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Xi He

Characterization of Key Signaling Pathways in Homeostasis and Cancer

For the Degree of Doctor of Philosophy by Published Work

Stowers Institute for Medical Research
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List of Abbreviations

5FU: 5-Fluorouracil
APC: Adenomatosis Polyposis Coli
APC: Antigen-Presenting Cell
APC<sub>min</sub>: An animal model in which a truncation mutation occurs at the 805 aa site of APC protein leading to loss of function of APC
Axin: A Wnt signaling inhibitor
Akt: A serine/threonine-specific protein kinase and downstream of PI3K.
Bad: BCL2 Associated Agonist of Cell Death
B-cell: B Lymphoid
BM: Bone Marrow
Bmi1: B Lymphoma Mo-MLV Insertion Region 1 Homolog, a Polycomb ring finger protein
BMP: Bone Morphogenic Protein
Bmpr: Bone morphogenic protein receptor
BrdU: 5-bromo-2′-deoxyuridine
Celecoxib: A selective Cox2 inhibitor
CAR: CXCL12-abundant reticular cell?
CBC: Crypt Base Columnar
CiDU: A BrdU analogue
CCL: Chemokine ligand
CCR: Chemokine receptor
Cdc42: Cell division cycle 42
CLP: Common Lymphoid Progenitor
CMP: Common Myeloid Progenitor
Cox: Cyclooxygenase
CRC: Colorectal cancer
CSC: Cancer Stem Cell
CSF: Clone stimulating factor
Csf1r: Clone stimulating factor1 receptor
CX3CR1: C-X3-C Motif Chemokine Receptor 1
DC: Dendritic Cell
DXR: Doxorubicin
DKK: Dickkopf-related protein, a Wnt inhibitor
EM: Electron Microscope
EP: Prostaglandin E receptor
CX3CR1: C-X3-C Motif Chemokine Receptor 1
DC: Dendritic Cell
DXR: Doxorubicin
DKK: Dickkopf-related protein, a Wnt inhibitor
EM: Electron Microscope
EP: Prostaglandin E receptor
ESC: Embryonic Stem Cell
FOP: For mutant TOP (see TOP)
FOXO: Forehead Box Transcription
Fzd: Frizzled, Wnt receptor
Gal: Galactosidase
GFP: Green Fluorescent Protein
GO: Gene Ontology
GSK3: Glycogen Synthase Kinase 3
HSC: Hematopoietic Stem Cell
HR-BC: Hormone Receptor-Positive Breast Cancer
ICM: Inner Cell Mass
ID2: Inhibitor of DNA Binding 2
IFNg: Interferon Gamma
ISC: Intestinal Stem Cell
JIP: Juvenile Intestinal Polyposis
JPS: Juvenile Polyposis Syndrome
Ki67: Nuclear protein that is associated with cellular proliferation
KO: Knock out
LacZ: LacZ β-galactosidase
Lef: LEF1 substitutes for TCF2, a Wnt signal repressor
Lgr5: Leucine rich repeat containing G protein-coupled receptor
LRC: Label Retaining Cell
Lrp: Lipoprotein receptor-related protein, a coreceptor for Wnt
LSC: Leukaemia Stem Cell
LTR: Long-Term Retaining
Luc: Luciferase
Ly6c: Lymphocyte antigen 6 complex, a monocyte marker
MC: Mesenchymal Cell
Mcl1: Myeloid Cell Leukaemia 1
MDC: Myeloid Derived Cell
MDSC: Myeloid Derived Suppressor Cell
Mφ: Macrophage
MPP: Multipotent Progenitor
mTOR: Mammalian Target of Rapamycin
Myc: Myc oncogene
Mx1: Interferon inducible promoter
Nfatc: Nuclear factor of activated T cells
NfκB: Rel/NF-κB transcription factors
NK cell: Natural Killer Cells
NSC: Neural Stem Cell
Noggin: A BMP inhibitor
NSCLC: Non-Small Cell Lung Cancer
P53: Tumor Protein 53
PCA: Principal Component Analysis
PDK: Phosphoinositide-dependent kinase-1
PGE2: Prostaglandin E2
PTGER4 (EP4): Prostaglandin E2 receptor 4
PI3K: Phosphoinositide 3-kinase
PIP: Phosphatidylinositol protein
PTEN: Phosphatase and Tensin homolog
p-PTEN: Phosphate Phosphatase and Tensin homolog
R26-LSL: A transcription stop site in the Gt (ROSA)26S or locus
RCC: Renal Cell Carcinoma
RNA-seq: RNA sequencing analysis
SCLC: Small Cell Lung Cancer
SCF: Stem Cell Factor
SEM: Scanning Electron Microscope
SMAD: Mediates TGF and BMP family of signaling transduction
SNO: Spindle-shaped N-cadherin*CD45* osteoblastic
TA: Transit Amplifying
T-ALL: T acute lymphoid leukemia
TAM: Tumor associated macrophage
TAFs: Tumor Associate Fibroblasts
TBA: Trabecular Bone Area
T-cell: T Lymphoid
TCF: T-cell Factor
TME: Tumor Microenvironment
Wnt: Wingless
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1. Overview
1.1 General introduction

1.1.1 Stem cell signaling in tissue health and cancer genesis

Stem cells play a pivotal role in maintaining tissue homeostasis and have garnered significant attention for their involvement in cancer initiation and progression. Here, I summarize key concepts to be addressed in the thesis aimed at unraveling the intricate signaling mechanisms that regulate both normal stem cells and cancer stem cells (CSCs) and shedding light on their impact on tissue health and cancer genesis.

Stem cells, which are characterized by their ability to self-renew and their capacity to give rise to various cell lineages and differentiated to mature cell type, are crucial for tissue maintenance. They reside within specialized microenvironments, called niches, that provide essential regulatory cues (Figure 1, upper panel). In reference to the niche, stem cells undergo asymmetric or symmetric division. Asymmetric division generates two daughter cells: one

![Figure 1: Comparison of the niches under normal and cancerous conditions.](image)

remains a stem cell if remains within the niche, while the other gives rise to progenitor cells.
that undergo non-self-renewal proliferation and subsequent differentiation. Symmetric division can produce either two daughter stem cells or two progenitor cells. These cells contribute to tissue regeneration, ensuring the continuity of normal physiological processes. However, dysregulation of stem cell development, such as in the case of unregulated proliferation, can lead to the formation of tumors (Figure 1, lower panel).

Understanding these processes has been a central focus of normal stem cell regulation and CSC dysregulation. Tumors can arise from uncontrolled proliferation of stem cells or progenitor cells with acquired self-renewal ability. The concept of CSCs has revolutionized therapeutic strategies by recognizing the pivotal role these cells play in tumor development.

My thesis aims to decipher some of the signaling pathways governing both normal and cancer stem cells, emphasizing the importance of maintaining a delicate balance between pro- and anti-proliferative signals. Perturbation of this balance can result in uncontrolled cell growth and impaired differentiation, ultimately leading to tumorigenesis.

To investigate the mechanism that governs stem cell proliferation and differentiation, I studied the behavior of cells in the intestinal and skin epithelium, as well as hematopoietic cells within the bone marrow. These tissues are characterized by high rates of turnover and significant regeneration capacity. In addition, these tissues and organs have been previously studied with known stem cell markers or methods to identify stem cells, thus making them ideal models for understanding stem cell regulation. Perturbation of the development of the bone marrow or intestine can lead to diseases such as leukemia and colorectal cancer respectively, making them valuable for studying the behavior of stem cells during cancer genesis.

1.1.2 Aims

**Rationale:** Stem cells reside in a niche, where intricate signaling maintains them in an undifferentiated state and ensures a balance between inhibition and promotion of proliferation. This leads to my general hypothesis: Perturbation of the control of proliferation, or the balance between proliferation and differentiation leads to overproduction of stem cells, resulting in the initial step of tumorigenesis. My thesis focused on testing this hypothesis via investigation of the following specific signaling pathways in Stem Cell regulation: I investigated
how disruptions in Wnt and BMP pathways impact stem cell behavior, potentially shifting their development toward increased proliferation at the expense of reduced differentiation.

I. **The PTEN-Akt pathway in converging BMP and Wnt signalling:** I examined the central role of the PTEN-Akt signalling pathway in mediating the interplay between Wnt and BMP signalling, with disruptions exacerbating Tumour-initiating stem cell (TSC) proliferation.

II. **The Cox2-PGE2-EP4 Pathway in TSC microenvironment:** I explored alterations in the Cox2-PGE2-EP4 pathway within the tumour microenvironment and how it fosters a niche that is conduct conducive to TSC survival and growth.

III. **Cross talk between TSCs and Tumour Microenvironment (TME):** I studied how the alterations in TME-associated BMP, PTEN-Akt, and Cox2-PGE2-EP4 signals promote TSC proliferation and self-renewal.

**Objectives:** By dissecting how these signalling pathways affect normal and cancer stem cells, my thesis seeks to deepen our understanding of stem cell regulation in healthy tissues and the dysregulation in cancer, thereby providing valuable insights into tumorigenesis. while also identifying potential therapeutic targets.

1.2 Specific introduction of the biological systems used in this work

1.2.1 Organization of the small intestine

Figure 2 illustrates the internal architecture structure of the intestine, which is essentially composed of laterally positioned crypts region medially positioned villi. Each crypt region primarily houses intestinal stem cells (ISCs), transit amplifying (TA) cells, and Paneth cells. In contrast villus comprises various differentiating epithelial cells as elaborated below:

- Enterocytes, characterized by apical microvilli on the surface, which absorb nutrients,
- Goblet cells, which are filled with mucus granules.
- Paneth cells are responsible for secreting anti-microbial peptides and are therefore packed with large granules.
- Enteroendocrine cells, which release neuropeptides.
The intestine comprises two primary structures: the crypt (lower part) and the villus (upper part). Intestinal stem cells (ISCs) give rise to transit amplifying cells, which are lineage progenitors that reside within the crypt. While most differentiated cells migrate into the Villus. Paneth cells are uniquely situated at the bottom of the crypt. A: Section, B: Cartoon. (Bjerknes and Cheng, 1999). ISC: Intestinal Stem Cell, TA: Transit Amplifying, MC: Mesenchymal Cell

The cell lineages in each villus are meticulously organized. During villus differentiation, there is a rapid medial migration from the lateral crypt to the villus tip, culminating in the cells being shed into the lumen at the villus’s apex. The cells shed into the lumen are constantly replenished by new differentiated cells originating from the crypt. As cells continuously migrate and mature, their position within the villus indicates their developmental stage. Therefore, within each villus-crypt unit, the intestinal stem cells, transient amplifying (TA) progenitors, functionally mature cells, and apoptotic cells occupy specific, identifiable regions (Figure 2)
1.2.2 Hematopoietic and hair follicle systems

Hematopoietic stem cells (HSCs) produce all the blood cell lineages, including erythroid, myeloid, lymphoid, and megakaryocyte. Marker-based cell sorting and transplantation in lethally irradiated recipients have been essential tools for assessing HSC function (Weissman and Baltimore, 2001). Quiescent HSCs, identified through the long-term label retaining cell (LRC) approach, predominantly reside in the endosteum (Wilson et al., 2008; Zhang et al., 2003b).

![Diagram illustrating the locations of stem cells in bone marrow and hair follicles.]

**A.** Hematopoietic stem cells in bone marrow reside in two distinct locations. Endosteal (inner bone surface) niches are enriched in more quiescent stem cells LRCs, while central marrow vessels are enriched in relatively more active and proliferating HSCs, supported by CARs. Hematopoietic stem cells (HSCs), LRCs (Label retaining cells), CARs (CXCL12-abundant reticular cells).

**B.** Hair follicle stem cells occupy various locations throughout the body. Quiescent cells are in the bulge; while active, proliferating stem cells reside in the hair germ.

In contrast, primed and active HSCs are enriched in the central marrow near arteriolar or sinusoidal niches (Kiel et al., 2005; Mendez-Ferrer et al., 2010) (Figure 3A).
Hair follicle stem cells were initially identified by the LRC approach in the bulge region, which is enriched with quiescent stem cells (Tumbar et al., 2004). These bulge stem cells don’t directly produce TA cells but give rise to active (or primed) stem cells in hair germ (Greco et al., 2009). The active stem cells in hair germ migrate to the dermal papilla and then produce TA cells, which differentiate into the hair shaft within hair matrix (Figure 3 B).

1.2.3 Tumor-initiating stem cells (or CSCs) and their microenvironment in Intestine

Intestinal tumorigenesis, encompassing the initial formation of adenoma, followed by the development of carcinoma, is a multifaceted cascade of events. It begins with localized

![Figure 4. Illustration showing the sequential accumulation of genetic mutations that leads to tumorigenesis Pathohistological sections of various adenoma stages visualized using H&E staining. (Cell Rep, 2021 Publication 10). APC: Adenomatosis Polyposis Coli, Cox2: Cyclooxygenase2. PTEN: Phosphatase and Tensin homolog, P53: Tumor Protein 53](image-url)
abnormal proliferative regions or abnormal hyperplastic structures and then progresses through invasion of surrounding tissue by tumor cells exhibiting immature characteristics (adenocarcinoma in situ). This is followed by deeper invasion into the muscular layer, resulting in invasive adenocarcinoma. Ultimately, metastasis takes place when cancer cells spread from the primary tumor and establish new tumors in distant tissues and organs (Figure 4).

During each phase of tumorigenesis, a combination of mutations and aberrant changes in signaling pathways leads to upregulated proto-oncogene activity and the inactivation of tumor suppressor function. These signaling pathways include Wnt- b-catenin, Kras and its downstream mitogen-activated protein kinase (MAPK), BMP/TGFβ, PTEN-regulated PI3K and AKT, and TP53 (Fearon and Vogelstein, 1990; Jackstadt and Sansom, 2016). A primary challenge to date has been finding a mouse colorectal cancer (CRC) model suitable for tumor/cancer studies, which requires the use of a large cohort of animals. For the study of TSC-Tumor microenvironment interaction, I selected Apc<sup>min/+</sup> mutant mice. These mice provide a straightforward tumor model, which consistently develop adenoma with a 100% occurrence rate, effectively mirroring human familial adenomatous polyposis (Groden et al., 1991; Miyoshi et al., 1992)

1.2.4 Tumor Microenvironment (TME) in the Intestine

In my 2004 report (Publication 2), I showed that BMP4 is expressed in mesenchymal cells located adjacent to label retaining ISCs, where it functions as a niche signal regulating ISC quiescence in the intestine. However, in the context of tumorigenesis, the mechanisms by which the TME enables TSC survival against therapeutic challenges, and subsequently supports tumor regrowth post-chemoradiotherapy, remain largely unclear (Li and Neaves, 2006). As depicted in Figure 5, the TME is composed of tumor-associated stromal cells (which support tumor structure and regulate its microenvironment), tissue-resident and recruited innate immune cells, together with adaptive immune T and B cells, as well as blood vessels, lymph, neurons, adipocytes, and microbiome. (Borovski et al., 2011; Quail and Joyce, 2017).
Emerging evidence indicates that macrophages play a pivotal role in the TME (Carvalho et al., 2018; Chen et al., 2018; Guadagno et al., 2018; Poh and Ernst, 2018). Macrophages, a type of leukocyte, possess innate immune functions such as phagocytosis and are critical for activating adaptive immunity. Consequently, macrophages exhibit significant heterogeneity in both their function and phenotype. In colon cancer solid tumors, particularly tumor-associated macrophages (TAMs), play a key role in the tumor microenvironment (TME). TMEs have been extensively studied to understand their impact on cancer progression and metastasis. Recent advancements in single-cell RNA sequencing (scRNA-seq) have further nuanced our understanding of TAMs in solid tumors, revealing a heterogeneity that extends beyond the binary M1/M2 classification (Wang et al., 2024). In mice, two distinct phenotypes of MDSCs have been observed: monocytic (M-MDSCs) and granulocytic (PMN-MDSCs), which have different roles and developmental pathways but both contribute to the tumor’s ability to evade the immune response.
response. MDSCs have been shown to suppress T cells, NK cells, dendritic cells, and macrophages, establishing them as potent suppressors of the immune response against tumors (Figure 6).

TAMs release key signals that promote cancer cell survival and proliferation, stimulate angiogenesis, suppress T cell accesses and activity against tumor cells (Gajewski et al., 2013; Grivennikov et al., 2010; Noy and Pollard, 2014; Qian and Pollard, 2010). However, the dynamics of the interactions between TAMs and TSCs and their role in the emergence of drug-resistance remained largely undefined. In many cancers, macrophages serve as one of the primary sources of PGE2. This compound is also produced by fibroblasts and malignant epithelial cells, especially during advanced stages of cancer development (Aoki et al., 2017; Harizi, 2013; Shao et al., 2006; Zelenay et al., 2015). The modulation and impact of PGE2 signaling on ISCs and CSCs in colorectal cancer however remain largely unexplored. See Publication10

In publication 10, I showed that in response to therapeutic stress, CSCs recruit tumor associate macrophages and monocytes (TAMMs) as their niche. TAMMs are the main source of PGE2, which in turn promotes CSC proliferation via the PGE2-EP4 signaling pathway. Furthermore, PGE2 is known to stimulate b-catenin activity, however, the underlying mechanism remained unknown. In this work, I found that that Akt/PKA (cAMP activated protein Kinase) phosphorylation can link PGE2/EP4 and b-catenin.

1.2.5 Immune phenotype in colorectal cancer (CRC)

Over the last decade, immune checkpoint blockade (ICB), has improved cancer treatment (Krummel and Allison, 1995). A significant challenge with immunotherapy is the limited T cell infiltration into the tumor. This subsequent review delves into the relationships between immune-response phenotypes across various cancer types. Based on the spatial distribution of CD8+ T cells within the tumor microenvironment, three distinct immunophenotypes can be identified: Immune inflamed, excluded, and desert (Figure 6).
❖ The immune inflamed category is defined by robust Interferon gamma (IFNγ) signaling, which promotes MHC-I gene expression and facilitates MHC-I mediated tumor antigen expression.

❖ The immune excluded category is defined by the presence of myeloid derived suppressor cells (MDSCs), that hinder CD8+ T cell infiltration to the tumor site through TGFβ and IL-10 signaling.

❖ The Immune desert category is characterized by high levels of fatty acid metabolism and dominant Wnt-β-catenin signaling, both of which impede the infiltration of CD8+ T cells into the tumor (Hegde and Chen, 2020).

Colorectal cancer (CRC) typically exhibits an “immune excluded’ immunophenotype, evident by its predominant TGFβ signaling and presence of MDSCs. In this case, CD8+ T cells accumulate at the tumor boundary region but fail to infiltrate into the tumor (refer to Figure 6).
Figure 6. Illustration showing the categories of immune responses to immune therapies.

Three immunophenotypes have been identified: Inflamed, Excluded, and Immune Desert. Inflamed immunophenotypes have numerous infiltrating but inactive immune cells, activate IFNγ signalling, and enhanced MHC-I gene expression, and is responsive to PD-1 therapy. The Excluded type, marked by MDSCs, prevents Cd8+ T cell infiltration into tumor through TGFβ and IL-10 signalling. The Desert type, which is dominated by fatty acid metabolism and Wnt-β-catenin, does not respond to Pd-1 therapy.

IFNg: Interferon Gamma, CD8T: Cd8 T Lymphocyte, TGFβ: Transforming Growth Factor-β, MDSC: Myeloid Derived Suppressor Cell, Wnt: Wingless
1.3. Introduction to signaling pathways involving stem cell regulation

Here, I briefly introduce the signaling pathways intricately involved in the regulation of stem cell behavior, encompassing self-renewal, proliferation, and differentiation processes. Moreover, I explore the crucial effects of dysregulation of these pathways, which can trigger tumorigenesis.

The formation of a complex multicellular organism from a single cell stands as one of the most remarkable processes in developmental biology. This process is under the tight control of dynamic signaling pathways, orchestrating proliferation, migration, differentiation, and cell interactions that lead to morphogenesis and organogenesis. Perturbation of the precise control of these developmental processes, such as in the case of hyper-proliferation, often leads to tumorigenesis.

1.3.1 The BMP pathway

BMPs are secreted molecules belonging to the transforming growth factor (TGF) family. More than 15 BMPs have been identified in mammals, and their activity is spatiotemporally dynamic (Mishina, 2003). According to established models (Figure 7), BMP ligands induce phosphorylation of type-I receptor a or b (1a or 1b) by type-II receptor (R-II) (Massague, 1998), and BMP signaling is then propagated via Smad transcriptional factors 1, 5, and 8, together with Smad4, which is then transduced from the cytoplasm into the nucleus.

In bone marrow, the BMP signal induces mesenchymal stem cells (MSCs) to undergo osteoblastic differentiation through the Bmpr1b receptor, which is predominantly expressed in MSCs (Chen et al., 1998).

In Publication 3, we investigated the effects of conditional (Mx1Cre: induced by IFNγ or polyI:polyC) inactivation of BMP receptor 1a (BMPR1a) in MSCs in mice, and observed that blocking BMP signaling via BMPR1a leads to ectopic trabecular bone formation. In addition, Mx1Cre is also expressed in hematopoietic stem cells (HSCs), but BmpR1a is not. Unexpectedly, however, in parallel with the bone phenotype, we observed an increase in the number of HSCs that was highly correlated with increased ectopic trabecular bone formation (Zhang et al.,
2003a). Where HSC resides in bone marrow was not previously known. This phenotype helped us to determine that HSCs, especially the deep-quiescent HSCs as identified by long-term DNA label retaining cell (LRC) assay, were mainly located in trabecular bone area (sponge-like bone structure beneath the growth plate of the femur) instead of central marrow covered by compact bone (main part of femur between trabecular bones).

These findings highlight the role of BMP signaling in controlling HSC niche size (specifically here the trabecular bone area), which in turn controls HSC numbers. So BMP has no effect on HSCs directly. This observation not only allowed us to identify the HSC niche in the endosteal region of trabecular bone, but also offered valuable insights into the effects of disrupting the BMP pathway on stem cells and their niche.

**Figure 7. A schematic illustration of the BMP signalling pathway as a series of sequential protein interactions**

BMP binding to R-II and R-I triggers R-II to phosphorylate R-I. Phosphorylated R-I acts as a serine-threonine kinase that phosphorylates and activates Smad1/5/8 proteins. Phosphorylated Smad1/5/8 forms a complex with Smad4, and this complex then translocated to the nucleus to regulate target genes. Noggin acts as an inhibitor of BMP by blocking the BMP binding sites on both R-I and R-II receptors, thereby modulating BMP activity within the pathway. BMP: Bone Morphogenic Protein, Bmpr: Bone Morphogenic Protein Receptor  
Smad: Mediates TGF and BMP family of signalling transduction,

### 1.3.2 The Wnt pathway

The evolutionarily conserved Wnt signaling pathway plays a critical role in determining cell fate during embryogenesis (Peifer and Polakis 2000). There are 19 Wnt ligands in mammals (Miller, 2002), and among these, Wnt3 are expressed in the epithelial Paneth and enteroendocrine cells of the intestinal differentiation cells. Wnt9a is expressed in the mesenchymal cells near
intestinal crypt region. In contrast, Frizzled receptors (Fzd5/7), along with the co-receptor LRP5/6, are predominantly expressed in ISCs and TA cells in the crypt (Figure 2) (Gregorieff et al., 2005).

During homeostasis, Wnt signaling components are expressed in the intestinal paneth cell and mesenchymal stem cell niche within the crypt region, where they have been shown to regulate stemness and cell fate decisions. APC forms a protein complex, facilitating phosphorylation of β-catenin at its N-terminus resulting in its degradation. When Wnt ligands bind to Frizzled receptors, this inhibits APC, resulting in cytosolic β-catenin accumulation, translocation to the nucleus, and activation of target gene transcription. Therefore, nuclear localization of β-catenin indicates active canonical Wnt signaling and is typically observed in the intestinal crypt. (Figure 8) (Batlle et al., 2002).

In this context, Wnt signaling, mediated by β-catenin, promotes cell proliferation. Mutations in the APC gene leads to a loss of inhibition of β-catenin, thus causing abnormal activation of β-catenin which results in intestinal polyposis (Miyoshi et al., 1992; Sancho et al., 2003).

In Publication 2, we investigated the impact of inactivation of BMP signaling on intestinal epithelial cells. We found that inhibition of BMP signaling, by knocking out BMPR1a, caused intestinal polyposis. We wondered whether this was related to Wnt signaling. Indeed, β-catenin

**Figure 8. A schematic illustration of the Wnt Signalling pathway as a series of sequential protein interactions.** Wnt signalling is initiated when Wnts bind to Frizzled receptors. The binding leads to recruitment of the phosphorylated co-receptor, Lrp6. This signalling transduces to the APC/Axin/GSK3 complex, which typically mediates the phosphorylation of b-catenin at its N-terminus. As a result, β-catenin stabilizes and subsequently translocates to the nucleus. Once in the nucleus, β-catenin associates with TCF/LEF to regulate downstream gene expression. Wnt: Wingless, Lrp: Lipoprotein receptor-related protein, a coreceptor for Wnt, APC: Adenomatosis Polyposis Coli, Axin: A Wnt signaling inhibitor, GSK: Glycogen Synthase Kinase, TCF: T-cell Factor, β-cat: β-catenin.
was observed to be over activated as indicated by its increased nuclear localization, leading to ISC expansion and tumorigenesis. This observation suggests that BMP can inhibit Wnt/β-catenin function, thus providing negative feedback to balance the positive role of Wnt/β-catenin in ISC proliferation. To better understand mechanistically how BMP and Wnt signaling crosstalk, we next explored the role of PTEN signaling.

### 1.3.3 The PTEN pathway

PTEN, an epithelial cell-enriched phosphatase regulated by TGFβ (potentially by BMP), is recognized as a tumor suppressor. Mutations in the PTEN gene have been identified in an array of cancers, including breast, glioblastoma, endometrial, ovary, prostate, melanoma, skin, and gastrointestinal cancers (Mutter, 2001). PTEN, as a negative regulator of phosphatidylinositol-3 kinase (PI3K), and inhibits formation of phosphatidylinositol-3,4,5-triphosphate (PIP3). PI3K in turn activates Akt (Wu et al., 2003), which primarily promotes cell-cycle progression by activating Cyclin D1 and suppressing p27kip1. Additionally, Akt impedes apoptosis through the inhibition of BAD and Caspases (Franke et al., 1997). Consequently, PTEN and phospho-Akt (P-Akt) exhibit an inverse relationship, as illustrated in Figure 9. In Publication 5 and 6, we uncovered that PTEN plays a crucial role in limiting the activation of ISCs and HSCs, determining lineage fate, and preventing tumorigenesis and leukemogenesis.

**Figure 9. A schematic illustration of the PI-3K signalling pathway as a series of sequential protein interactions.** Various growth factors can activate PI3K by binding to cognate receptors. Through PDK1, PI-3K activates Akt. PTEN plays a critical role in inhibiting PIP3, thereby suppressing Akt. Akt orchestrates multiple cellular processes, including proliferation and cell survival. This phosphorylated form of PTEN is associated with activated phosphor-Akt. PI3K:
Mutations in the gene encoding PTEN result in Cowden disease (CD) (OMIM #158350), which is characterized by intestinal polyposis in humans (Liaw et al., 1997). Similarly, mutations in the gene encoding BMPR1a are associated with Juvenile Polyposis Syndrome (JPS) (Zhou et al., 2001).

These observations suggested a potential link between PTEN, Akt, and BMP pathways in the context of intestinal polyposis. In prostate cancer cell lines, PTEN inactivation has been reported to activate Akt, leading to the nuclear accumulation of β-catenin (Persad, S. 2001). In our observation, I found that inactive PTEN (P-PTEN) activates Akt, which subsequently enhances β-catenin activity via directly phosphorylating β-catenin at Ser552 and Ser675 (My Publication 4, 2007). We further uncovered that, like TGFb, BMP also regulates PTEN activity. Inactivation of BMP signaling led to reduced PTEN activity and resulted in activation of PI3K-Akt signaling. Akt in turn enhanced β-catenin activity via directly phosphorylating C-terminal S552 and S675 of β-catenin (Publication 5).

1.3.4 The PGE2-EP pathway

Prostaglandin E2 (PGE2) is produced from arachidonic acid via the action of the two cyclooxygenase (COX) isoenzymes COX-1 and COX-2. PGE2 binds to its receptor EP1-4, initiating downstream signaling pathways involved in tissue injury repair (Jackstadt and Sansom, 2017; Miyoshi et al., 2017) (Figure 10) Cox-2 is predominantly expressed in myeloid immune cells and also expressed in many tumor cells. A previous retrospective study indicated that the combination of a COX-2 inhibitor with 5 fluorouracil (5FU, a chemotherapeutic agent) resulted in significantly improved survival of patients with colorectal cancer compared to 5-FU alone (Lin et al., 2006). Considering that chemotherapy with5-FU is effective in killing proliferating tumor cells (Longley et al., 2003), and is known to up regulate Cox-2 expressed in intestinal myeloid cell, I wondered whether a Cox2 inhibitor could target cancer stem cells (CSCs) and their tumor microenvironment (TME). To investigate this hypothesis, I selected the Apc<sup>Min</sup> mouse adenoma model (My Publication 10, 2021).
In colorectal cancer (CRC), elevated Prostaglandin E2 (PGE₂) levels due to the increased activity of COX-2 were observed (Lin et al., 2006). Either a Cox-2 inhibitor, celecoxib, or deletion of the Ptgs2 gene (which encodes COX-2) have been shown to substantially reduce the adenoma burden (Cherukuri et al., 2014). Additionally, 5FU has also been demonstrated to cause a regression of adenoma burden (Longley et al., 2003). However, in response to 5FU, it remains largely unclear whether and how the tumor microenvironment facilitates tumor stem cell survival from therapy challenge and supports subsequent tumor regrowth. The potential influence of COX-2 inhibition on tumor stem cell viability and proliferation has yet to be determined.

As a vital component of the TME, myeloid-derived cells (MDCs) are recognized for producing PGE2 (Publication 10). This compound is also produced by fibroblasts surrounding the tumor stem cells and transit amplifying malignant epithelial cells. PGE2 serves as a primary inflammation signal, facilitating post-injury tissue regeneration and promotes tumorigenesis in response to chemotherapy (Holohan et al., 2013; Montrose et al., 2015). Historically, PGE₂, acting through its receptors (EPs), has been identified as an activator of both Wnt- β-catenin signaling (Evans, 2009; Miyoshi et al., 2017) and PI3K/Akt signaling (Castellone et al., 2005; Hsu

![Figure 10. A schematic illustration of the PGE₂-EP4 Signalling pathway as a series of sequential protein interactions.](image)

ProstaglandinE2 (PGE₂) is produced by either cyclooxygenase 1 or 2. PGE₂ acts as a key inflammation signal, capable of enhancing Wnt and Akt signalling through its EP receptor. However, the connection between EP and β-catenin was not well-defined. Cox:

- Cyclooxygenase, EP: Prostaglandin E2 receptor,
- Akt: A serine/threonine-specific protein kinase
et al., 2017) (Che et al., 2017; Miyoshi et al., 2017). Yet, the relationship between EPs and β-catenin was not well-established in previous studies.

(Figure 10). In my Publication 5, we discovered that Akt can directly phosphorylate β-catenin and thus enhances β-catenin activity.

In Publication 10, we investigated the drug-resistance of adenoma and cross talk between therapy-resistant (Tr) TSC and TME. We observed that chemoradiotherapy eliminated a large portion of proliferating tumor cells including cycling TSCs. However, slow cycling TSCs are therapy resistant (Tr). In addition, TrTSCs recruited myeloid derived cells (MDCs) to their niche, which in turn promoted TrTSC proliferation via secreted PGE2 that signals via EP4 in TrTSCs. Upon PGE2 engagement, EP4 activated Akt, which in turn enhances β-catenin nuclear activity through directly phosphorylation of β-catenin (Publication 10).
2. Overview of Research

I have structured the description of published papers included in this thesis using the following format:

2.1 Summary of each publication - Research questions posed by papers
   2.1.1 Summary of 10 publications with main findings and critical questions addressed
   2.1.2 Summary table of the papers is then presented. This lists the papers and key facts about each one for reference, i.e., listing the short name, title, authors, contribution, year of publication, journal, citation, and impact factors.

2.2 Outline of the interrelationship between publications: I provide a commentary on the subject matter is then outline how the papers connect and contribute to the hypothesis.

2.3 Critical review of the retrospective state of knowledge and research in the field and how the work has contributed to the field.

This section reviews prospective and recent developments in the field and literature on this subject. Emphasis is given to new publications since the publication of my papers and how my paper has changed or contributed to the field. The contribution of each paper to the field is outlined along with a review of impact of the papers and associated activities.

2.4 Commentary on the reception of the publications, as indicated by citations and reviews, and the standing of the journals in which they were published.

This section reviews the impact of my papers, giving statistics on the number of citations, an outline of the journal or report or series containing the papers, internet statistics and social media coverage of this material.

This section is then followed by a list of my other publications, not forming the thesis, but included for the panel to review. These publications were not chosen to be included in the submission but help to show the range and depth of impact of my work in the fields of stem cell and cancer biology.

Finally, references referred to in this thesis in relation to the published papers are listed.

The thesis includes a copy of each paper as appendixes.
2.1 Summary of each publication. Research questions posed by papers.

Each paper begins with:
A statement highlighting the issue explored.
A general introduction to the material.
The research question of each paper is provided at the end of its description.
This format illustrates how each paper supports the overall hypothesis of the thesis.

A summary of the works to be submitted as the portfolio then follows a table summarizing key statistics about each paper.

The papers are arranged in 2 sections:
Part A contains papers that present my main findings and critical questions addressed.
Part B describes the relationships between the 10 papers, their contributions to the lab’s research, impact to the field, and comments and views by the fields.

2.1.1 Summary of 10 publications

P1: Research question connecting Paper 1 to the thesis hypothesis: What specific genes are expressed in stem cells that distinguish stem cells from progenitor and differentiated cells? Furthermore, can we identify the molecular basis that determines the multipotential capability of stem cells?

Back in 2000, there was considerable research interest in understanding how stem cells support tissue regeneration. In this study I reported the global molecular profiles of various stem cell populations in bone marrow, including quiescent and cycling HSCs, as well as common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) using microarray technology (Figure 11). The results revealed that Wnt 1, TCF3 serve as the ligand and transcription bind site respectively, for Wnt signaling (unclear- rewrite). Activin R which encodes for their cognate receptor, was identified among the key genes enriched in hematopoietic stem cells. We also learned from this paper that an open chromatin structure, which allows RNA polymerases accesses to initiate transcription, becomes gradually closed during lineage differentiation.
Alterations in chromatin structure are vital for the execution of genetic programs for stem cell proliferation and lineage fate determination. This paper revealed that HSCs maintain an open chromatin structure as a broad transcriptional accessibility state, facilitating their multilineage developmental potential.

I was the primary author, responsible for designing and conducting many of the experiments in this study. Additionally, I was the first person to introduce the use of Affymetrix GeneChip techniques for RNA array assays in hematopoiesis systems, including HSCs, MPP, CLP and CMP.


Figure 11. Schematic illustration of the distribution of hematopoietic and nonhematopoietic lineages. HSCs express multiple nonhematopoietic genes as well as hematopoietic genes. CLP express T lymphoid, B lymphoid and Natural killer lymphoid, but no myeloid genes. CMP express myeloid genes, no Lymphoid genes. This data indicates a stepwise decrease of lineage potential and lineage promiscuity during early hematopoiesis. Lineage promiscuity is distributed in a hierarchical and asymmetrical fashion. HSC: Hematopoietic Stem Cell, MPP: Multipotent Progenitor, CLP: Common Lymphoid Progenitor, CMP: Common Myeloid Progenitor, T-cell: T Lymphoid, B-cell: B Lymphoid, NK cell: Natural Killer Cells.
P2: Research question connecting this paper to the thesis hypothesis: Through genetically manipulate key pathways (such as BMP signaling pathways) known to be associated with certain inherited disorder (such as Juvenile polyposis syndrome—a pre-colorectal cancer) with tumor features, can we gain insight for understanding their roles in the regulation of stem cells?

Previously, through the study of families with rare autosomal dominant cancer syndromes, researchers gained significant insights into the signaling pathways associated with intestinal polyp formation and colorectal cancer. However, despite progress in identifying the relevant pathways, it remained largely unclear how these pathways regulate intestinal stem cell behavior. In this work (He et al., 2004), I demonstrated that blocking BMP signaling in mice via the conditional inactivation of Bmpr1a, perturbed the normal homeostasis of intestinal epithelial regeneration. I observed an expansion of both stem and progenitor cell populations, resulting in intestinal polyposis, reminiscent of human juvenile polyposis syndrome. I discovered that BMP signaling inhibited Wnt signaling, controlling the balance of stem cell self-renewal and proliferation. Notably, through the phosphatidylinositol-3 kinase–Akt pathway, I found Pten mediated the convergence of the BMP and Wnt pathways, with a common focus on regulating b-catenin activity. Consequently, BMP signaling plays a pivotal role in controlling the proliferation of ISCs and is a key mechanism for preventing crypt fission and for regulating crypt numbers (Figure 12). This study also revealed the role of BMP as a niche signal controlling ISC self-renewal and proliferation by antagonizing Wnt signaling. We used the BMP4 coding sequence and replaced it with the LacZ gene, such that the reporter reflects BMP4 expression (Lawson et al... Hogan, Denes & Dev 1999). These data offer insights into how ISC numbers are regulated and enhances our comprehension of the mechanisms underlying crypt fission, an important phenomenon in the clonal expansion of intestinal tumors (Figure 12).
I was the primary author and carried out the primary work for the project. Concept-wise, I reported the first niche signal, BMP, in the regulation of ISCs via antagonizing Wnt signaling in gut. In this work, I also revealed that expression of a BMP antagonist, Noggin, can coordinate with Wnt signaling to activate ISCs via inhibition of BMP signaling. This concept is the basis for today’s culture of organoids using Noggin. I was the first person to develop multicolor immunofluorescent staining in the intestinal field, and the methodology has now become widely utilized.

Xi C He, Jiwang Zhang, Wei-Gang Tong, Ossama Tawfik, Jason Ross, David H Scoville, Qiang Tian, Xin Zeng, Xi He, Leanne M Wiedemann, Yuji Mishina, and Linheng Li. BMP Signaling Inhibits...
DOI: [10.1038/ng1430](https://doi.org/10.1038/ng1430); Citations: 811 or [Google Scholar](https://scholar.google.com) 1233 (10/25/2023)

**P3: Research question connecting this paper to the thesis hypothesis:** Is the microenvironment (or niche) responsible for maintaining hematopoietic stem cells in adult bone marrow? If so, where is the niche?

The concept of a stem cell niche was first introduced by Ray Schofield (Schofield, 1978). While the stem cell niche has been described in the Drosophila ovary, the HSC “niche”—the regulatory microenvironment in which HSCs reside, and the mechanisms that control the number and quality of adult HSCs remained elusive. In this study (Zhang et al., 2003), in which I am a co-author, we demonstrated that trabecular bone is the primary location for quiescent HSCs in bone and bone marrow. Notably, the osteo-generating cells positioned at the endosteal (inner-bone) region play a key role in maintaining the quiescence of long-term (LT) HSCs. In this study, my contributions included performing bone marrow transplantations for analysis of HSC functions. Additionally, I created a novel multiple-color immunofluorescent staining technique and assay. Since then, this multiple-color immunofluorescent staining technique has become a standard technique in the field. This study revealed that stem cells (HSCs) are maintained in an undifferentiated state within a physically confined space in the bone marrow, known as the niche. The size of the niche determines the number of HSCs. This was a paradigm-shifting finding that revealed stem cells require extrinsic microenvironmental regulation.

DOI: [10.1038/nature02041](https://doi.org/10.1038/nature02041); Citations: 2241 or [Google Scholar](https://scholar.google.com) 3678 (10/25/2023)
P4: Research question connecting this paper to the thesis hypothesis: Does BMP signaling inhibit stem/progenitor cell activation and expansion, via antagonizing Wnt signaling? Does this also hold true for hair follicles in the skin?

While there were numerous prior studies of BMP signaling in hair follicle (HF) development, comparatively little attention had been given to epithelial stem cells in the skin. In particular, the mechanisms through which BMP signaling regulated epithelial stem cells and influenced HF tumorigenesis was largely unknown. In this study (Zhang et al., 2006) in which I am a co-author, we discovered that dynamic expression of Noggin in epithelial stem cells throughout the HF cycle results in the periodic inactivation of BMP signaling. This correlates with the activation and expansion of the numbers of epithelial stem cells during the early anagen phase. Additionally, we found that inhibiting BMP signaling by inducing the deletion of Bmpr1a in epithelial stem cells leads to their overproduction. The expansion of stem cells in hair follicles was due to predominant Wnt signaling following the deletion of Bmpr1a. The enhanced Wnt signaling was evidenced by increased nuclear-localization of β-catenin and concurrent Top-
Flash (Luciferase) signaling. The expansion of stem cells subsequently led to the formation of multiple units of hair follicles in the original hair follicle site and resulted in the development of Matricoma (cutaneous neoplasm). In contrast, the absence of Bmpr1a in HF progenitors/precursors disrupts hair shaft differentiation. Our study presented evidence suggesting the PTEN-Akt cascade is instrumental in bridging BMP and Wnt signaling in epithelial stem cells, particularly through the modulation of b-catenin activity. My Contributions to this paper as a co-author involved assisting in the development of the Mx1- Cre*Bmpr1afl/fl (Bmpr1a mutant) animal model, performing bromodeoxyuridine (BrdU) long-term labeling and pulse retaining assays during the hair follicle growth cycle.

The primary conclusion from this study is that proliferation and self-renewal of hair follicle stem cells is meticulously regulated by specific BMP niche signals. When BMP signaling is perturbed this can lead to tumorigenesis.

Jiwang Zhang, Xi C He, Wei-Gang Tong, Teri Johnson, Leanne M Wiedemann, Yuji Mishina, Jian Q Feng, Linheng Li. BMP signaling inhibits hair follicle anagen induction by restricting epithelial stem/progenitor cell activation and expansion. Stem Cells. 2006 Dec; 24(12):2826-39.

DOI: 10.1634/stemcells.2005-0544; Citation: 141 or Google Scholar 167 (10/25/2023)
P5: Research question connecting this paper to the thesis hypothesis: Can normal stem cells become cancer stem cells and how do these CSCs lead to tumor formation in the intestine?

In this study, I identified PTEN-deficient stem cells as the origin of polyposis and established a molecular mechanism through which coordinated PTEN-Akt and Wnt-β-catenin signaling regulates intestinal homeostasis. These findings help clarify the process by which a stem cell becomes a tumor-initiating cell and how these transformed cells subsequently drive tumor development. Furthermore, my research dissected the mechanism underlying intestinal tumorigenesis. In a variety of systems, including the intestine, nuclear localization of β-catenin is considered a pivotal event in stem cell activation. It was the dogma that accumulated β-catenin in the cytosol inevitably leads to its nuclear localization. However, given the critical role
of b-catenin in promoting stem cell self-renewal and proliferation, its nuclear localization should subject to a regulation. I aimed to elucidate the interaction between PTEN-Akt signaling and b-catenin in the context of nuclear localization and activity of β-catenin. Utilizing a liquid chromatography–mass spectrometry assay, we revealed that Akt phosphorylates β-catenin at the C-terminus Ser552. The sequence Arg-Arg-Thr-Ser (RRTS), which includes Ser552, forms a phosphoserine motif that presents an ideal site for Akt phosphorylation (Figure 15). To detect β-catenin phosphorylated by Akt at Ser552, we developed and characterized a phospho-specific antibody (anti p-β-cat-Ser552). Whole mount immunofluorescent staining subsequently demonstrated that cells with b-catenin phosphorylated on Ser552 initiate tumorigenesis either through crypt fission or the de novo formation of a crypt.

![Figure 15. Schematic illustration of the regulation of nuclear β-catenin by Wnt and Akt signaling suggesting a relationship between phosphorylation and nuclear activity of β-catenin.](image)

Nuclear localized forms of b-catenin are identified by the absence of phosphorylation at the N terminus, by C-terminal phosphorylation at Ser552, or both.


Findings from this study highlight the significance of two major signaling pathways in the development of oncogenic stem cells and offer insights into a possible genetic basis for their distinction from normal stem cells: specifically, PTEN loss and the subsequent nuclear accumulation of Akt-phosphorylated b-catenin.

I was the lead author and carried out the primary work on the project, which reported for the first time, a positive activation role for the phosphorylation of β-catenin at the C-terminus in
contrast to its N-terminal phosphorylation. The anti-p-b-catenin antibody developed in this study has been used by many labs in the field since then.

DOI: 10.1038/ng1928; Citation: 352 or Google Scholar 518 (10/25/2023)

P6: Research question connecting this paper to the thesis hypothesis: Can niche signals regulate the self-renewal of hematopoietic stem cells and influence lineage choices that lead to leukemia?

In this study (Zhang et al., 2006), in which I served as co-author, we examined the role of PTEN in the regulation of HSCs. We showed that inactivation of PTEN in bone marrow HSCs resulted in an increase in hematopoietic stem and progenitor cells (HSPCs), an increase in myeloid and T-lymphoid lineages, and the development of myeloproliferative disorder (MPD). This study highlighted the pivotal role of PTEN as a tumor suppressor. While the manuscript was in revision, the reviewers asked for additional experiments to determine whether P-PTEN associates with cycling cells, and I performed immunofluorescent co-staining of P-PTEN with Ki67, or with Cyclin D1, along with HSC markers, which is illustrated in Figure 1 of the paper. In summary, we demonstrated that PTEN plays essential roles in restricting the activation of HSCs, in lineage fate determination, and in the prevention of leukemogenesis (Figure 16).

Jiwang Zhang, Justin C Grindley, Tong Yin, Sachintha Jayasinghe, Xi C He, Jason T Ross, Jeffrey S Haug, Dawn Rupp, Kimberly S Porter-Westpfahl, Leanne M Wiedemann, Hong Wu, Linheng Li. PTEN maintains hematopoietic stem cells and acts in lineage choice and leukemia prevention. Nature. 2006 May 25; 441(7092):518-
DOI: 10.1038/nature04747; Citation: 652 or Google Scholar 975 (10/25/2023)
**Figure 16: PTEN regulates signal transduction and cancer development.**
a, AKT signalling is activated by growth factors and PTEN negatively regulates PI3K activity. This keeps cell division from becoming excessive or hyperproliferative. b, In the absence of PTEN, the proliferation of haematopoietic stem cells increases and leukaemia develops. This is presumably because hyperactivity of AKT leads to activation of mTOR with a subsequent increase in cell division. The checkpoints that would usually halt division of aberrant cells are overridden, so that there is an accumulation of mutations and eventually malignancies develop. Hyperactive AKT would also suppress FOXO, again causing hyperproliferation. In stem cells lacking PTEN, this proliferative stress leads to exhaustion of stem-cell numbers as self-renewal is reduced. c, The drug rapamycin inhibits the activity of mTOR and treatment of PTEN-deficient mice with rapamycin markedly restores stem-cell function and prevents the development of leukaemia. Rapamycin may also exert its effects through a direct inhibition of AKT activity. Figure from https://www.nature.com/articles/441418b/figures/1. PTEN: Phosphatase and Tensin homolog, p-PTEN: Phosphate Phosphatase and Tensin homolog, PI3K: Phosphoinositide 3-kinase, Akt: A serine/threonine-specific protein kinase, FOXO: Forehead Box Transcription, mTOR: Mammalian Target of Rapamycin

**P7: Research question connecting this paper to the thesis hypothesis:** Can HSCs strike a balance between routine blood production and long-term maintenance, especially under stress?

Hematopoietic stem cells (HSCs) are crucial for lifelong blood production. HSCs play a critical role in regenerating lost cells, while at the same time avoiding exhaustion and mutation accumulation. In this study (Haug et al., 2008), in which I am a co-first author, we identified two
stem cell subpopulations, based on N-cadherin expression, an adhesion molecule linked to HSC-niche interactions (Publication 3). HSCs with minimal/no N-cadherin expression exist in a ‘primed’ state ready for active proliferation and action. Meanwhile, those with N-cadherin expression act as ‘reserve’ HSCs with deep-quiescent state, functioning for long-term maintenance. This distinctive cell cycle state characteristic ensures a balance between blood regeneration and stem cell preservation, with each subpopulation having a distinct molecular signature. We observed that Igf2r was differentially expressed between reserve and primed HSCs and its upregulation in primed HSCs correlates with the downregulation of N-cadherin. Furthermore, loss of Igfr2 blocks the activation of HSCs as shown by our lab in a subsequent study (Venkatraman, et al., Nature 2013).

As co-first author I was responsible for the design of the research and I performed the primary biological and functional analyses, particularly those in Figure 1, Figure 2, HSC sorting in Figure 3, Figure 4, Figure 5 and Figure 6.

Figure 17. Model of Reserved and Primed HSCs. Properties and molecular signatures of reserved and primed HSCs. Reserved HSCs are adapted for long-term maintenance. Primed HSCs play a more active role in supporting hematopoiesis. Lost primed HSCs may be replaced from the larger reserve pool (bold forward arrow). Transition from primed to reserved may also occur (light reverse arrow). Flk2 LSK HSCs: the marker set Flk2 Lin Sca1 cKit+ for Hematopoietic Stem Cells. N-cad\textsuperscript{int}: N-cadherin intimidate level expression, N-cad\textsuperscript{low}: N-cadherin low level expression.

DOI: [10.1016/j.stem.2008.01.017](https://doi.org/10.1016/j.stem.2008.01.017), Citation:121 or Google Scholar [192](https://scholar.google.com/scholar?hl=en&btnG=Search&query=How%20do%20es%20combined%20PI3K/AKT%20and%20β-catenin%20signaling%20influence%20hematopoietic%20stem%20cell%20expansion%3F)&p=1 (10/25/2023)

**P8: Research question connecting this paper to the thesis hypothesis:** How does combined PI3K/AKT and β-catenin signaling influence hematopoietic stem cell expansion?

Building on my finding that Akt enhances b-catenin activity by directly phosphorylating b-catenin to promote intestinal stem cells expansion, we examined the combined effect of Akt and b-catenin on hematopoietic stem cell self-renewal and expansion. I contributed to this study (Perry et al 2011), as a co-author, by conducting bone marrow transplantations gene expression analyses, and anti-phospho-b-catenin S552 antibody staining assays in PTEN mutant mice. We hypothesized that self-renewal involves combining complementary cellular events: suppression of differentiation and prevention of apoptosis during proliferation. When β-catenin was activated alone, HSCs initially proliferated but then underwent apoptosis, as a result of exhaustion from over proliferation. In contrast, activation of PI3K/Akt signaling alone resulted in increased differentiation and the prevention of apoptosis. Simultaneous activation of both β-catenin and PI3K/Akt signaling expanded the number of HSCs by inhibiting differentiation and apoptosis in proliferating HSCs, notably within the Lin-Sca1+ c-Kit+, LSK population.

**P9: Research question connecting this paper to the thesis hypothesis:** What is the molecular mechanism underlying leukemia stem cells resistance to chemotherapy and immune escape? While Akt and β-catenin signaling can transiently promote HSC self-renewal. Uncontrolled stem cell expansion can lead to tumor development, and as seen in the intestine, PTEN; β-catenin double mutant mice develop T acute lymphoid leukemia (T-ALL). In this study (Perry et al.,
2020), which I co-authored, we delved into the molecular mechanism of leukemia stem cell (LSC) resistance to chemotherapy. Using our PTEN β-catenin T-ALL model, we discovered: 1) that chemotherapeutics like Nelarabine can spur LSC expansion while reducing leukemia blasts; 2) that low-dose doxorubicin, which inhibits Akt-driven β-catenin phosphorylation at S552, suppresses LSCs; and 3) that combining Nelarabine and low-dose doxorubicin optimized animal survival (Figure 29). Mechanistically, we discovered that β-catenin directly regulates multiple immune checkpoint genes including PD-L1, Tim3, and CD24 at a basal level. Akt phosphorylation of β-catenin boosts levels of these immune checkpoint genes, thus empowering LSCs with immune evasion capability (Figure 19). I conducted bone marrow transplantations and gene expression analyses.

**Figure. 19 Targeting Akt-activated β-catenin dependent immune escape in LSCs**

The cooperative effect of Wnt/β-catenin and PI3K/Akt signaling in resistance to anti-cancer therapies, including immune escape. In PTEN; β-catenin double mutant mice cooperative Akt: β-catenin signaling is essential for therapy-resistant LSCs.

A, Investigating the mechanisms underlying this resistance, we unexpectedly found that Akt-activated β-catenin binds to multiple IC genes, which are expressed on LSCs. 

B, In identifying DXR as an inhibitor of Akt: β-catenin interaction at low doses, we found that DXR could be repurposed as a targeted therapy for resistant LSCs, in part by inhibiting multiple ICs, particularly PD-1/PD-L1. Notably, LSCs but not blast cells exhibit unique properties of immune resistance, which can be reduced with low-dose DXR. LSC: Leukemia Stem Cell DXR: Doxorubicin, CD8 T-Cell: CD8 T lymphocyte.
DOI: 10.1038/s41556-020-0507-y; Citation: 63 or Google Scholar 82 (10/25/2023)

P10: Research question connecting this paper to the thesis hypothesis: How does the interaction between tumor-initiating stem cells (TSC)-and tumor associated microenvironment (TME) contribute to a pro-tumorigenic and immunosuppressive environment?
In this study (He et al., 2021), I investigated the mechanisms underlying therapy resistance that often leads to cancer relapse. I found that in response to chemoradiotherapy, slow-cycling stem cells, but not active cycling stem cells nor rapidly proliferating progenitor cells, survive. Subsequently, this population of therapy resistant TSCs (TrTSCs) recruited myeloid associated monocytes and macrophages (TAMMs) to their niche. TAMMs in turn form an immunosuppressive microenvironment and at the same time support TrTSCs proliferation. Mechanistically, I further showed that prostaglandin E2 (Pge2), a primary inflammation signal from myeloid derived cells, could activate Akt via its receptor EP4, thereby elevating β-catenin activity in intestinal adenoma stem cells. Given that Cox1/2 are essential for Pge2 synthesis, we utilized a Cox2-specific inhibitor, celecoxib, to suppress Pge2 and consequently reduce Akt-enhanced β-catenin activity. This work provides my basic research findings with clinical significance because it revealed how TSC-TME crosstalk promotes a pro-tumorigenic and immunosuppressive barrier (Figure 20).
I was the primary author and performed the majority of the experiments in this project. I applied a variety of technologies, including DNA label retaining, single-cell RNA sequencing (scRNA-seq) analysis, flow cytometry and electron microscopy (EM) to provide a comprehensive atlas of cellular components and signaling modules between TSCs and TME. I conducted in vitro and in vivo experiments to functionally characterize one of the identified signaling modules, the PGE2-EP4-mediated β-catenin axis. Finally, I examined the extent to which our findings in mouse adenoma may provide insight into human CRCs.

DOI: 10.1016/j.celrep.2021.109674; Citation: 20 or Google Scholar 24 (10/25/2023)
### 2.1.2 Summary Table

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<th>Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis</th>
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<th>BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt–β-catenin signaling</th>
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<td>Contribution</td>
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<td>PTEN-deficient intestinal stem cells initiate intestinal polyposis</td>
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<td>Cooperation between both Wnt/β-catenin and PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion</td>
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<td>primed states of hematopoietic stem cells</td>
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<td>9</td>
<td><strong>Overcoming Wnt–β-catenin dependent anticancer therapy resistance in leukemia stem cells</strong></td>
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| Paper 10 | **Tumor-initiating stem cell shapes its microenvironment into an** | Xi He, ... & Linheng Li | I was the primary author, and I established the experimental | 2021 | Cell Reports | Google Scholar 29 | 9.995 |
system for studying how tumor stem cells and the tumor microenvironment respond to chemoradiotherapy.

The novel finding arising from this work is that TSC actively shapes its microenvironment into an immunosuppressive niche.
2.2 Outline of the interrelationship between publications

2.2.1 Introductory narrative

My common research hypothesis is that several key developmental signaling pathways, namely BMP, Wnt, PTEN play essential roles in regulating homeostatic stemness properties in adults. Disruption of these key developmental signals furthered our understanding of the role of stem cells in development, and mechanisms by which abnormal stem cells (for example tumor-initiating stem cells), result in diseases such as cancer.

The work I have performed focused on defining the molecular and cellular mechanisms that regulate the formation, maintenance, and function of stem cells in hematopoietic, intestinal, and skin systems. Specifically, I aimed to map out key genes encoding signaling networks, including BMP, Wnt and PTEN, that control stem cell niche properties. (paper1)

I discovered that BMP antagonization of Wnt- β-catenin signaling is critical for intestinal stem cell maintenance, and that BMP signaling also inhibits epithelial stem and progenitor cell activation in the hair follicle. (paper2, paper 4). This highlighted the conserved and broad roles of specific signaling pathways in stem cell maintenance, behavior, and function.

Subsequently I discovered that PTEN loss-of-function, which affects BMP signaling, results in intestinal polyposis, providing an example that mis-regulation of normal developmental signaling leads to tumor disease. Furthermore, in this work I discovered that Akt can directly phosphorylate β-catenin and thus enhances β-catenin activity (Paper 5). Using the anti-p-β-catenin antibody, I found that p-β-catenin S552 marked tumorigenic stem cells in the intestine (paper 7).

Linking these pathways, I also participated in identifying the hematopoietic stem cell niche and in uncovering that co-operation between both Wnt/B-catenin and PTEN/PI3K/Akt signaling promotes murine hematopoietic stem cell self-renewal and expansion (paper3, paper6).
Based on my works, described above, I propose that stem cells escaping anticancer therapy utilize the same cellular and molecular mechanisms required during normal development. Consistent with this idea, I performed work showing that coordination of Wnt/β-catenin and PI3K/Akt signaling promotes hematopoietic stem cell self-renewal and expansion, while Wnt-AKT signaling drives resistance to therapy and immune escape in leukemia stems cells. (paper 8, paper 9)

Recently, I began focusing on studying solid tumors and TME and I was able to show that tumor-initiating stem cells shape their microenvironment into a pro-tumorigenic niche and an immunosuppressive barrier (paper 10). Collectively, my research broadly shows that intestinal stem cells (ISCs) and hematopoietic stem cells (HSCs) are regulated by exquisite signaling pathways that ensure maintenance of a quiescent state, control self-renewal, and proliferation. TSCs share some features of normal stem cells including self-renewal promoting signaling and supporting tumor regrowth upon damage, which I have exquisitely studied in each of my publications over the past decade. I have summarized my individual contributions to each project and publication.

I have divided my portfolio of 10 papers into four categories:

- Wnt and BMP Pathways in Stem Cell regulation
- The PTEN-Akt pathway in converging BMP and Wnt signalling.
- The Cox2-PGE2-EP4 Pathway in Tumor-initiating stem cell (TSC) microenvironment
- Cross talk between TSCs and Tumor Microenvironment (TME)

### 2.2.2 Relationship of the papers to this framework

Stem cells, with their self-renewal ability and multipotentiality, are essential for maintaining tissue homeostasis and injured tissue regeneration. Two decades ago, as the stem cell field gained momentum, many fundamental questions remained unanswered:

- What specific genes are expressed in stem cells?
- What mechanisms determine stem cell multipotentiality?
Where do stem cells reside in bone marrow and intestine?

What are the key signals that regulate stem cell self-renewal?

What mechanisms prevent stem cells from excessive proliferation?

What abnormal signals can lead to uncontrolled stem cell expansion to the extent that result in tumorigenesis?

Do tumor-initiating stem cells or cancer stem cells (TSC or CSC) operate under the same regulatory mechanisms as normal stem cells?

How do TSC/CSC resist chemotherapy and escape from immune challenge?

My research in publication 1 was among the earliest studies to understand the roles of specific genes expressed in stem cells that govern their stem cell properties. In that paper, I revealed that stem cells maintain a wide-open chromatin structure, allowing multiple lineage programs to access the DNA, thus serving as the molecular base of multipotentiality. In addition, several key genes were found to be highly expressed in stem cells: BMP, Wnt, FGF, and their corresponding receptors, which serve as candidate genes for my and other lab members’ subsequent research.

Publications 2, 3, 4 have collectively demonstrated a critical role for BMP signaling in the inhibition of stem cell self-renewal and proliferation, by antagonizing Wnt signaling in intestinal, bone marrow, and hair follicle cells. Loss of BMP inhibitory signaling led to over proliferation of stem cells, thus risking tumorigenesis. Furthermore, Bmpr1a mutant mouse model studies helped us identify a niche for HSCs, the first stem cell niche identified in mammals. An additional novel finding made in publication 2 was that PTEN controls PI3K/Akt pathway functions as a convergent point between BMP and Wnt signaling, and this links to subsequent publications 5 and 6.

Publications 5 and 6 further characterized the functional role of PI3K/Akt signaling via the PTEN knockout mouse model. The main findings include: 1) Akt directly phosphorylates Ser552/Ser675 of the C-terminus of β-catenin. In contrast to the N-terminus phosphorylation of
β-catenin by other kinases, that leads to degradation of β-catenin, the phosphorylation of Ser552/675 at the C-terminus of β-catenin facilitates nuclear localization and transcriptional activity of β-catenin. 2) Activation of Akt due to loss of PTEN enhances β-catenin activity, especially in stem cells of intestine and bone marrow, resulting in the development of intestinal polyposis and myeloid proliferative disorder. The discoveries in these studies were foundational for subsequent publications 8 and 9.

Publications 8 and 9 conducted studies on PTEN knockout and β-catenin transgenic (Ctnnb\textsuperscript{dEx3}) mouse models, in which both Akt and β-catenin are active. These studies uncovered: 1) that coordination of Akt and β-catenin signaling promotes stem cell self-renewal by promoting cell proliferation at the same time as inhibiting cell differentiation and preventing stem cell apoptosis; 2) that uncontrolled stem cell expansion could lead to tumorigenesis and leukemogenesis. Publication 9. Detailed analysis of PTEN: Ctnnb\textsuperscript{dEx3} double mutant mice, which develop T-ALL leukemia, led us to find the key role for Akt-β-catenin interaction in leukemia stem cells (LSCs), not only supporting self-renewal but also empowering LSCs with immune escape capacity by upregulating multiple immune checkpoint genes.

Publication 10. Discovered that TSC or CSC actively cross talk to the tumor-associated macrophages. Under stress, CSC can shape the macrophages into a tumor-promoting niche, via the PGE2-EP4-Akt-β-catenin signaling axis, and an immunosuppressive niche. Understanding the mechanisms underlying immune escape and immunosuppression provide insights for subsequent studies to develop drugs to overcome immune escape and immunosuppression. These studies are ongoing in our lab now.
(A) Stem Cell Niches
(B) Stem Cell Signals

P1, P2, P3, P7, P4

(C) Relationship Between Publication

Publication 1
Microarray analysis of cycling and differentiated HSC progenitors. Key roles for Wnt/BMP.

Publication 2
BMP inhibits self-renewal. Wnt promotes self-renewal. BMP acts via PTEN and Akt to regulate -Cat. PTEN regulates -Cat.

Publication 3
Discovery of new endosteal stem cell niche using Bmprela KO.

Publication 4
BMP loss leads to enhanced -Cat activity via increased Akt activity, resulting in stem cell expansion in hair follicle

Publication 5
PTEN/P3K/Akt specify cell fate. Akt directly enhances -Cat activity to promote stem cell proliferation.

Publication 6
PTEN prevents HSC activation. PTEN prevents leukaemia.

Publication 7
Discovery of co-existent quiescent and active HSC populations

Publication 8
Uncontrolled HSC growth in PTEN: -Cat double KO

Publication 9
-Cat increases immune checkpoint genes in leukaemia stem cells.

Publication 10
Cox2-PGE2-Ep4 signalling promotes intestinal tumour stem cell proliferation via Akt.

PGE2

Phosphoinositide 3-kinase (PI3K) regulates...
Figure 21: Illustration of the interrelationships between my selected papers.

(A) Categorisation of my 10 papers according to their relevance and three organ systems: intestine, hematopoiesis, and hair follicle. (B) Organisation of my 10 papers based on their relevance to the molecular signalling pathways: Bmp, PI3K, Wnt and Pge2. (C) Interrelationships between my 10 papers.

2.3 A critical review of the state of knowledge and research in the field and how each publication has contributed to the field

Publication (P)1: “Transcriptional Accessibility for Genes of Multiple Tissues and Hematopoietic Lineages is Hierarchically Controlled During Early Haematopoiesis”.

Stem cell research holds promise for tissue regeneration and gene therapies. However, our understanding of stem cell development, especially the genes influencing their behavior during early blood development remains uncomplete. Publication 1 used microarray technology to analyze gene expression profiles in purified quiescent HSCs, active-cycling HSCs, and their progenitors.

This study: 1) identified several key genes expressed in HSCs such as BMP and Wnt that facilitated our as well as the field’s subsequent studies; and 2) revealed that stem cells have a flexible open chromatin structure, essential for their diverse potential. This flexibility is reduced as they differentiate. This work provides insight into the molecular foundation of a stem cell’s diverse potential. Contributions to the field: I identified key candidate genes and their related signaling pathways in stem cells for my lab and other lab’s subsequent studies and provided a framework for understanding multipotentiality of stem cells.

Contributions of P1 to Stem Cell Research:

- December 2002
  Event: Poster Presentation by me.
Occasion: Annual American Society of Hematology (ASH), USA

Title: Accessibility for Multi-Tissue and Multi-Hematopoietic Lineage Genes is Hierarchically Controlled during Early Hematopoiesis.

- 2003
  1233-1139
  Authors: L. Li and Akashi, K
  Title: Unravelling the molecular components and genetic blueprints of stem cells

- 2004
  Publication: Journal of Data Science; 2(2004), 297-309
  Jie Chen, Xi He, and Linheng Li
  Title: Identifying the Patterns of Hematopoietic Stem Cells Gene using Clustering
  Methods: Comparison and Summary
  Details: This paper used HSPC profiling data from P1 and pioneered a statistical clustering analysis approach.

- Led to subsequent further discoveries and publications: In P1, we unveiled the predominant expression of BMP/Wnt/FGF and genes encoding their corresponding receptors within the HSC. This initial discovery led into more fundamental discoveries by fellows in our lab and subsequently by others in the field. For example, see BMP related publications 2, 3, 4 and Wnt related publications 2, 5, 8, 9, 10.

P2: “BMP Signalling Inhibits Intestinal Stem Cell Self-Renewal Through Suppression of Wnt/β-catenin Signalling”.

Previous genetic studies have shed light on the molecular pathways linked to intestinal polyp formation and colorectal carcinogenesis. For example, mutations of BMPR1A, SMADS and PTEN are associated with juvenile intestinal polyposis and Cowden syndrome. However, their impact on intestinal stem cell (ISC) behavior was poorly understood. In P2, I explored polyposis development, and revealed that the BMP and Wnt pathways were connected via PTEN/Akt signalling. Juvenile intestinal polyposis is an inherited cancer syndrome resulting from loss-of-
function mutations in BMP pathway components. Using a novel mouse model of juvenile polyposis syndrome (JPS), I uncovered signalling key regulators of ISC regulation and the mechanisms underpinning crypt fission, which are crucial for tumor expansion. My findings offer insights into the cellular behavior of polyps, which are precursors to the world’s third leading cause of cancer-related deaths. My contributions to the field as evidenced by the discoveries in this paper include being one of the first reports showing that BMP inhibits stem cell self-renewal and proliferation. This concept together with Hans Clevers’ work (Haramis et al., Science 2004) provided using Noggin to override BMP's inhibition of stem cell’s proliferation in the current widely used organoid culture method.

**Contributions of P2 to Stem cell Research:**

- **2004**
  
  Event: Invited Oral Presentation by PI
  
  Occasion: Harvard Club at Harvard University
  
  Title: BMP signalling inhibits intestinal stem cell self-renewal through suppression of Wnt/β-catenin signalling

- **2005**
  
  Event: Oral presentation based on this work by PI
  
  Occasion: International Society of Stem Cell Research (ISSCR)
  
  Title: Regulation of intestinal stem cells by BMP signalling.

- **2005**
  
  Grants awarded based on the papers.
  
  The following four grants were awarded to support our lab’s Intestinal stem cell research. These grants in which my role was listed role under key personnel were based on my 2004 and 2007 publications as well as my extended studies in the lab.

  National Institutes of Health

  **R01 DK070001**


  Title: Role of BMP Signalling in Intestine Stem Cell Development
2006
Event: Poster Presentation by me
Occasion: Digestive Disease Week (DDW).

Title: BMP Signalling Inhibits Intestinal Stem Cell Self-Renewal Through Suppression of Wnt/β-catenin Signalling.

2009
This paper demonstrates that Noggin could override BMP-imposed inhibition of intestinal stem cell proliferation. This laid the groundwork for employing Noggin in in vitro organoid culture.

2010
Li and Clevers, Science and Mira et al., Cell Stem Cell: Both publications reinforced the role of BMP signalling in maintaining the quiescence of various adult stem cells, including hair follicle, intestine, hematopoietic, and neural stem cells.

2013
Wang et al., Gastroenterology: Further research on using Noggin in the culture of both murine and human intestinal organoids.

P3: “Identification of the hematopoietic stem cell niche and control of the niche size”.

Studies of bone marrow have been pivotal to stem cell research, particularly hematopoietic stem cells (HSCs) which have defined many stem cell principles (Till & McCulloch 1961, Orkin 2000, Weissman et al. 2001). Yet, The HSC “niche”, it is in in vivo regulatory environment, and governance of adult HSC size - remains poorly understood. Given that BMP signaling has an essential role in inducing hematopoietic tissue during embryogenesis, we examined the roles of BMP signaling in regulating adult HSC development in vivo by analyzing mutant mice with a conditional inactivation of the BMP receptor type IA (BMPRIA). I as a co-author made major contributions to this work in which we identified the first stem cell niche in bone marrow and in mammals. This was paradigm-shifting work in the stem cell field, and it revealed a critical role for the microenvironment or niche in maintaining a stem cell’s stemness and in regulating stem cell fate.

Contribution of P3 to Stem cell Research:
2003:

**Award:** This P3 work won the Missouri Biotechnology Association Excellence in Life Sciences Award. Category: Basic Research

The award highlighted the importance of the stem cell niche, emphasizing its foundational role and guiding future investigations and potential application to the life science industry.

2005

**Feature article in Nature** by Kendall Powell: “Stem cells are engaged in constant crosstalk with their environment, biologists are fast realizing. So, the emerging field of regenerative medicine is now wrestling with the ecological concept of the niche”. (DOI: 10.038/435268a).

**P4:** “BMP signaling inhibits hair follicle anagen induction by restricting epithelial stem/progenitor cell activation and expansion”

Hair follicles (HFs) cyclically regenerate, providing a model to study stem cell behavior. Hair follicles have a stable component that includes sebaceous glands and the bulge area, and a dynamic cycle. HFs cycle through growth (anagen), regression (catagen), and rest (telogen). The bulge is home to epithelial stem cells (EP-SCs), which balance self-renewal and differentiation. These EP-SCs generate offspring that facilitate HF regeneration and BMP signaling regulates the HF cycle. Publication 4 showed that disrupting BMP type IA receptor (Bmpr1a) in EP-SCs leads to overproduction of HF stem cells and Matricoma formation, via unexpected activation of Wnt-β-catenin signaling.

**Contributions of P4 to Stem cell Research:**

- 2007

  Kobielak, Fuches et al., PNAS: Highlighted the role of BMP signalling in maintaining quiescent adult stem cells in the hair follicle.

**P5:** “PTEN-deficient intestinal stem cells initiate intestinal polyposis”
Current chemotherapy generally fails to totally eliminate cancer because it targets fast-proliferating malignant cells, but not the root cause—slow cycling cancer stem cells. A new perspective suggests that cancers arise from stem cell-driven processes that can renew and adapt themselves. The progression from mutations in stem cells to primary tumor initiation remains elusive. PTEN mutations are linked to Cowden syndrome, an inherited disorder with symptoms like Intestinal polyposis. Our study in P5 used a conditional PTEN mutant mouse model to investigate the role of PTEN-Akt signaling in transforming normal stem cells into cancerous ones, and how cancer stem cells initiate tumors. My contributions to the field include: 1) the discovery that constitutive activation of Akt due to loss of PTEN inhibition leads to over proliferation of ISCs; 2) that contrary to previous dogma that Akt indirectly regulates β-catenin activity via phosphorylation of Gsk3, I discovered that Akt can enhance the β-catenin activity by directly phosphorylating C-terminal S552 and S675 of β-catenin; and 3) I developed an anti-p-β-catenin antibody, which can mark tumor/cancer stem cells.

Contributions of this paper to Stem cell Research:

1. Development of Anti-p-β-catenin S552 antibody. During these studies I developed and characterized a phospho-specific antibody (anti-p-β-cat-S552) against the activated form of β-catenin, which is phosphorylated by Akt at Ser552. This reagent was critical in allowing us to detect tumour initiating cells in adenoma and adenocarcinoma. Today, this antibody has been shared with multiple labs throughout the world working on adenoma (see publications by the labs of T. Barrett, A. Nusrat, J. Yu, T. Steppenbeck, S. Henning, J. Perry, R. Sugimura), lymphoma (Zhang et al., 2010), and other groups (Qiu et al., 2010).

2. P-β-catenin S552 has been used to identify inflammation-driven, Akt-enhanced Wnt signalling. My work showing that Akt regulates β-catenin activity through direct phosphorylation of β-catenin at the Ser552, allows the field to use P-β-catenin S552 to identify inflammation-driven, Akt-enhanced Wnt signalling, as shown by other groups (Khan et al., 2013; Keefer et al., 2013; Koch et al., 2011).

3. Screening small molecules that inhibit p-β-catenin S552. Through high-throughput screening of a Food and Drug Administration-approved small-molecule library, we
identified doxorubicin as a candidate inhibitor of p-β-catenin S552. Our lab collaborated with KUMC to further show that doxorubicin inhibited phosphorylation at S552 of β-catenin by AKT. Low dosage doxorubicin was effective in inhibiting leukaemia stem cells in a pre-clinical study (Perry et al., 2020).

4. **Grants awarded based on the papers.**

The following three grants were awarded to support our lab’s Intestinal stem cell research. These grants on which I was listed as key personnel, were based on my 2004 and 2007 publications, as well as my extended studies in the lab.

Title: Role of BMP Signalling in Intestine Stem Cell Development
National Institutes of Health
U01-DK085507-01 (completed)
Active Project: 09/01/2009 – 08/31/2014

Title: Cellular, Molecular and Functional Characterization of Quiescent/Active Intestinal Stem Cells
U01 DK085507-02 (completed)
Active Project: 09/01/2015 – 08/31/2019

Cellular, Molecular and Functional Characterization of Quiescent/Active Intestinal Stem Cells
U01 DK085507-03 (active)
Active Project: 09/01/2015 – 08/31/2024

Title: Isolation and Characterization of Intestinal Stem Cells

**P6: “PTEN maintains hematopoietic stem cells and acts in lineage choice and leukaemia prevention”**.

In adults, balancing the quiescent and activated states of stem cells is crucial to ensure stem cell maintenance and continuous tissue regeneration. However, the molecular mechanisms that govern this equilibrium remain largely unexplored.
P6 demonstrated that PTEN inactivation disrupts HSC regulation, reducing the percentage of cells in the quiescent (G0) state, while concomitantly increasing the proportion of actively cycling cells, which collectively compromised the capacity to maintain LT-HSCs. We concluded that PTEN plays a pivotal role as a molecular regulator, guiding the forward (G0–G1) and backward (G1–G0) switch between the quiescent and activated states of LT-HSCs. I as a co-author of this work, made a major contribution to the field by uncovering a key pathway that can distinguish between normal stem cells and cancer stem cells.

Contributions of this paper to stem cell research:
This work together with another paper (Yilmaz et al., Nature 2006) linked PTEN/Akt signaling and cancer stem cell development, were pioneer studies in the field as evidenced by many subsequent papers that supported our initial findings. For example:

- Abnormal PTEN/Akt signalling regulates non-small cell lung cancer (Pérez-Ramírez et al., Pharmacogenomics 2015)
- PTEN deficiency reprograms neural stem cells toward stem-like cells in glioblastoma (Duan et al., Nature communication 2015).
- PTEN loss and Ras mutation cooperate in prostate cancer stem cells (Muholland et al., Cancer Research 2012).

P7. “N-cadherin Expression Level Distinguishes Reserved versus Primed States of Hematopoietic Stem Cells”.

Hematopoietic stem cells are predominantly found in a quiescent state. However, blood production is an active process with billions of blood cells generated each day. How quiescent stem cells continually support active blood production was incompletely understood.

This publication proposed the concept of co-existence of a ‘reserve pool’ of quiescent HSCs balanced with ‘active’ HSCs that support routine hematopoiesis.

Contributions of P7 to Stem cell Research:

- This concept, initially established in the hematopoietic field, allowed our lab to propose the same concept in the intestinal field (Scoville, He, and Li, Gastroenterology 2008) as
active cycling intestinal stem cells were discovered in 2007 (Barker et al., Nature 2007).

- In 2010, our lab PI Linheng Li, together with Hans Clevers published a prospective review extending this concept to stem cells in multiple tissues in mammals: “Coexistence of quiescent and active adult stem cells in mammals” (Li & Clevers, Science 2010).

P8: Cooperation between Wnt/β-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion.

Self-renewal allows stem cells to replicate throughout an organism’s lifespan, without losing developmental potential. Preventing differentiation is required for stem cell self-renewal. Conversely, imposing differentiation on proliferating stem cells—or apoptosis for stem cells that fail to properly differentiate—is critical to hematopoietic stem cell (HSC) homeostasis and cancer prevention. The stem cell pool is not static, however. During development or in response to acute stress, stem cells may transiently expand by symmetric division, where both daughter cells remain as undifferentiated stem cells. This paper used a hematopoietic stem and progenitor cell (HSPC)-specific conditional approach, driving both PTEN deletion and β-catenin activation. Through this study, we demonstrated for the first time that activation of either single pathway is insufficient to expand primitive HSCs. But in combination, both signaling pathways collectively drive the self-renewal and expansion of HSCs with long-term functional capacity.

Contributions of P8 to Stem cell Research:

This publication reported that coordination of Akt and β-catenin signaling can promote murine HSC self-renewal and support ex vivo HSC expansion. This provided the basis for a recent publication, Sakurai et al., Nature 2023, in which the authors screened small molecules that can activate human Akt and show this is one of the key steps required to maintain long-term ex vivo culture of HSCs.

P9: “Overcoming Wnt/β-catenin dependent anti-cancer therapy resistance in leukaemia stem cells”.
Leukaemia stem cells (LSCs) underlie cancer therapy resistance but targeting these cells remains difficult. My work in P9 shows that Wnt-β-catenin and PI3K-Akt pathways cooperate to promote tumorigenesis and resistance to therapy. Furthermore, this work also uncovered a mechanism underlying the immune escape of LSCs in which β-catenin directly binds to multiple immune check point genes, which are upregulated by Akt phosphorylation of β-catenin at S552.

Contributions of P9 to Stem cell Research:

- This work serves as the basis for translation into a clinical trial with registration no: NCT02914977. The clinical trial validated our findings in murine models and showed that low-dose of doxorubicin indeed reduces leukaemia stem cells in 50% of AML patients. Doxorubicin is a widely used chemotherapy agent.

P10. “Tumor-initiating stem cell shape its microenvironment into an immunosuppressive barrier and pro-tumorigenic niche”.

Prior studies focused on the tumor associated macrophages (TAMs), reported accumulating clinical observations that cancer stem cells (TSC/CSC) are more drug-resistant compared to the majority of cancer cells, and thus correlated well with cancer relapse. The work in P10 focused on: 1) identification of therapy resistant T/CSCs; 2) discovered that T/CSC actively cross talk with the TAM and shape the TAM into an immunosuppressive niche; 3) and revealed the key signaling pathways involved in mediating immunosuppression.

Contributions of P10 to stem cell and cancer research:

- Honors: I received a “Young Investigator Award”, at the 7th Midwest Tumor Microenvironment Meeting 2022
- I was invited to give an oral presentation for this study (P10) at the KUCC Early Phase Symposium 2021
- I was invited to give an oral presentation for this study (P10) at the 7th Midwest Tumor Microenvironment Meeting 2022
- This study attracted the attention of the American Association for Cancer Research (AACR). Our lab was invited to give two lectures at the 2022 annual meeting:
--Educational Session ED039 (04/09/2022): “The Evolving in the Cancer Stem Cell Niche at their 2022 AACR annual meeting”

--Major Symposium (04/10/2022): “Macroenvironmental and Microenvironmental Drivers of Cancer Stem Cell Generation”

- Based on my study, together with our NCB 2020 paper, our lab PI was invited to speak at a 2023 NCI special workshop focused on immune escape by cancer stem cells and immunosuppression by the TME.

- Based on my study, together with our NCB 2020 paper, our lab PI was invited to write a review article “Understanding and Overcoming Immunosuppression Shaped by Cancer Stem Cells. Cancer Res 83, 2096-2104.

2.4 Commentary on the reception of the publications, as indicated by citations and reviews, and the standing of the journals in which they were published

Although H-index has flaws and can be misused (statistics from Google Scholar may not be entirely accurate), it is a simple quantitative indicator that roughly measures the academic influence of scientists. My current H-index is 31 and my work has been cited over 13,000 times, reflecting the quality of my work and publications. However, the number of published articles and H-index are not the only indicators of impact. The contributions of individual scientists to the scientific research community are multifaceted. In addition to conducting research and publishing articles, teaching and educating people, another important aspect is providing useful scientific knowledge and materials to scientific peers, which is very important for pushing the field and experimental science forward.

Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early haematopoiesis

Blood Journal Editorial comment: Inside *blood* P:383 by Tariq Enver, Title: Blueprints for *blood*. “Described the global molecular profiles of various classes of highly purified murine stem and progenitor cells using microarray technology. Since the phenotype of any given cell is ultimately the product of the genes it expresses or has expressed during its lifetime, this approach is likely to yield significant insight into the molecular basis of “stemness.” The authors have distilled some digestible general principles that both echo and refine earlier ideas about molecular ground states…. This broad transcriptional accessibility in HSCs is sequentially restricted in MPCs, which display only hematopoietic priming through CMPs and CLPs that display myeloid-only and lymphoid only expression, respectively. 15 January 2003. 101(2)


BMP signalling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signalling
News and Views by Gijs van den Brink in the same issue of Nature Genetics

Title: Linking pathways in colorectal cancer

In this highlight, Dd. Van den Brink stated that “Studies of inherited cancer syndrome have implicated numerous signalling pathways in colorectal carcinogenesis, but the relationship between these signalling pathways remain poorly understood” and “He et al., provide us with a testable model in which three pathways (Wnt, BMP, Pten-Akt) linked to different polyposis syndromes cooperate to control ISC numbers and the rate of crypt fission”.


P3: Jiwang Zhang, Chao Niu, Ling Ye, Haiyang Huang, Xi He et al (2003)

Identification of the hematopoietic stem cell niche and control of the niche size

News and Views by I. Lemischka and K. Moore in the same issue of Nature:

Title: Interactive niches:

The microenvironment, or niche, in which stem cells reside controls their renewal and maturation. The niche that regulates blood-forming stem cells in adult animals has eluded researchers—until now!

P4: Jiwang Zhang, Xi C He et al (2006)

Bone Morphogenetic Protein signaling inhibits hair follicle anagen induction by restricting epithelial stem/progenitor cell activation and expansion

This work was reviewed and confirmed by Elaine Fuchs’s group.

(PNAS 2007: https://doi.org/10.1073/pnas.0703004104).

P5: Xi C He et al (2007)

PTEN-deficient intestinal stem cells initiate intestinal polyposis

“U.S. scientists have clarified how normal stem cells become cancer stem cells, as well as how cancer stem cells can cause the formation of tumors. Dr. Xi He and associate investigator Linheng Li, both with the Stowers Institute for Medical Research, studied the intestinal system in mice in which one of the human tumor suppressor genes, PTEN, had been deleted. They found that a loss of PTEN in intestinal epithelial cells accompanied by a loss of PTEN in stromal cells can lead to changes that may increase the number of stem cells and change their position or location. These changes result in crypt fission and budding and can lead to intestinal polyposis and uncontrolled tumor growth. This study showed that "cancer stem cells are a rare population in the tumor mass; that they are slow cycling, but more active than normal stem cells; and that cancer stem cells, and stromal insertions initiate the process of primary tumorigenesis."

P6: Jiwang Zhang, Justin C Grindley, Tong Yin, Sachintha Jayasinghe, Xi C He et al

PTEN maintains hematopoietic stem cells and acts in lineage choice and leukaemia prevention

News and Views by V. Janzen and D. Scadden in Nature:

Title: Good, bad, and reformed

The ability of stem cells to continuously supply vast numbers of cells is magnificent, but it can be devastating if it runs amok, as in some tumors. So, what makes a normal stem cell turn bad, and can it be redeemed?

Comments published in Haematology (2006)

“PTEN Scores a “10“ in Stem Cell Discrimination” by Peter Emmanuel:

" Don’t we wish sometimes that cancerous stem cells would just hold up a sign and say “Here I am?” Wouldn’t it make life a lot easier, especially for transplant physicians, if normal hematopoietic stem cells could be distinguished from malignant stem cells? Well, we’re not quite there yet but we may be starting to unlock some of the clues. Two mouse models recently reported in Nature implicate PTEN as a major regulatory switch in maintaining normal hematopoietic stem cell function. In their mouse model, Zhang
and colleagues (Publication 5) deleted exon 5 of the PTEN gene, which contains the lipid phosphatase domain, leading to PTEN deficiency. Their mice displayed a decline in the number of hematopoietic stem cells, a diminished self-renewal capacity, an increase in the number of colony-forming unit cells in the spleen and peripheral blood (with stable numbers of colony-forming unit cells in the bone marrow), a decrease in the number of common lymphocyte progenitors, and an increase in the peripheral blood monocytes and granulocytes. The result culminated in PTEN-deficient mice developing a myeloproliferative disorder. Further, the PTEN-deleted cells could be transferred to irradiated recipient mice with subsequent disease development, evidence that they are “cancer stem cells.”


**N-cadherin expression level distinguishes reserved versus primed states of hematopoietic stem cells.**

This work served as the basis for the review article published in Science (Li and Clevers, 2010).

P8: Perry, J.M., X.C. He et al (2011)

**Cooperation between both Wnt/{beta}-catenin and PTEN/PI3K/Akt signaling promotes primitive hematopoietic stem cell self-renewal and expansion**

Review by Science Daily—Science News:

[http://www.sciencedaily.com/releases/2011/09/110907220534.htm](http://www.sciencedaily.com/releases/2011/09/110907220534.htm) “All stem cells -- regardless of their source -- share the remarkable capability to replenish themselves by undergoing self-renewal. Yet, so far, efforts to grow and expand scarce hematopoietic (or blood-forming) stem cells in culture for therapeutic applications have been met with limited success. Now, researchers have teased apart the molecular mechanisms enabling stem cell renewal in hematopoietic stem cells isolated from mice and successfully applied their insight to expand cultured hematopoietic stem cells...”
P9: John M Perry, Fang Tao, Anuradha Roy, Tara Lin, Xi C He et al (2020)

**Overcoming Wnt-ß-catenin dependent anticancer therapy resistance in leukaemia stem cells**

P10: Xi He et al (2021)

**Tumor-initiating stem cell shapes its microenvironment into an immunosuppressive barrier and pro-tumorigenic niche.**
3. List of my publications and reviews not included in the thesis

As of October 2023, I have published 43 primary papers and 10 review articles.

❖ Collaboration, Mentoring, Training, and Supervision Bibliography

1) I provided stomach tissue sections of Bmpr1a KO mice to Dr. Offerhaus’ group for studying BMP signalling in gastric epithelia.


2) I trained Wang, Fenchao in ISC and Crypt isolation, histology, image analysis and intestinal scRNA assays. Fengchao is now a Professor in Chongqing University, China.


3) I trained Zhengrui Li in HSC isolation, flow cytometry, imaging and HSC RT-PCR of bone marrow. Zhengrui is currently a postdoctoral fellow at St Jude Children’s Hospital.


4) I, as direct supervisor of Donghua Liu, a graduate student, assisted with experimental design, assay development and analysis, and trained Donghua in HSC isolation, flow cytometry, and imaging of the Aorta, gonad, and mesonephros (AMG) region. Donghua is now an associate Professor in Harbin University School of Medicine.


5) I trained Pengxu Qian in HSC isolation, flow cytometry, and imaging of bone marrow. I also helped and coach him in RNA isolation and cDNA preparation for the Clip-seq assay. Pengxu is now a Professor in Zhejiang University, China.


6) I trained Ryohichi Sugimura (Sugimura et al., 2012) and Aparna Venkatraman
(Venkatraman et al., 2013) in HSC isolation, flow cytometry, and imaging analysis. I assisted with bone marrow transplantation experiments, developed, and designed research directions and performed trouble shooting of experiments.


7) I coached Yucai Xie and Tong Yin (co-first author) in HSC isolation, flow cytometry, and imaging analysis. I also assisted with the HSC homing assay. Xie is currently a researcher in Shanghai Jiao Tong University. Yin is an Associate Professor/Physician in Shanghai Jiao Tong University.


❖ Reviews


4. **Invited Oral and poster Presentations at Scientific Centres and Meetings**

I have presented around 20 posters and given 20 oral presentations at Scientific Centers and Meetings since December 2023

❖ **Oral Presentations**

- **March 2004**  Midwestern Developmental Biology 45th Annual Meeting, USA
- **June 2012**  Stem Cell Symposia Keio University, Japan
- **March 2014**  ISCC Spring Meeting, Bethesda MD, USA
- **June 2014**  FSC, SIMR, USA
- **March 2016**  Stem Cell Therapy-Facts and Myths, Heidelberg, Germany
- **September 2016**  ISCC Fall Meeting, Boston, USA
- **September 2016**  Wichita State University, Wichita, USA
- **October 2017**  FSC, SIMR, USA
- **June 2019**  FSC, SIMR, USA
- **October 2020**  FSC, SIMR, USA
- **January 2021**  FSC, SIMR, USA
- **October 2021**  KUCC Early Phase Symposium, USA
- **January 2022**  ISSCR, USA
- **May 2022**  Midwest Tumor Microenvironment Meeting, USA
- **January 2023**  The George Washington University, USA
- **May 2023**  Association of Biomolecular Resource Facilities Annual Meeting, USA
- **May, 2023**  SuperGroup Meeting, SIMR, USA
- **August 2023**  Open Mic, SIMR, USA
- **October 2023**  Translational Research Seminar Series, SIMR, USA
- **November 2023**  FSC, SIMR, USA

❖ **Poster Presentations**

- **January 2002**  Keystone Stem Cell Symposia, USA
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5. References


Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology 46, 1693-1703.


Cell stem cell 4, 280-282.


Cell 88, 435-437.


6. Copies of the Published Papers


42. PubMed PMID: 21890648


Copies of P1-P10 published papers as appendixes.