumorigenic status, which is parallel to the difference in property between embryonic carcinoma and normal embryonic stem cells (Chambers and Smith, 2004). In the small intestine, transformed cells are originally and mainly located at the bottom of the crypt, which is also considered to be the main location of cells that give rise to intestinal cancer (Barker et al., 2009).

The major challenge in the field of colon cancer stem cells is one characterized by the lack of a widely accepted specific cancer stem cell marker. Two groups independently reported the identification of a colorectal cancer stem cell based on the surface marker CD133 (Prom1) (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). Purified $CD133^+$ cells grew exponentially *in vitro* for long periods of time as undifferentiated tumor spheres. Limiting dilution analysis suggested that the tumor initiating cells enriched more than 200-fold in $CD133^+$ cells (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). However, as discussed above, CD133 (Prom1) is not likely a specific marker for normal intestinal stem cells, and whether it is a specific marker for colon cancer stem cell is also questionable. Shmelkov et al. demonstrated that $CD133^-$ and $CD133^+$ cells were equally

capable of tumor initiation, but the metastatic $CD133^-$ cells form more aggressive tumors in the xenograft model (Shmelkov et al., 2008). Clearly, other markers for colon cancer stem cells need to be identified and characterized.

Markers such as epithelial cell adhesion molecule (EpCAM) and CD44 have been used to isolate human colorectal cancer stem cells in a reproducible and consistent way. The isolated population was studied and demonstrated to have a certain gene expression profile, though variable, indicating correlation among expression of other stem cell markers such as CD44, CD133 and CD166 (Dalerba et al., 2007b; Snippert et al., 2009; Takaishi et al., 2009; Zhu et al., 2009). The normal intestinal stem cell markers, such as Lgr5, Bmi1 and DCAMKL-1, are also potential markers for colon cancer stem cells. In addition to its expression in intestinal stem cells and adenomas, Lgr5 is also expressed in the ovarian and hepatocellular carcinomas (McClanahan et al., 2006; Yamamoto et al., 2003). Further analysis of Lgr5 as a marker for cancer stem cells, particularly stem cells from human colon cancer, requires a specific antibody for immunohistochemistry and flow cytometry.

SUMMARY

Constitutive activation of Wnt/β-catenin signaling is a hallmark of colorectal cancer. Moreover, this signal transduction event also governs intestinal stem cell identity and tissue homeostasis. The concept of cancer stem cells implies that stem cells are the culprit of cancer origin and progression. The findings that Lgr5 is a stem cell marker and a Wnt target gene substantiate the importance of Wnt signaling in stem cell biology. Lgr5, together with other intestinal stem cell markers, provides surrogate index to track

the role of the Wnt pathway in intestinal stem cells and in tumorigenesis. Stem cells usually reside in a specialized niche. Communication between the niche and stem cell, such as through Wnt signaling, plays a critical role in stem cell self-renewal and differentiation. It is of great interest to investigate how cancer stem cells abduct the normal stroma for cancer growth. *In vitro* culture of a single Lgr5+ cell is able to recapitulate the basic organization of intestine epithelia. The requirement of Wnt and Notch signaling highlights the importance of these two pathways in regulating intestinal stem cells. This *in vitro* model will greatly facilitate research on intestinal stem cell biology and extend understanding of the role of stem cells in colon cancer initiation and progression.

Because of its roles in colon cancer and stem cells, Wnt signaling becomes a very attractive target for colon cancer prevention and therapeutics. It is expected that inhibiting Wnt signaling would inhibit colon cancer stem cells. From high throughput screenings, several small molecular inhibitors targeting the Wnt pathway have been identified. For example, CPG049090 inhibits the β-catenin/TCF interaction; IGC-001 inhibits the interaction of β-catenin/CBP (Emami et al., 2004; Lepourcelet et al., 2004); XAV939 and IWRs stabilize Axin (Chen et al., 2009; Huang et al., 2009). Wnt signaling is required for both tumorigenesis and stem cell self-renewal; how to inhibit cancer stem cells but not normal intestinal stem cells will be an exciting but challenging question.

CHAPTER 3: KLF4 IN THE INTESTININAL HOMEOSTASIS AND IN STEM CELLS

Krüppel-like factor 4 (KLF4) is a zinc finger protein and transcription factor, which is mainly expressed in the epithelia of the gut and the skin. Normal expression of KLF4 in intestinal epithelial cells that are fully differentiated suggests its importance in the proliferation-to-differentiation switch. KLF4 was found to be down regulated in colorectal cancer, highlighting its role as a tumor suppressor (Zhao et al., 2004). Also, it is one of the major four reprogramming factors that could induce pluripotent stem cells, indicating its function in stem cell self-renewal (Takahashi and Yamanaka, 2006).

KLF4 IN NORMAL INTESTINE AND COLON CANCER

KLF4 belong to the Krüppel-like zinc finger transcription factor family that plays important roles in many biological processes, and which is involved in regulating proliferation, differentiation and development. Members from this family contain three zinc fingers at the C terminus, which can bind DNA. They are named "Krüppel-like" because they are homologous to the Drosophila gene Krüppel.

KLF4 is expressed in gut and skin epithelial cells (Garrett-Sinha et al., 1996; Shie et al., 2000; Shields et al., 1996; Ton-That et al., 1997). The pattern of KLF4 expression in the intestine, which is gradually decreased from the luminal surface toward the base of the crypts, is consistent with its role in promoting differentiation and inhibiting proliferation (Shields et al., 1996; Zhang et al., 2006).

KLF4 knockout mice had a dramatic decrease in goblet cell numbers in the colon, indicating that KLF4 is required for lineage differentiation of goblet cells in early

development of the embryo intestine (Katz et al., 2002). However, further delineation of function of KLF4 is hampered due to early lethality of homozygous KLF4-/- mice, which die within 15 hours after birth. Questions remained as how KLF4 functions in adult mice. Recent study further defined the role of KLF4 in maintaining intestinal homeostasis, showing that KLF4 deletion from the intestine results in changes in maturation and differentiation of goblet cells, and that KLF4 deletion led to decrease in number of goblet cells in the adult intestine (Ghaleb et al., 2011).

KLF4 plays important roles in the proliferation-to-differentiation switch in the intestine, and it regulates fate of more than one cell types. The roles of KLF4 studied in the inducible mouse model studies will be discussed in the next chapter, which further delineate multifunction of KLF4 in regulating different cell types and maintaining the homeostasis in the intestine and colon cancer.

KLF4 is down regulated in colon cancer (Zhao et al., 2004), which is consistent with its role as a tumor suppressor. Moreover, KLF4 is down regulated in adenomas from $APC^{min/+}$ mice, which has been used as a typical model for tumorigenesis study in the intestine (Ton-That et al., 1997). $APC^{+/min}KLF4^{+/-}$ double heterozygotes have 59% more tumors than $APC^{+/min}$ mice alone (Ghaleb et al., 2007).

Several mutations were found in KLF4 (Zhao et al., 2004), indicating that the role of KLF4 as a tumor suppressor also applies in human colon cancer cell lines. Previous study also demonstrated the function of KLF4 in inhibiting tumor growth in xenograft mouse studies. Two colon cancer cell lines LS174T and DLD1 were used, both of which express doxycycline-inducible KLF4. Monitored tumor size over time of cell injection demonstrated that induced KLF4 inhibited tumor growth compared with non-induced mice (Zhang et al., 2006).

KLF4 IN STEM CELLS

KLF4 is one of the four major reprogramming factors that can induce mouse pluripotent stem cells (Takahashi and Yamanaka, 2006). Similar strategy was applied and succeeded in generating human pluripotent stem cells (Takahashi et al., 2007). From the 24 potential factors, authors selected out four factors including Oct4, Sox2, c-Myc and KLF4, and simultaneously induced the four proteins in fibroblast cells, which were induced to reprogram into pluripotent cells (iPS cells).

Further mechanism studies also indicated that KLF4 prevents differentiation of embryonic stem cells by directly interacting with Nanog promoter and regulating Nanog gene expression. Also, Oct4 and Sox2 were found to be directly interacting with KLF4 to promote cell reprogramming (Wei et al., 2009; Zhang et al., 2010). The mechanisms of formation of Oct4/Sox2/KLF4 complex in regulating self-renewal were illustrated in the studies of iPS cells. Although KLF4 seemed to be dispensable for human iPS cell generation, it might be important for facilitating reprogramming process (Jaenisch and Young, 2008; Yu et al., 2007).

As a multifunctional transcription factor, KLF4 may regulate many stem cell factors other than the four main regulators in the processes of reprogramming. The role of KLF4 in regulating stem cell marker Lgr5 and Bmi1 in the intestine will be discussed the chapter after next, which further implicates the importance of KLF4 in homeostasis and cell fate.

MECHANISMS OF KLF4 REGULATION AND WNT-KLF4 CROSSTALK

β-catenin regulates normal intestinal cell proliferation and inhibits differentiation in the crypt. The model was established about crosstalk between Wnt signaling and KLF4, which is predominantly expressed in differentiated epithelial cells (Zhang et al.,

2006). KLF4 was detected among those proteins that interact with β-catenin using affinity purification screening strategy. The observation that KLF4 interacts and inhibits Wnt/β-catenin signaling led to further finding that KLF4 interacts with β-catenin by binding to β-catenin transactivating domain. KLF4 was found able to inhibit proliferation and induce differentiation of intestinal cells. Xenograft mouse studies, using two stable colon cancer cell lines that express doxycycline-inducible KLF4, further demonstrated the function of KLF4 inhibiting tumor growth *in vivo* (Zhang et al., 2006).

As a zinc finger protein and transcription factor, KLF4 is acetylated by p300/CBP, which is critical for its role in transactivation of genes. Furthermore, it was found that KLF4 modulates acetylation of histone protein H4 at different target gene promoters, and its role as an activator or as a repressor depends on its interaction with co-activators or co-repressors. CBP and p300 can acetylate KLF4 and activates its transactivation on target genes, while HDAC3, as a co-repressor, can be recruited by KLF4 to repress target gene transcription (Evans et al., 2007).

In further investigation of mechanisms of KLF4 regulation on Wnt/β-catenin signaling, KLF4 was found to directly interact with the C terminus of β-catenin, which is critical for KLF4 inhibition on TOPFlash reporter. KLF4 inhibits β-catenin-mediated recruitment of CBP, thus inhibits acetylation of β-catenin as well as histone acetylation by CBP on Wnt target genes (Evans et al., 2010).

SUMMARY

In summary, the role of KLF4 in regulating cell proliferation and differentiation as well as stem cell fate might be through multiple pathways. First, it directly interacts with β-catenin and inhibits its recruitment of CBP, thus inhibits Wnt target gene activities

(Fig. 3A). Second, it recruits co-activators such as CBP and P300 to transactivate target gene activities. Finally, it interacts with co-repressors to inhibit target gene transcription (Fig. 3B).

As reviewed in Chapters 1 and 2, Wnt signaling plays important roles in intestine development and colon cancer. Chapter 3 reviewed the mechanisms of KLF4 regulation and its crosstalk with Wnt, along with its inhibitory role in colon cancer cell proliferation as well as its facilitating role in differentiation. However, little is known how KLF4 determines stem cell fate and regulates cell polarity. There remained a gap between the function of KLF4 in maintaining intestinal homeostasis and molecular mechanisms of Wnt-KLF4 regulation. The main hypothesis of this dissertation is that KLF4 regulates intestinal epithelial differentiation, proliferation and polarity; KLF4 directly binds and inhibits Bmi1 promoter and restrains its function in cell proliferation. To test the hypothesis of KLF4 regulating intestinal epithelial differentiation, proliferation and polarity, a group of studies will be demonstrated in Chapter 4 to test the effect of KLF4 depletion on morphological changes in the conditional knockout mouse intestine. Chapter 5 will review findings about Bmi1 and its correlation with stem cells and cancer. To test the hypothesis of KLF4 inhibition on Bmi1 and its activities, an array of analyses will be shown in Chapter 6, which studies the cooperative effect of Wnt and KLF4 on Bmi1 in colon cancer.

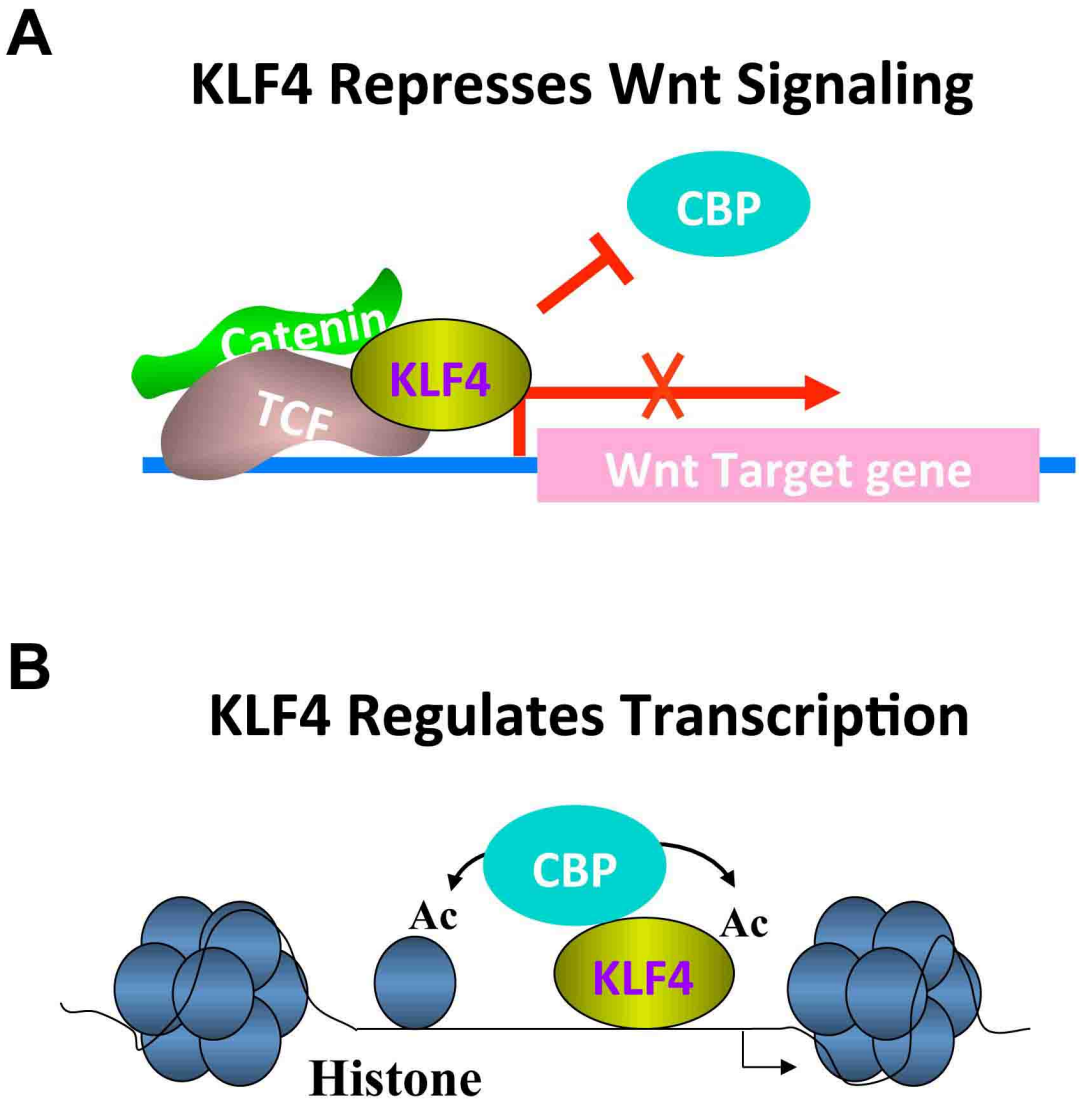

Figure 3: Mechanisms of KLF4 regulation. (A) KLF4 directly interacts with β-catenin and inhibits recruitment of CBP; Wnt target genes are turned off. (B) KLF4 interacts with co-activators or co-repressors to regulate transcript of target genes.

CHAPTER 4: KLF4 REGULATES INTESTINAL EPITHELIAL CELL MORPHOLOGY AND POLARITY *

Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor that plays a vital role in regulating cell lineage differentiation during development and maintaining epithelial homeostasis in the intestine. In normal intestine, KLF4 is predominantly expressed in the differentiated epithelial cells. It has been identified as a tumor suppressor in colorectal cancer. KLF4 knockout mice demonstrated a decrease in number of goblet cells in the colon, and conditional ablation of KLF4 from the intestinal epithelium led to altered epithelial homeostasis. However, the role of KLF4 in differentiated intestinal cells and colon cancer cells, as well as the mechanism by which it regulates homeostasis and represses tumorigenesis in the intestine is not well understood. In this study, KLF4 was partially depleted in the differentiated intestinal epithelial cells by a tamoxifen-inducible Cre recombinase. I found a significant increase in the number of goblet cells in the KLF4-deleted small intestine, suggesting that KLF4 is not only required for goblet cell differentiation, but also required for maintaining goblet cell numbers through its function in inhibiting cell proliferation. The number and position of Paneth cells also changed. This is consistent with the KLF4 knockout study using villin-Cre (Ghaleb et al., 2010). Through immunohistochemistry (IHC) staining and statistical analysis, I found that a stem cell and/or tuft cell marker, DCAMKL1, and a proliferation marker, Ki67, are affected by KLF4 depletion, while an enteroendocrine cell marker, neurotensin (NT), was not affected. In addition, KLF4 depletion altered the morphology and polarity of the intestinal epithelial cells. Using a three-dimensional (3D) intestinal epithelial cyst formation assay, I found that KLF4 is essential for cell polarity and crypt-cyst formation

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in human colon cancer cells. These findings suggest that, as a tumor suppressor in colorectal cancer, KLF4 affects intestinal epithelial cell morphology by regulating proliferation, differentiation and polarity of the cells.

INTRODUCTION

Colorectal cancer is the second most commonly diagnosed cancer among men and women and the second leading cause of cancer deaths in the United States (Jemal et al., 2009; Jemal et al., 2006). Different genetic variations could lead to abnormal epithelial development and polyp formation, which could be further induced to progression of colorectal carcinomas (Kinzler and Vogelstein, 1996). Wnt signaling plays an important role in early stages of colorectal carcinogenesis; abnormality in the gene APC or β-catenin leads to aberrant crypt formation (Giles et al., 2003; Polakis, 2000). Mutations in other oncogenes and tumor suppressor genes, such as K-ras and p53, also contribute to colorectal carcinogenesis(Kinzler and Vogelstein, 1996).

KLF4 is a zinc finger transcription factor initially found to be enriched in the epithelium of intestine and skin (Garrett-Sinha et al., 1996; Shields et al., 1996). Later, it was found in a variety of other tissues, such as thymus, cornea, cardiac myocytes and lymphocytes (Chiambaretta et al., 2004; Cullingford et al., 2008; Fruman et al., 2002; Panigada et al., 1999). KLF4 plays an important role in development and cell differentiation (Garrett-Sinha et al., 1996; Katz et al., 2002; Ton-That et al., 1997). In normal intestine, KLF4 is predominantly expressed in differentiated epithelial cells near the luminal surface and goblet cells in the crypts (Evans et al., 2010; Ghaleb et al., 2011). KLF4 is down-regulated in colorectal cancers and has been identified as a tumor

suppressor (Ghaleb et al., 2007; Zhang et al., 2006; Zhao et al., 2004). As one of the four factors that induce pluripotent stem cells, KLF4 plays a role in cell fate reprogramming and self-renewal of embryonic stem (ES) cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). The roles of KLF4 in differentiated intestinal cells are not well understood.

Mice homozygous for a null mutation in KLF4 had defects in terminal differentiation of goblet cells, while further study of KLF4 in mouse intestine was hampered due to early lethality of mutant mice (Katz et al., 2002). Using Villin-Cre recombinase system, another study found that conditional ablation of KLF4 from the intestinal epithelium led to failure of goblet cell differentiation (Ghaleb et al., 2011), which also highlights the role of KLF4 in maintaining intestinal epithelial morphology and homeostasis. Interestingly, depletion of KLF4 from two-week-old mice using vil-CreER, an inducible Cre recombinase, had no effect on goblet cell differentiation (Pellegrinet et al., 2011). The discrepancy may be due to differential expression of the villin gene in early and later stages of gut development (Maunoury et al., 1992). In this study, I analyzed the role of KLF4 in the adult intestine using an inducible Cre recombinase, which is driven by native promoter of KLF4.

MATERIALS AND METHODS

Transgenic mice and animal work

Mouse experiments were performed under the approval by the Institutional Biosafety Committee (IBC) and by the Institutional Animal Care and Use Committees (IACUC) of University of South Carolina (Proposal number 1573).

Transgenic mice were generated using a Cre recombinase derived from a bacterial artificial chromosome (BAC, RP23-322L22) containing mouse KLF4 gene (Lee et al., 2001). A Cre recombinase cDNA was fused with estrogen receptor gene and was inserted into KLF4 locus at the initiating codon, and the CreER gene transcription is under the control of KLF4 promoter. KLF4 knockout in KLF4/CreER (+/-)/KLF4 (flox/flox) double transgenic mice was induced by 100mg/kg tamoxifen intraperitoneally (i.p.) for 5 consecutive days at 4 weeks old. Expression of KLF4 as well as multiple genes in wild-type ($Klf4^{+/+}$) and knockout ($Klf4^{-/-}$) mice was analyzed 3, 5 or 30 days after induction by immunohistochemistry (IHC) staining of fixed intestine tissues.

Cell culture and 3D formation assay

Caco-2 human colonic epithelial cell line (Wang et al., 2002b) was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For 3D culture, approximately $1.5x10^5$ cells were embedded into 250µl of 80-90% matrigel. The 3D matrix was allowed to harden in a 24-well plate at 37 °C for 30 minutes, then 500µM of DMEM medium with 2% fetal bovine serum was added and cysts were allowed to form over 5-7 days at 37 °C.

LS174T colon cancer cell line (Zhang et al., 2006) was grown in RPMI medium (Mediatech) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Stable cell line LS174T-tet/on-KLF4 has been described previously (Zhang et al., 2006). LS174T-tet/on-KLF4 cells were plated at approximately $2x10^5$ cells per well in a 6-well plate. The following day, doxycycline (1 µg/ml) was added to the culture medium. After 24 h of incubation, cells were trypsinized and counted, then followed by 3D formation assay as indicated with Caco-2 cells.

Western Blotting

Cells were lysed in the appropriate volume of lysis buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, with protease inhibitors). The following antibodies were used: mouse anti-β-actin (Sigma, A1978), mouse anti-Flag (Sigma, F1804).

RT-PCR

LS174T-tet/on-KLF4 cells were plated at approximately $2x10^5$ cells per well in a 6-well plate. The following day, doxycycline (1 μg/ml) was added to the culture medium. After 48 h of incubation, RNA was isolated using the RNeasy kit (Qiagen). Reverse transcriptase PCR (RT-PCR) was performed as described previously (Zhang et al., 2006). The following primers were used: β-actin, 5′-CAACCGCGAGAAGATGAC-3′ and 5′-AGGAAGGCTGGAAGAGTG-3′; *IAP*, 5′-CCATTGCCGTACAGGATGGAC-3′and 5′-CGCGGCTTCTACCTCTTTGTG-3′; *p21*$^{Cip1/WAF1}$: 5′-CGACTGTGATGCGCTAATGG-3′ and 5′-AGAAGATCAGCCGGCGTTTG-3′; *LGR5*: 5′-CCTGCTTGACTTTGAGGAAGAC-3′ and 5′-ATGTTCACTGCTGCGATGAC-3′; *CD44*: 5′-CAGAATGGCTGATCATCTTG-3′ and 5′-CAAATGCACCATTTCCTGAG-3′; *LKB1*: 5′-GAGGAGGTTACGGCACAAAA-3′ and 5′-CTGTCCAGCATTTCCTGCAT-3′; *MARK2*: 5′-GCCAGAATCAAAAGCAAC and 5′-ATGATGTTTAGTGGGAGG-3′; *BMI1*: 5′-AGCAGAAATGCATCGAACAA-3′ and 5′-CCTAACCAGATGAAGTTGCTG-3′; *EPHB2*: 5′-AAAATTGAGCAGGTGATCGG-3′ and 5′-TCACAGGTGTGCTCTTGGTC-3′; *EPHB3*: 5′-AGCAACCTGGTCTGCAAAGT-3′ and 5'-TCCATAGCTCATGACCTCCC-3′.

Interference RNA, H&E staining, immunohistochemistry, PAS and AB staining

Interference RNA and immunohistochemistry were tested as described previously (Zhang et al., 2006). Lentiviral stocks were prepared using control shRNA or human KLF4 shRNA on pGIPz vector containing a marker of turbo GFP (Open Biosystems). H&E staining was performed based on standard protocol by Histology Laboratory of the Imaging Facility at University of Kentucky.

For immunohistochemistry staining, the following antibodies were used: KLF4 (Zhang et al., 2006), rabbit anti-human Lysozyme (Diagnostic BioSystems, RP 028-05), rabbit anti-DCAMKL-1 (Abgent, AP7219b), rabbit anti-Ki67 (Novus Biologicals, NB110-89717).

PAS staining was performed based on standard protocol using reagents of PAS Staining System from Sigma (395-B). Alcian Blue (AB) staining was performed according to standard protocol using Alcian Blue 8GX and Fast Red from Sigma (kindly provided by Dr. Tianyan Gao).

Immunofluorescent staining

Cells grown on cover glass were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, washed 3 times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then blocked in 5% goat serum in PBS at room temperature for 1 h. Cells were incubated with primary antibodies at room temperature for 2 hours. Antibodies used include rat anti-human CD49f (α6-integrin, BD Pharmingen, 555734) and rabbit anti-ZO-1 (Invitrogen, 61-7300). Then cells were washed 3 times with PBS and further incubated with Alexa-488-labeled anti-rat IgG (1:500) and Alexa-568-labeled anti-Rabbit IgG (1:500) diluted in PBS for 40 min. Nuclei were stained by DAPI

(Sigma). The cover glasses were washed, mounted on glass slides, viewed and photographed with an Olympus FW1000 confocal microscope.

RESULTS

KLF4 loss leads to change in number of goblet cells and morphology of the small intestinal epithelium

In order to test the function of KLF4 in adult intestinal epithelium cells, inducible KLF4 knockout (*Klf4*$^{-/-}$) mice were generated, which are KLF4/CreER (+/-) and KLF4(flox/flox) double transgenic. The Cre recombinase cDNA fused with tamoxifen-inducible estrogen receptor gene was inserted into BAC clone at the initiating methionine of KLF4 gene. Thus, the expression of Cre recombinase is driven by the KLF4 promoter in transgenic mice. Induction of KLF4/CreER (+/-) and KLF4(flox/flox) double transgene with tamoxifen led to activation of Cre recombinase. The KLF4 function in the skin was studied using this mouse model. KLF4 depletion resulted in a significant increase of hair follicle density, as well as changes of suprabasal cells from a single layer into multiple layers, which is indicating an inhibitory role of KLF4 in proliferation of mouse skin keratinocytes (Juan Li, 2011). In the small intestine, the Cre recombinase was predominantly expressed in the top of the villus, and which is recapitulating expression pattern of endogenous KLF4 (Fig. 4A). Tamoxifen-mediated Cre recombinase activation resulted in partial depletion of KLF4 when compared with non-induced transgenic mice (Fig. 4A). Haematoxylin and eosin (H&E) staining results indicated an increase in the number of secretory cells in *Klf4*$^{-/-}$ intestine; the position of these cells appeared to be dislocated compared with control intestine (Fig. 4B).

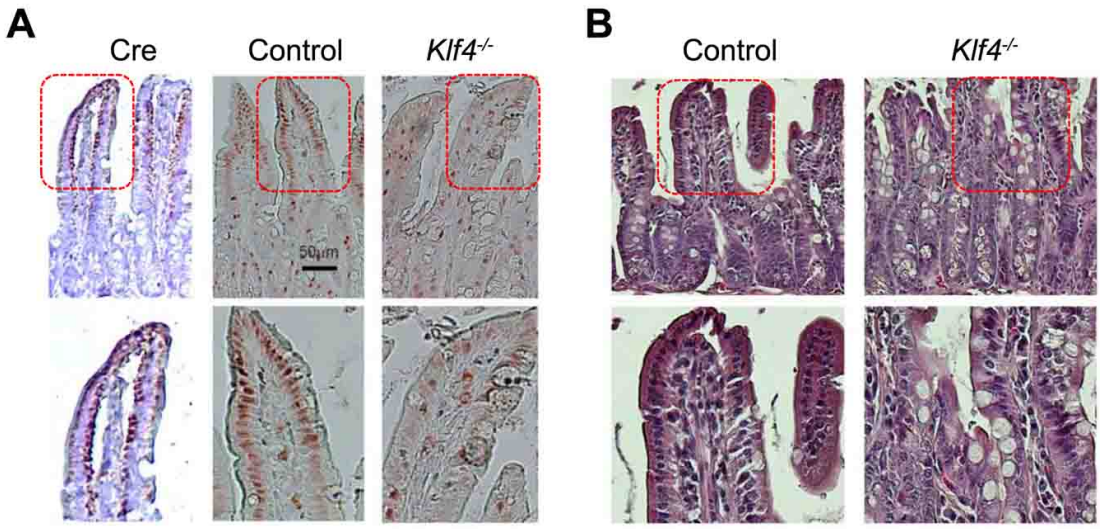

Figure 4: KLF4 loss leads to morphology change in the small intestinal epithelium. (A) Left: IHC staining for Cre recombinase in *Klf4*$^{-/-}$ small intestine. Right: IHC staining for KLF4 in control and *Klf4*$^{-/-}$ small intestine tissues. (B) H&E staining of control and *Klf4*$^{-/-}$ small intestine tissues.

To analyze the effects of KLF4 depletion on goblet cells, which are one of the secretory cell lineages in the small intestine, tissue sections were stained with both Periodic acid-Schiff (PAS) and Alcian Blue (AB), respectively (Fig. 5A left panel). An enlargement in size and an increase in the numbers of PAS and AB positive cells indicated an increase in goblet cell proliferation in small intestine of *Klf4*$^{-/-}$ mice (Fig. 5A right panel), which highlights the role of KLF4 in maintaining numbers of goblet cells in mature small intestine. Time point-specific changes in number of PAS positive cells due to tamoxifen treatment further indicated that KLF4 is critical for goblet cell number maintenance (Fig. 6). It is worth noticing that this result is distinct from the finding that KLF4 knockout leads to loss of Goblet cells in the colon (Katz et al., 2002), and that conditional ablation of KLF4 also leads to loss of goblet cells in the intestinal epithelium (Ghaleb et al., 2011). The difference is due to the stage of KLF4 knockout before or after

goblet cell differentiation. KLF4 depletion had no effect on neuroendocrine cells, as indicated by immunofluorescent staining for neurotensin (NT) (Fig. 5B), suggesting that function of KLF4 in small intestine is cell type-specific.

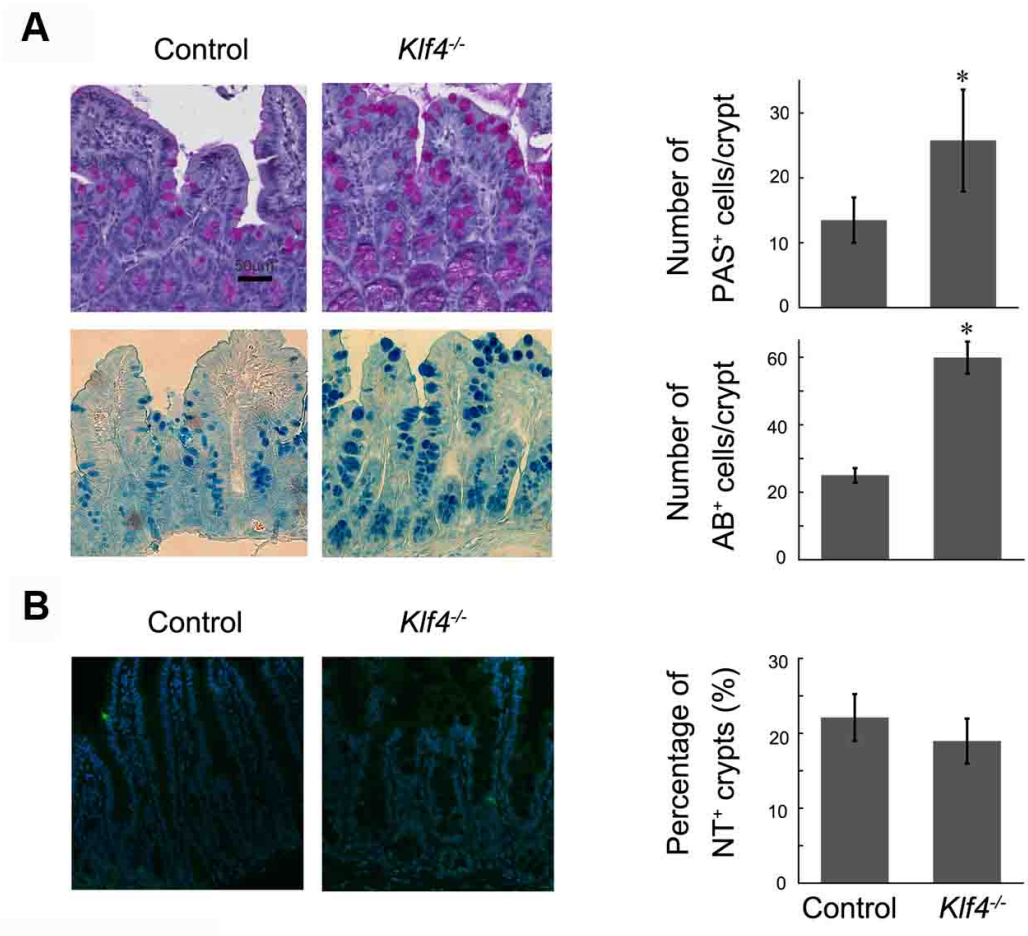

Figure 5: KLF4 loss leads to change in number of goblet cells and morphology of the small intestinal epithelium. (A) Small intestine treated with tamoxifen for 5 days were stained for Periodic acid-Schiff (PAS, top) and Alcian Blue (AB, bottom). (*, $P<0.05$) (B) Tissue slides from small intestine of control and *Klf4*$^{-/-}$ mice were stained for neurotensin (NT) antibody and detected by immunofluorescent antibody.

KLF4 ablation leads to abnormal proliferation and differentiation in small intestinal epithelium

In order to further examine the role of KLF4 in intestinal epithelial cells, the morphology change was analyzed in *Klf4*$^{-/-}$ mice compared with non-induced (Day 0) mice by H&E staining (Fig. 6): The average length of the crypt-villus axis was increased in *Klf4*$^{-/-}$ mouse intestine (Fig. 6, 7A). The number of secretory-like cells is increased; these cells either have larger volume of vacuoles or contain secreted granules like Paneth cells (Fig. 6). A large number of cell nuclei lost apical-basolateral polarity, which is typical of the wild-type enterocytes. Instead of a monolayer of well-oriented epithelial cells, *Klf4*$^{-/-}$ intestine had multiple layers of disorganized cells (Fig. 6, 7B). Positions of the secretory cells were changed; instead of sitting at the bottom of the crypt, the granule-containing cells dislocated upward in the crypts (Fig. 6, 7B). In order to confirm that the morphology change was not due to tamoxifen treatment, small intestine from wild-type (WT) mice treated with tamoxifen was stained as a control; they showed a normal morphology as non-treated transgenic mice.

The cell proliferation marker Ki67 was analyzed by IHC. The average length of Ki67^{+} region along the crypt-villus axis, as well as numbers of Ki67^{+} cells increased (Fig. 6, 7A-B), i.e., proliferation compartment of the intestine was expanded, indicating an increase in proliferation capacity in *Klf4*$^{-/-}$ mouse intestine. PCNA is another proliferation marker; and its change in response to KLF4 loss is consistent with the results from Ki67 staining (Fig. 6). This further highlights the role of KLF4 in inhibiting intestine proliferation.

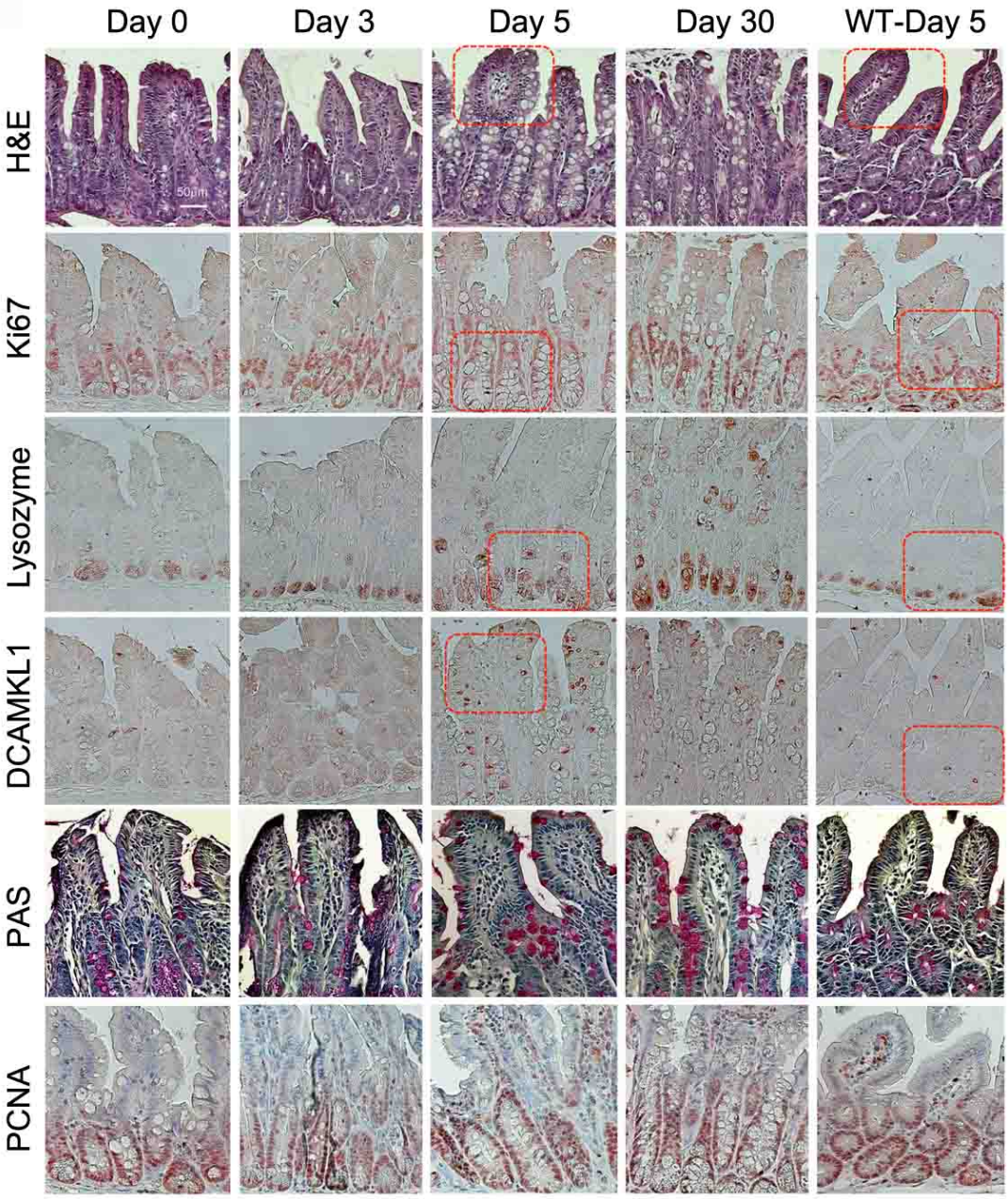

Figure 6: KLF4 ablation leads to changes in proliferation and differentiation pattern in small intestinal epithelium. Small intestine from *Klf4*$^{-/-}$ mice induced by tamoxifen for different time endurances were stained by H&E and PAS, and also immunohistochemistry staining was performed with anti-Ki67, anti-Lysozyme, anti-DCAMKL-1, and anti-PCNA antibodies respectively.

In addition to goblet cell staining, the role of KLF4 in intestinal cell proliferation was confirmed by staining for other cell types including Paneth cells and tuft cells. Tissue slides from both normal and *Klf4*$^{-/-}$ intestine were stained for lysozyme, which is a marker for Paneth cells (Fig. 6, 7B). A larger proportion of cells stained positive for lysozyme in small intestine from *Klf4*$^{-/-}$ mice compared with control mice, and these cells were dislocated through the crypt-villus axis, indicating that KLF4 loss also led to an increase in Paneth cell population and has an effect on position of these cells. This result re-emphasizes the role of KLF4 in controlling Paneth cells and strongly supports the finding from the KLF4 knockout study using villin Cre (Ghaleb et al., 2011).

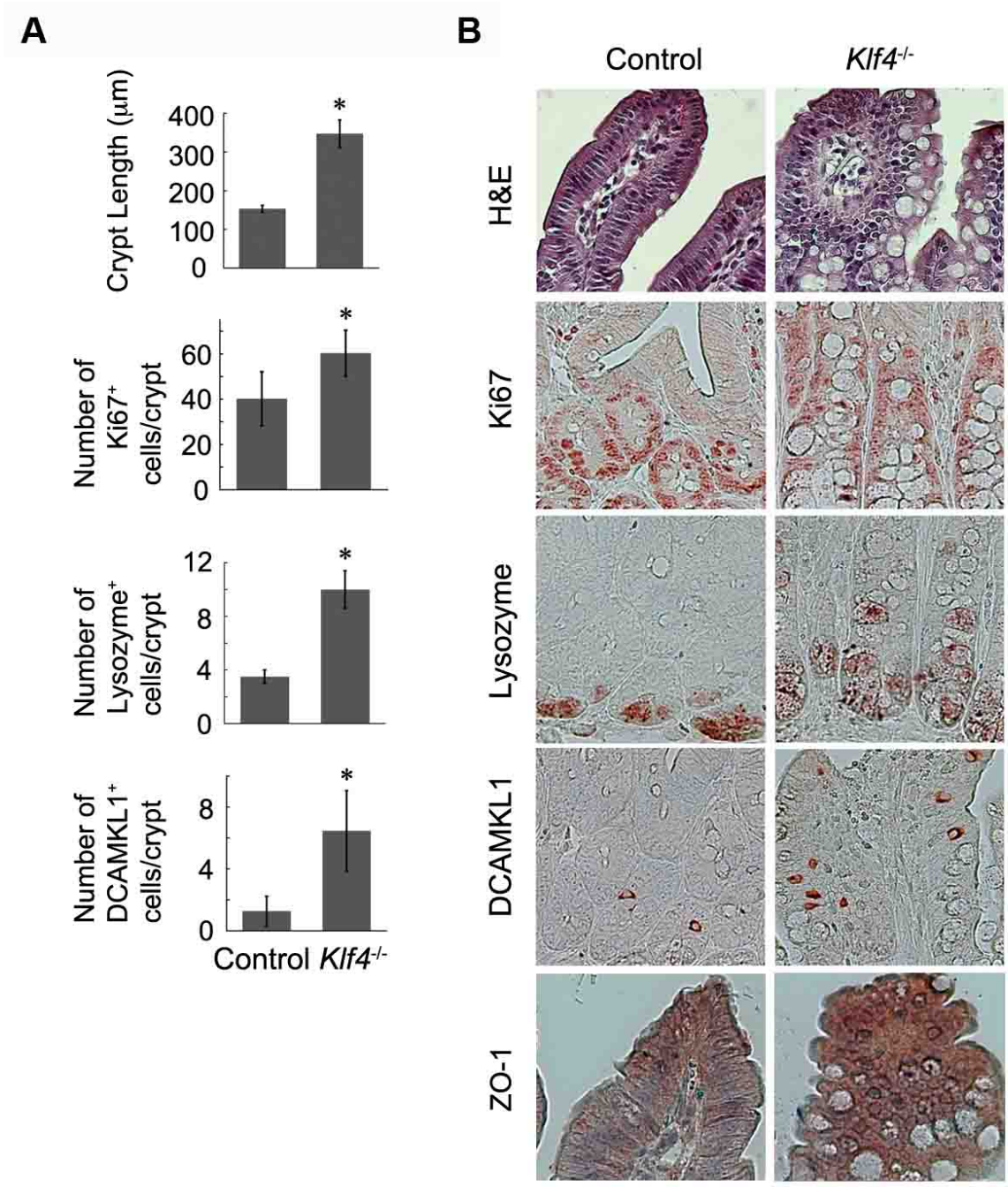

Figure 7: KLF4 ablation leads to abnormal proliferation and differentiation in small intestinal epithelium. (A) Statistic analysis of IHC staining results from Fig. 6. (*, $P<0.05$) (B) IHC staining from Fig. 6 in higher magnification of highlighted frames. Bottom panel: IHC staining with ZO-1 antibody in one-month knockout intestine tissue.

Based on the current model, small intestine is composed of the Paneth cell region (bottom of crypt), the stem cell zone (through +4 location), an amplification compartment (up to top of crypt) and a differentiation compartment (including crypt-villus junction) (Chen et al., 2008; Pinto and Clevers, 2005). To further analyze the effect of KLF4 on intestinal homeostasis, tissue sections from *Klf4*$^{-/-}$ mouse intestine were stained for stem cell and/or tuft cell marker DCAMKL-1 (Gerbe et al., 2009) (Fig. 6, 7). Surprisingly, DCAMKL-1 positive cells were increased in the *Klf4*$^{-/-}$ mouse small intestine along the villus (Fig. 7A), but were not restricted to the crypt base (Fig. 7B), indicating an increase in number of tuft cells due to loss of KLF4.

Based on the observation of changes in cell position as well as epithelial apical-basolateral morphology, I hypothesized that KLF4 is not only responsible for controlling cell differentiation and proliferation, but also cell polarity. As indicated by H&E staining, a great part of the cell nuclei lost polarity in *Klf4*$^{-/-}$ mouse small intestine. Paneth cells change their position due to depletion of KLF4. Meanwhile, most of the tuft cells that were positive for DCAMKL-1 also were out of direction in knockout mice (Fig. 6, 7B). In order to investigate the role of KLF4 in regulating cell polarity, I generated a three-dimensional (3D) epithelial cyst formation assay for Caco-2 cells, in which the morphological structure of cyst and apical-basolateral cell polarity can be examined *in vitro*.

KLF4 is essential for cell polarity and crypt-cyst formation in 3D culture of Caco-2 cells

Normally, Caco-2 cells with high polarity form lumen-containing cysts in matrigel-based 3D culture and show apical-basolateral polarity as indicated by ZO-1 as

an apical marker and α6-integrin as a basolateral marker, respectively (Fig. 8, bottom; Fig. 9, top); low- or no-polarity Caco-2 cells only form cysts without lumen (Fig. 9, bottom). Staining of α6-integrin and ZO-1 for Caco-2 cells in 2D culture is shown as control, indicating non-polarization of Caco-2 cells in 2D growth conditions (Fig. 8, top).

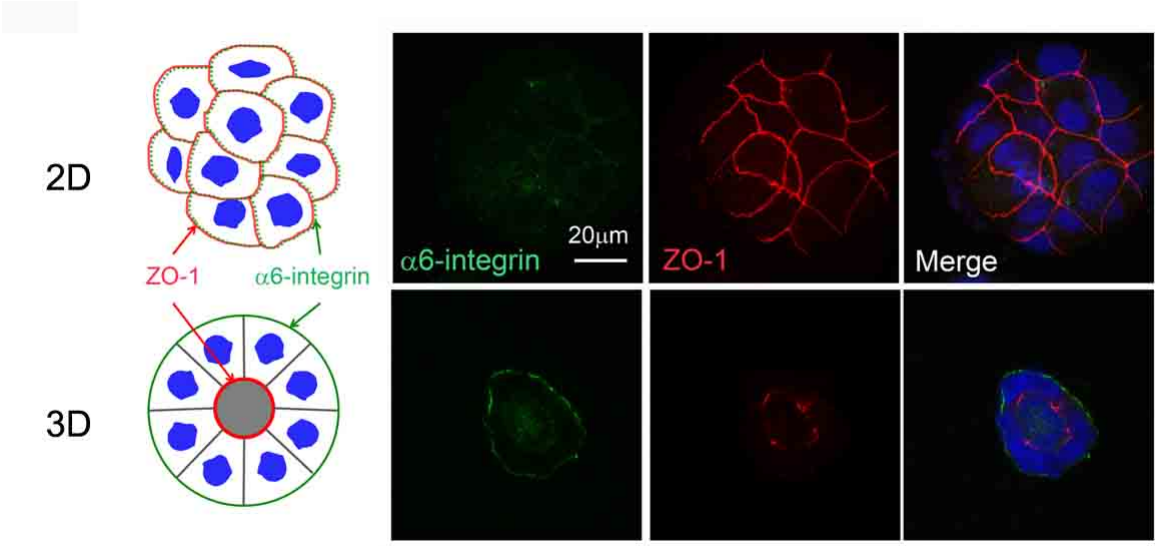

Figure 8: Immunofluorescent staining of Caco-2 cells in 2D and 3D culture with anti-α6-integrin and ZO-1 antibodies.

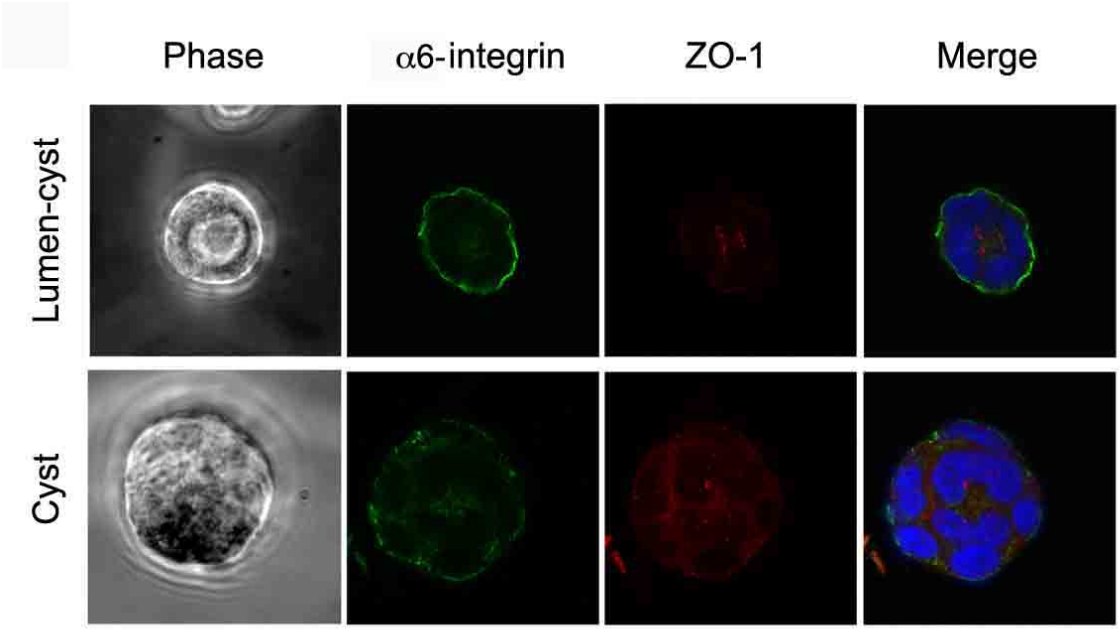

Figure 9: Caco-2 cells in 3D culture were stained for differentiation markers indicating cell polarity and cyst formation (definition of lumen-cyst versus cyst structures).

To determine the role of KLF4 in lumen-cyst formation, KLF4 was depleted in Caco-2 cells by siRNA and shRNA delivery approaches, respectively (Fig. 10A and 10B, left panels), followed by 3D formation assay. The number of lumen-cysts and total number of cysts were counted and the percentage of lumen-cyst was calculated to indicate the measure of cell polarity. I found that the efficiency of lumen-cyst formation was significantly reduced by siRNA and shRNA (Fig. 10A and 10B, right panels), suggesting that KLF4 is essential for cell polarity formation in the 3D culture of Caco-2 cells. To examine the role of KLF4 in apical-basolateral polarity in the intestine, I stained the knockout intestine tissues with ZO-1 antibody and found that KLF4 does regulate ZO-1 expression and distribution in the intestinal epithelial cells: instead of being highly expressed in the outer layer of epithelial cells surrounding the villus, KLF4 knockout

intestine had overexpressed ZO-1 in multiple layers of villus (Fig. 7B). This confirmed that KLF4 does not only regulate polarity formation of Caco-2 cells, it also regulates apical-basolateral polarity in intestinal epithelial cells.

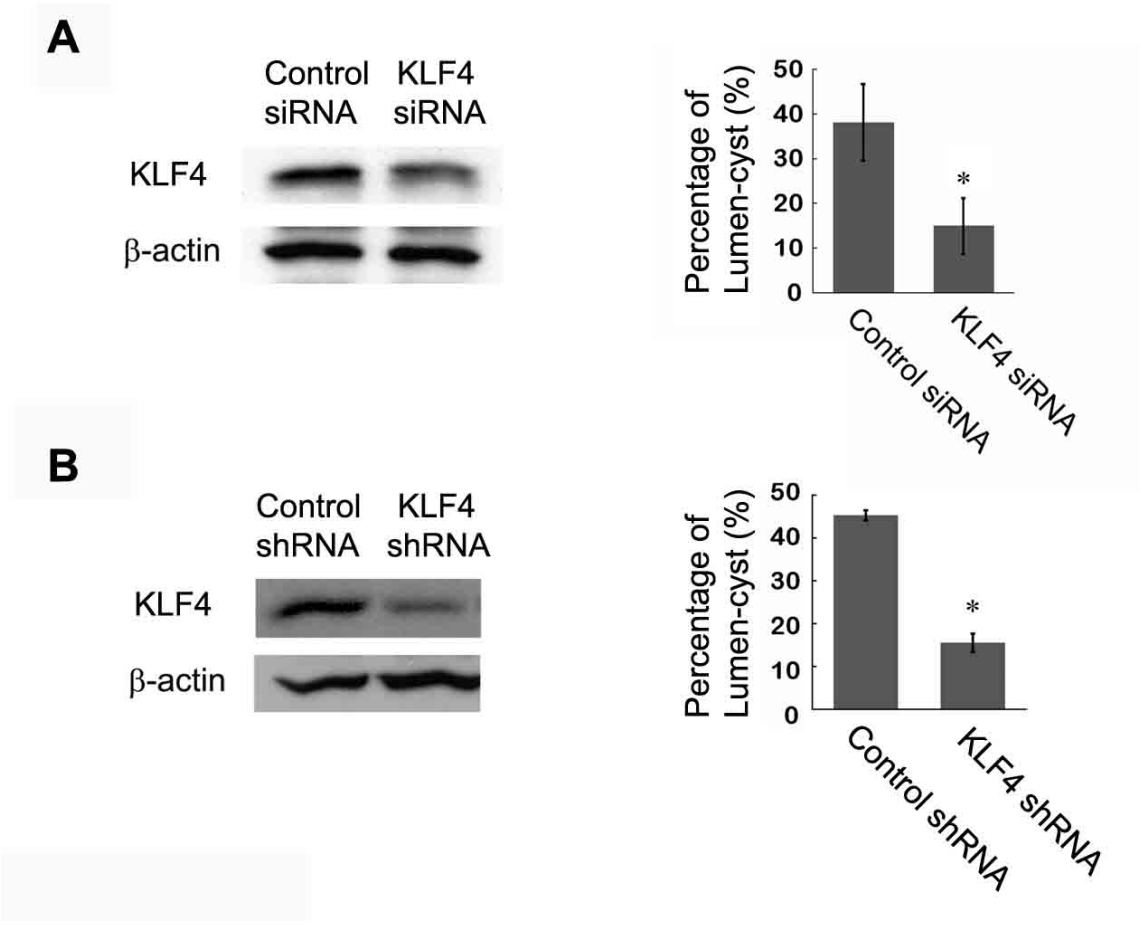

Figure 10: KLF4 is essential for cell polarity and crypt-cyst formation in 3D culture of Caco-2 cells. (A) Left: western blotting showing knockdown of KLF4 in Caco-2 cells. Right: statistical analysis of percentage of lumen-cyst formation in Caco-2 cell 3D cultures, comparing between control and KLF4 siRNA-transfected cell cultures. (*, $P<0.05$) (B) Left: western blotting showing expression of KLF4 in 293T cells co-transfected with human KLF4 and KLF4 shRNA plasmids. Right: statistical analysis of percentage of lumen-cyst formation in Caco-2 cell 3D cultures, comparing between control and KLF4 shRNA-infected cell cultures. (*, $P<0.05$)

KLF4 facilitates cell polarity and crypt-cyst formation in colon cancer cells

In order to confirm the role of KLF4 in facilitating cell polarity formation, 3D culture assay was performed in another colon cancer cell line to test whether KLF4 can enhance cyst formation *in vitro*. The LS174T-KLF4 stable cell line expresses KLF4 upon doxycycline induction (Zhang et al., 2006). LS174T cells seldom form cysts, even under 3D culture conditions. However, induction of KLF4 expression in LS174T cells significantly increased the chances of cyst formation in 3D culture (Fig. 11), indicating that KLF4 indeed enhances cell polarity and thus facilitates cyst formation in 3D formation assay.

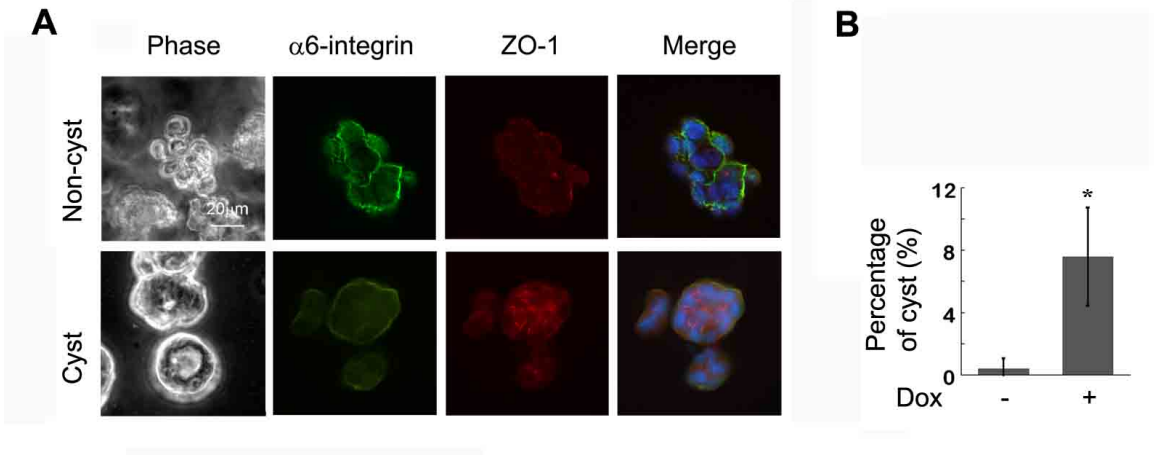

Figure 11: KLF4 facilitates cell polarity and crypt-cyst formation in colon cancer cells. (A) LS174 cells in 3D culture system were stained for differentiation markers indicating cell polarity and cyst formation. (B) Statistical analysis of percentage of cyst formation in LS174T cell 3D cultures, comparing between doxycycline (Dox)-induced and non-induced cells. (*, $P<0.05$)

In order to address the mechanism by which KLF4 regulates cell polarity both in a knockout mouse model and in a 3D culture system, a panel of cell fate and polarity-related genes were analyzed by semi-quantitative RT-PCR. KLF4 was induced by doxycycline in LS174T-KLF4 colon cancer cells (Fig. 12A). Though no significant changes were detected in DCAMKL-1 transcription, several polarity-related genes, *LKB1*, *EPHB2*, and *EPHB3*, were down-regulated. Intestinal stem cell markers *LGR5* and *CD44* were also down-regulated. As controls, the differentiation marker *IAP* and cell cycle inhibitor *P21*, which are known KLF4 target genes, were up-regulated by KLF4 (Fig. 12B). These findings suggest that KLF4 regulates epithelial cell polarity by regulating the transcription of multiple genes.

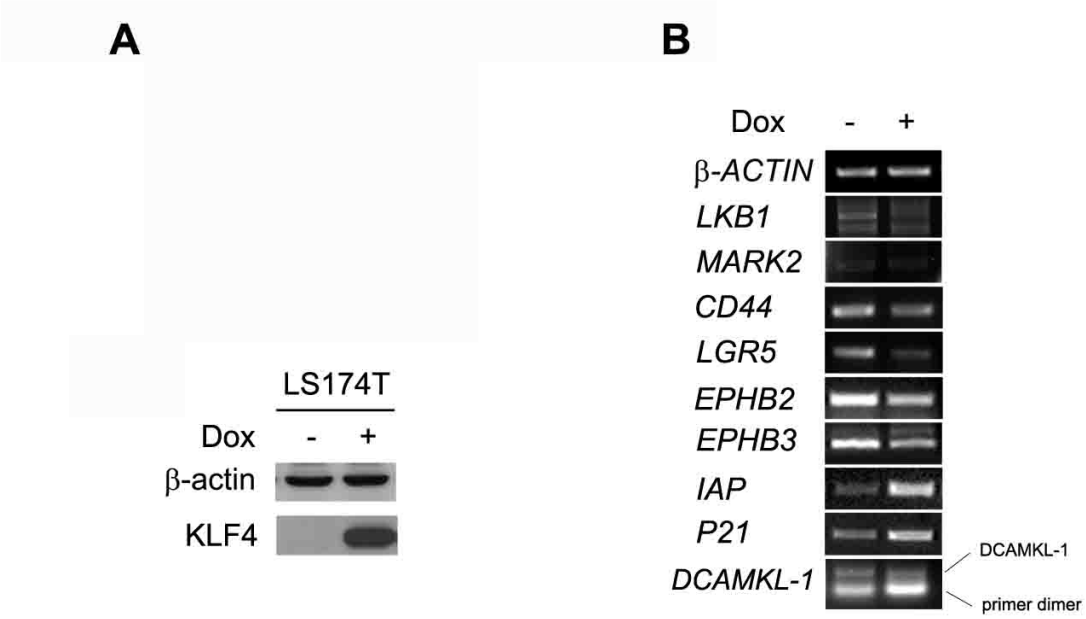

Figure 12: KLF4 regulates polarity-related genes in LS174T colon cancer cells. (A) Western blotting indicating expression of KLF4 in LS174T cell line with or without induction of doxycycline. (B) Semi-quantitative RT-PCR showing expression of genes related to KLF4-regulated cell polarity and related to Wnt signaling.

DISCUSSION

As an important regulator in intestinal cell differentiation during early development, KLF4 is also essential in maintaining normal homeostasis and morphology in adult intestine. Previous studies have deleted KLF4 in embryonic stages of mouse intestine; the terminal differentiation of goblet cells was decreased in these mice (Ghaleb et al., 2011; Katz et al., 2002). This study reported that in mature mouse intestine, partial depletion of KLF4 resulted in an increase in the number of goblet cells, indicating that KLF4 is required not only for goblet cell differentiation in early stages, but also for maintaining the number of differentiated goblet cells, probably by inhibiting cell proliferation. This is consistent with the observation that KLF4 is strongly expressed in goblet cells (Evans et al., 2010; Ghaleb et al., 2011; Ghaleb et al., 2007). I found that the average length of crypts was increased in KLF4-depleted small intestine, and the number of Ki67 positive cells was also increased. In agreement with previous findings, the number and position of Paneth cells had also changed (Ghaleb et al., 2011). DCAMKL-1 is a marker for tuft cells, and a potential marker for quiescent intestinal stem cells (May et al., 2008; May et al., 2009). I found that the number and position of DCAMKL-1 positive cells was also altered by KLF4 depletion. The changes in morphology and polarity of intestinal epithelial cells were confirmed by H&E staining. These data suggest that KLF4 plays a key role in maintaining normal intestinal homeostasis and morphology by regulating cell differentiation, proliferation and polarity. The roles of KLF4 in cell polarity were further analyzed in 3D culture, and several novel KLF4 target genes involved in cell differentiation and polarity were identified.

Results from this study suggested that tamoxifen-induced knockout of KLF4 is advantageous in tissue- and stage-specificity. KLF4 has been partially depleted in the villi of small intestine, where KLF4 normally predominantly expresses. In addition, the inducible knockout strategy allows normal development of small intestine in the early stage of development, which assures that lineage differentiation (i.e., the ability to differentiate Paneth and goblet cells) and intestine function is not affected by KLF4 depletion. The limitation of the mouse model is that KLF4 depletion is not complete. KLF4 is more efficiently deleted in differentiation cells, but less efficiently deleted in progenitor cells that have low levels of KLF4. The daughter cells differentiated from these progenitor cells may express high levels of KLF4. Thus, this mouse model can be used to study KLF4 function in differentiated cells, but is not suitable to study KLF4 function in cell fate determination during stem cell differentiation.

As to the function of KLF4 in cell proliferation, KLF4 plays a crucial role in maintaining the integrity of the cell cycle (Ghaleb et al., 2005). Low levels of KLF4 mRNA are essential for cell proliferation (Shields et al., 1996). In my study, the proliferating compartment of the intestine in *Klf4*$^{-/-}$ mice was increased while the total length of the villus-crypt axis turned out to be increased as well, suggesting the role of KLF4 in inhibiting outgrowth of the intestine villus-crypt beyond normal length. The numbers of goblet cells, Paneth cells and tuft cells were increased in KLF4 depleted small intestine, further suggesting that KLF4 inhibits proliferation of certain cell types and thus contributes to maintaining normal cell populations in the intestine. KLF4 also regulates the proliferation of stem cells and/or tuft cells, as indicated by DCAMKL-1 staining (Fig. 6, 7). In control mouse intestine, DCAMKL-1 positive cells were mainly located in the stem cell zone and amplification compartment; in *Klf4*$^{-/-}$ mouse intestine,

the number of DCAMKL-1 positive cells increased significantly in both the amplification and differentiation compartments. DCAMKL-1 has been suggested to be a marker for gastrointestinal stem cells and adenoma stem cells (May et al., 2008; May et al., 2009). However, others suggest that DCAMKL-1 only identifies tuft cells since they are not always located at the stem cell position, nor do they co-express with markers of any of the main lineages constituting the intestinal epithelium (Gerbe et al., 2009). The identity of DCAMKL-1 positive cells and the potential roles of KLF4 in intestinal stem cells remain to be determined.

Our previous work demonstrated that KLF4 crosstalks with Wnt signaling in the intestine (Evans et al., 2010; Zhang et al., 2006). Wnt signaling induces maturation of Paneth cells (van Es et al., 2005a) and mediates cell positioning in the intestinal epithelium (Batlle et al., 2002). The abnormal numbers and locations of Paneth cells could be partially due to enhanced Wnt signaling as a result of KLF4 depletion. The role of Wnt signaling in goblet cell is not clear. Goblet cell numbers were decreased by either activation of Wnt signaling through APC deletion or inhibition of Wnt signaling by DKK1 overexpression (Pinto et al., 2003; Sansom et al., 2004). Notch signaling also regulates goblet cells (Pellegrinet et al., 2011). It is possible that the differentiation and proliferation of goblet cells are regulated by multiple signaling pathways and different developmental stages.

Based on the observation of changes in cell position and apical-basolateral polarity in *Klf4*$^{-/-}$ intestine epithelia, together with results from the 3D intestinal epithelial cyst formation assay, I demonstrated that KLF4 regulates intestinal epithelial cell polarity

in addition to cell differentiation and proliferation, thus affecting morphology and homeostasis of the intestine.

Several genes that regulated cell polarity were repressed by KLF4, including *LKB1*. As a 'master' regulator of cell polarity, LKB1 was reported to induce complete polarity in intestinal epithelial cells; depletion of *LKB1* in Caco-2 cells led to impairment of spontaneous polarization (Baas et al., 2004a; Baas et al., 2004b). Recently, it was reported that that CDX2 deficiency leads to abnormal apical-basal polarity in intestinal epithelial cells (Grainger et al., 2010) and that CDX2 deficiency leads to elevated expression of LKB1 (Gao and Kaestner, 2010). Since KLF4 expression is dependent on CDX2 in human colon cancer cells (Dang et al., 2001), my finding is consistent with these reports and suggests that KLF4 regulates cell polarity through multiple genes, including *LKB1*.

In summary, the results from this study and previous studies suggest that KLF4 has multiple functions. In the early embryonic stage, KLF4 induces goblet cell differentiation in intestinal epithelium; throughout intestinal development, KLF4 maintains homeostasis of normal intestinal growth and keeps epithelial cells from over-proliferation. Meanwhile, KLF4 regulates apical-basolateral polarity of the intestinal epithelial cells. After all, the intestinal homeostasis and morphology are regulated by multiple factors, including KLF4 and its target genes.

CHAPTER 5: BMI1 AND HISTONE REGULATION IN CANCER CELL EPIGENETICS

Bmi1 belongs to the Polycomb group (PcG) gene family, which was originally discovered in Drosophila, and was also implicated in mammalian Homeobox (Hox) gene regulation. There are two main Polycomb repressive complexes: Polycomb repressive complex 2 (PRC2) methylates H3 histone protein on Lys27 (H3K27), which is a marker for gene silencing; while Polycomb repressive complex 1 (PRC1) is mainly responsible for recognizing the H3K27 mark and maintaining the state of gene silencing.

BMI1 IN THE POLYCOMB COMPLEXES

PRC2 has four core subunits, and its signature activity is to add up to three methyl groups to the target Lysine residue H3K27 by its methyltransferase activity. Trimethylated H3K27 (H3K27me3) is the major form of methylated H3K27 in the PcG silencing process. The H3K27me3 modification provides a docking site for PRC1, which recognizes the trimethylated Lysine on H3 although it is not sufficient for PRC1 targeting (Simon and Kingston, 2009).

PRC1 also has four core proteins, including RING1B (also known as RNF2) and B lymphoma Mo-MLV insertion region 1 (BMI1), although there are orthologues of these core proteins found residing in the complex. The mechanisms of PRC1-mediated gene silencing include: (1) blocks to the binding of key transcription factors at the promoter of target genes; (2) blocks to RNA polymerase association with the promoter of target genes; (3) blocks to initiation or elongation of target gene transcription (Simon and Kingston, 2009). One of the key mechanisms is H2A-mediated RNA polymerase pausing

in the middle of the transcription (Stock et al., 2007). This work demonstrated that elongation of RNA polymerase is correlated with the presence of ubiquitinated H2A, which process is impaired in a way that depends on PRC1 components. Enforced PRC-mediated H2A ubiquitination led to release of poised RNA polymerase and subsequent gene depression. And authors proposed this model to allow ES cells to self-renew while retain the ability of multi-lineage generation.

Bmi1 belongs to the PRC1 complex, and was found to play an essential role in ubiquitination of H2A and silencing of Hox genes. Bmi1 and Ring1A cooperate in the PRC1 complex to facilitate H2A ubiquitination. By reconstitution of the PRC1 complex and subcomplexes, both proteins were demonstrated to positively regulate H2A ubiquitination. Knockout Bmi1 and Ring1A resulted in significant loss of H2A ubiquitination, suggesting an *in vivo* model for Bmi1 and Ring1A function in H2A ubiquitination, and that both proteins contribute to the E3 ligase activity. Bmi1 depletion also led to upregulation of Hoxc13 expression, further supporting the mechanism that Bmi1 regulates gene silencing through mediating H2A ubiquitination and chromatin remodeling (Cao et al., 2005). Similar results from another reconstitution assay also support the finding that Bmi1 is required for H2A ubiquitination and that H2A ubiquitination regulates Bmi1-mediated gene silencing (Wei et al., 2006). These findings provide important implications for defining the role of Bmi1 participating in cancer and stem cell self-renewal, in the context of histone modification, chromatin remodeling and epigenetic regulation of gene expression.

BMI1 AND POLYCOMB IN STEM CELLS

It was found that Bmi1 and Mph1/Rae28 are highly expressed in primitive hematopoietic cells. Mice with mutations in Bmi1, Mel-18, Mph1/Rae28 suffer from deficiencies in the hematopoietic system, and both Bmi1 and Mph1/Rae28 deficient stem cells are defective in their proliferation and self-renew capacity (Lessard and Sauvageau, 2003; Park et al., 2003).

The PcG proteins govern stem cell fate, which was first demonstrated in Bmi1-deficient mice, which had loss of hematopoietic cells as well as cerebellar neurons (van der Lugt et al., 1994). The hematopoietic stem cell activities are regulated by Bmi1 as a component of the PRC1 complex, which requires and directly interacts with another Polycomb protein Mph1/Rae28 (Ohta et al., 2002). Mph1/Rae28 is also highly expressed in ES cells and was found to be downregulated during the process of differentiation (Fortunel et al., 2003; Loring et al., 2001). Not only the PRC1 complex, PRC2 as well as the contact between PRC1 and PRC2 are also essential for Polycomb silencing in the early development processes (Valk-Lingbeek et al., 2004).

How do Bmi1 and Polycomb protein complexes regulate cell states through modulating gene silencing/activating states? First, expression of Bmi1 may function as a transcription factor to repress activity of the tumor suppressor INK4A/ARF locus, to enhance the proliferation capacity and self-renewal (Smith et al., 2003). Second, Bmi1 demonstrated an important role in progenitor cell maintenance and expansion by facilitating respond to proliferation induction. This was through crosstalk with sonic hedgehog (Shh), a major developmental signaling pathway (Leung et al., 2004). Third, as a highly expressed gene among other stem cell markers, Bmi1 may play a role in

suppressing global gene expression, among which differentiation genes are suppressed at a higher extent.

A model of bivalent chromatin structure partially explain the mechanisms under which PRC complexes regulate chromatin status with co-existence of active transcription marker – methylation on histone H3 lysine 4 (H3K4) and repressed transcription marker – methylation on histone H3 lysine 27 (H3K27). Genome-based histone modification analysis in ES cells revealed a strong correspondence between the genomic sequence and histone methylation, while this correlation is weaker in differentiated cells. By comparing genomic DNA hybridization assay, a total of 343 sites of H3K4 methylation and 192 H3K27 methylation sites across the bivalent domains in ES cells.

The study proposed that the bivalent domains consist of large sections of H3K27 methylation harboring smaller portions of H3K4 methylation. ES cell differentiation assay demonstrated that bivalent domains are mostly related to ES cells and they are prone to resolve upon differentiation. These different regions coincide with transcription factors that are expressed at very low levels. These bivalent domains play a role in silencing developmental genes while keep them poised for later-on activation in ES cells (Bernstein et al., 2006).

PcG proteins suppress expression of master developmental regulatory genes such as homeodomain-containing transcription factors and pluripotency genes. Upon differentiation, many of these genes become activated based on stage of development and proliferation. Thus, PcG proteins including Bmi1 are playing such a crucial role in stem cell regulation in a context-dependent manner – when the cells are in the stem cell stage, committed stage, or terminally differentiated stage (Schuettengruber and Cavalli, 2009).

BMI1 AND POLYCOMB IN CANCER

High levels of Bmi1 expression are linked to many types of human malignancies including lymphoma, acute myeloid leukemia (AML), colorectal carcinoma, liver carcinoma, breast carcinoma, medulloblastoma, prostate cancer, non-small cell lung cancer, head and neck cancer, and globlastoma (Cao et al., 2011).

As an oncogene, Bmi1 targets INK4A/ARF locus and initiate tumorigenesis, which contributes to cell cycle activity change, proliferation maintenance as well as differentiation prevention (Cao et al., 2011; Haupt et al., 1993; Itahana et al., 2003). Bmi1-mediated cell cycle regulation is essential for life span extension and proliferation involving p16 regulation (Itahana et al., 2003). In addition, as a regulator of INK4A/ARF, Bmi1 cooperates with c-Myc to enhance proliferation and transformation of mouse embryonic fibroblasts (Jacobs et al., 1999b). Through the INK4A locus, Bmi-1 was reported to regulate cell proliferation and senescence (Jacobs et al., 1999a). However, transformation of breast cancer cells with co-overexpression of Bmi1 and activated H-Ras (RasG12V) are due to dysregulated growth pathways that are independent of the INK4A/ARF locus (Hoenerhoff et al., 2009). Therefore, the role and mechanisms of Bmi1 in cancer cell regulation is in a context dependent manner.

Bmi1 not only plays crucial roles in regulating stem cell states, but is also considered an important target for cancer cell manipulation. As mentioned above, Bmi1 is playing an essential role in hematopoietic stem cells. Not only that, Bmi1 activity is also necessary for the self-renewal of leukaemic stem cells (Lessard and Sauvageau, 2003). Authors reported that Bmi1-deficient leukaemia cells failed to induce leukaemia after transplantation. This work demonstrated a Bmi1-mediated pathway that is crucial for proliferation and self-renewal of both normal haematopoietic stem cells and leukaemic stem cells.

More recent works also demonstrated that Bmi1 is required for leukemic stem and progenitor cells, and that high levels of Bmi1 could facilitate establishing of aggressive leukemia in mice, compared with cells with lower levels of Bmi1 (Schuringa and Vellenga, 2010; van Gosliga et al., 2007). And more interestingly, Bmi1 has been demonstrated to be collaborating with Twist1 to induce epithelial-mesenchymal transition (EMT), which is a crucial program during the process of cancer invasion and matastasis (Singh and Settleman, 2010; Yang et al., 2010). Twist1 and Bmi1 cooperate to downregulate E-cadherin and p16INK4A to promote EMT and tumor initiation. Collectively, Bmi1 plays a critical role in tumor stem cell regulation and tumor initiation.

BMI1 CROSSTALKS WITH SIGNALING PATHWAYS

Various signaling pathways are involved in cancer cell proliferation and stem cell self-renewal. Wnt, Shh, Notch and PTEN pathways are among those well-known events that take place in both normal and cancer stem cells. The crosstalk between Bmi1 and those well-known signaling pathways has drawn great attention especially in studies in the stem cell and cancer field (Pardal et al., 2003; Valk-Lingbeek et al., 2004).

In terms of cooperative effect of Wnt and Bmi1 signalings in control of stem cell self-renewal, both pathways turn out to play essential roles in promoting proliferation and inhibiting cell death. As positive downstream effectors of Wnt signaling, c-Myc and cyclin D1 promote proliferation, survival or migration of stem cells. While as negative downstream target of Bmi1, CDKN2A-encoded cyclin-dependent kinase inhibitors, INK4A and ARF, block proliferation through activation of Rb and p53 pathways, respectively (Jacobs et al., 1999a). Based on the concurrency of Wnt activation and Bmi1

expression in intestinal epithelial stem cells and colon cancer, as well as other stem cell events and characters (Pardal et al., 2003), questions were raised: whether and how Bmi1 and Wnt correlate with each other in stem cell regulation? The next chapter of this dissertation will be discussing the correlation and crosstalk between Wnt/β-catenin/KLF4 signaling and Bmi1 in colon cancer studies, with focus on the role of KLF4 in transcriptional regulation on Bmi1, as well as the function of Bmi1 in cell proliferation and colorectal tumorigenesis (Yu et al., 2012a).

CHAPTER 6: BMI1 REGULATION BY BETA-CATENIN AND KLF4 AND ITS IMPLICATION IN COLON CANCER *

B lymphoma Mo-MLV insertion region 1 (Bmi1) is a Polycomb group (PcG) protein important in gene silencing. It is a component of Polycomb Repressive Complex 1 (PRC1), which is required to maintain the transcriptionally repressive state of many genes. Bmi1 was initially identified as an oncogene that regulates cell proliferation and transformation, and is important in hematopoiesis and the development of nervous systems. Recently, it was reported that Bmi1 is a potential marker for intestinal stem cells. Since Wnt signaling plays a key role in intestinal stem cells, study of this section analyzed the effects of Wnt signaling on Bmi1 expression. I found that Wnt signaling indeed regulates the expression of Bmi1 in colon cancer cells. In addition, the expression of Bmi1 in human colon cancers is significantly associated with nuclear β-catenin, a hallmark for the activated Wnt signaling. Krüppel-like factor 4 (KLF4) is a zinc finger protein highly expressed in the gut and skin. Previous study found that KLF4 crosstalks with Wnt/β-catenin in regulating intestinal homeostasis. Here I demonstrate that KLF4 directly inhibits the expression of Bmi1 in colon cancer cells. I also found that Bmi1 regulates histone ubiquitination and is required for colon cancer proliferation *in vitro* and *in vivo*. My findings further suggest that Bmi1 is an attracting target for cancer therapeutics.

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INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer world-wide, maintaining a high death rate the past ten years (Jemal et al., 2011), thus great attention was drawn upon mechanisms that lead to tumorigenesis in gastrointestinal epithelial cells. Aberration of Wnt/β-catenin signaling pathway is one of the major causes of tumorigenesis (Giles et al., 2003; Kinzler and Vogelstein, 1996), yet it is not well known how Wnt pathway crosstalks with other signaling pathways in intestinal homeostasis and cancer initiation. Krüppel-like factor 4 (KLF4), a zinc finger protein highly expressed in the gut and skin, was recently found interacting with β-catenin/TCF complex to repress Wnt signaling and inhibit tumor growth (Evans et al., 2010; Zhang et al., 2006). KLF4 is one of the four factors that induce pluripotent stem cells, thus playing a crucial role in stem cell regulation (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). In a normal intestine, KLF4 inhibits proliferation of crypt progenitor cells and regulates the differentiation of goblet and Paneth cells (Ghaleb et al., 2011; Katz et al., 2002).

Intestinal stem cells are located in the bottom of crypts. Currently, the +4 label-retaining cells (LRC) model and the crypt base columnar cells (CBC) model suggest there are several stem cells per crypt to populate the entire crypt (Scoville et al., 2008; van der Flier and Clevers, 2009). Since Wnt signaling plays essential roles in both normal intestinal stem cells and colon cancers, it was hypothesized that colon cancer is initiated from intestinal stem cells or progenitor cells (van de Wetering et al., 2002). Lgr5 (or Gpr49) is a leucine-rich repeat-containing G protein-coupled receptor; it is a Wnt target gene as well as an intestinal stem cell marker specific for CBC (Barker et al., 2007). In Wnt signaling-induced adenomas, the expression of Lgr5-EGFP was restricted to a small population of cells, suggesting that stem cells or progenitor cells are maintained in these

tumors, supporting the cancer stem cell concept in colorectal tumorigenesis (Barker et al., 2009).

Another potential stem cell marker, B lymphoma Mo-MLV insertion region 1 (Bmi1), belongs to the Polycomb group (PcG) gene family which functions in gene silencing through chromatin modifications. Bmi1 is predominantly expressed in the +4 cells in the crypt (Sangiorgi and Capecchi, 2008). Bmi1 was initially identified as an oncogene that regulates cell proliferation and transformation (Haupt et al., 1991; Jacobs et al., 1999b). It was later found to play an important role in hematopoiesis and development of the nervous system (van der Lugt et al., 1994). Bmi1 is also crucial for self-renewal of stem cells and cancer initiation (Kang et al., 2007; Lessard and Sauvageau, 2003; Sangiorgi and Capecchi, 2008; Valk-Lingbeek et al., 2004). The role of Bmi1 in controlling cell proliferation and self-renewal might be through its function as a Polycomb group (PcG) protein, which facilitates histone modification and regulates gene silencing (Rajasekhar and Begemann, 2007; Wang et al., 2004a; Wei et al., 2006).

In order to get a deeper insight into the function and regulation of these stem cell markers, I analyzed the effects of Wnt signaling and KLF4 on the expression of Bmi1. I found that Wnt signaling enhances while KLF4 inhibits the expression of Bmi1. Bmi1 is required for colon cancer cell proliferation, and it is up-regulated in primary human colon cancers. The mechanisms of Bmi1 function and regulation in colon cancer were examined in this study.

MATERIALS AND METHODS

Cell lines, lentiviral transduction, proliferation tumor xenograft assay, Hematoxylin and Eosin (H&E) and Alcian Blue staining

LS174T colon cancer cell line (Zhang et al., 2006) was grown in RPMI medium (Mediatech) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Stable cell line LS174T-KLF4 has been described previously (Zhang et al., 2006).

Bmi1 cDNA (Origene, CS116894) was cloned into lentivirus vector pCS-CG-hPGK (kindly provided by Dr. Tianyan Gao). Control shRNA and Bmi1 shRNA cloned in pLKO.1 vectors with puromycin resistance selection marker were from Sigma. Lentiviral stocks were prepared as previously described (Qin et al., 2007). LS174T cells were infected with lentivirus carrying pCS-CG-hPGK control vector and pCS-CG-hPGK-Flag-Bmi1, respectively; LS174T and/or HT29 cell lines were infected with lentivirus carrying pLKO.1-control shRNA and pLKO.1-Bmi1 shRNA, respectively.

For proliferation assay, stable cell lines were seeded as 2.5 x 10^4/well in 12-well plates and counted at appropriate times using cell viability analyzer (Beckman Coulter, Vi-Cell XR).

For xenograft assay, HT29 stable cell lines (1 x 10^6) were injected subcutaneously into both flanks of athymic nude mice as described previously (Evers et al., 1991; Wang et al., 2002a). Tumor growth was analyzed twice weekly. Tumor xenografts were harvested and embedded in paraffin after three weeks. Alcian Blue (AB) staining was performed based on standard protocol using Alcian Blue 8GX and Fast Red from Sigma (kindly provided by Dr. Tianyan Gao). Hematoxylin and Eosin (HE) staining was performed by the Histology Laboratory of the Imaging Facility at University of Kentucky.

Western blotting and histone extraction

Cells were lysed in appropriate volume of lysis buffer (50 mm HEPES, 100 mm NaCl, 2 mm EDTA, 1% glycerol, 50 mm NaF, 1 mm Na_3VO_4, 1% Triton X-100, with protease inhibitors). For histone extraction, cell pellet from lysate residues were re-suspended with 0.2N HCl and shaken at 4°C overnight. The extract after centrifuge contained histones to be tested. The following antibodies were used: mouse anti-β-actin (Sigma, A1978), mouse anti-Flag (Sigma, F1804), mouse anti-Bmi1 (Millipore, 05-637), mouse anti-β-tubulin (Developmental Studies Hybridoma Bank, E7) and rabbit anti-uH2A (Millipore, 05-678).

Semi-quantitative RT-PCR and quantitative Real-Time PCR

LS174T-KLF4 cells were plated at approximately 2 x 10^5 cells per well in a 6-well plate. The following day, doxycycline (1 μg/ml) was added to the culture medium. After 48 h of incubation, RNA was isolated using the RNeasy kit (Qiagen). Reverse transcriptase PCR (RT-PCR) was performed as described previously (Zhang et al., 2006). The following primers were used: β-actin, 5′-CAACCGCGAGAAGATGAC-3′ and 5′-AGGAAGGCTGGAAGAGTG-3′; *CTNNB1*: 5′-TCTGTGTTGTTTTATGCCAT-3′ and 5′-CCATCCCTTCCTGTTTAGT-3′; *LGR5*: 5′-CCTGCTTGACTTTGAGGAAGAC-3′ and 5′-ATGTTCACTGCTGCGATGAC-3′; *BMI1*: 5′-AGCAGAAATGCATCGAACAA-3′ and 5′-CCTAACCAGATGAAGTTGCTG-3′; *RING1A*: 5′-CCATCAAGACCGAGTGCTTA-3′ and 5′-ACATCCTTCTCCATCCCCTTC-3′; *RING1B*: 5′-CAATGGCAATTGATCCAGTA-3′ and 5′-TGGTTTGATTCACCTTTGCT-3′.

Real-Time PCR was performed according to standard protocols using TaqMan Gene Expression Assays (Applied Biosystems) including control eukaryotic 18S rRNA (Hs99999901_s1) and Bmi1 (Hs00180411_m1).

Interference RNA, ChIP assay, luciferase reporter assay and immunohistochemistry (IHC) staining on TMA slides

Interference RNA, chromatin immunoprecipitation (ChIP) assay, luciferase reporter assay and immunohistochemistry (IHC) were tested as described previously (Evans et al., 2007; Zhang et al., 2006).

Colon cancer tissue array from Biomax (CO482, Rockville, MD, US) was deparaffinized and IHC performed using standard protocol.

For ChIP assay, the following primers were used: Cyclin B1: 5′-TCTTGCCCGGCTAACCTTTCCAGG-3′ and 5′-TTCCGCCGCAGCACGCCGAGAAGA-3′; Bmi1: 5′-GGCGGCCGGGAGAAAGAAAGAAC-3′ and 5′-AGGGCGGCCTGGGAATTAGTGTC-3′.

For IHC staining, the following antibodies were used: KLF4 (Zhang et al., 2006), mouse anti-Bmi1 (Millipore, 05-637) and rabbit anti-β-catenin (Sigma, c2206).

Statistical analysis

Descriptive statistics were calculated and bar graphs and line plots were generated to summarize cell proliferation, luciferase intensity qRT-PCR, tumor volume across cell culture conditions and between xenograft groups. Two-sample t-test was employed to compare luciferase levels and analysis of variance for comparison of cell proliferation between shRNA groups and days of measurement. Linear mixed models were employed

for comparison of tumor growth rate over time between control versus shRNA groups. Finally, analyses of IHC total scores (sum of intensity and percent staining) on colorectal cancer tissue specimens included calculation of Spearman correlation coefficient to assess correlations between Bmi1, KLF4 and β-catenin and nonparametric tests for comparison across grade and stages of colorectal tumors.

RESULTS

Bmi1 is over-expressed in colon cancer tissues.

Wnt/β-catenin plays a central role in normal intestinal development; deregulation of Wnt signaling leads to colon cancer. Wnt signaling regulates the self-renewal of intestinal stem cells and may also regulate the colon cancer stem cells. Lgr5, one of the potential stem cell markers, is specifically expressed in the crypt base columnar (CBC) cells in the intestine and has been identified as a target of Wnt signaling (Barker et al., 2007; Yamamoto et al., 2003). Bmi1 is a novel stem cell marker expressed in the +4 cell at the bottom of crypt. To determine whether the expression pattern of Bmi1 correlates with β-catenin during colorectal progression, tissue microarrays (TMA) were stained with antibodies against Bmi1, β-catenin and KLF4 (Fig. 13). TMA slides contained normal tissue samples and three stages of colorectal tumor tissue samples. Duplicated cores per case were analyzed, making a total of 20 cases of colonic carcinoma and four cases of colonic normal tissue from necroscopy. Evaluation of the staining was based on the percentage of positive cells (nuclear staining) in each tissue core as well as intensity of the positively stained cells.

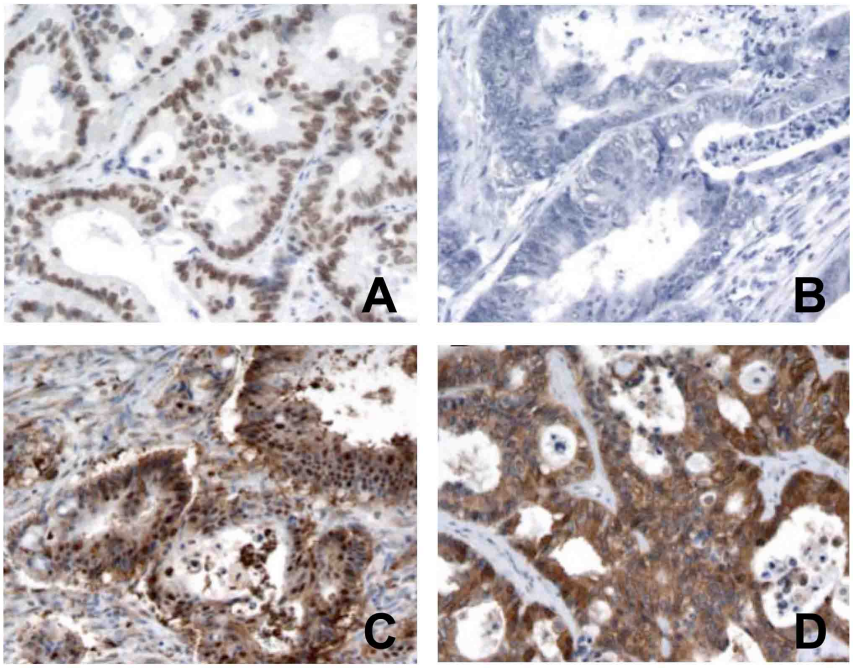

Figure 13: Immunostaining of Bmi1 and β-catenin in colon cancer tissue microarrays (TMA). (A) Colorectal cancer (CRC) with strong nuclear immunoreactions of Bmi1 (immunoreactivity score of 6 = percentage score of 3 and intensity score of 3). (B) CRC with no immunoreactions of Bmi1 (immunoreactivity score of 0 = percentage score of 0 and intensity score of 0). (C) CRC with strong nuclear localization of β-catenin. (D) CRC with strong cytoplasmic immunoreaction but no nuclear localization of β-catenin.

Both β-catenin expression and Bmi1 expression are significantly higher in colon cancer tissues than normal tissues. A positive correlation between nuclear β-catenin and Bmi1 was found in all tissue cores (Spearman Correlation Coefficient = 0.51825, p=0.0113) (Table 1-3).

Table 1: Statistical analysis of correlation of Bmi1 and β-catenin in colon cancer tissue microarrays

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Observations		
	TOTB_CAT	**TOTBMI**
TOTB_CAT	1.00000 23	0.51825 0.0113 23
TOTBMI	0.51825 0.0113 23	1.00000 23

Table 2: Evaluation score of TMA slides from colon cancer patient.

Core Number	KLF4 Percentage	KLF4 Intensity	β-catenin Percentage	β-catenin Intensity	Bmi1 Percentage	Bmi1 Intensity	Grade
A1	3	2	0	0	0	0	1
A2	3	2	0	0	3	2	1
A3	0	0	2	1	0	0	1
A4	0	0	2	1	0	0	1
A5	0	0	1	1	0	0	1
A6	0	0	1	1	0	0	1
A7	3	3	2	3	3	2	2
A8	3	3	2	3	3	2	2
B1	0	0	1	1	3	2	2
B2	0	0	1	1	3	2	2
B3							2
B4	0	0					2
B5	0	0	0	0	0	0	2
B6	0	0					2
B7	1	2	1	1	3	2	2
B8	1	2	1	1	3	2	2
C1	1	2	3	2	3	3	1-2
C2	1	1	2	2	3	3	1-2
C3	0	0	0	0	0	0	2
C4	0	0	0	0	0	0	2
C5	0	0	3	3	2	2	2

C6	0	0	3	3	2	2	2
C7	0	0	1	1	0	0	2
C8	0	0	1	1	0	0	2
D1	1	1	0	0	0	0	2
D2	1	1	0	0	0	0	2
D3	0	0			0	0	2
D4	0	0	2	2	0	0	2
D5	0	0	2	2	0	0	2
D6	0	0	2	2	0	0	2
D7	1	1	0	0	0	0	3
D8	1	2	0	0	0	0	2
E1	3	3	1	1	1	2	3
E2	3	3	1	1	0	0	3
E3	3	1	2	3	1	2	3
E4	3	1	2	3	1	3	3
E5	3	3	3	2	2	2	2-3
E6	3	3	3	2	2	2	2-3
E7	3	2	3	2	0	0	3
E8							

Note:
Percentage: none=0; <10%=1; 10-50%=2; >50%=3
Intensity: none=0; weak=1; intermediate=2; strong=3
For KLF4 expression in normal tissues, percentage=2 and intensity=2.

Table 3: Statistical analysis of TMA scores from Table 2.

Grade	N Obs	Variable	N	Minimum	Lower Quartile	Mean	Median	Upper Quartile	Maximum
0	4	TOTKLF	4	4.00	4.00	4.00	4.00	4.00	4.00
		TOTB_CAT	4	0.00	0.00	0.00	0.00	0.00	0.00
		TOTBMI	4	0.00	0.00	0.00	0.00	0.00	0.00
1	3	TOTKLF	3	0.00	0.00	1.67	0.00	5.00	5.00
		TOTB_CAT	3	0.00	0.00	1.67	2.00	3.00	3.00
		TOTBMI	3	0.00	0.00	0.83	0.00	2.50	2.50
2	13	TOTKLF	13	0.00	0.00	1.23	0.00	2.50	6.00
		TOTB_CAT	12	0.00	0.00	2.46	2.00	4.25	6.00
		TOTBMI	12	0.00	0.00	2.08	0.00	5.00	6.00
3	4	TOTKLF	4	4.00	4.50	5.25	5.50	6.00	6.00
		TOTB_CAT	4	2.00	3.50	4.25	5.00	5.00	5.00
		TOTBMI	4	0.00	0.75	2.25	2.50	3.75	4.00

Wnt/β-catenin signaling regulates Bmi1 expression in colon cancer cells.

To test whether Bmi1 is a target of Wnt/β-catenin signaling, LS174T colon cancer cells were treated with β-catenin siRNA and expression levels of Bmi1 were analyzed. I found that knockdown of β-catenin by siRNA decreased the levels of Bmi1 mRNA (Fig. 14A). As expected, β-catenin siRNA also inhibited the transcription of known Wnt target, Lgr5. To confirm this result, I performed a reporter assay using a luciferase gene driven by Bmi1 promoter (Guo et al., 2007). Overexpression of β-catenin increased the Bmi1 promoter activity (Fig. 14B).

In a generated stable colon cancer cell line that contains doxycycline (Dox) inducible dnTCF (dominant-negative TCF), wild type TCF function was inhibited and Wnt signaling was attenuated (Zhang et al., 2006). I found that expression of dnTCF in LS174T colon cancer cells inhibited the expression of Bmi1 at both protein levels (Fig. 14C) and mRNA levels (Fig. 14D). This suggests the Wnt/β-catenin signaling regulates Bmi1 expression through a TCF/LEF dependent mechanism. TCF/LEF binds a specific sequence in the promoter of its direct target (van de Wetering and Clevers, 1992); however, no consensus TCF/LEF binding site was found in the promoter region of Bmi, suggesting that Wnt/β-catenin signaling may regulate Bmi1 gene indirectly, probably through other β-catenin target genes.

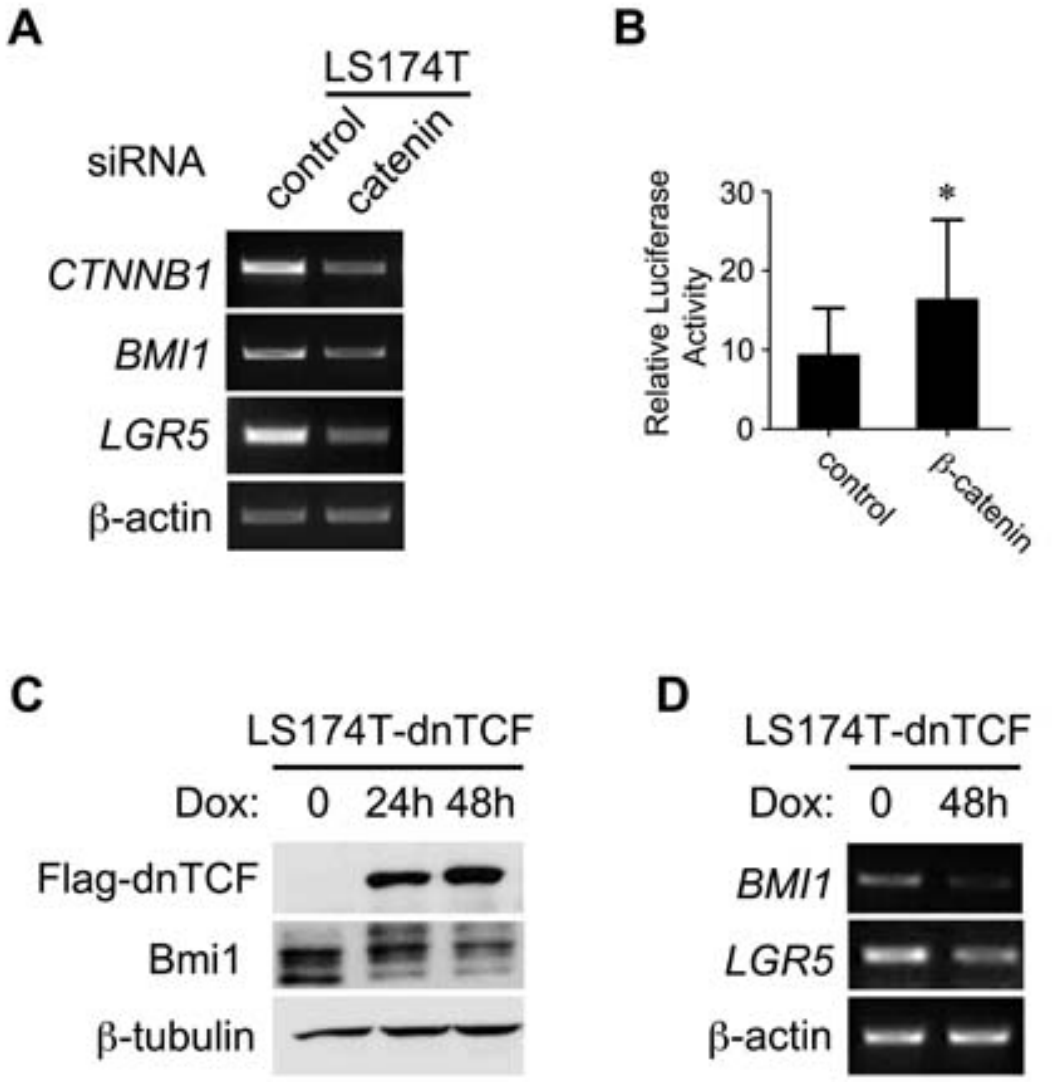

Figure 14: Bmi1 is regulated by Wnt/β-catenin. (A) Semi-quantitative RT-PCR of Wnt target genes in LS174T colon cancer cells after transfected with control/ β-catenin siRNA oligos. (B) Luciferase reporter assay testing the effect of β-catenin on Bmi1 promoter activity (*, P=0.0306). (C) Western blot showing expression of Bmi1 and Lgr5 in LS174T cells after induction of dominant negative TCF (dnTCF) in doxycycline (Dox)-inducible cells. (D) Semi-quantitative RT-PCR showing transcription of Bmi1 and Lgr5 in LS174T cells with induced dnTCF.

KLF4 directly binds Bmi1 promoter and regulates Bmi1 expression in colon cancer cells.

As a tumor suppressor protein in colon cancer (Zhao et al., 2004), KLF4 crosstalks with Wnt/β-catenin and represses β-catenin-mediated gene expression (Zhang et al., 2006). To test whether KLF4 regulates Bmi1 expression, I performed a reporter assay and demonstrated that Bmi1 promoter activity was suppressed by KLF4 in LS174T colon cancer cells (Fig. 15A). To analyze the effects of KLF4 on endogenous Bmi1 expression, LS174T-KLF4 cells were treated with doxycycline for 24 and 48 hours to induce KLF4 expression. Bmi1 expression was examined by western blot and semi-quantitative RT-PCR analysis. Both protein levels and RNA levels of Bmi1 were decreased upon KLF4 expression (Fig. 15B, 15C). To confirm this result, I analyzed Bmi1 expression by Real-Time PCR. Again, the mRNA levels of Bmi1 were significantly decreased by KLF4 (Fig. 15D). Collectively, these data strongly suggest that KLF4 represses Bmi1 expression at transcription level in colon cancer cells.

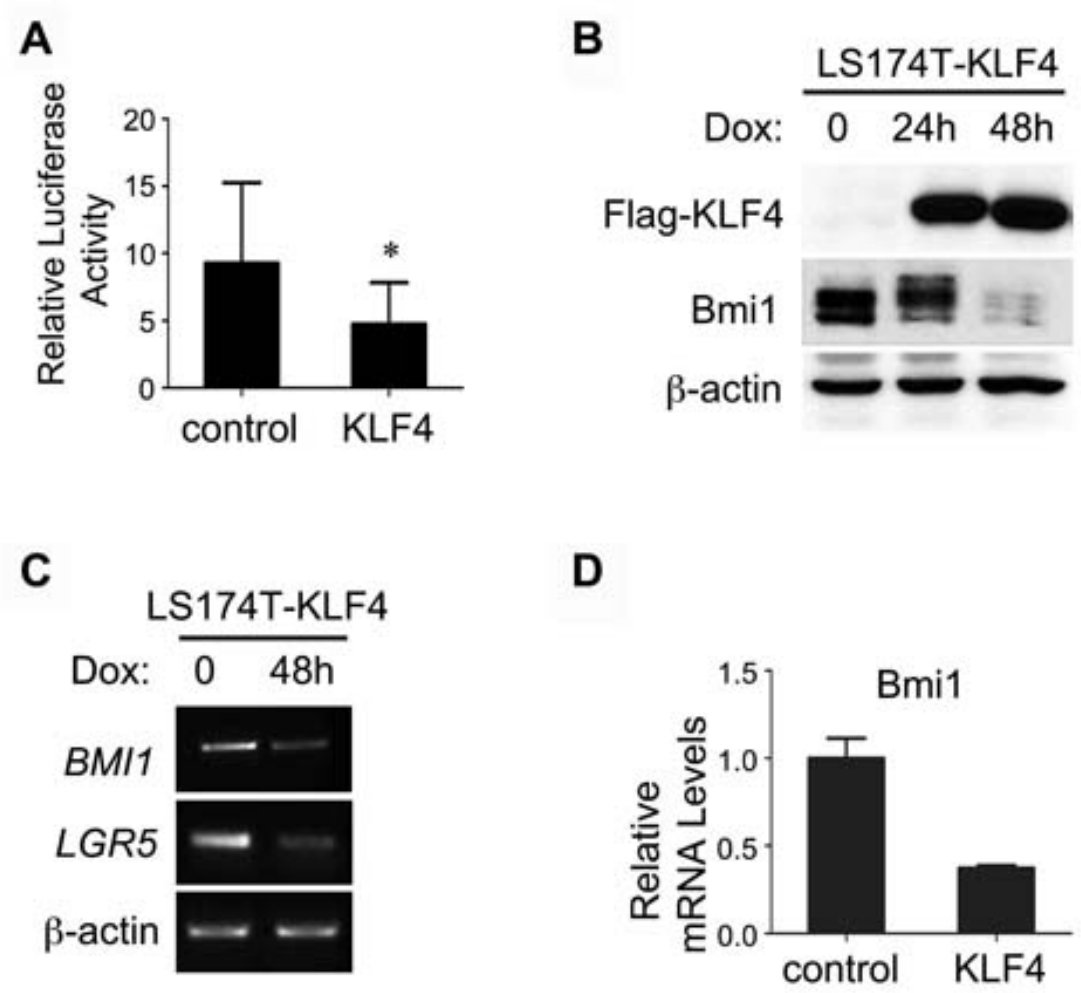

Figure 15: Bmi1 is regulated by KLF4. (A) Luciferase reporter assay testing the effect of KLF4 on Bmi1 promoter activity (*, P=0.0228). (B) Western blot showing expression of Bmi1 in LS174T cells expressing Dox-inducible KLF4. (C) Semi-quantitative RT-PCR testing the transcription of Bmi1 and Lgr5 in LS174T cells after induction of KLF4 in Dox-inducible cells. (D) Real-Time RT-PCR indicating mRNA level of Bmi1 expression under the effect of Dox-inducible KLF4 expression.

To determine if KLF4 binds to Bmi1 promoter in colon cancer cells, chromatin immunoprecipitation (ChIP) assay was performed using an anti-KLF4 antibody in LS174T-KLF4 cells. Cyclin B1, a known KLF4 target gene, was used as a positive

control. PCR analysis with KLF4 specific primers demonstrated that KLF4 did bind to Bmi1 promoter, and the binding was increased upon doxycycline-induced KLF4 expression (Fig. 16A). To quantify the occupation of KLF4 on Cyclin B1 and Bmi1 promoters, I analyzed the relative intensity of ChIP-PCR bands using Alpha Innotech AlphaView software. Integrated intensity values showed that the binding of KLF4 with both Cyclin B1 promoter and Bmi1 promoter are significantly different between KLF4-induced and non-induced cells (Fig. 16B-C).

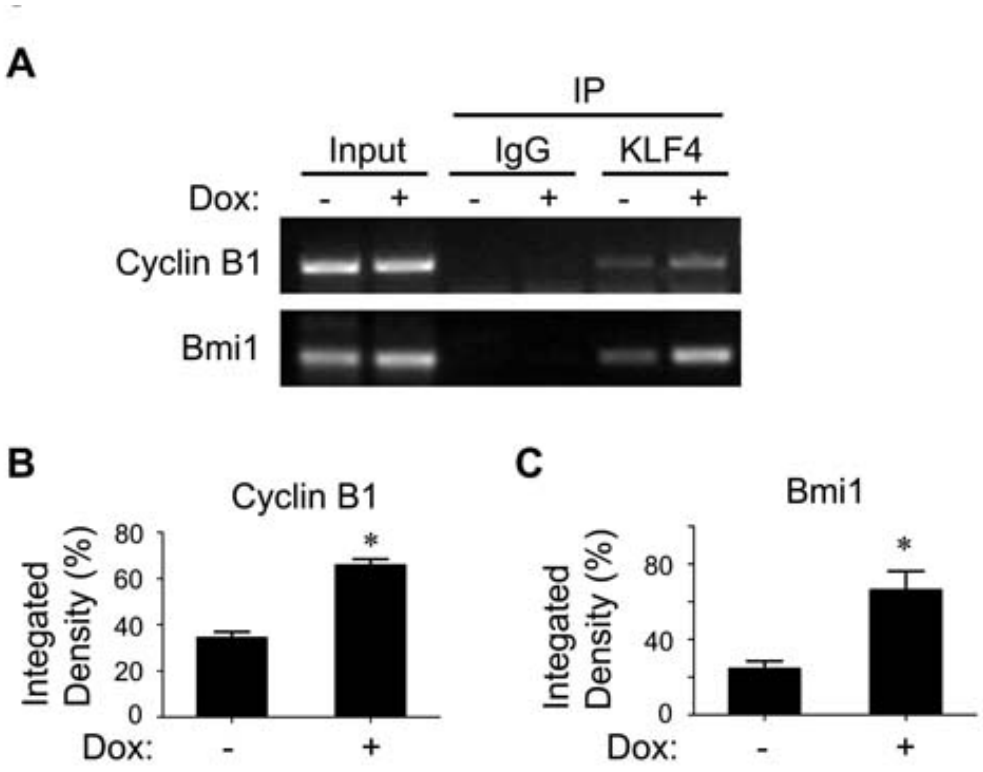

Figure 16: KLF4 interacts with Bmi1 promoter in colon cancer cells. (A) ChIP assay testing interaction of KLF4 on Bmi1 promoter. IgG and KLF4 were used to precipitate chromatin DNA fragments as indicated; and DNA fragments from both Dox-induced (+) and non-induced (-) cells were used respectively. (B) and (C) Quantification of relative intensity of KLF4 binding with Cyclin B1 (B) and Bmi1 (C) promoter, respectively.

The minimal essential binding site for KLF4 is 5'-G/AG/AGGC/TGC/T-3' (Brown et al., 2005; Shields and Yang, 1998). There is a c-Myc binding site and two putative sequences similar to KLF4 binding sequences in the Bmi1 promoter (Fig. 17A). Luciferase reporter assay was performed to test the effect of KLF4 on Bmi1 promoter with c-Myc binding site-mutation or with deletion of the two putative KLF4 binding sites. Results showed no significant change in Bmi1 activity in response to KLF4 after mutation of c-Myc binding site, indicating that KLF4 inhibits Bmi1 independent of c-Myc. To my surprise, Bmi1 promoter depleted of the two putative KLF4 binding sites still responded to KLF4, suggesting that KLF4 inhibits Bmi1 by interacting with Bmi1 promoter but the direct interaction is not through these sites (Fig. 17B).

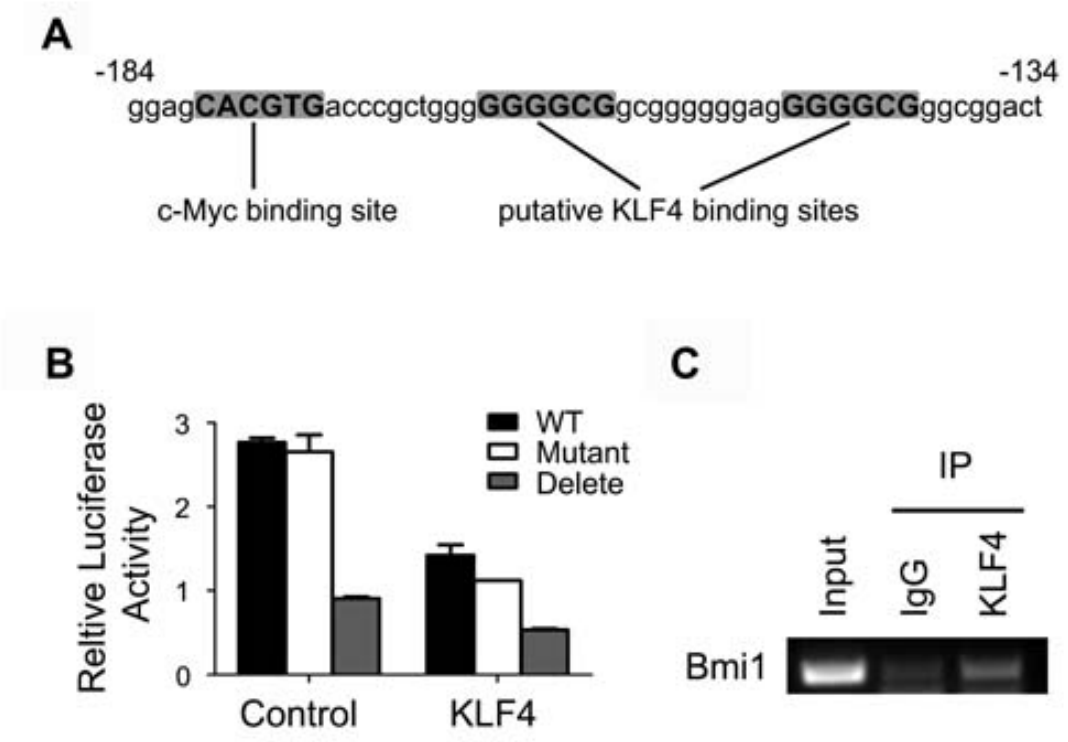

Figure 17: KLF4 directly binds Bmi1 promoter and regulates Bmi1 promoter activity in colon cancer cells. (A) Promoter region of Bmi1 gene with a c-Myc binding site and two putative KLF4 binding sites. (B) Luciferase reporter assay testing the effect of KLF4 on wild type (WT) Bmi1 promoter, Bmi1 promoter with a mutation in c-Myc binding site (Mutant), or Bmi1 promoter deleted of two putative KLF4 binding sites (Delete) indicated in 3D. (C) ChIP assay testing interaction between Flag-KLF4 and Bmi1 promoter, both of which were overexpressed in 293T cells by co-transfection. IgG and KLF4 were used to precipitate chromatin DNA fragments as indicated.

To test the specificity of KLF4 binding with Bmi1 promoter construct with deletion of the two putative KLF4 binding sites, ChIP assay was performed with 293T cells, which were co-transfected with Flag-KLF4 and Bmi1 promoter. Consistent with the luciferase assay, binding of KLF4 was still detected on the mutated Bmi1 promoter (Fig. 17C), suggesting that the GGGGCG sites are not required for KLF4 binding, and that the promoter sequence -233-0 is sufficient for KLF4 binding.

KLF4 inhibits Bmi1-mediated histone ubiquitination.

As a member of Polycomb group protein (PcG), Bmi1 is required for histone H2A ubiquitination and thus regulates gene silencing (Cao et al., 2005; Rajasekhar and Begemann, 2007; Wang et al., 2004a; Wei et al., 2006). Knockdown of Bmi1 resulted in a decrease in H2A ubiquitination, which is consistent with previous reports (Fig. 18A). It has been reported that c-Myc regulates Bmi1 (Guo et al., 2007). As a control, c-Myc siRNA also decreased H2A ubiquitination. In order to test whether the inhibition of Bmi1 expression by KLF4 also leads to loss of H2A ubiquitination, the levels of ubiquitinated H2A were analyzed in LS174T cells that express doxycycline-induced KLF4. Interestingly, KLF4 expression significantly decreased the levels of ubiqutylated H2A while the total levels of H2A were not affected (Fig. 18B), suggesting KLF4 inhibits H2A ubiquitination, which is regulated by the Bmi1 complex.

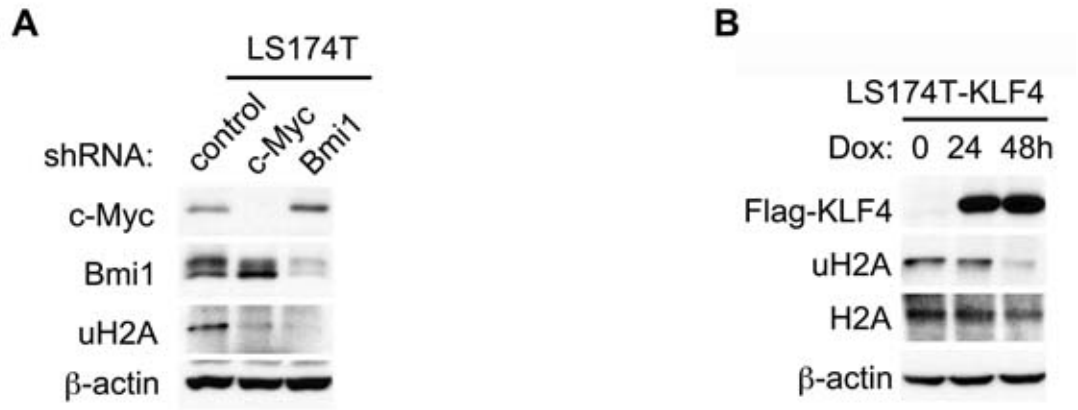

Figure 18: KLF4 inhibits Bmi1-mediated H2A ubiquitination. (A) Western blot indicating expression of Bmi1 and ubiquitinated H2A (uH2A) in control and Bmi1 shRNA-expressing LS174T cells. (B) Level of uH2A in LS174T cells with Dox-induced KLF4 expression.

To test whether Bmi1 overexpression can rescue cell proliferation repressed by KLF4, I established a stable cell line, LS174T-KLF4-Bmi1, which overexpresses Flag-tagged Bmi1 in addition to doxycycline-induced Flag-tagged-KLF4 (Fig. 19A). In the control cell line (LS174T-KLF4), expression of KLF4 repressed Bmi1 expression (Fig. 19A, left panel). In LS174T-KLF4-Bmi1 cells, the expression of Bmi1 was not repressed by KLF4 (Fig. 19A, right panel).

Consistent with previous report (Zhang et al., 2006), expression of KLF4 inhibited the growth of LS174T cells (Fig. 19B). Bmi1 overexpression led to increase in growth rate of LS174T cells (Fig. 19B), indicating the role of Bmi1 in promoting colon cancer cell proliferation. This result is also consistent with the effects of the shRNA study on inhibiting colon cancer cell proliferation (Fig. 20A and 20B).

I found that Bmi1-mediated increase in cell proliferation was sequestered by KLF4; and KLF4-induced inhibition on cell proliferation was not rescued by Bmi1 expression (Fig. 19B). These results indicated that Bmi1 overexpression is not sufficient to rescue KLF4-mediated growth inhibition. I hypothesize that KLF4 regulates cell proliferation through multiple mechanisms.

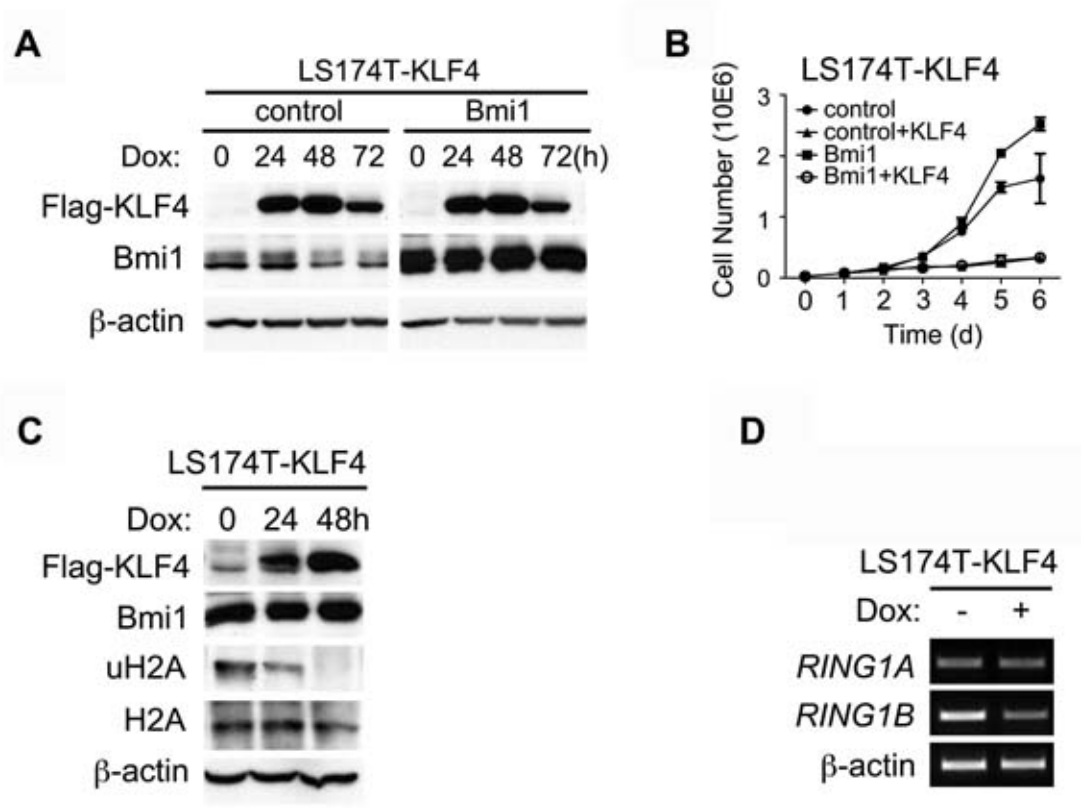

Figure 19: KLF4 inhibits Bmi1-mediated H2A ubiquitination but not through Bmi1 only. (A) Western blot showing expression of Bmi1 in LS174T cells expressing Dox-inducible KLF4, which were infected with control or Bmi1-carrying lentivirus, respectively. (B) Growth curves of LS174T cell lines that express Bmi1 and/or Dox-inducible KLF4 ($P<0.0001$ between KLF4 vs Control, Bmi1 vs Control, and Bmi1+KLF4 vs. Bmi1; $P=0.7454$ between Bmi1+KLF4 vs. KLF4). (C) Protein expression of Bmi1 and level of uH2A in the stable LS174T cell line under the combined effect of Bmi1 overexpression and inducible KLF4 expression. (D) Semi-quantitative RT-PCR testing transcription of other Polycomb complex members under the effect of Dox-induced KLF4 expression.

To further test the mechanism how KLF4 regulates Bmi1-mediated H2A ubiquitination, I compared the ubiquitination status of H2A among KLF4-expressing cells, Bmi1 expressing cells and KLF4/Bmi1-double expressing cells. I found that Bmi1-mediated H2A ubiquitination could be attenuated by KLF4; however, KLF4-mediated

inhibition of H2A ubiquitination cannot be rescued by Bmi1 overexpression (Fig. 19C). These results implicated that KLF4 inhibits H2A ubiquitination by more than one mechanism; other factors in the Bmi1 complex might also be regulated by KLF4.

To test this hypothesis, the mRNA levels of *RING1A* and *RING1B*, components of the Bmi1-polycom repressive complex, were analyzed by semi-quantitative RT-PCR. I found that the mRNA levels of *RING1B* but not *RING1A* were repressed by induced KLF4 expression in LS174T cells (Fig. 19D). It is of great interest to further study the mechanisms how KLF4 regulates H2A ubiquitination complexes as well as their functions in cell proliferation and tumorigenesis.

Bmi1 is essential for colon cancer cell proliferation *in vitro* and *in vivo*.

Since Bmi1 acts as an oncogene that regulates cell proliferation and transformation of several types of cancers (Haupt et al., 1991; Jacobs et al., 1999a), I tested whether Bmi1 also regulates proliferation of colon cancer cells. Bmi1 shRNA was transfected into different colon cancer cell lines through lentivirus infection. Cell proliferation assay indicated that Bmi1 down-regulation inhibited the growth of all tested colon cancer cell lines, including HT29 (Fig. 20A), LS174T cells (Fig. 20B) and KM20 (data not shown).

To determine the role of Bmi1 *in vivo*, I generated stable HT29 colon cell lines that express control shRNA or Bmi1 shRNA. These cells were injected subcutaneously into the flank of athymic nude mice. The tumors were measured twice a week for three weeks. I found that Bmi1 depletion significantly inhibited xenografted tumor growth (Fig. 21A and 21B), indicating that Bmi1 is essential for colon cancer progression.

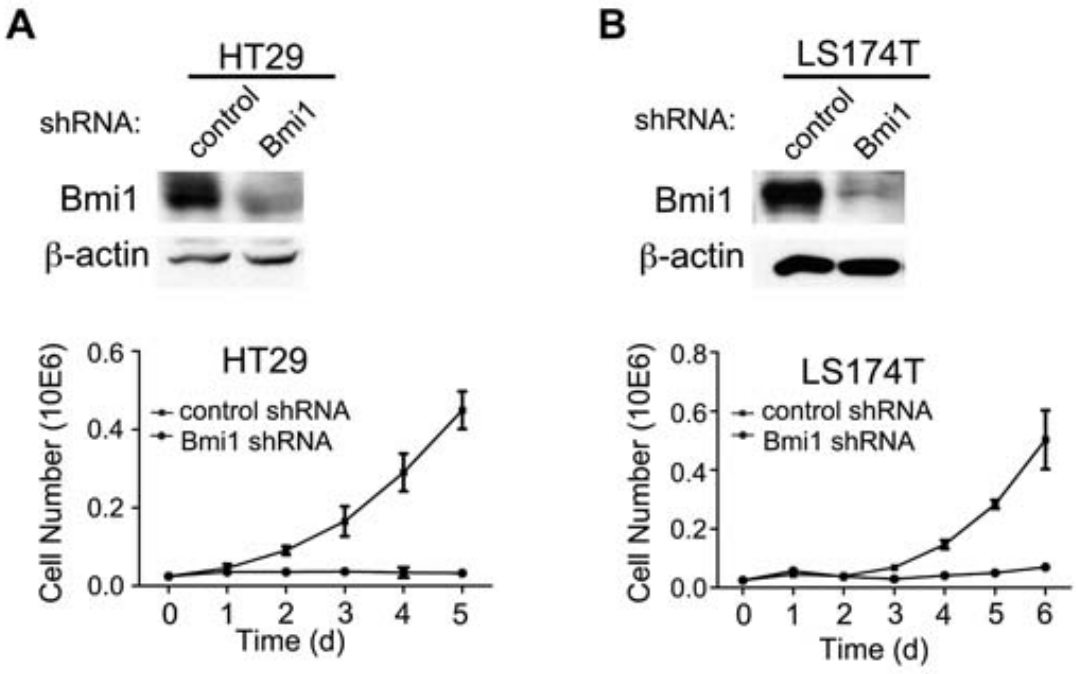

Figure 20: Bmi1 is essential for colon cancer cell proliferation. Growth curves of the stable cell lines that express lentivirus- infected control/Bmi1 shRNA. Bmi1 expression levels were indicated in stable HT29 (A) and LS174T (B) cell lines with lentivirus-induced shRNA compared to control shRNA ($P<0.0001$ between groups).

The tumor sections were analyzed by H&E staining and Alcian Blue (AB) staining (Fig. 21C). The Bmi1 shRNA treated tumors had significant increase levels of AB stainings, which is a marker for glycoprotein mucin (Fig. 21C). AB staining is also used to identify goblet cells in the normal intestine and used as a marker for colon cancer cell differentiation (van Es et al., 2005b). My finding suggests that Bmi1 down-regulation inhibits tumor progression. Previous work has demonstrated that KLF4 induced mucin expression in colon cancer xenografts (Zhang et al., 2006). The increase in AB positive cells in Bmi1 shRNA treated colon cancer xenografts is consistent with the hypothesis

that KLF4 represses Bmi1, inhibits proliferation and regulates differentiation in colon cancer.

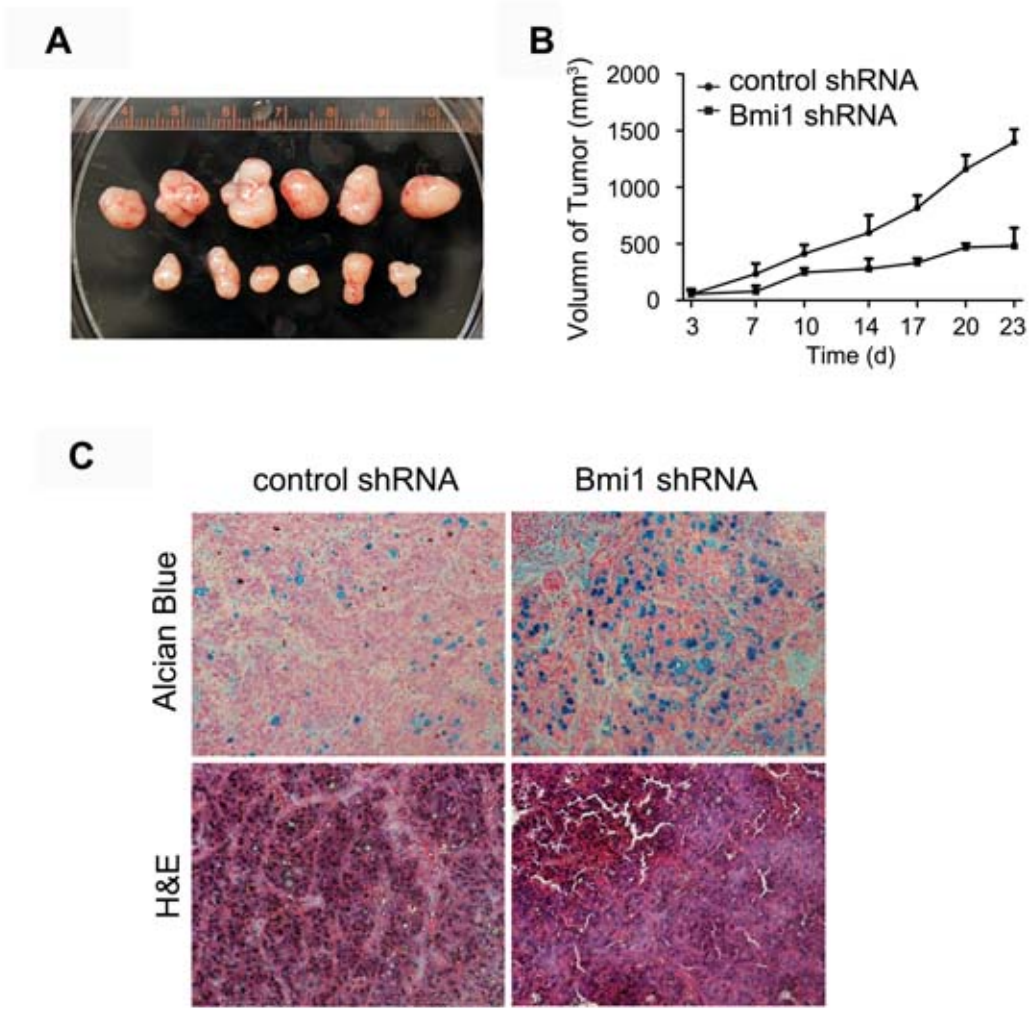

Figure 21: Bmi1 is essential for tumor formation. (A) Tumors from control (top) and Bmi1 (bottom) shRNA xenograft mice 23 days after cell injection. (B) Growth curve of tumors from control and Bmi1 shRNA xenograft mice (P=0.0002 between groups). (C) Alcian Blue (AB) and Hematoxylin and Eosin (H&E) staining of tumor tissues from control and Bmi1 shRNA xenografts.

DISCUSSION

As a Polycomb repressive protein, Bmi1 regulates a pool of genes and plays important roles in stem cell regulation and tumorigenesis. Activation of Wnt/β-catenin signaling is a hallmark of colorectal cancer; it interacts with many other signaling pathways in regulating both normal intestinal stem cells and cancer stem cells. This study delineated the mechanisms of Bmi1 regulation in colon cancer cells. I found that Wnt/β-catenin signaling enhances Bmi1 transcription and KLF4 represses Bmi1 transcription. KLF4 also represses H2A ubiquitination by inhibiting the Bmi1 complex. These findings suggest that Bmi1 is regulated by multiple mechanisms in colon cancer, and is essential for colon carcinogenesis.

Bmi1 and Wnt/β-catenin signaling overlap roles in stem cell self-renewal, including hematopoietic stem cells (Lessard and Sauvageau, 2003) and intestinal stem cells (Sangiorgi and Capecchi, 2008), and are important links between stem cell and cancer (Valk-Lingbeek et al., 2004). Wnt signaling regulates the expression of many stem cell markers, such as Lgr5 (Barker et al., 2009). Since Bmi1 is also a stem cell marker for the intestine, it is not surprising that Wnt/β-catenin signaling regulates the expression of Bmi1. Based on the TMA analysis of human colon cancer samples, the nuclear levels of β-catenin and Bmi1 have significant positive correlation in all tissue cores. My results are consistent with the well-known function of Wnt/β-catenin signaling in colon caner and the role of Bmi1 as an oncogene in many tissues. However, there is no consensus β-catenin/TCF binding site in the promoter region of Bmi1 gene. No binding between β-catenin/TCF and the Bmi1 promoter was detected by ChIP assay (not shown), suggesting that Wnt signaling indirectly regulates Bmi1 expression, probably through another β-catenin target. c-Myc is a well-known target of Wnt pathway (He et al., 1998). Within the Bmi1 promoter region, there is a c-Myc binding site and Bmi1 is a bona fide target of c-

Myc oncoprotein (Guo et al., 2007). It is possible that Wnt/β-catenin signaling regulates Bmi1 through c-Myc, which is an important mediator of Wnt signaling in colon cancer (Sansom et al., 2007).

In contrast, KLF4 directly binds the promoter of Bmi1 and represses Bmi1 expression. Although my finding from the ChIP assay suggests that the promoter sequence -233-0 is sufficient for KLF4 binding, no direct binding sequence was detected within this region. We cannot rule out the possibility that KLF4 indirectly binds -233-0 through another transcription factor or KLF4 binds additional site in Bmi1 promoter beyond this region. The role of KLF4 in stem cells and cancer is very complicated. KLF4 acts as a tumor suppressor in many cancers but may also act as a context-dependent oncogene (Rowland et al., 2005). KLF4 inhibits cell proliferation and induces cell differentiation; however, it is one of the key factors required for iPS cell self-renewal.

KLF4 is down regulated in most tumors but is also unregulated in a number of tumors, suggesting that the expression and function of KLF4 is dependent on the context of different tumors. For example, it has been suggested that the KLF4 acts as a tumor suppressor or oncogene depending on the status of p53, Ras and $p21^{CIP1}$ (Rowland et al., 2005). In the TMA study, the correlation between KLF4 and Bmi1 is not clear, because the level of KLF4 varies across grades 1, 2 and 3 in tumor tissues. KLF4 protein is most highly expressed in grade 3 of tumor tissues, probably because of additional genetic or epigenetic changes that altered KLF4 expression (Table 2).

Bmi1 is a member of the Polycomb complex that plays important roles in chromatin remodeling and gene silencing. I found that KLF4 repressed both Bmi1 and *RING1B*, another member of this complex, and repressed H2A ubiquitination. It was previously reported that KLF4 interacts with p300 and regulates histone acetylation (Evans et al., 2007). Regulating histone ubiquitination is novel function of KLF4. KLF4

acts as both transcriptional activator and repressor; it is not clear if Bmi1 can also act as a direct activator of transcription. It is important to further investigate the physiological roles of histone ubiquitination in cancer and stem cell biology.

shRNA knockdown experiments suggest that Bmi1 is required for colon cancer cell proliferation *in vitro* and *in vivo*. Interestingly, depletion of Bmi1 by shRNA not only inhibited cell growth, but also facilitated cell differentiation in xenograft tumors, as analyzed by mucin staining by AB. Mucin is a marker for goblet cells, which are regulated by KLF4 and Notch signaling. Inhibition of Notch signaling using a g-secretase inhibitor resulted in goblet cell differentiation in adenomas of Apc^{Min} mice (van Es et al., 2005b). Previous works have shown that KLF4 induced goblet cell differentiation in colon cancer xenografts (Zhang et al., 2006); this is consistent with the role of KLF4 in Bmi1 repression. The expression of KLF4 is also regulated by Notch pathway in the intestine (Ghaleb et al., 2008; Zheng et al., 2009); it will be interesting to learn if Notch signaling interacts with Bmi1 in the intestine. The demonstrated role of Bmi1 in xenograft tumor growth is consistent with previous report that overexpressed KLF4 inhibited xenograft tumor growth (Zhang et al., 2006) and with the finding that KLF4 inhibits Bmi1 as discussed above.

Our findings demonstrate that Bmi1 is deregulated in colon cancer by multiple factors. Bmi1 could be used as a marker for colon cancer diagnostics. My findings also demonstrate that Bmi1 is essential for colon cancer cell proliferation by regulating histone H2A ubiquitination. It is important to note that Bmi1 knockout mice are viable (Molofsky et al., 2005; van der Lugt et al., 1994), suggesting that Bmi1 is an ideal therapeutic target for human cancers, including colon cancer.

CHAPTER 7: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

GENERAL CONCLUSIONS

As a fundamental transduction pathway in regulating many of the cancer types, Wnt/β-catenin signaling and its crosstalk with other stem cell factors has provided profound mechanisms underlying normal development and cancer. The activity of /β-catenin, which is the hallmark of Wnt signaling, is over-expressed in many cancer types including colon cancer. This implicates the pro-survival role of Wnt activity in tumorigenesis (Giles et al., 2003), which is consistent with the original finding of Wnt overexpression in mammary tumors (Nusse and Varmus, 1982). However, Wnt cascades do not work alone to promote cell proliferation and overgrowth, nor are their functions restricted within a particular scope. Wnt not only cooperate with several major signal transduction pathways such as Notch, BMP, PI3K, EGF, K-Ras and Shh pathways, it also interacts with transcription factors including KLF4 (Zhang et al., 2006).

As a critical transcription factor and reprogramming factor, KLF4 plays more roles than just a tumor suppressor does. Functional activities of KLF4 start as early as during the embryogenesis, which contributes to directing cell lineage differentiation, regulating cell maturation, as well as restricting cell proliferation. KLF4 binds to promoters of different genes in a series of transduction pathways and thus functions in manipulating transcription of target genes. The underlying mechanisms are majorly through competitive inhibitory binding with co-repressors or co-activators of the gene promoter. KLF4 crosstalk with Wnt/β-catenin is by transcriptional inhibition of β-catenin recruitment of CBP as well as inhibition on CBP-mediated β-catenin acetylation (Zhang et al., 2006).

As an oncogene, a Polycomb group protein, and a stem cell marker, Bmi1 not only indicates advanced stage of cancer and poor survival, but also manipulates a large group of genes through regulating histone modification, chromatin remodeling, and gene silencing status. As many other oncoproteins, Bmi1 expression has been analyzed in many types of cancers and is a good object when manipulated in mouse model studies for development of targeted therapies for cancer cure. In addition, analysis of normal and cancer tissues has been greatly facilitated using Bmi1 as a protein marker to identify the cells with stem cell characters (Sangiorgi and Capecchi, 2008).

In the context of colon cancer, this dissertation firstly discusses about functional role of KLF4 in regulating proliferation, differentiation pattern and cell polarity and maintaining homeostasis of the small intestine. The original finding that KLF4 depletion in mouse small intestine led to change in numbers of Goblet cells, Paneth cells and stem cells brought about the hypothesis that morphology change was due to loss of function of KLF4 in regulating proliferation, differentiation and cell polarity. Both studies from the conditional knockout mouse model and the 3D culture studies demonstrated KLF4 to be a regulator of cell fate and number, as well as polarity.

In the context of Wnt/β-catenin crosstalk with KLF4, this dissertation also focuses on investigating the role of KLF4 mediating colon cancer cell proliferation; and mechanisms mainly involve Bmi1 and its function in histone modification. As the well known Wnt target gene and intestinal stem cell marker Lgr5, Bmi1 expression turned out to be affected both by Wnt and by KLF4 in my primary study. Profound mechanisms had been further investigated in colon cancer cells as well as xenograft mouse models. KLF4 is a critical regulator by binding to Bmi1 promoter and inhibiting its activity. As to the function in histone modification, KLF4 demonstrated to inhibit Bmi1-mediated H2A ubiquitination. However, Bmi1 is not the only histone regulator in the Polycomb

repressive complex 1 (PRC1) that is affected by KLF4. Other components such as Ring proteins are regulated by KLF4 as well.

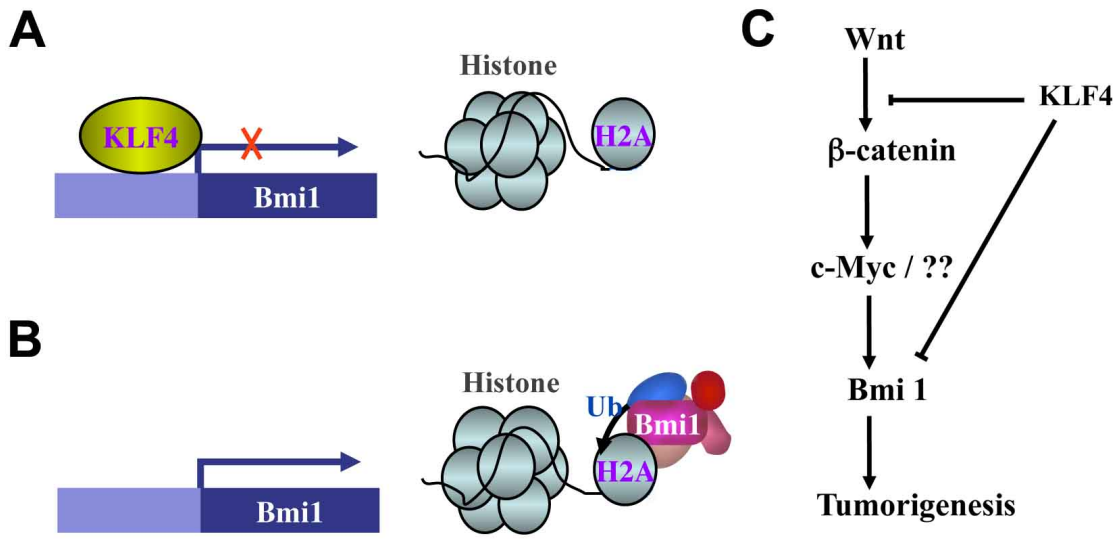

Figure 22: Model of KLF4 regulation in Bmi1-mediated cell proliferation. (A) Bmi1 regulates expression of a group of genes through modulating H2A ubiquitination. Under normal condition, KLF4 inhibits promoter of Bmi1, which maintains the homeostasis and inhibits tumor growth. (B) Dysregulated KLF4 results in hyper-activation of Bmi1, leading to abnormal proliferation and tumor growth. (C) Chart of Wnt-KLF4-Bmi1 regulation in colorectal tumorigenesis.

The overall findings of this dissertation are summarized as shown in Fig. 22. Bmi1 regulates expression of a group of genes through modulating H2A ubiquitination. Under normal condition, KLF4 inhibits promoter of Bmi1, which maintains the homeostasis and inhibits tumor growth (Fig. 22A). However, dysregulated KLF4 results in hyper-activation of Bmi1, leading to abnormal proliferation and tumor growth (Fig. 22B). And the cooperative effect of Wnt-KLF4 manipulates cell proliferation and tumorigenesis through a Bmi1-mediated mechanism (Fig. 22C).

FUTURE DIRECTIONS

In my previous study of optimizing culture conditions with chemical-defined medium for hES cells (Liu et al., 2006), p53 pathway was found to be extraordinarily active in human ES cells. Different from mouse ES and other cell types, hES cells undergo transcription-independent mitochondria apoptotic pathway under DNA-damage conditions, which make them very susceptible to DNA-damage-induced spontaneous apoptosis and differentiation. The finding of effect of p53 down-regulation provided great insight of improving survival and self-renewal of hES cells by targeting p53 pathway (Qin et al., 2007). It is worthwhile testing whether the stem cells residing at the crypt bottom of small intestine also partake transcription-independent apoptotic pathway, which would provide implications for targeted therapies for intestinal and colorectal cancers.

In addition to p53-mediated apoptotic pathways, Wnt/β-catenin and KLF4 pathways as well as Bmi1-mediated epigenetic pathways, it is interesting to investigate other potential pathways that are involved in regulating and maintaining homeostasis of the small intestine.

Study of primary cancer cells

Stem cell isolation has been challenging using traditional cell sorting with markers such as CD133, because single cells are difficult to grow or self-renew in culture medium, and CD133 is not likely a specific marker for colon cancer stem cells (Shmelkov et al., 2008). Using the established model of primary tumor cell culture, studies of Wnt/KLF4 pathway in stem cell regulation and colorectal tumorigenesis could be performed to further delineate the mechanism of cancer initiation and progression.

Primary tumor cell lines could be prepared from colon cancer patients based on protocol as described (Kondo et al., 2011).

The primary cell line preparation is based on the following procedure. Tumor tissues from patient are to be isolated and immediately put in DMEM, rinsed and minced into small pieces, digested with a combination of mild digestion enzymes, which effectively dissociates the tissue pieces into organoids. Digested tissue pieces will be then filtered through 500um filter; the flow-through will be re-filtered and pieces between the size of 40-500 will be collected, followed by suspension culture in hESC medium to keep the cell actively dividing and self-renewing. Maintaining the cell-cell contact and activation of growth-supportive pathways are key conditions that support the primary cell growth. Even within 2 days of culture, the cells form very nicely shaped spheroids called colon tissue originated spheroids (CTOSs) (Fig. 23).

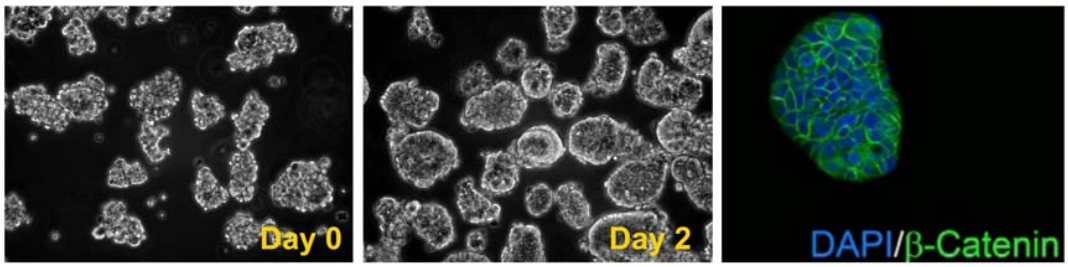

Figure 23: Growth of colon tumor originated spheroids (CTOSs) in Day 0 and Day 2 of suspension culture in human ES cell medium.

In addition to suspension culture, CTOSs from colorectal tumor tissues are also capable of growing in three-dimensional (3D) culture, which is composed of reconsitituional collagen solution and covered by hESC medium (Fig. 24). 3D culture system is capable of maintaining the CTOS growth for weeks to months depending on

cases of patients; and 3D culture can support a higher growth rate than suspension culture. Conditions could be optimized using alternative way of Matrigel 3D culture method, which is supportive to Caco-2 cell growth and lumen structure formation (Yu et al., 2012b). This model can be used to develop assays for cancer stem cell study as well as drug screening for cancer therapies.

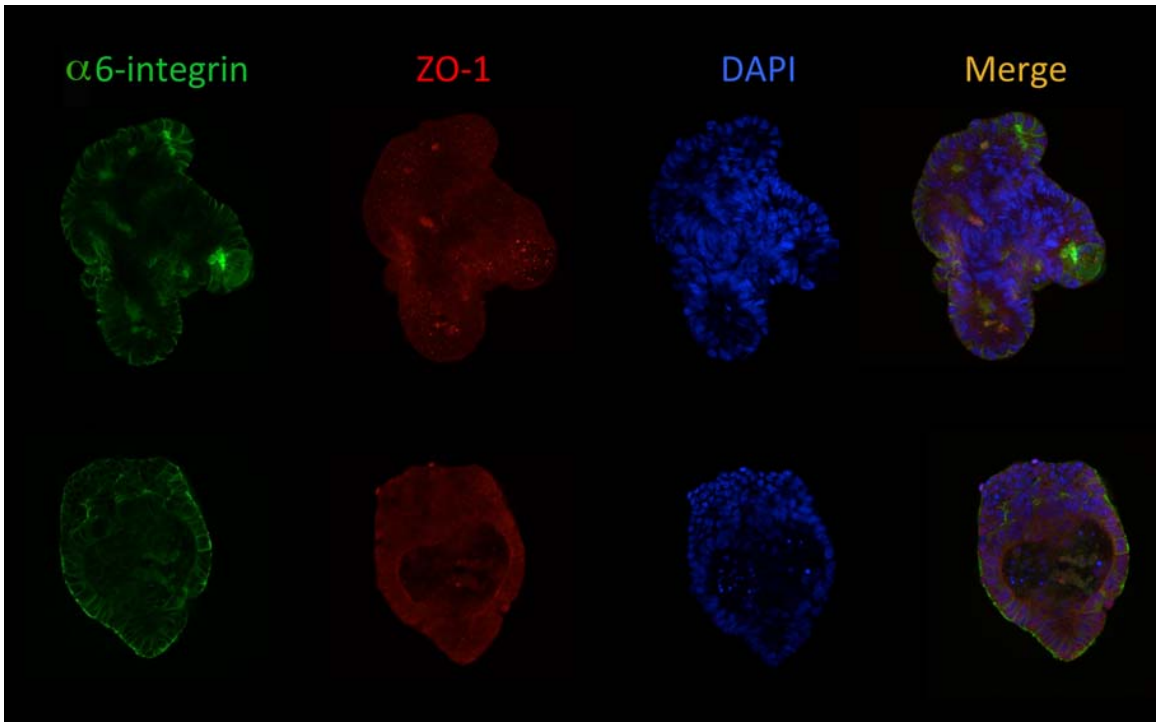

Figure 24: Immunofluorescent staining of colon tumor originated spheroids (CTOSs) with α6-integrin and ZO-1 antibody, after 2-month suspension cultures in human ES cell medium (top panel) and collagen 3D culture (bottom panel), respectively.

It is interesting to study mechanisms under which normal stem cells differ from cancer stem cells, and how cancer originate *via* abnormality in stem cell regulation. Investigations in the field of cancer research with relate to stem cell biology have become more interesting and of greater value in terms of development of targeted therapies for

cancer. Therefore, future directions of my study will not only focus on stem cell mechanisms, but also applications in cancer stem cells.

Study of Wnt/β-catenin/KLF4-Bmi1 in other cancer types

Signaling pathways involved in cancer and stem cell regulation may apply in more than one cancer type. And thus I would extend the study of Wnt/KLF4 signaling pathway and Bmi1 regulation to other cancer types. Study of KLF4 in normal epithelial cells and carcinogenesis extends to the skin tumorigenesis study, which demonstrated that KLF4 depletion in the skin led to increased cell proliferation and facilitated tumorigenesis (Li et al., 2012). It is of great interest to know whether the mechanisms of Wnt/KLF4-Bmi1 regulation also apply in other cancer types for example breast cancer, especially in controlling the status of stem cells.

Studies of epithelial-mesenchymal transition during cancer progression provide implications for mechanisms of stem cell regulation especially in breast cancer. Both normal mammary stem cells and breast cancer stem cells express markers associated with EMT (Mani et al., 2008). The metastatic gene Slug cooperates with stem cell gene Sox9 to regulate normal mammary stem cells and breast cancer stem cells (Guo et al., 2012). Due to lack of efficient technique to isolate stem cells from stromal cells or myoepithelial cells, the defining factors and mechanisms underlying self-renewal, dedifferentiation and malignancy remain elusive. Crosstalk of signaling pathways from interactive microenvironment also contributes to the complications in searching for origins of cancer.

Since Wnt signaling regulates self-renewal and differentiation state in many types of cancers including breast cancer, it is important to study how Wnt regulates stem cells during breast cancer initiation, progression and metastasis. Wnt/β-catenin crosstalks with

KLF4 to regulate intestinal homeostasis (Zhang et al., 2006). Thus, Wnt, KLF4 and other signaling pathways involved in stem cell and cancer cell regulation are worth investigating. It is possible that Wnt and KLF4 regulate metastasis genes in mammary stem cells and breast cancer stem cells.

As a Polycomb group protein and an oncogene, Bmi1 might be targeting one or more of the stem cell genes (slug, snail, twist, sox9, sox10…) by regulating their histone status. And/or, as a stem cell marker, Bmi1 might play a parallel roll with Slug and Sox9 to manipulate stem cell state and transition between self-renew and differentiation.

Investigation could be performed to test whether Wnt, KLF4 and Bmi1 regulate functions of the stem cells using MaSC assay *in vivo* and *in vitro* as described (Guo et al., 2012), and to test whether it is correlated with Wnt/β-catenin and KLF4 (assays as described) (Zhang et al., 2006). In addition, tissue array could be used to analyze the expression of β-catenin, KLF4, Bmi1 and other stem cell genes comparing normal mammary tissue with breast cancer tissue. Gene expression analysis could be made to compare the profile of signaling pathways involved in normal mammary cells and breast cancer cells. Xenograft assay and metastasis analysis could be performed to test the ability of breast cancer cells to initiate tumor with the effect of Wnt, KLF4, Bmi1 and other stem cell regulators.

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Publications

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Posters

Tianxin Yu and Chunming Liu. Mechanism of Bmi1 Regulation in Colorectal Tumorigenesis. (2010) Digestive Disease Week conference

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Tianxin Yu, Juan Li, Ren Xu, Xi Chen, Wen Zhang, Timothy Wang, Walden Ai, Chunming Liu. Krüppel-like factor 4 Regulates Intestinal Epithelial Cell Morphology and Polarity. (2011) Digestive Disease Week conference, selected poster presentation; also selected as poster presentation on University of Kentucky Department of Molecular and Cellular Biochemistry Summer Research Conference

Tianxin Yu, Xi Chen, Wen Zhang, Deannon Colon, Jiandang Shi, Dana Napier, Piotr Rychahou, Wange Lu, Eun Y. Lee, Heidi L. Weiss, B. Mark Evers, Chunming Liu. Regulation of Bmi1 by β-catenin and KLF4 and its Implication in Colon Cancer (2012). American Association for Cancer Research Annual Meeting. Recognized as top 2.5% Distinguished Poster Presentation

Invited Speeches

Jun Yang, Wen Zhang, Xi Chen, Tianxin Yu, Jianhang Jia and Chunming Liu. Identify and Characterize a Regulatory Subunit of PP2A that Regulates PP2A-mediated b-catenin Dephosphorylation. (2009) Selected oral presentation on Wnt Meeting

Tianxin Yu. Mechanism of Bmi1 Regulation in Colorectal Tumorigenesis. (2010) Oral presentation on University of Kentucky Department of Molecular and Cellular Biochemistry Summer Research Conference

Tianxin Yu. KLF4 regulates intestinal epithelial cell morphology and polarity. (2011) Invited presentation on Markey Cancer Center Research Day.

Tianxin Yu. Krüppel-like factor 4 Regulates Intestinal Epithelial Cell Morphology and Polarity. (2011) Oral presentation on University of Kentucky Department of Molecular and Cellular Biochemistry Summer Research Conference

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