UNIVERSITY OF CALIFORNIA, SAN DIEGO

Wnt Signaling and Developmental Logic in Human Stem Cell Populations

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

Ian Joseph Huggins

Committee in Charge:

Professor Karl Willert, Chair
Professor Jerold Chun
Professor Terry Gaasterland
Professor Larry Goldstein
Professor Alysson Muotri

2016

The Dissertation of Ian Joseph Huggins is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2016

To Sara
You are ever the love of my life and the song of my heart.

To my children
You are my greatest delight and hope for the future.

To my parents, Jennie and Mark
For your unfailing love and unshakeable confidence in me.

To my Grandma, Marian Budge
Who read to a little boy by night and taught him to love raccoons, squirrels, honeybees, hummingbirds and everything under the sun.

To my Grandma, Mary Dawn Huggins
Whose loving words and deeds are with me always.

UNLESS someone like you
cares a whole awful lot,
nothing is going to get better.
It's not.

Dr. Seuss

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AAV	Adeno-associated Virus
APC (Fluorophore)	Allophycocyanin
APC (Protein)	Adenomatous Polyposis Coli
bp	Base Pairs
Btd	Buttonhead
Buf	Wnt Buffer
cDNA	Complementary Deoxyribonucleic Acid
ChIP	Chromatin Immunoprecipitation
ChIP-Seq	Chromatin Immunoprecipitation followed by Sequencing
CHIR	Small Molecule Glycogen Synthase Kinase Inhibitor CHIR98014
CM	Conditioned Medium
cm	Centimeter
CNS	Central Nervous System
Cre-ER	Cre Recombinase Estrogen Receptor Fusion Protein
CRISPR	Clustered Regularly Interspersed Palindromic Repeats
DE	Definitive Endoderm
Del	Deletion
dHet	Deletion Heterozygote
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dPBS	Dulbecco's Phosphate Buffered Saline
DVL	Dishevelled

E8	Essential 8 Culture Medium
EGFP	Enhanced Green Fluorescent Protein
EGR	Early Growth Response
E*n*	Embryonic Day *n*, where *n* is the number of days
F12	Ham's F12 Medium
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FLAG	Flag™ Peptide
FZD	Frizzled
GEO	Gene Expression Omnibus
GFP	Green Fluorescent Protein
GIMP	Gnu Image Manipulation Program
GSK	Glycogen Synthase Kinase
GST	Glutathione S Transferase Alpha Peptide
H1	WA01 (H1) Human Embryonic Stem Cell Line
H9	WA09 (H9) Human Embryonic Stem Cell Line
hESC	Human Embryonic Stem Cell
hESC-CM	Human Embryonic Stem Cell Conditioned Medium
hiPSC	Human Induced Pluripotent Stem Cell
hNSC	Human Neural Stem Cell
hPSC	Human Pluripotent Stem Cell
HUES7	HUES7 Human Embryonic Stem Cell Line
iFGFR	Fibroblast Growth Factor Receptor Inhibitor PD166866

iHet	Insertion Heterozygote
Ins	Insertion
iPSC	Induced Pluripotent Stem Cell (Any Species)
IRES	Internal Ribosomal Entry Site
iSP5	Doxycycline-Inducible SP5
kbp	Kilobase Pairs
kD	Kilodalton
KLF	Kruppel-Like Family
KMOS	KLF4, c-Myc, OCT4 and SOX2
KOS	KLF4, OCT4 and SOX2
M	Molar
MCM	Mouse Embryonic Fibroblast Conditioned Medium
MEF	Mouse Embryonic Fibroblast
mESC	Mouse Embryonic Stem Cell
mL	Milliliter
mM	Millimolar
mm	Millimeter
MMTV	Murine Mammary Tumor Virus
mRNA	Messenger Ribonucleic Acid
MuLV	Murine Leukemia Virus
nM	Nanomolar
OPC	Oligodendrocyte Progenitor Cell
PCR	Polymerase Chain Reaction
pM	Picomolar

PSC	Pluripotent Stem Cell (Any Species)
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNA-Seq	Ribonucleic Acid Purification followed by High Throughput Sequencing
RPKM	Reads per Kilobase per Megabase Mapped
RQ	Relative Quantity
sgRNA	Single Guide Ribonucleic Acid
sh	Short Hairpin
SID	mSin3a-Interacting Domain
SP1/5	SP1 and SP5
Sp5-Cre	Cre Recombinase under the control of the Sp5 Promoter
SP5dZF	SP5 with deleted Zinc Finger
TGF-β	Transforming Growth Factor Beta
TSS	Transcription Start Site
µL	Microliter
µM	Micromolar
µm	Micrometer
VEGF	Vascular Endothelial Growth Factor
Wg	Wingless
Wnt	A portmanteau of Wingless and Int-1
WT	Wild Type
YFP	Yellow Fluorescent Protein

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

Chapter 2, in part, is currently being prepared for submission for publication of the material. Huggins, Ian J.; Willert, Karl. I am the primary investigator and author of this material. I would like to further acknowledge the following individuals and organizations: Eric O'Connor, Karl Marquez, and Jesus Olvera of the UC San Diego Human Embryonic Stem Cell Core Facility for help and expertise in human pluripotent stem cell culture and cell sorting; Steven Head and his staff at the Scripps Research Institute Next Generation Sequencing Core Facility for library preparation and sequencing of samples for RNA-Seq and ChIP-Seq; Sonal Naik and Aritra Dey of the UC San Diego Stem Cell Genomics Core for invaluable advice and assistance with single cell gene expression experiments; Bing Ren and Ah Young Lee for sharing equipment, reagents and technical advice regarding ChIP-Seq; Rick Meyers and Kim Newberry for advice and expertise regarding ChIP-Seq; Nathan Kumar for advice and guidance in the generation of definitive endoderm. This work was supported by the California Institute for Regenerative Medicine. IJH was supported by a grant from the California Institute for Regenerative Medicine (TG2-01154).

Additionally, I would like to thank several individuals (mostly in order of acquaintance) who have helped me throughout my time in graduate school:

Antonio Fernandez, for being a top-notch lab manager, scientist, and friend, and for teaching me the ropes of human embryonic stem cell culture and genetic manipulation. Mat Bauer, for regularly providing purified Wnt protein and helping me figure out recombinant DNA in my early days, and for continued friendship and advice afterward. Nathan Kumar, who walked the bed of coals a few steps ahead of me and answers the question, "What do you want to be when you grow up?" with the

response, "Happy!". Dave Brafman, for providing helpful feedback, giving great experimental advice and more than one ride home after a long day in the lab. Holly Aur, Christine Phung, Catherine Phung, and Nikita Shah, whose friendship always made the lab a great place to be. Jason Ross, for help with critical experiments and for continued friendship. Bia Freitas, for friendship, advice and antibodies. Noel Moya, for ensuring the lab had a proper appreciation for Alexander the Great, the Stoics (especially Diogenes), the Romans, Renaissance art, pirates, and Cuban music. Stephanie Soltero, for true friendship many years after leaving the lab for bigger and better things. Terry Gaasterland, for her infectious enthusiasm for all things sequencing and for introducing me to the awesome possibilities of bioinformatics. Kayla Ruggiero, my first student in the lab, whose boundless enthusiasm for science served as a constant reminder of the privilege to work in an exciting field. Kelly Ross, for her contagious love of *schmidtea mediterranea* and *Gallus gallus*. Brett Henninger, who worked tirelessly to optimize antibodies that would be critical to the success of my work. Tomas Bos, for companionship, advice, and a wealth of good jokes and funny stories. Cleber Trujillo, for friendship, mouse work, and invaluable advice. Anne Conway, whose embodiment of hard work, dedication, and determination has inspired us all at some point. Stephanie Grainger, for friendship, laughs, a few airport rides, and more than a few memorable field trips. Jenna Richter, for friendship, enthusiasm and reminding me that grad school is not really so bad. Kelli Chase, my second student, who was a quick study with golden hands and incredibly productive during her short time in the lab. Emily Smith, for constant encouragement and determination. Colleen Stoyas, for friendship, encouragement, and critical reading of so many resumes. Julia Nussbacher, for friendship and for

helping me figure out how to use the Tapestation. Ah Young Lee, for helping me to figure out ChIP-Seq when I was at the end of my rope. Alan Rubin, my third student, for dogged perseverance and pursuit of his goals in the face of adversity, and teaching me that all things happen in their own time. Ana Ramos, for helping me to optimize SP5 immunostains. Angeline Puranen, my fourth student, for dedication, laser focus, hard work, and perseverance. Charlotte Rossdam and Christina Jessen, visiting students from Germany whose brief stays in our lab led to friendship and mountains of data. Olivia Gaylord, my final student, for working hard to discover her passion and for making tons of transgenic cell lines. Finally, I would like to acknowledge everyone else who has helped me along the way. The many family and friends who have shown continued love and support have helped me in ways that are difficult to put down in writing. It wouldn't have been the same without you.

Last I would like to thank Karl, my advisor, for patience, guidance, encouragement and support through technical challenges, scientific successes, and life's important milestones that took place during my time in his lab.

VITA

2008	University of Utah Bachelor of Science, Biology, *cum laude* *Bioscience Undergraduate Research Fellow*
2009-2016	University of California, San Diego Biomedical Sciences Graduate Research Program UCSD Stem Cell Program Sanford Consortium for Regenerative Medicine Graduate Research
2009-2010	Genentech Foundation Predoctoral Research Fellow
2012-2014	CIRM Predoctoral Research Fellow
2016	University of California, San Diego Doctor of Philosophy, Biomedical Sciences

PUBLICATIONS

Huggins IJ, Willert K. The Transcription Factor SP5 Amplifies Wnt Signals in Human Pluripotent Stem Cells by Repressing Wnt Antagonists. *In preparation*.

Fernandez A*, **Huggins IJ***, Perna L, Brafman D, Lu D, Yao S, Gaasterland T, Carson DA, Willert K. The WNT receptor FZD7 is required for maintenance of the pluripotent state in human embryonic stem cells. *PNAS USA*. 2014 Jan 28;111(4):1409-14. doi: 10.1073/pnas.1323697111. Epub 2014 Jan 13.

Rai K, **Huggins IJ**, James SR, Karpf AR, Jones DA, Cairns BR. DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and gadd45. *Cell*. 2008 Dec 26;135(7):1201-12. doi: 10.1016/j.cell.2008.11.042.

ABSTRACT OF THE DISSERTATION

Wnt Signaling and Developmental Logic in Human Stem Cell Populations

by

Ian Joseph Huggins

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

Professor Karl Willert, Chair

Human development is complex. Countless molecular events guide a single cell and its daughters through processes like cell division, differentiation and even death to produce a free-living organism that comprises trillions of cells, multiple organs and a complex brain capable of adaptation, emotion and invention. To achieve this end, cells have evolved a highly complex system of signals to communicate with each other throughout development and into adulthood. The Wnt signaling pathway is key among such developmental signals, and is required for multicellular animal life. The role of the Wnt signaling pathway in early human development is poorly understood. Using human pluripotent stem cells (hPSCs) and human neural stem cells (hNSCs), I have undertaken a series of studies designed to

elucidate the functions of the Wnt pathway in these models of early human development.

In Chapter 1, I introduce hPSCs, their derivatives (such as hNSCs) and their potential as a model system. I discuss the Wnt signaling pathway and its roles in development and disease. Finally, I introduce the transcription factor SP5, which I discovered is an important target of Wnt signaling in hPSCs and hNSCs.

In Chapter 2, I describe high throughput RNA sequencing expression analysis of Wnt3a-treated hPSCs. I identify *SP5* as a key transcriptional target and characterize its binding genome-wide by chromatin immunoprecipitation followed by sequencing. I demonstrate that SP5 functions by repressing Wnt antagonist genes and that this activity restricts the formation of definitive endoderm in Wnt-treated hPSCs.

In Chapter 3, I describe the effects of Wnt pathway activation on pluripotency and differentiation to early germ layers. I further discuss the novel role of Wnt signaling as a negative regulator of EGR1 expression.

In Chapter 4, I demonstrate the effects of Wnt signaling on hNSC multipotency and differentiation. I describe high throughput RNA sequencing expression analysis of Wnt3a-treated hNSCs. I identify FGF family members and *SP5* as key transcriptional targets and demonstrate their roles promoting hNSC multipotency and proliferation.

In Chapter 5, I discuss the broader implications of my findings and speculate on the subject and results of future work in the field.

Chapter 1 - Introduction

1.1 Defining the role of Wnt signaling and SP5 in hPSCs

Human pluripotent stem cells (hPSCs) promise to speed the advent of the age of regenerative medicine, which aims to treat disease and heal the body by understanding and harnessing the body's ability to develop, maintain and repair itself. These cells, capable of self-renewing cell divisions and differentiation into all cell types of the body, represent a potentially revolutionary tool for both modeling and eventually treating diseases and injuries of all body systems. Before this potential is to be fully realized, a more complete understanding of the nature of these cells and the processes that govern their behaviors is required. All cells are molecular machines that integrate biochemical signals from both the extracellular and intracellular environment to make decisions based on a molecular logic whose language we are only beginning to understand. One of these key "signaling pathways" that governs the behavior of hPSCs and their derivatives from gastrulation through the life of an organism is known as the Wnt signaling pathway.

Here, I have undertaken biochemical, genetic, and genomic studies of the Wnt signaling pathway in hPSCs, human neural stem cells (hNSCs), and other cell types in an attempt to understand the role this pathway plays in important cellular decisions, especially self-renewal and differentiation. I have utilized advanced methodologies such as RNA-Seq and ChIP-Seq to identify key roles of Wnt signaling in the self-renewal and differentiation of hPSCs and hNSCs. In particular, I have identified a previously unidentified positive feedback regulation mechanism present in Wnt-responsive stem cells, but not other cell types. This positive feedback regulation centers on the Wnt target gene *SP5*, which encodes a member of the SP/KLF family of transcription factors whose primary function has been defined as repression of SP1

target gene expression (Fujimura et al., 2007). Critically, I have revealed several Wnt antagonist genes to be targets of SP1 and SP5 by ChIP-Seq, suggesting that SP5 serves to sensitize stem cells to Wnt signals by repressing these antagonists.
In order to more fully understand stem cell behavior and the workings of the Wnt signaling pathway, I have focused on using tissue culture models of early human development to perturb the Wnt pathway and measure changes in cell behavior.

1.2 hPSCs and their derivatives

The technology to derive and maintain hPSCs in artificial culture systems is relatively new (Thomson et al., 1998), owing partly to the difficulty of identifying culture conditions that promote self-renewal over spontaneous differentiation. This challenge was overcome by culturing the cells in the presence of mouse embryonic fibroblast cells that acted as supportive "feeders", secreting undefined factors that promoted the maintenance of pluripotency. Since the first derivation of human embryonic stem cells (hESCs), a subset of hPSCs that are derived from the inner cell masses of human blastocysts (**Figure 1-1A**), several important milestones have been cleared: The ability to culture hPSCs in the presence of a chemically defined serum replacement (Amit et al., 2000) reduced variation between batches of culture medium. Feeder-free culture of stem cells with MEF-conditioned medium on a purified protein substrate (Xu et al., 2001) represented another advance that greatly facilitated experimentation using standard molecular biology methodologies such as Western Blot and quantitative real-time PCR (qPCR). The advent of chemically defined culture media and growth substrates (Chen et al., 2011; Ludwig et al., 2006) and small molecules to promote the survival of enzymatically dissociated hPSCs (Watanabe et al., 2007) facilitated the application of standard methods as well as advanced clonal

genetic engineering approaches. Importantly, the derivation and expansion of hPSCs in entirely xeno-free culture conditions, which lack undefined animal products of any kind, enables the potential transplant of hPSC-derived cells and tissues to human patients. We currently find ourselves in an era of great opportunity as the power of this model system is only now beginning to be realized.

PSCs derived from human embryos are incredibly useful for modeling human development and disease, but their application suffers from critical drawbacks: the ethical and legal framework for work with embryo-derived cells and tissues is often restrictive to the point that the use of some hESC lines is limited to the study of one germ layer and its derivatives; hESC-derived cells may be difficult to match to patients in the eventual case of cell transplantation therapies. The ability to derive hPSCs from adult tissues circumvents both of these problems because they do not require the destruction of an embryo, and patient-specific hPSCs would be a perfect match in cell transplantation therapies. The demonstration that mouse somatic cells such as skin fibroblasts could be induced to pluripotency by the retroviral introduction of 4 defined factors (Takahashi and Yamanaka, 2006) advanced the study of PSCs (**Figure 1-1B**). These induced pluripotent stem cells (iPSCs) closely resembled mouse ESCs in terms of marker expression, trilineage differentiation potential, and importantly, the ability to contribute to chimeric embryos and the germline. Human somatic cells were demonstrated to be amenable to reprogramming shortly afterward (Takahashi et al., 2007). It is important to note that while hiPSCs are very similar to hESCs in marker expression and differentiation potential, they differ significantly at the level of the transcriptome (Chin et al., 2009) and epigenome (Lister et al., 2011), indicating that four factor reprogramming incompletely recapitulates the hESC

phenotype. Nevertheless, these cells have proven invaluable as a source of material for the study of genetic disease with cellular and molecular detail never before imaginable, and hold the promise of one day providing an unlimited source of patient-matched hPSCs for the development of cell transplantation therapies.

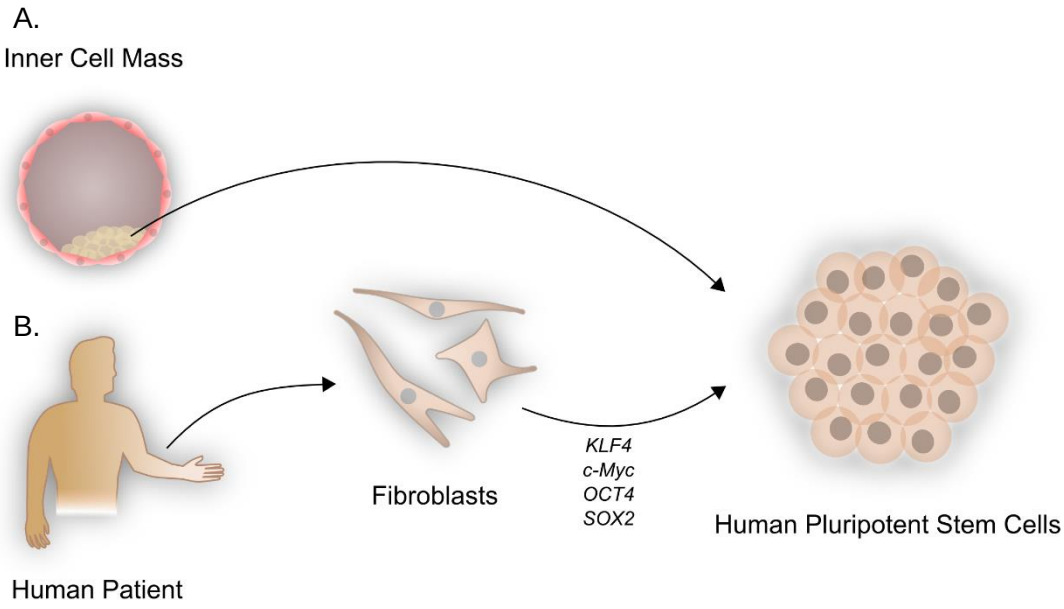

Figure 1-1: Derivation of Human Pluripotent Stem Cells (hPSCs)

A. Human Pluripotent Stem Cells can be derived from the inner cell mass of an early blastocyst stage embryo. **B.** Fibroblasts generated from patient biopsies can be reprogrammed through the addition of four defined factors, giving rise to induced pluripotent stem cells.

Early human embryonic development has historically been a poorly-accessible subject of investigation. The advent of hPSC technology enables diverse studies that delve into the processes that govern organogenesis in humans. Cell types representative of the three germ layers, endoderm, mesoderm, and ectoderm are now routinely derived from hPSCs in laboratories across the world. Some cell and tissue types readily form during spontaneous random differentiation of hPSCs, while others are less easily obtained. Lessons from developmental biology have informed efforts to generate more elusive cell types in greater homogeneity than is possible through spontaneous differentiation. So-called "directed differentiation" promises to provide an unlimited supply of tissue for developmental studies, drug screening and transplantation, but remains incompletely understood; there are still many lessons to learn.

Much work has been done to understand the principles underlying the generation of functional tissues from hPSCs. I will now briefly discuss some of the successes researchers have had in generating specific cell types as well as some of the challenges that lay ahead. First, I will discuss derivatives of the endodermal lineage. Type I Diabetes results when an autoimmune response attacks and eventually kills a patient's pancreatic β cells. In theory, these cells could be derived from hPSCs and transplanted as a long-term therapeutic option superior to regular manual glucose monitoring and accompanying insulin injections. Early work demonstrated that in some hPSC lines it was possible to generate definitive endoderm as well as early pancreatic cells capable of producing the hormones insulin and glucagon (D’Amour et al., 2005). The protocol was severely limited in its ability to coax multiple hPSC lines to efficiently generate pancreatic progenitors, and only

following maturation in animal hosts were true mono-hormonal insulin producing cells developed (Matveyenko et al., 2010), and even then only in some cases. Eventually, the ability to generate functional β cells *in vitro* was achieved (Pagliuca et al., 2014) and represents a major milestone on the path to unleashing the potential of regenerative medicine. Other endodermal cell types have been derived as well, including hepatocytes (Hannan et al., 2013), intestine (Spence et al., 2011) and lung (Huang et al., 2014). The ability to generate these cell types will lead to important gains in our understanding of human development and tissue homeostasis that will in turn lead to improved therapies, quality and length of life.

Next, I will discuss the advances made in studying tissues derived from the mesoderm. Perhaps the greatest success in this area is the development of functional beating cardiomyocytes, the muscles of the heart. Several protocols have emerged, but a consistent feature of efficient cardiomyocyte generation is activation of the Wnt signaling pathway (Lian et al., 2012; Tran et al., 2009). There are major challenges that must be overcome before *in vitro* generated cardiomyocytes can be used as a source of cells for therapies, however. For example, while it is trivial to generate these cells with high efficiency (>90%), the resulting cardiomyocytes are not all of one type. Some are similar to atrial cardiomyocytes while others are more representative of ventricular cardiomyocytes. Another major hurdle is the development of mature cardiomyocytes, as those that arise through *in vitro* differentiation display a very immature phenotype. Cell transplantation therapies and even drug testing will require pure populations of mature ventricular cardiomyocytes (Hartman et al., 2015; Yang et al., 2015). The generation of these cells represents a

significant challenge to the regenerative medicine community and much work remains to be done to discover the cues that will coax them into being.

The development of functional hematopoietic stem cells (HSCs) from hPSCs represents another major hurdle in the generation of mesodermal tissues. HSCs are the cells of the bone marrow that are capable of reconstituting all cells of the blood. HSC transplant often represents the treatment of last resort for patients suffering with diseases ranging from leukemia to sickle cell disease, but matched HSCs are often difficult to obtain. The generation of HSCs from hPSCs, especially patient-derived hiPSCs, would be a great advance in this area and could potentially save thousands of lives every year. While researchers have long been able to generate hematopoietic progenitor and derivative cell types (Kouskoff et al., 2005), the generation of *bona fide* HSCs capable of reconstituting the hematopoietic system has not been reported. This will likely remain a high priority for the stem cell community for many years until the challenge is overcome.

Similar challenges arise in the generation of ectodermal tissues, especially those of the central nervous system. While researchers have long been able to generate neural precursor cells from hPSCs (Zhang et al., 2001), it was many years before robust protocols for their generation emerged (Chambers et al., 2009). Many researchers use the so-called "dual SMAD inhibition" protocol developed by Chambers el al., but often the resulting hNSCs are representative of a wide range of developmentally distinct neural stem cells including forebrain, midbrain, hindbrain, and spinal cord. The Wnt signaling pathway has been shown to regulate the patterning of hNSCs *in vitro*, where its level of activity in a given hNSC reflects the patterned state of that cell (Moya et al., 2014). This knowledge has proven useful, but

the ability to generate highly pure populations of regionally specific neurons and glia is still a significant challenge. Neurons derived from hPSCs *in vitro* offer a wonderful chance to study human diseases in a dish (Reviewed in Chailangkarn et al., 2012; Young and Goldstein, 2012), but the most promising use of these cells will be for cell-replacement therapies. Parkinson's disease, which results from a loss of dopaminergic neurons in the Substantia Nigra, could possibly be treated by transplant of *in vitro*-derived dopaminergic neurons (Barker et al., 2015). Studies have shown promise in primates (Redmond et al., 2007; Wakeman et al., 2014), but human studies will be more difficult to conduct, as they rely solely on functional outcomes, leaving mechanism of action (or failure) more difficult to assess. Others are attempting to use transplanted neural stem cells to treat diseases and injuries such as stroke (Reviewed in Diamandis and Borlongan, 2015), spinal cord injury (Reviewed in Ramer et al., 2014) and Alzheimer's disease (Reviewed in Tong et al., 2015).

In conclusion, these tissues will allow unprecedented studies of human tissues in the context of development, disease and potential therapies. Drug tests in mice are notorious for poor translation to human studies. Human tissues derived *in vitro* represent a potentially useful tool to surmount the challenges of studying disease in lower vertebrates. Ultimately, the long-term goal of hPSC research is to improve our understanding of human development and leverage that knowledge to treat disease and improve quality of life in ways unthinkable to previous generations of scientists and clinicians.

1.3 Wnt Signaling

1.3.1 Wnt signaling in development and stem cells

Embryogenesis is a staggeringly complex process that requires the precise coordination and concerted efforts of continuously dividing and differentiating cells. To properly orchestrate the development of a multicellular organism, cells must continuously send and receive signals that are integrated via complex signaling pathways. These signals may promote proteomic and transcriptional changes, or homeostasis. Ontogeny is highly conserved across phyla, especially during early stages of development, suggesting an ancient origin of the more basic and critical signaling systems that cells must employ to form the first basic patterns: head and tail, front and back, left and right, inside and outside, etcetera. By investigating the mechanisms by which cells organize their behaviors in early development, we will gain increased understanding of the processes that regulate the formation of organs and organisms, as well as the causes of disease. This knowledge will be instrumental going forward as we seek to develop the next generation of cell and gene therapies.

The Wnt signaling pathway is among the most ancient and conserved animal signaling pathways (Cadigan and Nusse, 1997; Nusse, 2001; Wikramanayake et al., 2003). At the transcriptional level, Wnt signaling drives the upregulation of TCF/LEF target gene expression by stabilizing β-catenin, which is ordinarily degraded in a constitutive fashion by a "destruction complex" which includes APC, AXIN and GSK-3β. This complex phosphorylates β-catenin, which leads to its ultimate degradation by the proteasome. Wnt signals are relayed through the secretion of lipidated, glycosylated Wnt protein ligands (Willert et al., 2003). Upon secretion, Wnt proteins may engage the Lrp and Fzd family of cell surface receptors. These receptors transduce the signal through interaction with the Dvl family of proteins, which are subsequently phosphorylated and recruit the destruction complex to the membrane,

deactivating it. This allows cytoplasmic accumulation of β-catenin, which translocates to the nucleus where it displaces the TLE family of co-repressors and ultimately drives the transcription of TCF/LEF target genes (**Figure 1-2**). The Wnt pathway plays instrumental roles throughout development, in tissue homeostasis, and in many cancers (Klaus and Birchmeier, 2008) and is thus of critical importance in the study of human development and the practice of regenerative medicine.

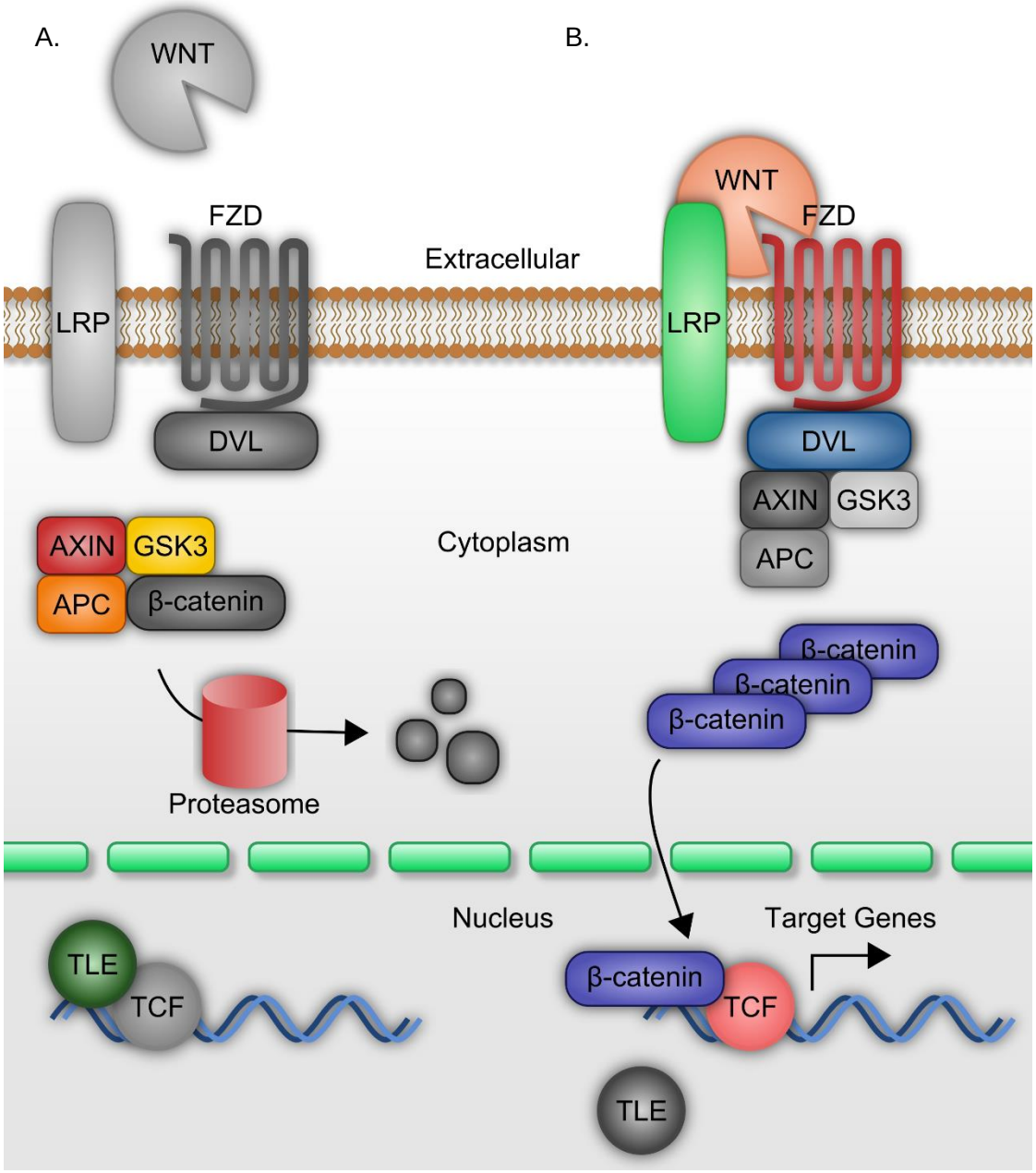

Figure 1-2: The Wnt Signaling Pathway

A. In the absence of a Wnt ligand, the destruction complex phosphorylates β-catenin, leading to its ubiquitination and degradation by the proteasome. TCF/TLE complexes repress Wnt target genes in the nucleus. **B.** When a Wnt ligand binds its receptors, DVL recruits the destruction complex, inactivating it. β-catenin accumulates in the cytoplasm, translocates to the nucleus, displaces TLEs, and activates transcription of TCF/LEF target genes. Active pathway components are in color; inactive components are in grey.

Adapted from
http://web.stanford.edu/group/nusselab/cgi-bin/wnt/sites/default/files/Wntsignaling.png

Wnt signaling is required for axis formation and gastrulation in the early vertebrate embryo (Biechele et al., 2011; Liu et al., 1999b), and is subsequently indispensable in many critical developmental processes. Wnt3 patterns the primitive streak (Yoon et al., 2015) and regulates the expression of *Brachyury*, which encodes a transcription factor that is critical in that process and downstream processes (Rivera-Pérez and Magnuson, 2005). The numerous specific roles of Wnt signaling throughout development exceed the scope of this dissertation and are reviewed extensively in the literature (Clevers, 2006; Nusse, 2005).

A central theme of Wnt signaling is the control of stem cell behavior. Wnt signaling has been shown to regulate the stem cells of many tissues, including intestine (Pinto et al., 2003), skin/hair follicle (Nguyen et al., 2009; Snippert et al., 2010), brain (Lie et al., 2005), mammary gland(Zeng and Nusse, 2010), and blood (Reya et al., 2003). The exact mechanisms whereby Wnt signaling maintains stem cells and in some cases drives differentiation are poorly understood and warrant further investigation.

Components of the Wnt signaling pathway have also been shown to promote pluripotency in hPSCs (Fernandez et al., 2014). This role for Wnt signaling fits its activities in other stem cell populations, but is the subject of much debate within the field. Others have insisted that Wnt signaling primarily serves to drive hPSC differentiation, not self-renewal (Davidson et al., 2012). These discrepancies are possibly explained by dosage-specific effects of Wnt signaling, with Wnts acting as morphogens that drive pluripotency at low concentrations and promote differentiation at high concentrations, but this question has so far been incompletely addressed. In a

role that supports the notion that Wnt signaling promotes pluripotency and maintenance of stem cell identity in hPSCs, Wnt signaling is required for reprogramming of human fibroblasts to hiPSCs (Ross et al., 2014). This suggests a possible instructive role for Wnt that promotes a pluripotent or stem-like state generally. A deeper and more comprehensive understanding of the effects of Wnt signaling in hPSCs is a critical need in the field.

1.3.2 Genetics and Biochemistry of the Wnt Pathway

As with many important discoveries in biology, Wnt signaling was first glimpsed through the study of *Drosophila melanogaster* genetics with the identification of the *Wingless* gene (Sharma and Chopra, 1976), a *Drosophila* Wnt homolog. Later, through the study of oncogenic retrovirus biology, *Int-1*, later known as *Wnt1*, was discovered to be activated by spontaneous insertion of the mouse mammary tumor virus (MMTV) in several clones derived from transformed cells in culture (Nusse and Varmus, 1982). Due to technological limitations and the lack of a fully sequenced mouse genome, it took several years before the sequence of the *Int-1* gene was deduced (Fung et al., 1985; van Ooyen and Nusse, 1984). Once that sequence was finally obtained, it bore no resemblance to those of any other sequenced genes at the time. One of the most salient features of the gene was the presence of a signal sequence, indicating that *Int-1* likely encoded a secreted protein, a feature that set it apart from then-discovered viral and cellular oncogenes like *myc* and *fos*.

Work understanding the other components of the pathway was slow, but a major breakthrough occurred that led to a rapid expansion in knowledge. It was found that the drosophila homolog of mouse *Int-1* is identical to *wg*, identifying a highly

conserved signaling pathway (Rijsewijk et al., 1987). In honor of this shared heritage, workers in the field proposed a new nomenclature: Wnt, which is a portmanteau of *Wingless* and *Int-1* (Nusse et al., 1991). Studies in *Drosophila* had revealed many other genes in epistatic relationships with *Wg*, and those components were found to be conserved in the mouse. The so-called "segment polarity" genes *Armadillo* (β-catenin), *Arrow* (LRP), *Dishevelled* (DVL), and *Wingless* were found to be critical mediators of Wnt signaling. The discovery that the mammalian *Int-1* and fly *wg* genes were homologs enabled the translation of years' worth of rich genetic studies in the fly that had yielded several genes in the *wg* pathway and their epistatic relationships in the pathway.

The discovery that the Frizzled family of genes encoded Wnt receptors was a major advance for understanding the pathway (Bhanot et al., 1996). At the time, the lack of soluble Wnt protein was a considerable challenge that hampered the development of cellular assays. *Drosophila* tissue culture presented a system that could be used as a workaround: by obtaining soluble Wg protein from *Drosophila* S2 cells, Clone 8 cells derived from *Drosophila* imaginal disc cells can be induced to stabilize Armadillo protein in culture. Bhanot et al. observed that S2 cells transfected with Dfz2, a cloned *Frizzled* homolog, also stabilize Armadillo. They further demonstrated that several mammalian homologs of Frizzled also bound to Wg protein, demonstrating the high degree of evolutionary conservation of these co-adapted signaling molecules across hundreds of millions of years of evolution. The identification of the Wnt receptor opened the door to the identification of cytoplasmic signaling components that interact with FZD. They correctly predicted that Dishevelled protein could interact with Frizzled receptors and thereby transduce Wnt

signals, a finding that was hinted at by earlier research that put Dsh in the same pathway as Fz (Krasnow et al., 1995). Intriguingly, it was observed that Wg stimulation of C8 cells led to Dsh phosphorylation by CK2 (Willert et al., 1997). While it was initially suspected that phosphorylation was required for Wg pathway activation, it was demonstrated that overexpression of Dfz2 leads to Dsh phosphorylation, but not Armadillo accumulation. The role of Dsh/DVL phosphorylation is unclear even at the time of writing of this dissertation.

A critical tool for the study of the Wnt pathway in mammalian cell culture models has been the purification of soluble, active Wnt protein. Wnt proteins are highly insoluble due to high N-terminal hydrophobicity and previously unknown post-translational modification through palmitoylation. Purification of active Wnt protein was elusive for many years. The key to purifying active Wnt protein was the realization that all purification steps required detergent to maintain solubility (Willert et al., 2003). Importantly, the palmitoylation of Wnt proteins occurs on a conserved cysteine residue (C77 in mouse Wnt3a; C51 in *Drosophila* Wnt8) that is present in all Wnt proteins. C77A mutant Wnt3a is secreted as efficiently as WT protein, but becomes markedly less hydrophobic and fails to activate signaling in conditioned medium. Transfected cells are able to activate signaling likely in an autocrine fashion, but activity is greatly reduced, indicating that palmitoylation of Wnt ligands is essential to their basic biological functions. Other studies revealed palmitoylation at S209 and demonstrated that this modification is required for proper Wnt protein trafficking and secretion (Takada et al., 2006). The importance of this modification was driven home when the first crystal structure of a Wnt ligand engaging a Fzd receptor was solved. Wnt proteins engage the cysteine-rich domain of Fzd directly through the palmitate

residue at S209 (Janda et al., 2012). Thus, the feature of Wnt proteins that renders them incredibly hydrophobic and difficult to purify is also required for their biological activity. Importantly, purified Wnt proteins can be used to activate the Wnt signaling pathway in tissue culture, which is highly advantageous when compared to the use of conditioned medium, which is poorly defined and may contain confounding factors. This application has enabled a new generation of studies of the role of the Wnt signaling pathway in development and disease, especially with regards to the elucidation of the role of the Wnt signaling pathway in early human development modeled by hPSCs.

1.3.3 Wnt signaling in cancer

An important idea that gained strength from studies of Wnt signaling is that developmental pathways are often repurposed or aberrantly regulated in several cancers (Ibsen and Fishman, 1979; Rubin, 1985). This hypothesis was supported early on by frequent observation of tumors composed of highly disorganized, undifferentiated cells that differ markedly in morphology from the tissue of origin. Notably, undifferentiated tumors are often associated with poor prognosis (Garcia-Rostan et al., 2001; Guo et al., 1988; Umbas et al., 1994), which may reflect key characteristics of cancer stem-like cells: resistance to genotoxic stress; quiescent and activated states; invasiveness and ability to enter circulation and metastasize to distant sites throughout the body. The cells that drive the advance of highly-aggressive cancers often express developmental and stem cell-specific markers and bear striking similarity to their early developmental counterparts. Frequently, mutations in the Wnt pathway are found in highly aggressive cancers, both inherited and spontaneous.

In addition to the initial discovery of *Int-1* in mouse mammary tumors, abnormal Wnt signaling has been found to drive cancer initiation, maintenance, and progression in several tissues including breast (Reviewed in Prosperi and Goss, 2010), colon (Reviewed in Sebio et al., 2014), brain (Rheinbay et al., 2013), ovary (Yoshioka et al., 2012), and lung (Stewart, 2014).

Diverse aspects of the Wnt pathway are found to be mutated or misregulated in cancer. Components of the Wnt signaling pathway are fairly easily subdivided into positive and negative regulators. In Wnt-driven cancers, negative regulators are often mutated to a loss of heterozygosity, whereas positive regulators are frequently activated (Polakis, 2012). Perhaps the most famous Wnt pathway-driven cancer is familial colon cancer characterized by mutations in the *Adenomatous Polyposis Coli* (*APC*) gene, which normally functions as a key member of the destruction complex that targets β-catenin for proteasomal degradation in the absence of a Wnt signal (Rubinfeld et al., 1993; Su et al., 1993). 2 deleterious alleles of the *APC* gene are required for the initiation of tumorigenesis, and indeed these biallelic inactivations are present in the vast majority of colon cancers, both familial and sporadic. Other negative regulators of the Wnt signaling pathway are also known tumor suppressors, including AXIN1 and AXIN2, which cooperate with APC to recruit GSK3β and βTrCP, proteins that are required to target β-catenin for proteasomal degradation (Polakis, 2012).

Positive regulators of Wnt signaling are also demonstrably mutated in some cancers. β-catenin undergoes activating mutations that prevent its phosphorylation and subsequent ubiquitination and degradation in an estimated 1-10% of spontaneous colon cancers (Klaus and Birchmeier, 2008). These mutations are

generally N-terminal and frequently involve canonical phosphorylation sites at Ser45, Thr41, Ser37, Ser33, and nearby residues (Polakis, 2000).

While the mechanisms of tumorigenesis downstream of Wnt signaling are incompletely understood, it is clear that persistent signaling can promote entry into the cell cycle through the expression of targets like c-Myc (He et al., 1998) and CyclinD1 (Shtutman et al., 1999; Tetsu and McCormick, 1999). Continuously cycling cells are then prone to further genetic hits that may drive increased malignancy and aggressiveness, indicating that mutations of critical Wnt signaling components represent an important initiatory step down the road to full blown malignant cancers.

Because the Wnt signaling pathway so often misregulated in human cancers, it may be a suitable target for intervention in some cases. Indeed, at the writing of this dissertation, several drugs targeting the Wnt pathway in cancer are in ongoing clinical trials (**Table 1-1**). Cancers that aberrantly express FZD receptors are especially attractive for therapy because it has been demonstrated that Wnt signaling can be effectively blocked with antibodies that target these proteins and interfere with Wnt ligand binding (Fernandez et al., 2014).

As with drugs that target other signaling pathways that govern stem cell behavior, a critical aspect of any compound that targets the Wnt pathway is low off-target toxicity. Care must be taken to balance positive anti-tumor effects with the negative effects on the Wnt-dependent processes in healthy tissues, especially stem cell maintenance and tissue regeneration. Any number of unwanted side effects could be theorized based on the role of Wnt signaling in various tissues, including skin lesions, bleeding, hair loss, depression, or memory loss. Strategies to avoid unwanted side effects would include tumor-targeted antibodies that recognize so-

called "oncofetal" protein epitopes that are highly expressed during development, in cancer, but not in adult tissues. Because Wnt-driven cancers generally carry mutations to other signaling pathways as well, personalized combination therapies that target these additional pathways in a patient-specific manner are preferable. This method of treatment allows a lower dose of Wnt-targeting drug to achieve similar or perhaps better efficacy.

In short, the Wnt signaling pathway is an ancient signaling pathway that is critical for the proper development of metazoan animals. Wnt signaling is required for stem cell maintenance and regeneration of many tissue types and represents a possible therapeutic avenue to treat injuries and disease that are refractory to current approaches. Interestingly, Wnt signaling is a key player in many cancers and may also represent a therapeutic avenue to treat patients with previously terminal disease. These facts demonstrate that the Wnt pathway warrants the intense study and investigation it receives from members of the genetics, developmental biology, stem cell, and cancer research communities, and serves as a basis for my research to uncover the roles of Wnt signaling in hPSCs.

Table 1-1: Drugs targeting the Wnt pathway in cancer that are currently in clinical trials (clinicaltrials.gov)

Drug	Type	Mechanism
PRI-724	Small Molecule	Blocks β-catenin/CBP interaction, inhibiting transcription of target genes
Genistein	Small Molecule	Upregulation of SFRP2 (Zhang and Chen, 2011), which binds Wnt ligands and prevents signaling
OMP-18R5	Biologic	Anti-FZD7 monoclonal antibody, blocks pathway activation by Wnt ligands
OMP-54F28	Biologic	Recombinant FZD8 Cysteine Rich Domain binds Wnt ligands and prevents signaling
OTSA101-DTPA-90Y	Radio-Biologic	Anti-FZD10 monoclonal antibody conjugated to toxic Yttrium-90 radioisotope

1.4 SP5

1.4.1 Introduction

1.4.1.1 Identification of SP5 as a Wnt target in hPSCs and selection for study

The primary focus of my studies in the Wnt signaling pathway has been to identify important transcriptional targets in human stem cells, especially hPSCs and hNSCs. In order to identify strongly regulated target genes of the Wnt pathway in these cell types, I treated cells with purified recombinant Wnt3a protein, extracted RNA, and performed RNA high-throughput sequencing following bioinformatic analysis. The criteria for selecting a target for investigation were as follows: 1. Target must be significantly and highly upregulated (e.g. > 2-fold) following Wnt treatment at 12, 24, or 48 hours. 2. Target must be a direct target of Wnt/β-catenin signaling as defined in the literature. 3. Poorly-understood targets with potentially high-impact functions were given priority. Using these selection criteria, I identified a handful of potentially interesting subjects for study. Among the most attractive was the gene encoding the transcription factor SP5, which was highly upregulated in both hPSCs and hNSCs following Wnt3a stimulation for 12, 24, and 48 hours. The literature revealed that this gene met another important criterion as a known target of Wnt signaling (Weidinger et al., 2005). Of note, the literature surrounding SP5 was strikingly bare, which suggested there was still much to learn about this enigmatic transcription factor. Because SP5 satisfied these criteria so thoroughly, I embarked on the study of this protein as the major undertaking of my dissertation research.

1.4.1.2 SP/KLF family of transcription factors

Although little is known of SP5, it belongs to a highly-studied and well-known family of transcription factors, the SP family, which is itself a sub-family of the larger

SP/KLF family. This family of transcription factors is characterized by the presence of a highly conserved zinc finger DNA-binding motif that recognizes a canonical DNA sequence motif known as a "GC Box", with sequence "GGGCGG (Gidoni et al., 1984). Upon recognition of this motif, these factors bind to chromatin and recruit machinery to drive the transcription of target genes. Often, GC Box motifs are found in so-called "TATA-less" gene promoters (Boam et al., 1995), whose transcription is driven independent of the presence of a TATA-Box sequence motif, as their name suggests. It is now believed that the majority of genes are transcribed from promoters such as these (Beishline and Azizkhan-Clifford, 2015), indicating far-reaching transcriptional roles for the SP/KLF family of transcription factors.

SP1, the first discovered SP family member (Dynan and Tjian, 1983), is ubiquitously expressed (Saffer et al., 1991) and is considered to be a housekeeping gene that regulates the transcription of many important basic metabolic genes. SP1 is required for embryonic development in mouse, but not for cell survival *per se* (Marin et al., 1997). Much work has gone into understanding the roles of SP1 in diverse processes ranging from development (Saffer et al., 1991), metabolism (Dittmer et al., 1994; Schäfer et al., 1997), cell cycle regulation (Kim et al., 1992; Owen et al., 1998), cancer (Beishline and Azizkhan-Clifford, 2015), and fibrosis (Kim et al., 2013; Zhu et al., 2012). Other SP family transcription factors have been identified through molecular cloning techniques and are involved in a wide array of functions throughout development (**Table 1-2**).

KLF family members have also been shown to play interesting roles in development and are structurally distinct from SP family members with the exception of the highly conserved C-terminal C2H2 Zinc Finger DNA binding motif (Suske et al.,

2005). The first KLF family member identified, EKLF, was cloned by subtractive cloning to enrich for genes expressed in an erythroid leukemia cell line, but not in monocytes (Miller and Bieker, 1993), and was shown to recognize a sequence motif "CCACACCCT". Treichel et al. have suggested that Sp5 may represent an ancestral or bridging member between families because it possesses the characteristic Buttonhead domain of SP family members, but the proline-rich N-terminal domain of the KLF family (Treichel et al., 2001). Later studies suggested that Sp8 may be ancestral owing to its functional roles in mouse that are highly similar to those of *Buttonhead* (Treichel et al., 2003).

Genetic and genomic analysis in vertebrates and invertebrates has clarified the evolutionary history of the SP family of transcription factors. A genomic analysis of several species by Schaeper et al. suggest the existence of three SP family "clades": the SP1-4 clade, the SP5/btd clade, and the SP6-9 clade. Interestingly, the basal metazoan *N. vectensis* possesses only 3 SP family genes, present as tandem duplications on one chromosome, that appear to be ancestral founders of the three SP family clades present in vertebrate and invertebrate genomes (Schaeper et al., 2010). In the human genome, *SP3*, *SP5* and *SP9*, genes representative of these three clades, are located in close proximity to each other and are also closely linked to the ancestral HOXD cluster. All other family members exist as duplicates adjacent to corresponding derived HOX gene clusters (**Figure 1-3**). Importantly, the first hypothesized chromosomal duplication eventually lost the *SP5* ortholog before subsequent duplications to form the family as it exists in mammals today.

Table 1-2: The roles of SP family transcription factors in development and tissue homeostasis (Adapted from Zhao and Meng, 2005)

Factors	Expression	Major phenotypes in mouse knockouts	Functions in other vertebrate species	References
Sp1	Ubiquitous	Growth retardation, prenatal lethality	Unknown	(Saffer et al. 1991; Marin et al. 1997)
Sp2	Ubiquitous	Unknown		
Sp3	Ubiquitous	Growth retardation, death at birth; tooth, bone and hematopoietic defects	Unknown	(Bouwman et al. 2000; Gollner et al. 2001b; Van Loo et al. 2003)
Sp4	CNS, liver, lung, kidney, heart, gonads, intestine, and tooth bud	Increased postnatal mortality; smaller body; sterile	Unknown	(Hagen et al. 1992; Supp et al. 1996; Nguyen-Tran et al. 2000; Gollner et al. 2001a)
Sp5	Mesoderm precursors and derivatives; posterior neuroectoderm and derivatives	No morphological changes; increased taillessness in Sp5–/–; T/+double mutants	Mesoderm induction and development of midbrain and hindbrain boundary in zebrafish	(Harrison et al. 2000; Tallafuss et al. 2001; Treichel et al. 2001; Ossipova et al. 2002; Zhao et al. 2003)
Sp6	Developing teeth; caudal neuropore; limb buds; hair follicles	Unknown	Unknown	(Nakamura et al. 2004)
Sp7	Osteoblasts	Death at birth; failure in ossification;	Unknown	(Nakashima et al. 2002)
Sp8	CNS, limb	Neural tube closure failure; shorter tail; shorter limbs; death at birth	Inhibiting secondary neurogenesis in zebrafish; required for limb/fin outgrowth in chick and zebrafish	(Bell et al. 2003; Beermann et al. 2004; Kawakami et al. 2004; Penberthy et al. 2004)
Sp9	In specific domain of the CNS; limb	Unknown	Required for limb/fin outgrowth in chick and zebrafish	(Kawakami et al. 2004)

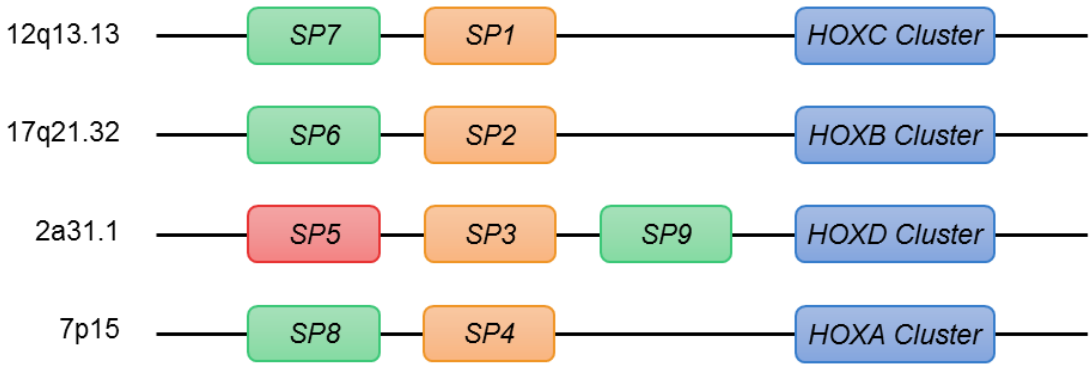

Figure 1-3: Linkage and evolutionary relationships of human SP family genes.

SP family members are color-coded based on ancestral clade: SP1-4 (Orange); SP5/Btd (Red); SP6-9 (Green). HOX gene clusters are color-coded Blue. (Adapted from Suske et al., 2005)

1.4.1.3 Discovery of Sp5 and knockout in the mouse

The near-simultaneous identification and cloning of mouse *Sp5* by 2 groups underscores the importance of the SP family of transcription factors to the field of developmental biology. Each group employed a different means to identify novel genes. Harrison et al. utilized subtractive cloning to identify genes abundant in the primitive streak when compared to early embryonic ectoderm and endoderm. *Sp5* cDNA was enriched and identified as a novel cDNA through sequencing (Harrison et al., 2000). Treichel et al. were specifically searching for new members of the SP family of transcription factors and utilized degenerate PCR primers to amplify relatives of the C2H2 zinc finger region of *Sp1* from mouse genomic DNA. The amplified fragment was then used as a probe to identify matching cDNA clones from a E8.5 mouse cDNA library (Treichel et al., 2001). Both groups characterized Sp5 as a novel member of the SP family of transcription factors and revealed a strikingly dynamic expression pattern using RNA *in situ* hybridization. Harrison et al. went a step further and targeted a LacZ cassette to the *Sp5* locus, generating a faithful reporter and disrupting the *Sp5* gene. Surprisingly, no overt developmental or reproductive phenotype was observed in $Sp5^{LacZ/LacZ}$ animals. Because the expression pattern of *Sp5* was reminiscent of the pattern of *Brachyury* (*T*) expression, they speculated that these two genes could function in the same pathway. Indeed, when $Sp5^{LacZ/LacZ}$ animals were crossed with heterozygous mutants of the *T* gene, an enhanced tailless phenotype was observed, indicating an important genetic interaction between these genes in posterior patterning and development of the spine (Harrison et al., 2000). These observations led to the suggestion that redundancy with other members of the SP family of transcription factors could explain the apparent lack of phenotype in *Sp5*

null animals, a hypothesis that would be validated more than a decade later when Dunty et al. demonstrated that *Sp5*$^{LacZ/LacZ}$ *Sp8*$^{\Delta/\Delta}$ double mutant mice display no expression of *T* in the tail and phenocopy Wnt3a$^{-/-}$ mice (Dunty et al., 2014), indicating a key role for these two transcription factors in tissue patterning downstream of Wnt signaling.

1.4.1.4 SP5 is a Wnt target that represses SP1 target genes

Harrison et al. speculated that *Sp5* gene expression was likely regulated by a critical developmental signaling pathway such as the FGF or Wnt signaling pathways. This prediction was based on the overlapping expression domains of *Sp5* and components of these pathways throughout mouse development (Harrison et al., 2000). Work in zebrafish demonstrated that *sp5* and *sp5l*, homologs of mouse *Sp5*, are direct targets of Wnt/β-catenin signaling in early development where they are involved in tail development and patterning of mesoderm and neuroectoderm (Thorpe et al., 2005; Weidinger et al., 2005). In humans it was demonstrated that *SP5* is upregulated in colon cancer downstream of the Wnt pathway (Takahashi et al., 2005). Later work by Fujimura et al. would demonstrate that the *Sp5* gene is also a direct target of Wnt signaling in mouse neural stem cells, and that it acts as a transcriptional repressor of Sp1 target genes (Fujimura et al., 2007). Using a Gal4 reporter system, they fused various domains of Sp5 to Gal4 and demonstrated repression with three distinct domains, dubbed R1, R2, and R3 (Amino acids 1-76, 223-297, and 379-398 respectively). They further demonstrated that R1 contains an mSin3a-interacting domain (SID) motif, that purified GST-Sp5R1 is capable of precipitating *in vitro* translated mSin3a and that Sp5-FLAG pulls down mSin3a-Myc in cellular lysates. Importantly, they noted that deletion of the SID in R1 led to modest transcriptional

activation, suggesting that in some cases, Sp5 may act in this capacity. Finally, they showed that overexpression of Sp5 in mouse primary neurospheres lead to 2-fold and greater downregulation of 107 genes, 90 of which were either known Sp1 target genes or contained Sp1 binding sites in the proximal promoter region (500 bp upstream through 1 bp downstream of transcription start site), strongly suggesting that the function of Sp5 is to mediate Wnt signaling effects in target tissues by repressing Sp1 target genes.

1.4.2 SP5 is a developmentally important Wnt target

1.4.2.1 Interspecies conservation

SP5 is highly conserved in vertebrates (**Figure 1-4**), suggesting a meaningful contribution to organismal fitness that is subject to strong selective pressure. Of note, the amino acid identity among mammalian SP5 homologs approaches 100 percent (**Figure 1-4A**). Conservation between mammals and lower vertebrates such as *X. tropicalis* and *D. rerio* is lower, but it should be noted that the C2H2 zinc finger domains remain highly conserved (**Figure 1-4B**), indicating that the DNA binding specificity of SP5 homologs is under strong positive selection. The lower degree of overall conservation could possibly be explained by the fact that both *X. tropicalis* and *D. rerio* genomes contain two *SP5* homologs instead of one, in contrast to mammals and birds. This opens the door for overlapping but potentially divergent roles for each homolog and would allow for genetic drift in one homolog when its activity is still compensated for by the other.

A.

	Homo sapiens	*Macaca mulatta*	*Canis lupus familiaris*	*Rattus norvegicus*	*Mus musculus*	*Pan troglodites*	*Bos taurus*	*Gallus gallus*	*Xenopus tropicalis*	*Danio rerio*
Homo sapiens	100%	99%	98%	97%	97%	96%	91%	90%	71%	66%
Macaca mulatta	99%	100%	98%	98%	98%	95%	92%	89%	71%	66%
Canis lupus familiaris	98%	98%	100%	97%	97%	94%	91%	89%	70%	66%
Rattus norvegicus	97%	98%	97%	100%	100%	93%	90%	89%	70%	65%
Mus musculus	97%	98%	97%	100%	100%	93%	90%	89%	70%	65%
Pan troglodites	96%	95%	94%	93%	93%	100%	89%	86%	68%	64%
Bos taurus	91%	92%	91%	90%	90%	89%	100%	83%	65%	62%
Gallus gallus	90%	89%	89%	89%	89%	86%	83%	100%	74%	70%
Xenopus tropicalis	71%	71%	70%	70%	70%	68%	65%	74%	100%	75%
Danio rerio	66%	66%	66%	65%	65%	64%	62%	70%	75%	100%

B.

```
H. sapiens      CHVPGCGKVYGKTSHLKAHLRWHTGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFACPECGKRFMRSDHLAKHVKTH 378
M. Mulatta      CHVPGCGKVYGKTSHLKAHLRWHTGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFACPECGKRFMRSDHLAKHVKTH 378
C. Lupus Fam.   CHVPGCGKVYGKTSHLKAHLRWHTGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFACPECGKRFMRSDHLAKHVKTH 378
R. Norvegicus   CHVPGCGKVYGKTSHLKAHLRWHTGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFACPECGKRFMRSDHLAKHVKTH 378
M. Musculus     CHVPGCGKVYGKTSHLKAHLRWHTGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFACPECGKRFMRSDHLAKHVKTH 378
P. Troglodites  CHVPGCGKVYGKTSHLKAHLRWHTGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFACPECGKRFMRSDHLAKHVKTH 378
B. Taurus       CHVPGCGKVYGKTSHLKAHLRWHTGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFACPECGKRFMRSDHLAKHVKTH 422
G. Gallus       CHIPGCGKVYGKTSHLKAHLRWHTGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFVCPECGKRFMRSDHLAKHVKTH 351
X. Tropicalis   CHIPGCGKVYGKTSHLKAHLRWHSGERPFICNWLFCGKSFTRSDELQRHLRTHTGEKRFVCPECGKRFMRSDHLAKHVKTH 354
D. Rerio        CHIPGCGKVYGKTSHLKAHLRWHSGERPFVCNWLFCGKSFTRSDELQRHLRTHTGEKRFVCPDCCKRFMRSDHLAKHVKTH 344
                **:******************:*****:****************************.**:* ***************
```

Figure 1-4: Conservation of SP5 in vertebrate species.

A. Heatmap of pairwise conservation of SP5 among several vertebrate species. Percent identity between species is given. White denotes least identity whereas blue denotes greatest identity. (ClustalW2) **B.** Sequence alignment of all 3 SP5 C2H2 Zinc Finger domains (blue boxes) across vertebrate species. An asterisk (*) denotes perfect conservation across species; a colon (:) denotes highly similar amino acid properties; a period (.) denotes weakly similar amino acid properties; a space ( ) denotes a position that is not conserved across all species examined. (ClustalW2)

1.4.2.2 Mouse developmental studies

Harrison et al. were first to visualize the complex and dynamic expression pattern of *Sp5* throughout development using whole-mount RNA *in situ* hybridization (Harrison et al., 2000). They observed that *Sp5* expression originated in the primitive streak and persisted in the tailbud throughout the process of axial elongation. Transverse sections revealed *Sp5* expression in the primitive ectoderm and mesoderm, an observation supported by Treichel et al., who further noted that *Sp5* is expressed in the definitive endoderm upon gut formation as well as the notochord, where single *Sp5*-positive cells are surrounded by *Sp5*-negative cells (Treichel et al., 2001).

Interestingly, *Sp5* expression appears in two waves: an early wave that arises during gastrulation, described above, and a secondary wave that occurs throughout organogenesis, notably in the brain, somites, and limb buds (Treichel et al., 2003). Expression in the brain is first evident in the prospective mesencephalon (midbrain) at embryonic day 8.0 and persists there through the 12-somite stage (**Figure 1-5**). There is also expression in the diencephalon, the caudal portion of the prosencephalon (forebrain). This expression pattern is very similar to the expression of *Wnt1* in the early mouse brain, which plays a critical role in the development of the midbrain and hindbrain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), and suggests a possible role for Sp5 downstream of Wnt signaling in this tissue, where it may mediate the effects of the Wnt pathway in this process. Curiously, the drosophila gene *Buttonhead*, an *SP5* ortholog, is required for the proper patterning of the head, suggesting a conserved role that dates to a common ancestor of vertebrates and invertebrates.

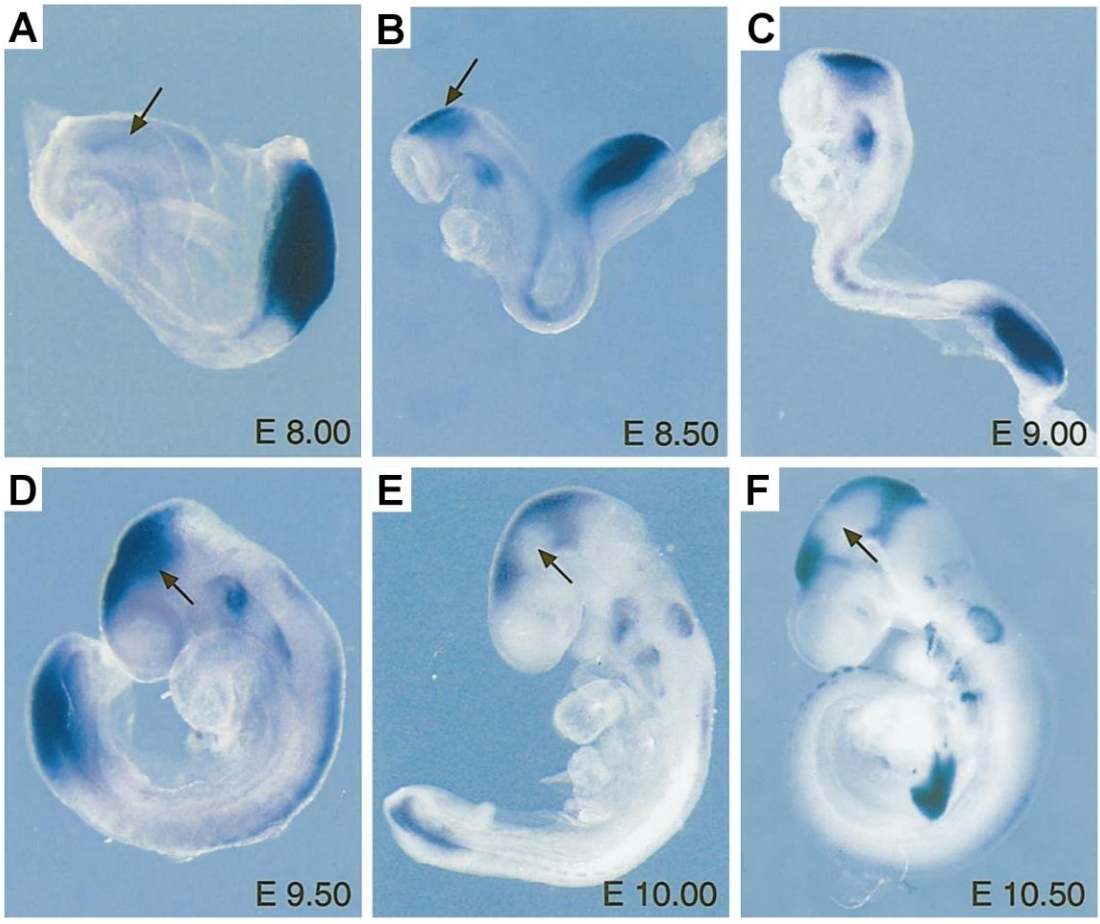

Figure 1-5: Sp5 expression in mouse brain development

A. Faint staining of the mesencephalon (black arrow) and strong staining of the tailbud. **B.** Strong staining of the mesencephalon and diencephalon (black arrow), tail, and somites. **C, D.** Strong staining in mesencephalon, dorsal diencephalon and lateral diencephalon (black arrow). **E, F.** Gradual clearance of Sp5 message from the lateral diencephalon (black arrow). (Adapted from Treichel et al., 2001)

Perhaps the most influential finding in the early work surrounding *Sp5* in the mouse was the lack of an apparent phenotype in double-knockout animals (Harrison et al., 2000). This observation has likely contributed to a paucity of literature centered on *SP5* and led to a drought in the development of useful tools, especially high quality antibodies. Harrison et al. were quick to point out that *Sp5* is indeed an important patterning factor by demonstrating that *Sp5*$^{LacZ/LacZ}$ *T*$^{+/-}$ animals show enhancement of the *T* phenotype, an abnormally shortened tail. They correctly predicted that functional redundancy with other as-yet unpublished SP family transcription factors could explain the fact that SP5$^{LacZ/LacZ}$ animals demonstrate apparently normal development, fertility, and lifespan. The work done by Dunty et al. demonstrated that in some regards, *Sp5* and *Sp8* act redundantly, with double mutants displaying a phenotype highly reminiscent of *Wnt3a* mutants (Dunty et al., 2014). One key observation by this group was that Sp5$^{LacZ/LacZ}$ animals do indeed display a very subtle phenotype with low penetrance: the tails of these animals display a characteristic "kink" at a frequency of 2-3%. Tail kinks are observed in Wnt3a$^{+/-}$ animals at a similar frequency. Since *Wnt3a* and *Sp5* are both expressed in the primitive streak, Dunty et al. tested whether compound mutants would display enhanced penetrance of this phenotype. Indeed, they observed that *Wnt3a*$^{+/-}$ animals heterozygous or homozygous for *Sp5*^{LacZ} displayed elevated penetrance of this phenotype, indicating that *Sp5* is a downstream factor in Wnt-mediated patterning of the tail (Dunty et al., 2014). They continued their analysis by examining the contribution of *Sp8* in mouse development. *Sp8*$^{\Delta/\Delta}$ mice suffer severe truncation of the posterior axis and limbs and die at birth (Treichel et al., 2003), but *Sp8*$^{+/\Delta}$ animals develop normally without any overt phenotype, and display tail kinks at a very low

frequency of about 2%, suggesting that both *Sp8* and *Sp5* are required for proper patterning of the developing tail. *Sp5*$^{Lacz/LacZ}$ *Sp8*$^{+/\Delta}$ animals have kinked tails 33% of the time, again indicating a key role for these factors in promoting proper development. Notably, both *Sp5*$^{LacZ/LacZ}$ and *Sp8*$^{\Delta/\Delta}$ mutant mice develop normally until embryonic day 9.5, but *Sp5*$^{LacZ/LacZ}$ *Sp8*$^{\Delta/\Delta}$ die between embryonic day 13.5 and 14.5 and completely lack *T*$^{+}$ neuromesodermal progenitor cells, phenocopying *Wnt3a*$^{-/-}$ mice in this regard and indicating that these factors cooperate as downstream effectors of Wnt signaling in the mouse (Dunty et al., 2014).

1.4.2.3 Zebrafish and Xenopus developmental studies

Studies in fish, amphibians, and reptiles have helped to shed additional light on the role of *SP5* and its homologs downstream of Wnt signaling. Importantly, both Zebrafish and Xenopus have 2 homologs of *SP5*, which may demonstrate different activity or specificity. Tallafuss et al. used degenerate PCR to identify new homologs of the *Drosophila btd* gene and were able to recover a clone that recapitulated *btd* function when expressed in *btd* mutant flies under the control of the proper promoter. They designated this gene *bts1* (later renamed *sp5a* in recognition of its homology to other vertebrate *SP5* homologs) and demonstrated its requirement for the induction of *pax2.1* expression during early zebrafish development, but their studies fell short of proving a requirement for this gene in the establishment of the midbrain-hindbrain boundary (Tallafuss et al., 2001). Later studies of *sp5l*, the zebrafish paralog of *sp5a*, presented evidence that this gene encodes a protein that mediates the effects of *wnt8* during early zebrafish development by patterning the mesoderm and neuroectoderm, noting the importance of this gene in the following processes: posteriorization of

neuroectoderm; restriction of the dorsal mesoderm; proper hindbrain patterning (Thorpe et al., 2005).

Ossipova et al. identified 2 *SP5* paralogs in *Xenopus*, originally termed *XSPR-1* and *XSPR-2* and later renamed *sp5* and *sp5l*. In a fascinating glimpse of divergent evolution of paralogous genes, they demonstrated that *sp5* is expressed predominantly in tissues associated with the second wave of *Sp5* expression in mouse, and that *sp5l* is expressed in tissues associated with the first wave, indicating that together, both *Xenopus* paralogs of mouse *Sp5* together recapitulate its expression (Ossipova et al., 2002). Park et al. showed that *Xenopus sp5* is expressed in the presumptive neural crest, where it is a critical regulator of early neural crest induction and proper development of craniofacial cartilage, pigmentation, and the dorsal fin (Park et al., 2013).

These studies of *SP5* homologs in fish and frog demonstrate the critical role of these genes in the proper development of lower vertebrates. They reveal that the domains of *SP5* homolog expression are highly conserved across vertebrate species but raise the question of why mammalian *SP5* homologs are seemingly dispensable for proper development. In the mouse, it seems that evolution has favored *Sp8* for the heavy lifting of development and that *Sp5* is involved in critical fine-tuning of gene expression. Though the effects of *Sp5* mutation are superficially subtle, evolution has also ensured a high degree of conservation of this key regulator of animal development. Clarifying its function and identifying its targets remains a critical challenge for understanding the role of *SP5* in human embryonic development and beyond.

1.4.3 SP5 in disease and cancer

1.4.3.1 Role of SP/KLF family members in cancer

As critical regulators of the transcriptome, SP/KLF family members are critical for healthy development and tissue homeostasis. In that vein, it is not surprising that these factors are mutated or misregulated in cancers, which often develop as a result of inappropriate developmental pathway activation. Many studies have focused on SP1 and its homologs, which are often considered to be ubiquitously expressed, but whose expression may vary greatly by tissue (Saffer et al., 1991). *SP1* is frequently overexpressed in gastric tumor cells relative to healthy tissue (Wang et al., 2003), and high levels of SP1 correlate with high expression of *VEGF*, which is also associated with reduced survival in gastric cancer patients (Yao et al., 2004). This impact on *VEGF* expression and has also been reported in pancreatic adenocarcinomas, where Shi et al. reported high levels of SP1 protein in tumor samples expressing high levels of VEGF, and a strong negative effect on expression from the *VEGF* promoter when the SP1 recognition site was genetically ablated in several pancreatic cancer cell lines (Shi et al., 2001). Furthermore, it has been demonstrated that other SP transcription factors, including SP2, SP3 and SP4 can also drive increased expression from the *VEGF* promoter *in vitro* (Abdelrahim et al., 2004). Elevated SP1 protein levels in invasive breast carcinoma have been demonstrated to drive increased expression of uPAR, which in return drives increased expression of *SP1* in a positive feedback loop that may progressively enhance tumorigenicity (Zannetti et al., 2000). In addition, SP1 expression has been demonstrated to be elevated in colorectal cancers, where it can drive the expression of DNA repair machinery (Hosoi et al., 2004).

Much work has also gone into understanding the role of KLF family members in cancer. Notably, KLFs have been demonstrated to play a role as both tumor suppressors and oncogenes in a cell- and tissue-specific fashion (Reviewed by Bureau et al., 2009). Klf4, first identified in gastrointestinal tissue as a factor that maintains the differentiated state of enterocytes and prevents their proliferation, is down-regulated in intestinal tumors of mice and humans (Ton-That et al., 1997; Xu et al., 2008). It has been suggested that diminished expression of *KLF4* in intestinal tumors not only correlates with, but is drive by enhanced β-catenin/TCF signaling (Flandez et al., 2008). KLF6 is also a known tumor suppressor in colorectal cancers, where mutated forms lose the ability to induce p21 and thereby stall cell division (Reeves et al., 2004). Interestingly, KLF5 has been shown to promote tumor cell proliferation and often plays an opposing role to KLF4 in intestinal cell proliferation (Reviewed by Ghaleb et al., 2005), which serves to strengthen the paradigm that transcription factors in the same family often achieve the ends of development and homeostasis by striking a balance between "on" and "off" regulation of key genes. In a tissue culture screen of the SP/KLF family of transcription factors for repressors of proliferation downstream of oncogenic KRAS expression, it was demonstrated that some function in this capacity, including SP5, SP8, KLF2, KLF3, KLF4, KLF11, KLF13, KLF14, KLF15 and KLF16, highlighting the complexity underlying the roles of SP/KLF family members in cancer (Fernandez-Zapico et al., 2011).

1.4.3.2 SP5 is upregulated in several human cancers

SP5, a potent target of Wnt signaling in some tissues, is highly expressed in multiple human cancers. Using DNA microarray expression analysis, Chen et al.

observed extremely high *SP5* expression in hepatocellular carcinoma, gastric cancer, and colon cancer when compared to healthy tissues (Chen et al., 2006).

1.4.3.3 Possible roles for SP5 in cancer

Chen et al. investigated possible roles for *SP5* in cancer by using an inducible overexpression system to introduce SP5 protein into MCF7 cells, which do not normally express detectable levels of SP5 (Chen et al., 2006). Using DNA microarrays, they were able to identify several misregulated genes, some of which were upregulated, and some of which were downregulated. Upregulated genes included the cell cycle regulator *CDC25* and a known oncogene, *MYBL1*, both which are expressed downstream of known SP1 regulatory regions, suggesting an activating role for SP5 in this cellular context. Importantly, they also noted strong downregulation of *p21*, an interesting finding which was later repeated by Fujimura et al. as a result of *Sp5* overexpression in mouse neural stem cells. Of note, they performed a Gal4 fusion reporter assay to identify activating and repressing domains of SP5, similar to the experiments later performed by Fujimura et al (Fujimura et al., 2007). In contrast with these later results, Chen et al. find that the N-terminal region of SP5 can activate reporter gene transcription in 293 cells, while an internal repressor domain can interfere with this activity. This is in contrast with the results of Fujimura et al., who observed three distinct repressor domains throughout the length of mouse Sp5 protein, and only hints of a cryptic activation domain internally. This discrepancy highlights the limitations of artificial protein fusion systems to predict repression versus activation activity of transcription factors at various endogenous loci and cell types. While it is useful to have a transcriptional framework when

studying the effects of a transcription factor genome-wide (e.g. activator versus repressor), in the end the effects on key genes need to be validated individually.

1.4.4 SP5 in stem cells

Very little is known of the role of *SP5* in stem cells of any type. The main focus of my dissertation work has been to identify the function of this transcription factor in hNSCs and hPSCs, but there is some evidence to suggest that *SP5* is expressed downstream of Wnt signaling in healthy adult stem cells. Understanding the mechanisms of SP5 function and identifying the downstream transcriptional networks in one cell type may help to shed light on the function of this protein in adult tissue-specific stem cells and in cancer. Unfortunately, little data exists on the level of *Sp5* expression in well-studied Wnt-maintained stem cell compartments in the mouse such as the skin, intestine, blood, brain, and muscle. Notable exceptions, discussed below, have helped to shed light on the biology of this protein.

Much research has gone into identifying Wnt-responsive stem cells *in vivo*, and the discovery that *Lgr5* expression marks such cells in several tissues (stomach, intestine, liver, breast, skin) has led to an expanded understanding of the molecular mechanisms that drive stem cell behavior downstream of Wnt signals (Reviewed in Koo and Clevers, 2014). *Lgr5* encodes a transmembrane protein that serves as a receptor for R-spondins, protein agonists of the Wnt pathway that serve to enhance signaling rather than activate it. Interestingly, *Lgr5*-positive stem cells in the mouse stomach demonstrate upregulation of several known Wnt target genes, including *Sp5* (Barker et al., 2010). Future studies of *Lgr5*-positive stem cells in other tissues may also demonstrate increased levels of Sp5 protein. Oligodendrocyte progenitor cells (OPCs), which produce new myelinating cells following brain injury, have been shown

to respond in vivo to Wnt signaling, which is pathologically upregulated following ischemia. In response to this insult, OPCs upregulate many of the same Wnt target genes that are expressed in colon cancer, hinting at stereotypically parallel modes of action across vastly different tissues (Fancy et al., 2014). Interestingly, *Sp5* was among the genes upregulated by OPCs following ischemic injury, and mice deficient in Sp5 demonstrated premature differentiation of these cells, suggesting that Sp5 may normally function to promote stem cell identity downstream of Wnt signaling in healthy tissues.

1.4.4.1 SP5 is a Wnt target gene in hPSCs and hNSCs

In my studies, I have discovered that *SP5* is a target of Wnt signaling in some human cell types. Using RNA-Seq transcriptional profiling, I have demonstrated that *SP5* is upregulated in hNSCs and hPSCs following Wnt treatment. This observation makes sense in the face of vertebrate developmental data, which shows high levels of *Sp5* transcript in the primitive streak (Harrison et al., 2000; Treichel et al., 2001), which forms as a consequence of Wnt-driven axis formation and gastrulation, and in the developing brain, where Wnt signaling is known to play critical roles in both patterning the cortex (McMahon and Bradley, 1990), expanding early cortical stem cell populations (Woodhead et al., 2006), and driving neuronal differentiation of late cortical stem cell populations (Munji et al., 2011). While the role of SP5 in these cell types remains unclear, I have attempted to shed light on the biological significance of its expression in tissue culture models.

1.4.4.2 Importance of understanding the role of SP5 in human biology

In conclusion, an improved understanding of the role of SP5 in human biology is desperately needed. Given its dynamic expression throughout development and in

adult stem cell populations, and its potential role in promoting Wnt-mediated carcinogenesis, an improved understanding of SP5 and its function in humans may yield improved diagnostics and treatments for diseases that are incurable by current standards. In addition, understanding the complex processes that underlie development will be necessary for pioneering work in regenerative medicine, especially in tissue engineering, where developmental signaling pathways instruct the organization of functional tissue from a small number of disorganized stem and progenitor cells. To that end, I have undertaken research aimed at understanding the role of the Wnt signaling pathway in hPSCs and hNSCs, and especially the function of the transcription factor SP5 in these cells.

Chapter 2 - The role of the transcription factor SP5 in human pluripotent stem cells

2.1 Abstract

Throughout multicellular animal development, the cells of the embryo must coordinate their actions to ensure that a multitude of developmental processes are all executed in a spatiotemporally correct fashion, ultimately resulting in the generation of healthy organisms. Macromolecular protein signaling pathways represent one of the main avenues of communication utilized by cells throughout development and into tissue homeostasis. A prime example among many such pathways is the Wnt signaling pathway, which is fundamentally required for processes ranging from axis formation, gastrulation, germ layer formation and patterning, expansion of cell compartments, and cellular differentiation. In order to better understand the role of this pathway in early human development, we have utilized *in vitro* culture of human pluripotent stem cells (hPSCs) to model the molecular events downstream of Wnt pathway activation. Using RNA-Seq, we identified transcriptional changes in hPSCs treated with recombinant Wnt3a protein at several different timepoints. Wnt-treated hPSCs recapitulate several aspects of early human development, indicated by distinct waves of gene expression enriched for markers of gastrulation, the primitive streak, and early germ layer formation. Interestingly, we identified *SP5*, which encodes a GC Box binding transcriptional repressor, as one of the most highly upregulated transcripts in Wnt-treated hPSCs. Because SP5 can repress targets of the ubiquitously expressed SP1, this finding suggests that Wnt signaling may exert influence over a large previously unappreciated branch of the transcriptome during early human development. We found that SP5 protein accumulates in the nuclei of Wnt-treated hPSCs, where it competes with SP1 for binding in the promoter regions of many genes, some of which are known antagonists of Wnt signaling. We further

demonstrate that SP5 negatively regulates the expression of these Wnt antagonists, leading to enhanced Wnt signaling throughout early development. Finally, we demonstrate that SP5 restricts the formation of definitive endoderm from hPSCs, likely by increasing endogenous Wnt pathway activation through this mechanism. These findings demonstrate a novel role for Wnt signaling and the transcription factor SP5 in early human development.

2.2 Introduction

Human development is a staggeringly complex process that requires the transformation of a single celled zygote into a macro-scale organism composed of trillions of cells. In order to reach this goal, it is necessary for the cells of the early embryo, which are essentially identical at first, to break symmetry and orchestrate the cascade of cell division and differentiation that will ultimately generate all of the tissues in the organism in their proper places and times. Because it is absolutely critical for cells to properly understand their place and identity within the new organism, they have developed sophisticated input/output mechanisms for integrating signals from their external environments and for sending messages out to neighboring cells. Cells often use secreted macromolecules and their highly co-evolved receptors to activate biochemical signaling cascades and feedback loops in this capacity. Throughout development, cells signal to one another to determine emerging identities, cell cycle cadence, or even to initiate apoptosis. Slight modifications to these stimuli or their associated responses may have drastic effects, necessitating tight control and strict regulation. Paradoxically, this is evidenced both by the high degree of conservation of these molecules and pathways across species as well as by the rare beneficial elaborations produced throughout evolution that have

led to the myriad forms of animal life on the planet today. The Wnt pathway is one such signaling mechanism, which is employed by cells and organisms repeatedly throughout development and in tissue homeostasis to achieve these ends.

The Wnt signaling pathway is of ancient origin, conserved throughout metazoans and a cornerstone of vertebrate development, where it is required to coordinate and often execute numerous critical steps throughout this process (Reviewed in Cadigan and Nusse, 1997; Cadigan and Peifer, 2009). Wnt signaling induces the first body axis and is required for gastrulation (Liu et al., 1999b), following which it is crucial for the emergence and patterning of several tissues (Berge et al., 2008a; Kiecker and Niehrs, 2001; Morkel et al., 2003). These processes are well-studied in lower vertebrates and other animals, but are not easily studied in a human system.

Human pluripotent stem cells (hPSCs), derived from the inner cell masses of blastocyst-stage embryos or through epigenetic reprogramming of somatic cells by defined factors, possess the remarkable capabilities of endless self-renewing cell divisions and the potential to differentiate into cellular derivatives of all three germ layers (Takahashi et al., 2007; Thomson et al., 1998). The study of these cells promises insight into the mechanisms of early human development and organogenesis with the potential to revolutionize medicine through improved drug screening, disease modeling and cell-based therapies, but much work remains before these goals will be realized (Chailangkarn et al., 2012; International Stem Cell Initiative et al., 2007; Lister et al., 2011; Young and Goldstein, 2012).

The roles of the Wnt pathway in early human development parallel those studied more thoroughly in other vertebrate models. The lessons learned from

ontogenetic studies in fish, frogs, birds and mice have informed the derivation of numerous cell types from hPSCs, indicating that the developmental functions of many signaling pathways, including Wnt signaling, are highly conserved. Nevertheless, many aspects of human development are unique and not easily tractable in simple model organisms. A more complete understanding of early human development necessitates a truly human model system.

Therefore, to improve our understanding of the molecular events that take place during early human embryonic development, we have employed hPSCs to model the role of Wnt signaling in gastrulation, the formation of the primitive streak, and the development of the early germ layers. To identify key targets of Wnt pathway activation in hPSCs, we treated cells with purified Wnt3a protein, collected RNA and performed RNA-Seq. Here, we describe the identification of the transcriptional repressor SP5 as a key target of Wnt signaling in hPSCs, and the discovery that it potentiates Wnt signals by repressing SP1 target genes. We performed ChIP-Seq analysis to identify targets of SP1/5 in hPSCs and quantify changes in binding following Wnt3a treatment. We identified several known antagonists of the Wnt signaling pathway as targets of SP1/5 in hPSCs and performed quantitative RT-PCR on single Wnt3a-treated cells and on *SP5* mutant cells to confirm that they are negatively regulated by SP5. Finally, we examined the role of *SP5* downstream of Wnt signaling in early endodermal differentiation and determined that SP5 inhibits the formation of definitive endoderm by enhancing Wnt signaling in a cell-autonomous manner. Thus, this chapter describes the mechanism by which SP5 mediates the effects of Wnt signaling in early human development modeled in hPSCs.

2.3 Results

2.3.1 RNA-Seq identifies differentially expressed genes following Wnt3a treatment

To identify the transcriptional targets of Wnt signaling genome-wide in hPSCs, we subjected the cells to a timecourse of treatment with Wnt buffer or purified recombinant mouse Wnt3a. RNA was isolated, reverse transcribed, and sequenced. Sequencing reads were mapped to the genome, quantified, and differential expression was calculated. Greater than 500 genes were significantly differentially regulated in at least one timepoint relative to cells treated with Wnt buffer. Because Wnt signaling is crucial for early developmental events such as gastrulation and the formation of germ layers, we hypothesized that treatment with Wnt3a should yield changes in gene expression representative of some or all of those events. To identify groups of genes with similar expression patterns, we clustered genes by expression levels at each timepoint to reveal patterns of gene upregulation and downregulation (**Figure 2-1A**). This analysis revealed distinct waves of gene expression, suggesting that Wnt-treated hPSCs undergo several developmental changes over the course of 48 hours. Prominent clusters included a group of late genes upregulated at 48 hours that included markers of definitive endoderm (*CXCR4, DKK1, SOX17*) and the tailbud (*FGF17*, *ID1*) (**Figure 2-1A, group i)**, which was accompanied by striking morphological changes (**Figure 2-1B**); a group of mid-late genes upregulated at both 24 and 48 hours that included primitive streak markers (*LEFTY1*, *SP5*, *WNT3*) and markers of primitive endoderm (*FOXA2*, *CER1*) (**Figure 2-1A-ii**); a group of genes upregulated primarily at 24 hours and downregulated again by 48 included mesendodermal markers (*GSC*, *MIXL1*, *NODAL*, *T*) and *AXIN2*, a direct target and negative feedback regulator of the Wnt pathway (**Figure 2-1A-iii**); a final cluster of genes that were steadily downregulated following Wnt3a treatment and included

markers of pluripotent stem cells (*FZD7*, *SOX2*) and ectoderm (*POU3F1*, *SOX3*) (**Figure 2-1A-iv**). These waves of gene expression indicate that high dosage Wnt3a treatment of hPSCs drives a process akin to gastrulation and generates cells reminiscent of the primitive streak, mesoderm, and endoderm, while restricting the emergence of ectodermal cells, consistent with literature reports that Wnt signaling promotes mesendodermal fates in hPSCs (Blauwkamp et al., 2012; Davidson et al., 2012).

In order to more deeply probe the molecular events downstream of Wnt signaling in hPSCs, we chose to examine the role of one particular upregulated target, the transcription factor SP5. Because *SP5* is highly upregulated in response to Wnt signaling in hPSCs and because it can act as a repressor of SP1 target genes (Fujimura et al., 2007), it potentially brings those genes under the control of Wnt signaling during crucial processes in early embryogenesis. We therefore sought to further understand the role of SP5 downstream of Wnt signaling in hPSCs. First, in order to confirm that *SP5* is a target of Wnt signaling in hPSCs, the expression of *SP5* mRNA following treatment with several dosages of Wnt3a was confirmed by qPCR (**Figure 2-1C**). *AXIN2*, a direct target of the Wnt signaling pathway (Lustig et al., 2002), was included as a control for pathway activation. At the highest dose of Wnt3a, *SP5* was upregulated several hundredfold. Interestingly, *AXIN2* transcript levels were only modestly upregulated, suggesting important differences in transcriptional regulation in the respective promoter regions of these two genes. Because SP5 is a known transcription factor, we hypothesized that it acts in this capacity in hPSCs and therefore sought to determine the subcellular localization of SP5 protein. Immunofluorescence staining of buffer- and Wnt3a-treated hPSCs

revealed abundant SP5 staining in the nuclei of positive Wnt-treated cells and no staining in buffer-treated cells (**Figure 2-1D**), indicating that SP5 likely functions as a transcription factor in hPSCs and their early derivatives. To better determine the kinetics of SP5 protein induction, we performed Western blot analysis on samples from hPSCs treated with a timecourse of Wnt3a protein, which revealed that SP5 protein accumulates to detectable levels 6 hours post Wnt3a treatment and continues to accumulate through 24 hours post treatment (**Figure 2-1E**).

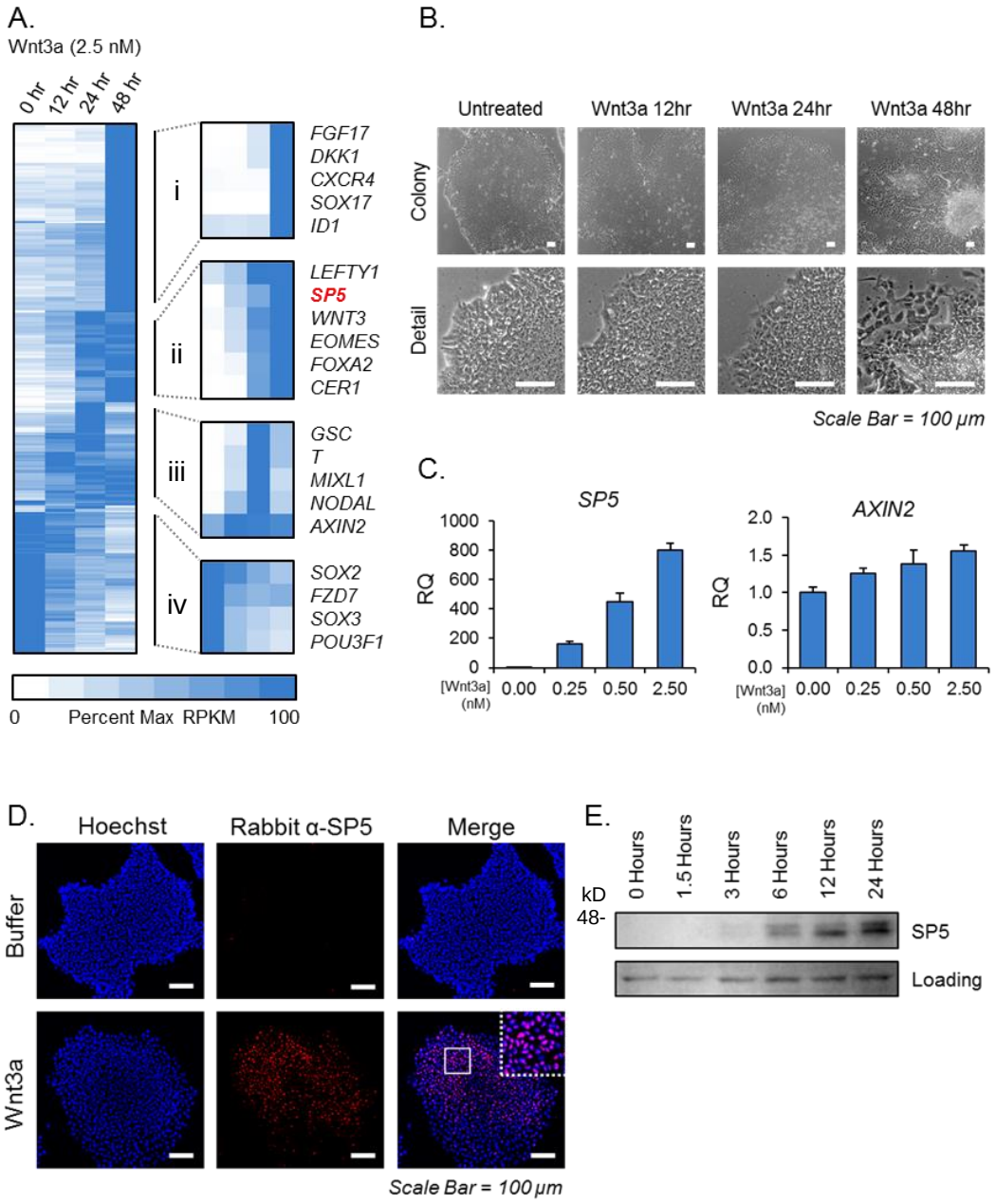

Figure 2-1: *SP5* is a Wnt target gene in hPSCs

A. Clustered heatmap of differentially expressed genes in hPSCs following Wnt3a treatment for 0, 12, 24 or 48 hours. Expression given as percent maximum (0%, white; 100%, blue). **B.** Phase contrast images of Wnt3a treated hPSCs. **C.** qPCR validation of *SP5* and *AXIN2* gene expression following Wnt3a dose response. **D.** Immunofluorescence of fixed hPSCs stained with Hoechst (blue) and Rabbit α-SP5 (red). (Scale bar = 100 µm) **E.** Western blot timecourse of SP5 protein expression in Wnt3a-treated hPSCs.

2.3.2 Global decrease in SP1 binding following Wnt treatment of hPSCs

Next, we sought to determine the genome-wide distribution and targets of SP5. We employed chromatin immunoprecipitation followed by sequencing (ChIP-Seq) with anti-SP5 antibodies to identify SP5 targets. Due to poor anti-SP5 antibodies, we also performed ChIP-Seq with anti-SP1 antibodies previously validated by the ENCODE project. We reasoned that decreased SP1 binding is a good surrogate for SP5 binding for the following reasons: first, SP1 and SP5 have been shown to bind the same sequence and to compete for binding *in vitro* (Fujimura et al., 2007); second, *SP1* expression levels in hPSCs remain constant across 48 hours of Wnt treatment in our RNA-Seq dataset; third, in the same dataset, no other member of the SP/KLF gene family is dynamically regulated to the extent of *SP5* (**Figure 2-2A**). We therefore generated chromatin from hPSCs treated with buffer or Wnt3a for 24 hours and performed ChIP using anti-SP1 and anti-SP5 antibodies. Purified DNA was sequenced and mapped to the genome and peaks were called. In order to determine the most common binding motifs of SP1/5 in hPSCs, we performed motif analysis on called peaks. This analysis revealed that the most overrepresented transcription factor binding motif in our datasets was the canonical SP1 binding sequence, or GC box (**Figure 2-2B**). We next sought to determine the genomic distribution of SP1/5 peaks. Peaks were localized mainly in or near transcription start sites (TSS), introns, or intergenic regions (**Figure 2-2C**), indicating that these peaks mostly fall within the likely promoter regions of nearby genes. Next, we set out to determine the distribution of SP1/5 binding sites within peaks and calculated the average density of the GC box motif within 500 base pairs upstream and downstream of the peak centers. The data revealed that on average, this motif is

found at the centers of peaks (**Figure 2-2D**), suggesting that the called peaks map to *bona fide* SP1/5 binding sites in the genome. We hypothesized that SP5 mediates the effects of Wnt signaling in hPSCs by competing with SP1 for binding in promoter regions genome-wide, resulting in decreased expression of nearby genes. We therefore predicted that SP1 binding in SP1/5 peaks should diminish following treatment with Wnt3a, accompanied by a concomitant increase in SP5 binding. In order to assess whether SP1/5 binding within peaks changes following Wnt3a treatment, we calculated the average change in read depth for SP1 and SP5 across all SP1/5 peaks. In agreement with our prediction, this analysis revealed decreased binding of SP1 and increased binding of SP5 at peak centers genome-wide (**Figure 2-2E, F**), suggesting that SP5 is able to exert transcriptional influence downstream of Wnt signaling in hPSCs.

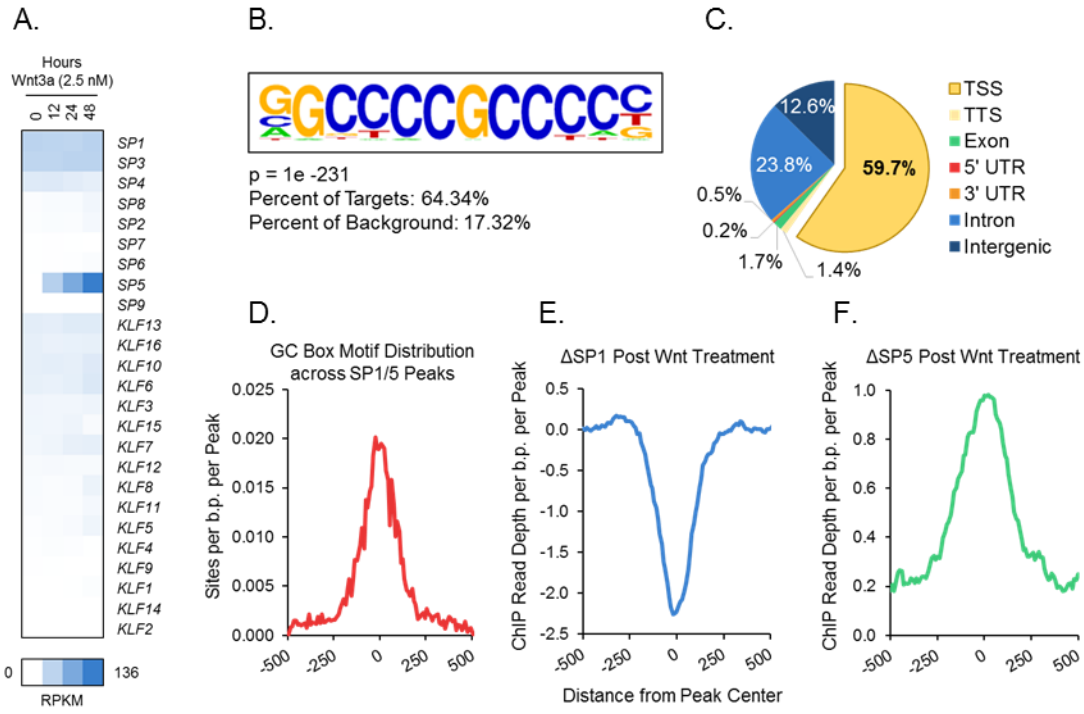

Figure 2-2: SP1/5 binding dynamics in hPSCs following Wnt3a treatment

A. Heatmap of gene expression of SP/KLF family members, sorted by family and order of highest RPKM at 0 hours of Wnt3a treatment. RPKM = 0, white; RPKM = 136, blue. **B.** Motif analysis of SP1/5 peaks. **C.** Genomic distribution of SP1/5 peaks. **D.** Average GC Box distribution across all SP1/5 peaks. **E.** Change in SP1 ChIP-Seq read depth following Wnt3a treatment. **F.** Change in SP5 ChIP-Seq read depth following Wnt3a treatment.

2.3.3 SP5 drives decreased expression of known Wnt antagonists

We next sought to identify SP1/5 targets of potential interest to Wnt signaling and early development. Assuming that SP1/5 regulate the transcription of proximal target genes, we annotated all peaks with the identity of the nearest gene. We then sought groups of genes involved together in similar biological processes. Interestingly, we identified several genes known to encode antagonists of the Wnt pathway as potential targets of SP1/5 in hPSCs (**Figure 2-3A**). Because SP5 is a known transcriptional repressor (Fujimura et al., 2007), we hypothesized that it functions in hPSCs in part to downregulate Wnt antagonists, sensitizing cells to Wnt signals and acting as a Wnt pathway fine-tuning mechanism throughout early developmental processes. We therefore sought to determine how *SP5* expression relates to the expression of Wnt antagonists. First, we sought to determine the effect of SP5 in single cells. We treated hPSCs with Wnt3a for 24 hours and performed single cell qPCR for Wnt antagonists, *SP5*, and markers of pluripotency and early lineage commitment. We found that *SP5* expression is negatively correlated with the expression of several Wnt antagonists within single cells (**Figure 2-3B**), a finding that supports our hypothesis that SP5 represses the transcription of these genes. We next took a genetic approach to study the effects of SP5 in hPSCs. Using CRISPR/Cas9-mediated mutagenesis, we used paired single guide RNAs (sgRNAs) to excise the C2H2 zinc finger coding region of the *SP5* gene (**Figure 2-4A**), which would result in the production of a truncated protein unable to bind DNA. We screened dozens of clones utilizing a PCR-based genotyping strategy and identified homozygous deletion mutant lines, termed *SP5dZF* (**Figure 2-4B-D**). We expanded two clones, A2 and E11 for further analysis. To confirm the mutant status of SP5 protein in these clones,

we performed Western blot analysis on lysates from CHIR98014-treated cells. Probing with anti-SP5 antibodies, we detected reactive bands of lower molecular weight than wild type SP5 protein in lysates from CHIR-treated, but not vehicle-treated *SP5dZF* cells consistent with truncated SP5 protein (**Figure 2-4E**). Because our hypothesis predicts that SP5 acts to repress Wnt antagonists, we next sought to determine whether *SP5dZF* cells exhibit increased expression of these genes relative to wild type (WT) cells following induction of the Wnt pathway. We performed a Wnt pathway activation timecourse on WT and *SP5dZF* cells by treating them with CHIR98014. Gene expression analysis by qPCR revealed that expression of Wnt antagonists is significantly upregulated in *SP5dZF* cells relative to WT (**Figure 2-4F**), suggesting that an important role of *SP5* is to act as a positive feedback regulator of the Wnt signaling pathway by driving downregulation of Wnt antagonists. Critically, SP5 appears to target multiple Wnt antagonists that act at many levels of the Wnt pathway, implying that small decreases in expression of individual antagonist genes likely contribute meaningfully to the intracellular level of Wnt pathway activation.

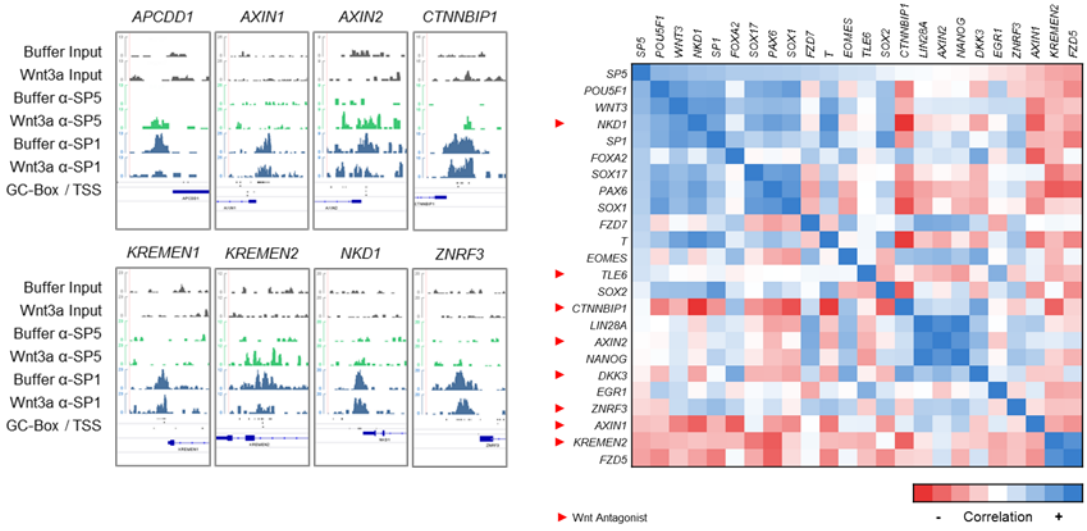

Figure 2-3: SP1 and SP5 bind Wnt Antagonist Promoters

A. ChIP-Seq tracks for Buffer and Wnt3a treated cells. Input (grey), anti-SP5 (green), anti-SP1 (blue). **B.** Pearson correlation of genes expressed in single cells. Wnt antagonists highlighted by red arrowheads. Correlation is given as a heatmap: blue, positive; white, zero; red, negative.

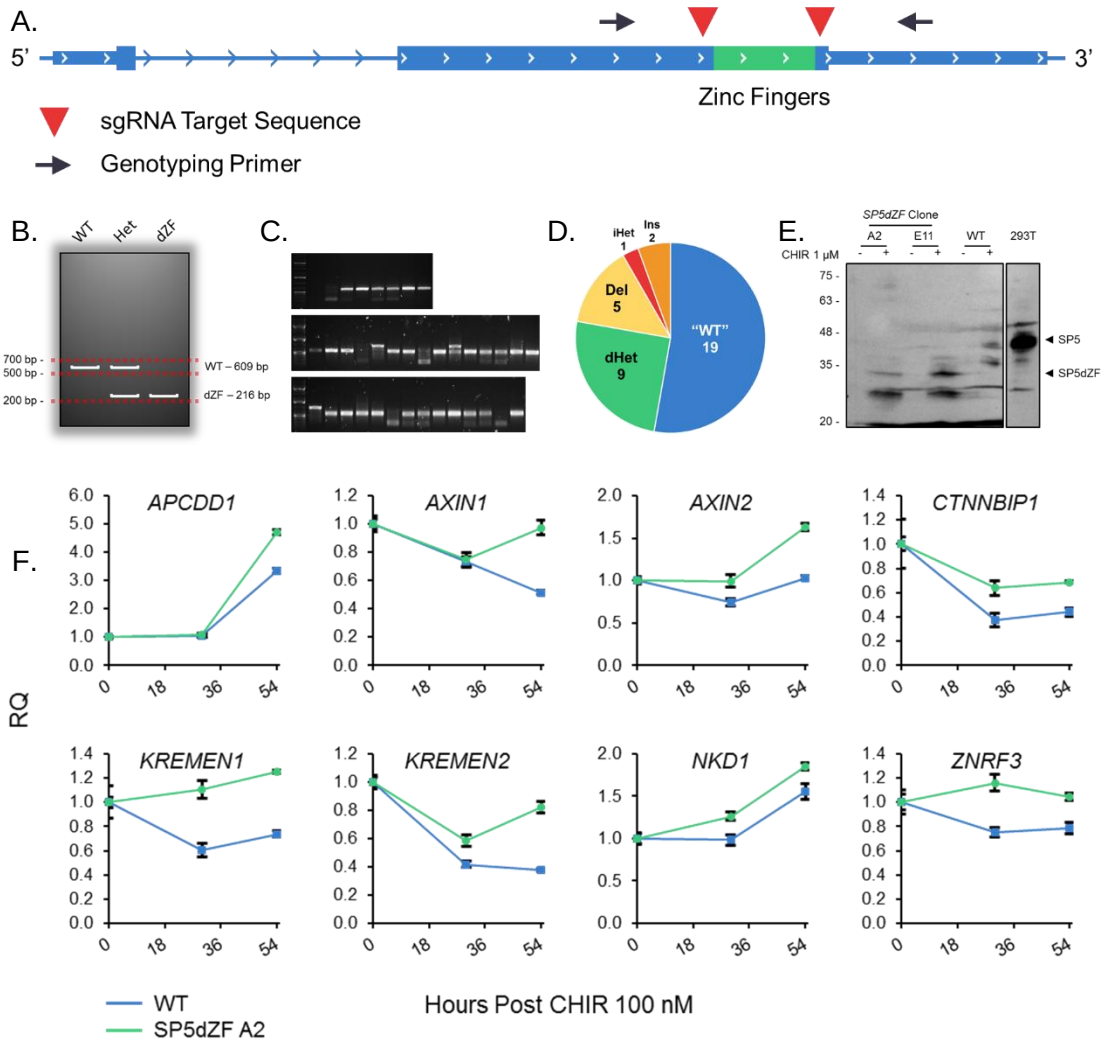

Figure 2-4: Differential Expression of Wnt Antagonists in WT and SP5dZF Cells.

A. Strategy for CRISPR/Cas9 mutagenesis of the *SP5* gene. Red arrowheads indicate sgRNA cognate sequences. Gray arrows indicate PCR primer sequences. **B.** Schematic for interpreting clone genotyping. **C.** Genotyping PCR of the SP5 Zinc Finger locus in 36 mutant clones. Molecular weight markers - bright bands at 0.5, 1.5 and 5.0 kbp **D.** Quantification of mutation type. "WT", one band at 609 bp; Del, one band at 216 bp; dHet, One band at 609 bp and one at 216 bp; Ins, one band at approx. 1 kbp; iHet, One band at 609 bp and one at 1 kbp. **E.** Western Blot analysis of SP5 protein expression in *SP5dZF* clones (A2, E11) and WT. SP5 overexpressed in HEK293T cells serves as a positive control. Molecular weight (kD) is given at the left. **F.** Timecourse of Wnt antagonist gene expression in WT and *SP5dZF* (clone A2) mutant cells following CHIR98014 100 nM for 0, 30 or 54 hours. WT, blue; *SP5dZF*, green.

2.3.4 *SP5* and *AXIN2* are differentially induced

We previously noted that *SP5* is induced several hundredfold in response to Wnt pathway activation in hPSCs, whereas *AXIN2*, often considered a universal target of the Wnt pathway (Reviewed in Logan and Nusse, 2004; MacDonald et al., 2009), was very poorly induced by comparison (**Figure 2-1A**). We reasoned that perhaps *SP5* may also be a universal target of the Wnt pathway and provide a more sensitive readout for Wnt pathway activation. To determine whether SP5 is a universal target of the Wnt pathway, we treated several established cell lines of various origins with Wnt3a for 24 hours and performed qPCR for *AXIN2* and *SP5*. Immediately it became clear that some cell lines preferentially upregulated either *AXIN2* or *SP5* to a much higher degree than the other. By calculating the ratio of *SP5* induction relative to *AXIN2* induction following Wnt pathway activation, we identified several classes of cell lines stratified by factors of 10: Those where *SP5* / *AXIN2* was 100 or greater; 10 or greater; 1 or greater; 0.1 or greater; 0.01 or greater (**Figure 2-5A**). Although most cell lines tested modestly upregulated both genes in response to Wnt pathway activation, those cells with the highest *AXIN2* induction (H1299, H1299, T47D) demonstrated very poor induction of *SP5*. Conversely, cell lines with high levels of *SP5* induction (H1 hESC, HUES7 hNSC, NCCIT, PA1) exhibited relatively lower induction of *AXIN2* (**Figure 2-5B**). These results suggest that *AXIN2* and *SP5* may both be universal targets of the Wnt pathway, but that neither provides a definitive readout of the level of Wnt pathway activation. Interestingly, cells with high *SP5* induction potential were all undifferentiated cell types including hESCs, two teratocarcinoma cell lines, and hNSCs, suggesting that *SP5* may be a marker of Wnt-sensitive stem cells, where it potentially functions to repress the expression of *AXIN2*.

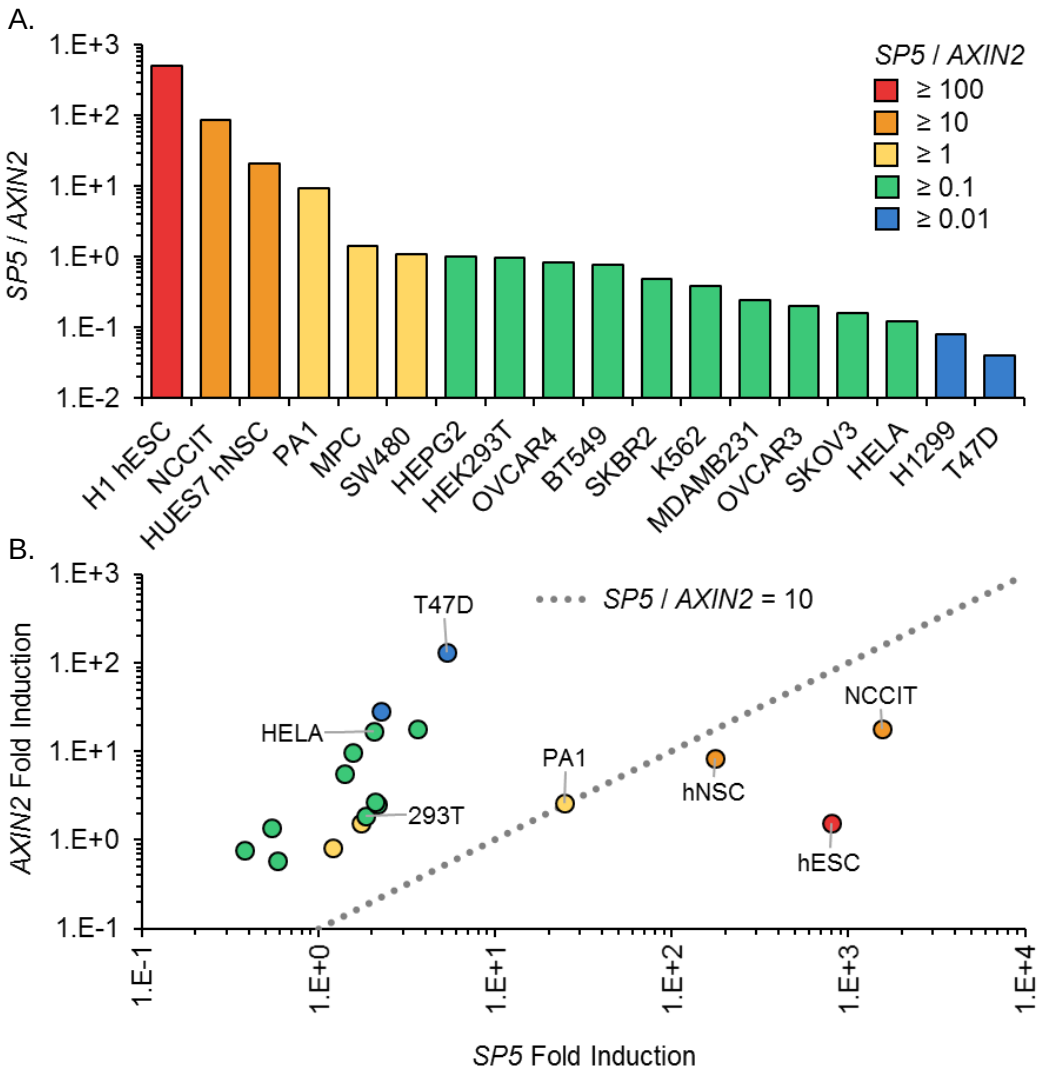

Figure 2-5: Bimodal induction of AXIN2 and SP5 across many cell lines

A. Ratio of SP5 induction to AXIN2 induction following 24 hours of Wnt3a treatment is given for many cell lines. B. Scatterplot of AXIN2 expression versus SP5 expression following 24 hours of Wnt3a treatment for cell lines enumerated in A. The point at which SP5 induction is 10-fold that of AXIN2 is given by a dashed gray line.

2.3.5 SP5 expression impairs endoderm formation through enhanced Wnt signaling

We next sought to determine the implications of SP5 expression in the context of early human development. Because *SP5* is upregulated in Wnt3a-treated hPSCs at the same time as many genes involved in gastrulation and the formation of the primitive streak, we hypothesized that *SP5dZF* cells would display differences in differentiation propensity. Furthermore, if SP5 functions to amplify Wnt signals, we hypothesized that *SP5dZF* cells would display phenotypes indicative of impaired Wnt signaling. In order to determine the effect of SP5 on hPSCs downstream of Wnt-mediated differentiation, we subjected WT and *SP5dZF* cells to a timecourse of CHIR98014 treatment. RNA was isolated and reverse transcribed, and expression of markers of endoderm (*SOX17*), mesoderm (*T*) and ectoderm (*SOX1*) was assessed. Interestingly, *SP5dZF* mutants displayed nearly identical expression patterns of *T* and *SOX1* to those of WT cells, but displayed much higher upregulation of *SOX17* (**Figure 2-6A**), indicating that SP5 may play a role to restrict the formation of endoderm downstream of Wnt signaling. To further investigate the role of SP5 in the formation of definitive endoderm, we used transduced hPSCs with retroviruses to overexpress or silence *SP5* while also expressing GFP. By transducing cells at a low MOI, we were able to generate mixed populations of GFP+ and GFP- cells for each construct. These populations were then differentiated toward definitive endoderm, stained with anti-CXCR4-APC antibodies, and analyzed by flow cytometry. By comparing the fraction of CXCR4+ cells between GFP+ and GFP- populations, we demonstrated that *SP5* overexpression led to reduced CXCR4 staining, whereas *SP5* knockdown increased it (**Figure 2-6B**). In both cases, control vectors (GFP only) displayed equal CXCR4 staining in both GFP+ and GFP- populations. Because SP5

is able to repress Wnt antagonists, we hypothesized that SP5 restricts endoderm by amplifying Wnt signals. We therefore set out to determine whether increased Wnt pathway activation can inhibit the formation of definitive endoderm. To test our hypothesis, we subjected *SOX17-GFP* H9 reporter hESCs to directed endodermal differentiation, including a CHIR98014 dose response during days 2 and 3 (**Figure 2-6C**). Indeed, the relative fraction of GFP+ cells was reduced with increasing Wnt pathway activation (**Figure 2-6D**), suggesting that SP5, may drive decreased endoderm formation by increasing Wnt pathway activation.

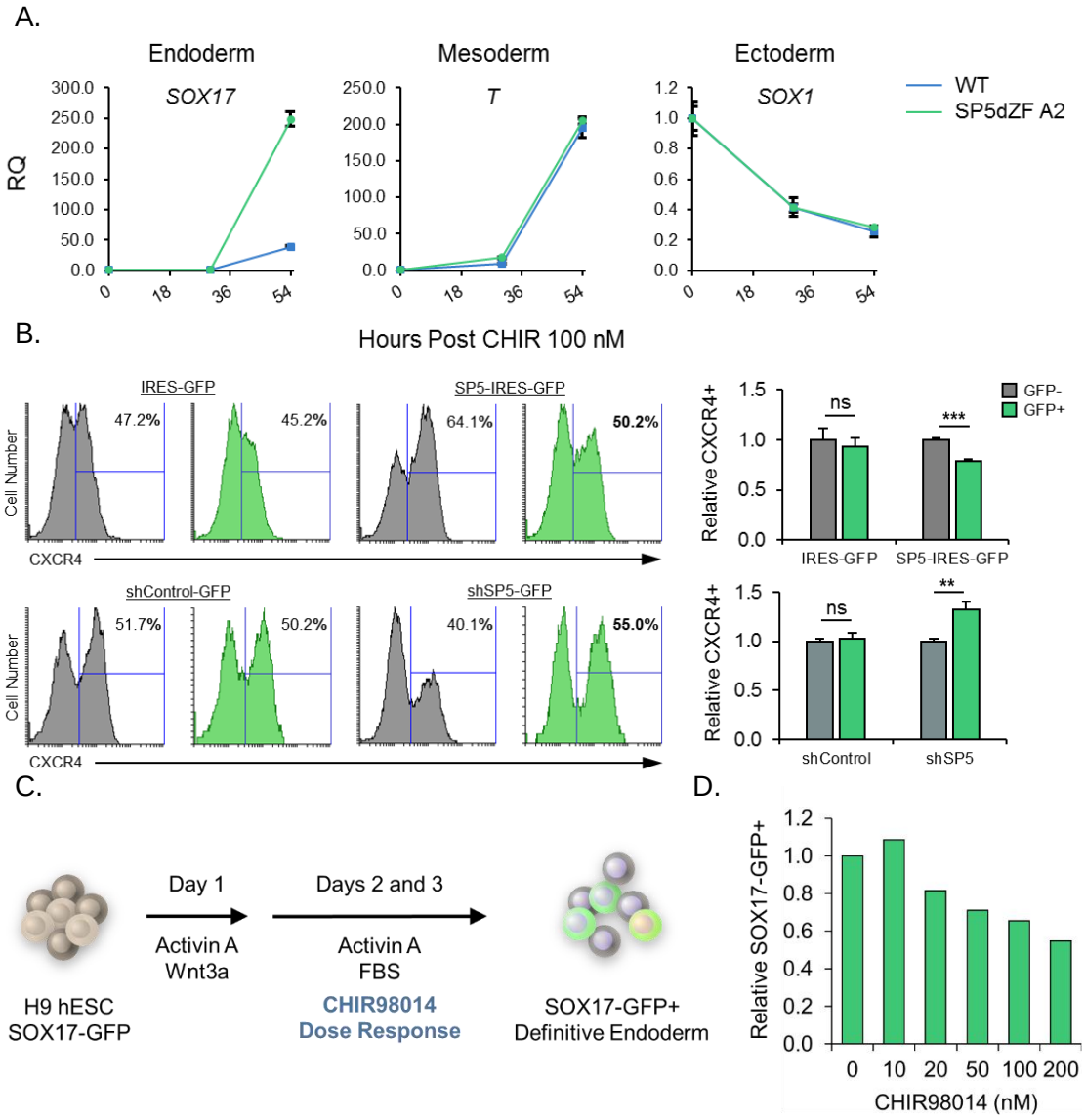

Figure 2-6: SP5 inhibits the formation of definitive endoderm from hESCs.

A. Effect of SP5 mutation on germ layer gene expression following CHIR98014 treatment. WT, blue; SP5dZF, green. **B.** Effect of SP5 overexpression and knockdown on definitive endoderm formation. Following anti-CXCR4-APC staining, GFP- (grey) and GFP+ (green) populations were analyzed by flow cytometry. Representative histograms shown. Quantification of three biological replicates are given as bar graphs where percent positive is normalized to GFP-. ** = $p < 0.01$; *** = $p < 0.001$. **C.** Experimental outline for testing the effect of Wnt pathway activation on the formation of definitive endoderm, marked by GFP in SOX17-GFP H9 reporter hESCs. **D.** Effect of CHIR98014 doses on definitive endoderm formation.

2.4 Discussion

In this study, we set out to identify the molecular circuitry downstream of the Wnt signaling pathway in hPSCs. Utilizing RNA-Seq, we identified several categories of differentially regulated genes. We observed that *SP5*, which encodes a transcriptional repressor, is highly upregulated following Wnt3a treatment, and hypothesized that SP5 represses target genes in response to Wnt signaling in hPSCs. We demonstrated that SP5 binds the promoter regions of SP1 target genes and drives decreased SP1 binding at those sites genome-wide. Using ChIP-Seq, we demonstrated that several known Wnt antagonists are targets of SP1 and SP5. Furthermore, cells with mutant SP5 that cannot bind DNA exhibit increased expression of these antagonists following Wnt pathway induction. Finally, we showed that SP5 interferes with the formation of definitive endoderm from hPSCs, possibly by increasing the level of Wnt pathway activation in a cell-autonomous fashion. Together, these findings suggest that Wnt signaling functions in early human development not only by driving the expression of TCF/LEF/β-catenin target genes, but also by repressing SP1 target genes through SP5.

Wnt treatment of hPSCs led to the upregulation of many genes associated with the earliest stages of development, including gastrulation, the formation of the primitive streak, mesendoderm, and definitive endoderm. This finding is consistent with roles for Wnt signaling in the literature, where it is shown to play roles in all of these processes (D'Amour et al., 2005; Liu et al., 1999b; Martin and Kimelman, 2008). We also observed decreased expression of genes associated with the early ectoderm and neurogenesis. In a study of the effects of endogenous Wnt signaling in hPSCs, Blauwkamp et al. reported that relatively high levels of pathway activation

corresponded with predisposition towards mesendodermal lineages and against neural lineages (Blauwkamp et al., 2012), a finding that is substantiated by our result. While our findings parallel and confirm those obtained from embryological studies of the role of Wnt signaling in the development of other model organisms, to our knowledge they represent the first transcriptome-wide study of hPSCs treated with purified Wnt protein and will provide a fertile field for future studies of the Wnt pathway in human development.

We confirmed that the transcription factor SP5 is a target of Wnt signaling in hPSCs. This finding is supported by observations in the literature that SP5 is a direct target of the Wnt pathway in other contexts, including early the primitive streak and early neuromesodermal patterning (Dunty et al., 2014; Weidinger et al., 2005), neural development (Fujimura et al., 2007) and colon cancer (Takahashi et al., 2005). Furthermore, we observed that among the entire SP/KLF family of transcription factors, none displays robust induction to the degree observed for *SP5*, suggesting a unique role for this factor downstream of Wnt pathway activation. We went on to demonstrate through ChIP-Seq that SP5 binds many of the same regions as the ubiquitously expressed SP1 genome-wide and that upon Wnt3a stimulation, SP5 accumulates at these sites while SP1 binding is diminished, a finding consistent with work by Fujimura et al. that demonstrated direct competition for binding of radiolabeled oligonucleotides by SP1 and SP5 (Fujimura et al., 2007). The majority of these binding sites were found in or near gene promoter regions and possessed canonical GC Box motifs, indicating that these are *bona fide* SP1/5 binding sites. Importantly, this finding strengthens the notion that the Wnt signaling pathway exerts transcriptional regulatory influence on a broad subset of gene targets through

mechanisms other than TCF/LEF/β-catenin mediated transcription, further underscoring its importance as a regulator of transcriptional events that take place throughout development and into homeostasis.

Our finding that several Wnt antagonists, many of which are directly regulated by Wnt signaling, also exhibit SP1/5 binding in their promoter regions, introduced the possibility that SP5 participates in a positive Wnt signaling feedback loop. We used single cell gene expression profiling in Wnt-treated hPSCs to demonstrate a negative correlation between the expression of *SP5* and Wnt antagonists at the cellular level. Furthermore, *SP5dZF* mutant hPSCs exhibit abnormally high expression of these antagonists relative to WT cells following Wnt3a treatment. Together, these findings indicate that a key role for *SP5* in development is to potentiate Wnt signals, consistent with previously published roles for homologs of *SP5* and the closely related *SP8* throughout vertebrate development where mutants and morphants display phenotypes that closely resemble Wnt mutant phenotypes (Dunty et al., 2014; Treichel et al., 2003; Weidinger et al., 2005). While it is likely that other targets of SP5 contribute to its importance downstream of Wnt signaling in development, it is also probable that aberrant upregulation of Wnt antagonists can drive premature attenuation of Wnt signals critical for proper development.

We sought to determine whether *SP5* is a so-called "universal" Wnt target gene, similar to the well-known *AXIN2*. Interestingly, among many cell lines surveyed, only hPSCs, hNSCs, NCCIT, and PA1 cells displayed strong *SP5* induction following Wnt3a treatment. This finding is consistent with the observation that *Sp5* expression is restricted mainly to the primitive streak, tailbud, and neural tissue of the developing mouse (Treichel et al., 2001) and suggests that it plays similar roles in humans.

Furthermore, cell lines with strong *SP5* induction display only very weak *AXIN2* induction, consistent with the hypothesis that SP5 functions in part to repress Wnt antagonists in some cell types. Other cell types demonstrate robust induction of *AXIN2* and modest or no induction of *SP5*. We propose a model where Wnt-responsive stem cells throughout development and in mature tissues upregulate *SP5* in response to Wnt signals while neighboring cell types predominantly upregulate Wnt antagonists such as *AXIN2*. In this model, stem cells are especially sensitive to Wnt ligands, and surrounding tissue types are more resistant, helping to prevent aberrant pathway activation. This paradigm allows for improved targeting of a Wnt signal to specific cell types on top of other established mechanisms such as physical location, expression of cell surface receptors, and expression of intracellular and extracellular Wnt agonists and antagonists.

Finally, we demonstrated a function of *SP5* in a human developmental context by measuring the expression of germ layer markers following Wnt pathway activation in WT and *SP5dZF* cells. Interestingly, we observed that both WT and *SP5dZF* cells express equal levels of *T* and *SOX1* following CHIR98014 (100 nM) treatment, but *SP5dZF* cells exhibit a substantial increase in *SOX17* gene expression, indicating that *SP5* functions to restrict the formation of definitive endoderm downstream of Wnt signaling. Strengthening this hypothesis is our observation that *SP5* overexpression during directed differentiation to definitive endoderm leads to decreased staining for the endoderm marker CXCR4, while *SP5* knockdown has the opposite effect. This role for SP5 fits with previously published reports that *Sp5* expression in early mouse development is highly restricted to the primitive streak and only partially expressed in the definitive endoderm (Treichel et al., 2001). To determine whether this effect could

be explained by the level of Wnt pathway activation during definitive endoderm differentiation, we differentiated *SOX17-GFP* H9 hESCs to definitive endoderm and included a CHIR98014 dose response for the final 2 days of differentiation. We demonstrated that GFP expression is inversely correlated with CHIR dosage, indicating that Wnt pathway activation inhibits the formation of definitive endoderm following induction. This observation is consistent with reports indicating an early requirement for Wnt signaling and later inhibitory effects during the derivation of definitive endoderm from hPSCs (D'Amour et al., 2005; Naujok et al., 2014). Together, these observations suggest a model where Wnt pathway activation drives the expression of SP5, which represses Wnt antagonists and augments Wnt pathway activation. Increased levels of Wnt pathway activation in turn drive further restriction of the formation of definitive endoderm in early human development, ensuring the proper patterning and development of early embryonic tissues (**Figure 2-7**).

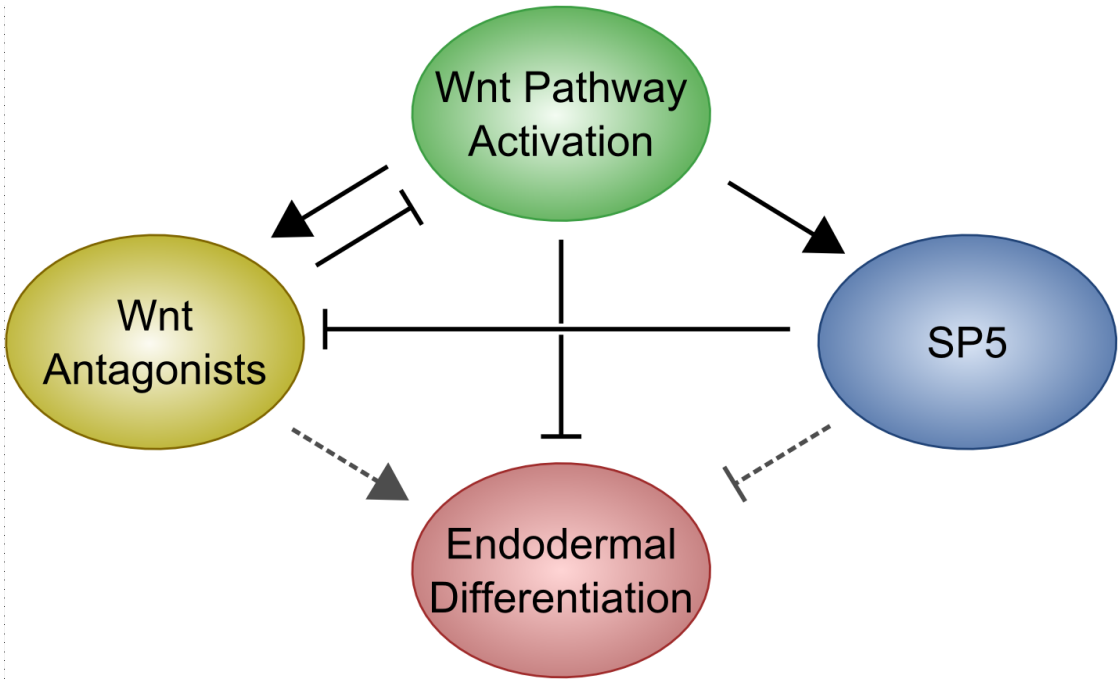

Figure 2-7: Model for SP5 function downstream of Wnt signaling in hPSCs.

Following Wnt pathway activation of hPSCs, upregulation of SP5 drives downregulation of Wnt antagonists, leading to increased cellular levels of Wnt pathway activation, which restricts or delays endodermal differentiation. Dashed grey arrows represent the effects of Wnt antagonists and SP5 by proxy of their effect on the level of Wnt signaling.

In conclusion, we have identified key molecular events downstream of Wnt signaling in an hPSC model of early human development. Our work clarifies the role of the transcription factor SP5 in early human development by demonstrating that it plays an important role as a repressor of Wnt antagonists, sensitizing stem cell populations to Wnt signals. This finding will inform future studies of Wnt-responsive stem cells throughout development, in mature tissues and cancer. Wnt-responsive stem cells may possibly be identified by *SP5* expression, and conditional targeted manipulation of *SP5* in tissue stem cells may shed light on the fitness advantages conferred by this gene.

Acknowledgments

Chapter 2, in part, is currently being prepared for submission for publication of the material. Huggins, Ian J.; Willert, Karl. I am the primary investigator and author of this material. I would like to further acknowledge the following individuals and organizations: Eric O'Connor, Karl Marquez, and Jesus Olvera of the UC San Diego Human Embryonic Stem Cell Core Facility for help and expertise in human pluripotent stem cell culture and cell sorting; Steven Head and his staff at the Scripps Research Institute Next Generation Sequencing Core Facility for library preparation and sequencing of samples for RNA-Seq and ChIP-Seq; Sonal Naik and Aritra Dey of the UC San Diego Stem Cell Genomics Core for invaluable advice and assistance with single cell gene expression experiments; Bing Ren and Ah Young Lee for sharing equipment, reagents and technical advice regarding ChIP-Seq; Rick Meyers and Kim Newberry for advice and expertise regarding ChIP-Seq; Nathan Kumar for advice and guidance in the generation of definitive endoderm. This work was supported by the

California Institute for Regenerative Medicine. IJH was supported by a grant from the California Institute for Regenerative Medicine (TG2-01154).

Chapter 3 - Effects of Wnt signaling on gene expression in human pluripotent stem cells

3.1 Abstract

Wnt signaling is a critical regulator of pluripotent stem cell self-renewal and differentiation. In hPSCs, the exact roles of Wnt signaling are poorly understood and often contested. Wnt signaling has been shown to drive differentiation of hPSCs, but has also been shown to exist endogenously at low levels in these cells, where it modulates germ layer differentiation propensity and does not adversely affect the pluripotent state, but rather promotes it. Indeed, other work has demonstrated requirements for Wnt signaling components in the maintenance of the pluripotent state and for cellular reprogramming to pluripotency. Here, I describe work aimed at dissecting the various roles of Wnt signaling in hPSCs by examining the effects of Wnt pathway activation using fine-grained dose response experiments. I demonstrate that low levels of endogenous Wnt signaling promote the pluripotent state. I further show that higher levels of Wnt signaling promote differentiation, but that this differentiation can be blocked or delayed by the inhibition of PORCN, an enzyme required for the secretion of active Wnt proteins, suggesting the presence of an endogenous positive feedback loop in hPSCs. I further demonstrate dose-dependent activities of Wnt pathway activation by CHIR98014 in the early differentiation of hPSCs toward endodermal and mesodermal fates. In the second half of this chapter, I present work that demonstrates a role for Wnt signaling in regulating the expression of the transcription factor EGR1. I identify culture conditions that promote expression of this factor and then show that short term Wnt pathway activation can suppress it. I demonstrate that conditioned medium from densely-cultured hPSCs can induce EGR1 expression in other cells. Finally, I demonstrate that Wnt-mediated regulation

of EGR1 expression is cell type specific and occurs in hPSCs, but not in the cancer cell line MDA-MB231.

3.2 Introduction

The Wnt signaling pathway is required for normal animal development, where it plays many important roles. Wnt proteins are secreted lipid-modified glycoproteins that mediate signaling by binding cell surface receptors and thereby activate intracellular signaling cascades. While the hydrophobic lipid modification of Wnt proteins is suggestive of poor solubility, considerable evidence exists to demonstrate that they are able to diffuse relatively long distances *in vivo*, signaling to cells several cell diameters away from the point of origin. In this regard, Wnt proteins have been shown to act as morphogens, which exert dose-dependent effects on target cells. In a simple model of protein diffusion, this translates into high doses of Wnt protein for cells proximal to the source and lower doses for distal cells. Using the developing *Drosophila* wing as a model, Zecca et al. observed that Wg protein, a *Drosophila* Wnt homolog, was able to signal to target cells at a distance (Zecca et al., 1996). Importantly, when wild type Wg protein was substituted for by a membrane-bound version, distal cells no longer expressed Wg target genes, indicating that soluble Wg protein signals to distal cells by diffusing through the intracellular space. Later work aimed at visualizing extracellular Wg confirmed this hypothesis and demonstrated a true gradient of protein emanating from the imaginal disc (Strigini and Cohen, 2000). The ability of these hydrophobic proteins to traverse such relatively large distances is explained by their association with lipoprotein particles that act as long range carriers (Panáková et al., 2005). Such morphogen gradients have also been demonstrated in *C. elegans* (Coudreuse et al., 2006). Importantly, Wnt protein gradients also pattern

vertebrate tissues. Studies in *Xenopus* neural development revealed that Wnts act as morphogens to pattern the neural tube (Kiecker and Niehrs, 2001; Roelink and Nusse, 1991) and regulate limb outgrowth (Gao et al., 2011).

In vitro models of early development have also demonstrated roles for Wnt signaling gradients in hPSCs (Blauwkamp et al., 2012), the patterning of the early mammalian primitive streak (Berge et al., 2008b) and early stage hPSC-derived neural rosette stem cells (Moya et al., 2014). One strength of the hPSC model system is the availability of small molecule and recombinant protein modulators of the Wnt pathway that can be titrated into cultures in carefully controlled doses. In the first half of this chapter, I leverage these powerful tools in order to study of the effects of low and high levels of Wnt pathway activation in hPSCs.

In culture, hPSCs represent a snapshot of early human development. Culture conditions have been refined and tuned over the years to maintain the pluripotency of hPSCs, which remain forever on the verge of spontaneous differentiation and the initiation of developmental programs. Importantly, hPSC culture medium must be replaced daily to resupply cells with nutrients and especially growth factors that promote the pluripotent state. Furthermore, hPSCs likely secrete growth factors into the culture medium that may drive spontaneous differentiation, which places increased emphasis on the importance of regular medium replenishment. Finally, hPSCs often exhibit exceptionally rapid growth in culture, especially when cultured in medium based on the E8 formulation (Chen et al., 2011). Care must be taken to passage hPSCs regularly to avoid overgrowth and spontaneous differentiation. Little work has been done to address the molecular character of hPSCs as they transition from pluripotent to differentiating following depletion and/or conditioning of the growth

medium in dense cultures. In the second part of this chapter, I have examined the expression of the transcription factor EGR1 as hPSCs in culture reach high density and have sought to determine what role, if any, is played by Wnt signaling in regulating the expression of this transcription factor. Using the small molecule GSK3β inhibitor CHIR98014, I was able to demonstrate that Wnt pathway activation negatively regulates the induction of EGR1 in densely cultured hPSCs.

3.3 Results

3.3.1 Morphogen effects of Wnt signaling on hPSC pluripotency and differentiation

3.3.1.1 IWP2 treatment drives decreased expression of pluripotency markers

First, in order to determine whether endogenous Wnt signaling affects the expression of pluripotency markers in human embryonic stem cells, I subjected them to treatment with increasing doses of IWP2, a compound that inhibits the activity of PORCN, an intracellular acyltransferase required for the secretion of active WNT proteins (Takada et al., 2006). In order to ensure efficient blockade of Wnt secretion, I plated the cells in E8 culture medium on Day 1 and fed them with E8 + IWP2 each subsequent day until harvest (Day 7). I observed that expression of *AXIN2* and *SP5* was attenuated in a dose-dependent fashion following IWP2 treatment (**Figure 3-1A**), suggesting that Wnt signaling is active in hPSCs. More interestingly, treatment with IWP2 led to dose-dependent decreases in expression of several markers of pluripotent stem cells including *FZD7*, *POU5F1* and *NANOG* (**Figure 3-1A**), leading me to speculate that Wnt signaling may play a role in the maintenance and differentiation of hPSCs. Furthermore, this dose-dependent decrease in gene expression raised the possibility that Wnt proteins act as morphogens in the context of early human embryonic development as modeled in hPSCs.

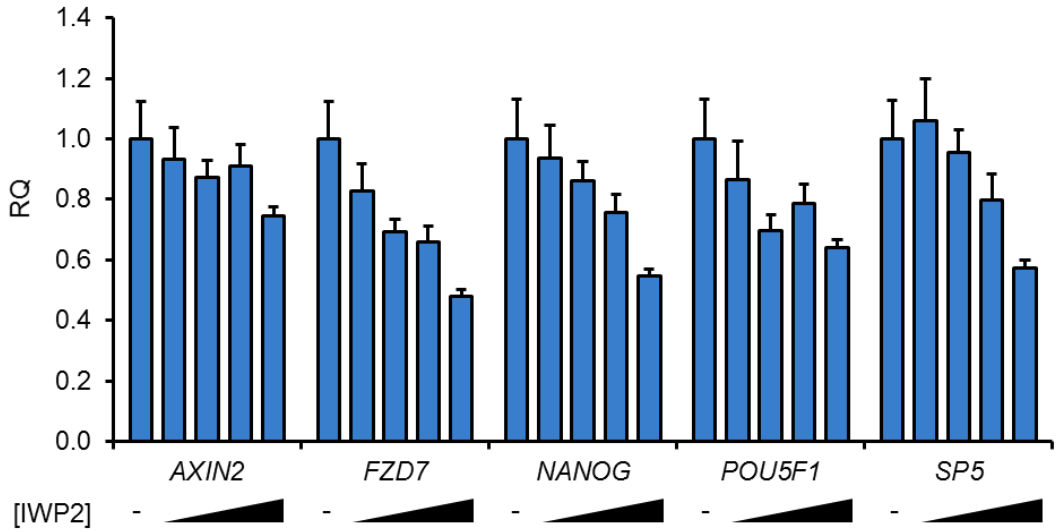

Figure 3-1: Effect of IWP2 treatment on hPSC pluripotency.

A. hPSCs were cultured in the presence of increasing doses of IWP2 (0, 50, 100, 250, 500 nM) for the duration of 1 passage. Gene expression is given as relative quantity (RQ) versus expression in control treated cells (0 nM IWP2).

3.3.1.2 IWP2 treatment blocks Wnt-mediated differentiation

I next set out to test the hypothesis that Wnt signaling can both promote and antagonize expression of hPSC pluripotency markers in a dose dependent fashion. In order to identify concentrations of Wnt3a that promote either self-renewal or differentiation measured by expression of *POU5F1*, I seeded cells and treated them for the duration of the passage with a wide range of concentrations of purified Wnt3a protein. Furthermore, to reduce the influence of so-called "non-canonical" Wnts, I replicated these treatment conditions in the presence and absence of IWP2. As expected, both *AXIN2* and *SP5* expression steadily increased in a dose-dependent fashion following Wnt3a treatment (**Figure 3-2A**), though *SP5* expression appeared to peak and diminish at concentrations higher than 1,000 pM. More interestingly, *POU5F1* expression appeared to peak at 250 pM Wnt3a, which was followed by a sharp decline in expression at higher concentrations (**Figure 3-2A**), a result that is indicative of roles for Wnt signaling in both self-renewal and differentiation of hPSCs. Intriguingly, in the presence of IWP2, induction of *AXIN2* and *SP5* was diminished but not abolished. Furthermore, IWP2 treatment blocked the severe downregulation of *POU5F1* that I observed at similar doses of Wnt3a in IWP2-untreated cells (**Figure 3-2A**). These results imply the existence of a positive feedback loop that relies on the secretion of Wnt proteins downstream of Wnt pathway activation in hPSCs. Indeed, in previous work I had identified *WNT3* as a target of Wnt signaling in hPSCs (**Figure 2-1A**), indicating that this feedback loop is important for early Wnt-mediated events such as gastrulation and germ layer formation.

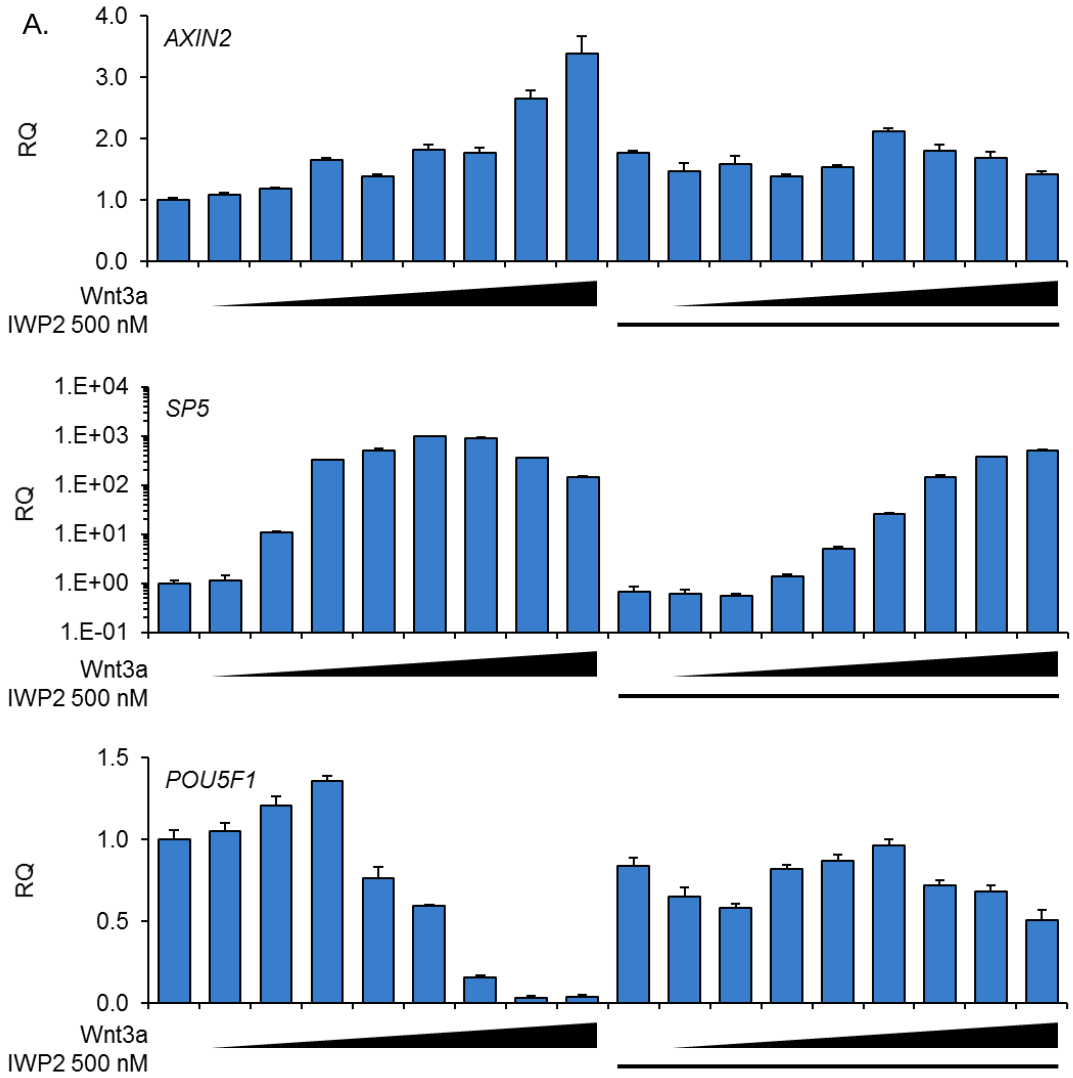

Figure 3-2: Wnt3a drives hPSC differentiation in a dose-dependent manner

A. Expression of *AXIN2*, *SP5* and *POU5F1* measured by qPCR following Wnt3a treatment (0, 50, 100, 250, 500, 1000, 2500, 5000, 10000 pM) with IWP2 500 nM (black bar) or without.

3.3.1.3 Dose-dependent effects of Wnt pathway activation

I next set out to determine the dose-dependent effects of Wnt pathway activation on hPSC morphology and differentiation. I seeded cells and treated them either for 24 hours or continuously throughout the passage with various doses of CHIR98014. I imaged the cells daily to determine the amount of time before continuous Wnt pathway activation drives morphological differentiation, which I define here as the loss of compact, epithelial colonies and the appearance of multiple cell types. All treatment conditions save buffer only promoted differentiation by Day 6, but only 1000 nM CHIR promoted morphological differentiation in as little as 24 hours (**Figure 3-3A**). Interestingly, different doses of CHIR also led to the emergence of morphologically different cell populations, strengthening the argument that Wnt signaling behaves in a morphogen-like manner in models of early human development. I next assayed gene expression in these cells. I observed that markers of pluripotency (*NANOG*, *POU5F1* and *SOX2*) were downregulated by low doses of CHIR98014 at 24 hours post treatment, and by all doses following continuous treatment (**Figure 3-3B**). Expression of AXIN2 increased with dose over both 24 hours and continuous treatment, but cells treated continuously demonstrated extremely elevated expression. SP5 expression was higher during acute treatment, but remained high under continuous treatment (**Figure 3-3B**). Germ layer markers demonstrated very interesting expression patterns: *SOX17* levels peaked at low levels of continuous treatment. *T* levels were highest at high doses and track remarkably well with *SP5* expression. *SOX2*, which marks pluripotent cells and neural lineages but may also be indicative of certain patterned endodermal populations, was expressed only with no treatment and at a mid-high dose following continuous

treatment (**Figure 3-3B**). These observations are again consistent with the hypothesis that Wnt signaling has important dose-dependent effects on hPSCs and their progeny.

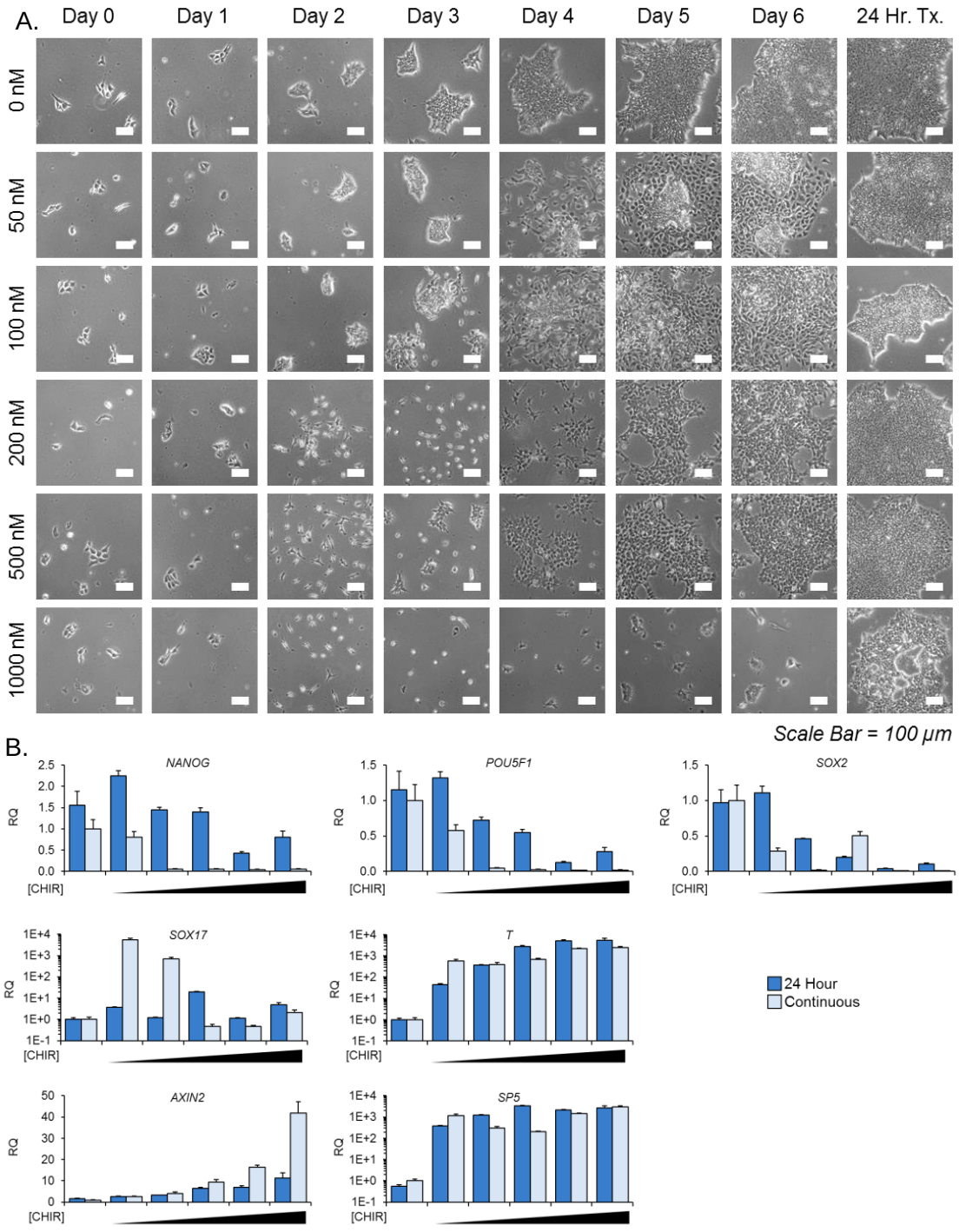

Figure 3-3: Dose-dependent effects of Wnt signaling on hPSCs.

A. Effect of CHIR dose response on hPSC morphology. **B.** Effect of CHIR dose response on expression of pluripotency and germ layer markers. 24 hour treatment (dark blue) and continuous treatment (light blue) shown.

3.3.2 Wnt pathway activation modulates EGR1 expression in hPSCs

Next, I sought to determine the effect of Wnt signaling on EGR1 expression in hPSCs. The gene encoding the transcription factor EGR1 was identified by several independent groups searching for immediate-early gene products following serum stimulation of cultured cells (Chavrier et al., 1988; Christy et al., 1988; Sukhatme et al., 1987). *EGR1* mRNA is upregulated in response to growth factor stimulation in several cell types and was originally thought to be a mediator of rapid growth in stimulated cells (Sukhatme, 1990), but has since been shown to play myriad roles in cell culture and in organismal homeostasis, notably in the regulation of hematopoietic stem cell maintenance and migration (Min et al., 2008), macrophage lineage specification and differentiation (Nguyen et al., 1993), learning and memory (Dragunow, 1996), expansion of neural stem and progenitor cells (Alagappan et al., 2013) and inhibiting cellular reprogramming to pluripotency (Worringer et al., 2014).

I have previously demonstrated by RNA-Seq that *EGR1* transcript is downregulated in hPSCs (data not shown) and hNSCs (**Figure 4-3A**) following Wnt3a treatment, suggesting potential regulation by the Wnt pathway. Such a link has not been demonstrated in the literature and represents an important advance in our knowledge of how this important transcription factor is regulated during early human developmental events. I sought to probe deeper into the role of the Wnt signaling pathway in the regulation of this important transcription factor in hPSCs.

3.3.2.1 EGR1 expression with increased hPSC density

Human embryonic stem cells cultured in defined feeder-free conditions do not normally express high levels of *EGR1* message or EGR1 protein. After observing inconsistent expression of the protein in protein lysates from multiple experiments, I

resolved to determine the exact repeatable conditions leading to the production of EGR1 protein. I found that critical variables across experiments included initial cell seeding density and harvest time. To determine whether cell density influences EGR1 protein levels, I seeded hESCs at various densities and harvested them 6 days post passage. Interestingly, I observed that EGR1 protein expression was high in lysates from densely plated cells and virtually undetectable in lysates from sparsely plated cells (**Figure 3-4A**). Cells cultured at low initial densities (10,000 - 20,000 cells per well) displayed normal morphology and formed healthy, compact colonies, while cells cultured at higher initial densities (40,000 - 60,000 cells per well) formed looser colonies whose cells failed to pack tightly in an equal amount of time (**Figure 3-4B**). Notably, disorganized cellular morphology was tightly correlated with EGR1 protein expression, suggesting that induction of this protein may be coupled to the earliest stages of cellular differentiation in hESCs. Because cell morphology and EGR1 expression could be explained by final cell density rather than initial plating density, I sought to determine whether late harvest of cells seeded at low density would result in EGR1 expression. I observed the expression of EGR1 in lysates from cells seeded at 20,000 cells per well when harvested one day later than usual (**Figure 3-4C**), suggesting that EGR1 protein expression relates to the final density of cells culture rather than the initial density.

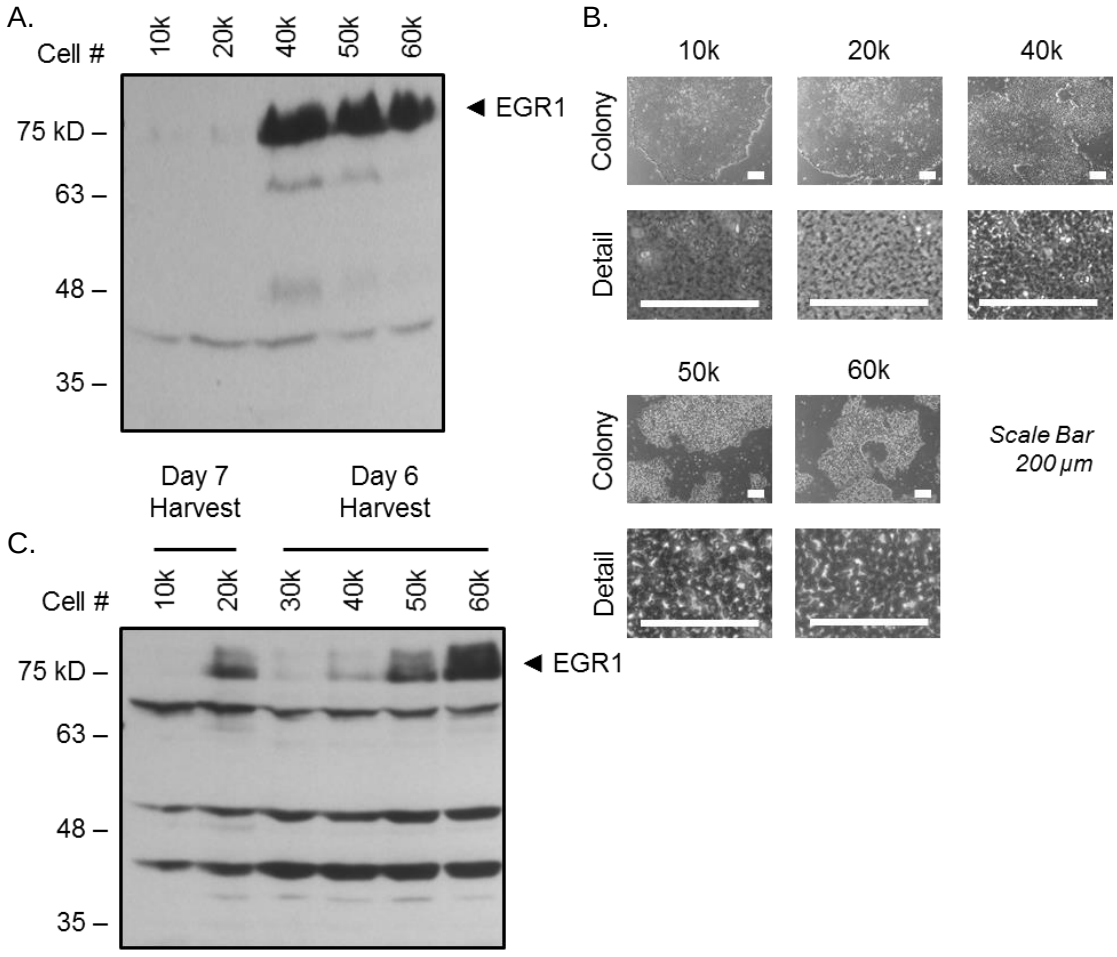

Figure 3-4: EGR1 expression increases with cell density

A. Western blot analysis of EGR1 protein levels in hESCs seeded at varying densities. **B.** Phase contrast microscopy of hESCs seeded at varying densities. Scale bar = 200 µm. **C.** Western blot analysis of EGR1 protein levels in hESCs seeded at varying densities and harvested on two different days.

3.3.2.2 CHIR treatment blocks EGR1 induction

My next goal was to determine whether activation of the Wnt pathway affects the level of EGR1 protein in hESCs. The cells were seeded densely to stimulate EGR1 protein production. The cells were then treated with various doses of CHIR98014 either chronically (6 days) or acutely (24 hours). I observed that EGR1 protein levels were diminished by CHIR98014 treatment in a dose-dependent fashion at both 6 day and 24 hour timepoints (**Figure 3-5A**). Furthermore, I observed that while colony morphology of low dose and untreated colonies was loose and disorganized, compact colony morphology was exceptionally well preserved following 24 hours of CHIR98014 treatment (**Figure 3-5B**), a result that suggests that Wnt pathway activation may delay differentiation for a short time.

3.3.2.3 EGR1 is induced by hESC conditioned medium

I next asked whether EGR1 protein expression is regulated by the state of the culture medium. I prepared a dense 10 cm plate of hESCs (230,000 cells - equivalent to 40,000 cells per well of a 6-well plate) and collected conditioned medium (hESC-CM) on day 6, when EGR1 protein is normally abundant. This medium was then sterile-filtered through a 0.45 μm filter to prevent carryover of cellular material and debris. Next, I prepared a 5 wells of 6-well plate with hESCs seeded at normal density (20,000 cells per well) and one well at high density (40,000 cells) as a control for EGR1 induction. On day 5, I treated the cells with hESC culture medium mixed with increasing fractions of hESC-CM and analyzed EGR1 expression by Western blot. EGR1 protein was induced in a dose-dependent manner by hESC-CM (**Figure 3-6A**), suggesting that the condition of the culture medium regulates its expression.

Furthermore, the morphology of hESC-CM-treated colonies was disorganized and loose, similar to that of cells cultured at high densities (**Figure 3-6B**).

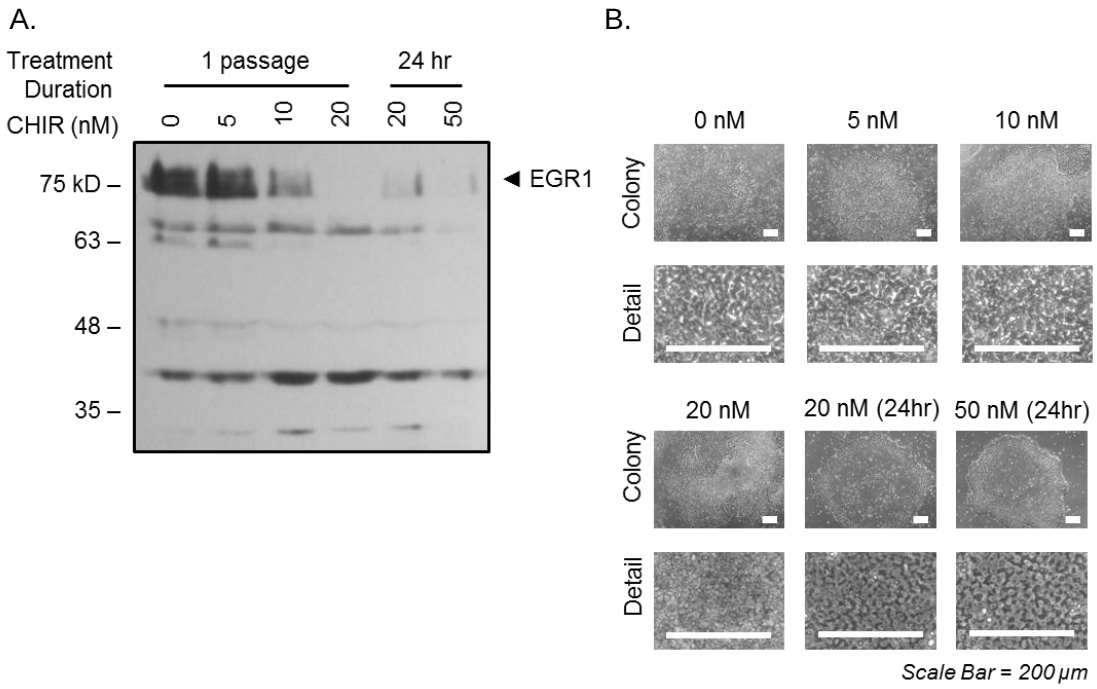

Figure 3-5: The effect of Wnt pathway activation on EGR1 protein expression

A. Western blot analysis of EGR1 protein levels in lysates from densely cultured hESCs treated with various doses of CHIR98014 for either the duration of the passage or for 24 hours only. **B.** Phase contrast microscopy of densely cultured hESCs treated with various doses of CHIR98014 for either the duration of the passage or for 24 hours only. Scale bar = 200 µm.

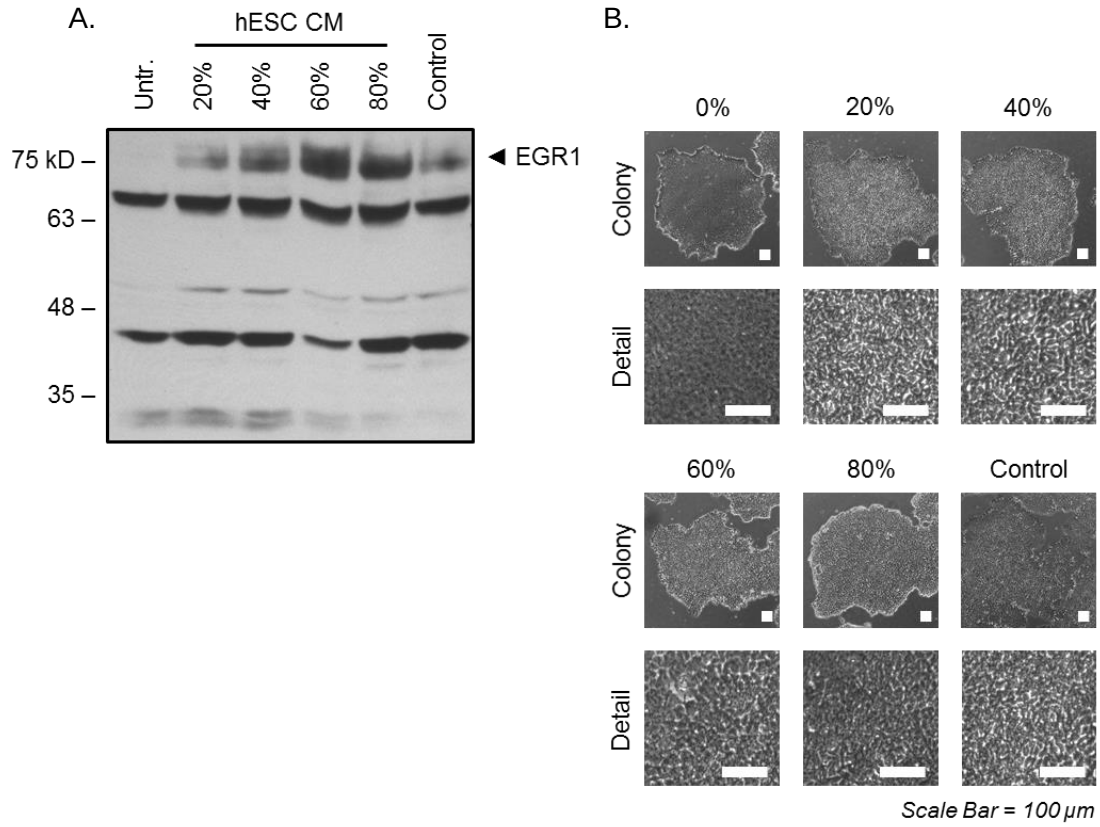

Figure 3-6: Induction of EGR1 protein in hESCs by hESC-conditioned medium

A. Western blot analysis of EGR1 protein in lysates from cells treated with various concentrations of hESC-CM. Control lysate from densely-seeded cells. **B.** Phase contrast microscopy images of colonies treated with various concentrations of hESC-CM. Scale bar = 100 µm.

3.3.2.4 Wnt signaling does not block EGR1 induction in MDA-MB231 cells

Finally, I sought to determine whether Wnt pathway activation drives diminished EGR1 protein in a different context. Classically, EGR1 is induced in cells that have been subject to serum starvation followed by serum stimulation. To determine whether Wnt signaling is able to regulate EGR1 expression in serum-stimulated cells, I utilized a breast cancer cell line, MDA-MB231, a triple-negative cancer line that expresses high levels of the Wnt receptor FZD7 and is Wnt-responsive. First, in order to determine the time of peak EGR1 protein expression, I subjected cells to serum starvation for 24 hours and then began a timecourse of serum stimulation with medium containing 10% fetal bovine serum. EGR1 protein appeared 60 minutes post stimulation and rapidly diminished (**Figure 3-7A**), indicating that between 1 to 2 hours post stimulation was the ideal timeframe for the assay. Next, to determine whether Wnt pathway activation is able to block the expression of EGR1 protein, I subjected cells to serum starvation for 24 hours and then stimulated for 0, 1, or 2 hours with 10% fetal bovine serum. Additionally, cells were either pre-treated with Wnt3a or buffer control for 24 hours (at the same time as starvation) or co-treated at the same time as serum stimulation. I interrogated EGR1 protein levels by Western blot and determined no change in lysates from cells either pre- or co-treated with Wnt3a protein (**Figure 3-7B**), indicating that Wnt signaling does not regulate EGR1 expression following serum stimulation of MDA-MB231 cells.

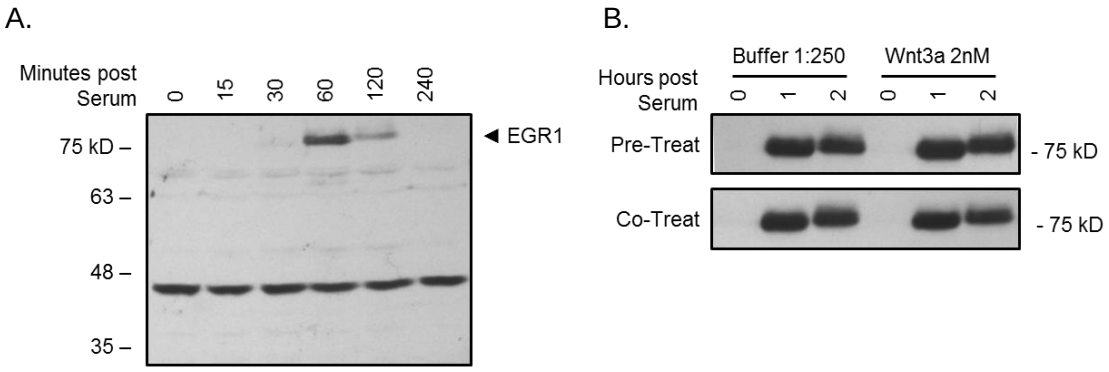

Figure 3-7: Effect of Wnt3a treatment on EGR1 induction in MDA-MB231 cells

A. Western blot analysis of EGR1 protein in lysates from serum-stimulated MDA-MB231 cells. **B.** Western blot analysis of EGR1 protein in lysates from serum-stimulated MDA-MB231 cells either pre-treated or co-treated with purified Wnt3a protein.

3.4 Discussion

In this chapter, I demonstrated previously unappreciated aspects of Wnt-mediated modulation of gene expression in hPSCs. My findings can be broken up into two major categories: First, I demonstrated that Wnt proteins act as morphogens and have dosage-specific effects the expression of markers of pluripotency and differentiation in these cells. Second, I demonstrated that EGR1 protein is expressed in densely-cultured hPSCs and that its expression is strongly attenuated by Wnt signaling.

Wnt proteins act as morphogens on hPSCs in culture. This is an important finding and helps to reconcile apparently disparate findings in the field. Work by Davidson et al. suggested that Wnt signaling promotes the differentiation of hPSCs (Davidson et al., 2012). In the study, the authors demonstrate by flow cytometry and qPCR that hPSCs gradually lose pluripotency after several passages when cultured in the presence of conditioned medium (CM) from Wnt3a-producing mouse L cells. This effect is rescued, but only partially, by treatment with the Tankyrase inhibitor XAV939, which enhances β-catenin degradation by stabilizing AXIN protein. They go on to show that treatment with Wnt3a CM drives cells towards mesodermal lineages, but they also claim that inhibiting Wnt signaling has no effect on self-renewal and pluripotency, a result that is strikingly at odds with my findings in this chapter, and the finding that the Wnt receptor FZD7 is required for the pluripotency of hPSCs (Fernandez et al., 2014). This discrepancy can possibly be explained through the use of poorly defined conditioned media not only for the induction of Wnt pathway activation but also for the maintenance of hPSCs in the pluripotent state. In my work, and in the work published by Fernandez et al., hPSCs were cultured in chemically

defined E8 medium (Chen et al., 2011). It may be that MEF CM or L cell CM contain modulators of pluripotency and differentiation that mask the effect of Wnt pathway inhibition following small molecule treatment. Work by Blauwkamp et al. went on to suggest that endogenous Wnt signaling is important for generating an equilibrium of cells that are predisposed to neural or mesendodermal lineages (Blauwkamp et al., 2012), but also stops short of demonstrating a requirement for Wnt signaling in the maintenance of the pluripotent state of hPSCs.

Here, I have shown that endogenous Wnt signaling at low levels modulates the expression of pluripotency markers in these cells. I further demonstrated that high levels of Wnt signaling drive a positive feedback loop that promotes cellular differentiation, which can be blocked by the addition of IWP2 to the culture medium. These findings relied on the use of purified recombinant Wnt3a protein, whose concentration in culture media may be tightly controlled. The ability to quantitatively dose Wnt3a allowed for the discrimination between effects of very large doses (differentiation) and small doses (self-renewal), which reconciles the observations of Davidson et al., Fernandez et al. and Blauwkamp et al. Thus, the Wnt signaling pathway is not dispensable for the maintenance of pluripotency; rather, a low level of pathway activation defines and preserves pluripotent cells, which are primed for differentiation and require only a small increase in pathway activation to activate a positive feedback loop that initiates processes reminiscent of gastrulation and the formation of the primitive streak.

I further demonstrated that long-term Wnt pathway activation by CHIR98014 results in dose-dependent differential expression of the germ layer marker genes *SOX17* (endoderm) and *T* (mesoderm). *SOX17* expression reaches its high point at

low doses while *T* expression is highest at high doses. Cellular morphology is also heavily influenced by the level of Wnt pathway activation and is highly variable in treated cells from one day to the next, indicative of the underlying cellular differentiation taking place in the culture. Nakanishi et al. demonstrated the induction of primitive streak like cells from both mESCs and hESCs (Nakanishi et al., 2009), and showed a similar dose-dependent induction of *T* following Wnt pathway activation. Their data also demonstrate higher expression of *Foxa2*, an endodermal marker, at midrange doses of Wnt3a, with a sharp decrease at the highest dose, indicating that excessive Wnt signaling inhibits the formation of endoderm from the primitive streak. These findings are in concordance with those of Naujok et al., which confirmed an inhibitory effect of Wnt pathway activation on definitive endoderm by increasing concentrations of CHIR99012, another small molecule agonist of Wnt signaling (Naujok et al., 2014). My work fills a gap between these previous findings by showing dose-dependent effects of CHIR98014 on the expression of markers of mesoderm and endoderm (**Figure 3-8A**).

Another key finding from my work was the regulation of EGR1 protein expression in hPSCs by Wnt signaling. *EGR1* was originally identified as a highly upregulated transcript in cells following growth factor stimulation or re-entry into the cell cycle (Chavrier et al., 1988; Christy et al., 1988; Sukhatme et al., 1987). That EGR1 was cloned and often studied in the context of cell growth established a line of thinking that influenced the study of this protein for many years (Sukhatme, 1990). In this study, I found that EGR1 protein is surprisingly absent or lowly expressed in protein lysates from hESCs under normal culture conditions which include a high concentration of Fibroblast Growth Factor in the culture medium. Instead, EGR1

becomes abundant only once the density of cells within the culture becomes very high. This finding is apparently at odds with the classical view of EGR1 as a driver of cellular proliferation akin to other immediate-early genes such as *c-fos* and *c-jun*. Importantly, other roles for EGR1 have emerged, especially as a factor driving differentiation of stem cells. Critically, it was found that Egr1 protein is rapidly induced in pluripotent mouse P19 embryonal carcinoma cells following retinoic acid mediated differentiation (Edwards et al., 1991), suggesting that EGR1 may be a critical regulator of this process. An analogous process may be taking place in hESCs as culture density increases and the growth factor milieu is fundamentally altered as some factors such as FGF2 degrade and other factors are secreted by the cells. Others have noted similar roles for EGR1 in differentiation and negative regulation of proliferation. Using mouse cell culture models of hematopoiesis, Nguyen et al. identified a requirement for Egr1 in the differentiation of macrophages and found that it restricts hematopoietic cells to this lineage (Nguyen et al., 1993). Furthermore, EGR1 has been shown to suppress oncogenic transformation of cell lines in culture and to negatively regulate their proliferation in a DNA binding dependent fashion (Huang et al., 1994, 1995). It was later determined that EGR1 drives the expression of TGF-β1, which suppresses the growth and transformation of some cell types (Liu et al., 1996, 1999a). In this context, my finding that Wnt signaling can negatively regulate EGR1 is unsurprising. Wnt signaling plays an important role in the initiation and maintenance of cancers in multiple tissue types and likely promotes the expansion of undifferentiated stem-like cells. Downregulation of a potential tumor suppressor like EGR1 would make aberrant Wnt pathway activation more dangerous. Along this line of reasoning, it stands that if EGR1 is upregulated in response to

differentiation cues in hESCs, activation of the Wnt pathway may acutely block, delay or alter this process (**Figure 3-8B**).

Finally, my finding that Wnt pathway activation fails to alter EGR1 protein induction in MDA-MB231 cells indicates that this function of the Wnt pathway is cell type specific. Interestingly, the *EGR1* promoter contains several binding sites for SP1 and EGR1 protein, suggesting the possibility of autoregulation but also introducing the intriguing possibility of regulation by SP5. As discussed in Chapter 2, SP5 is highly upregulated in hPSCs following Wnt pathway activation and is a known transcriptional repressor. Furthermore, its induction seems to be specific to Wnt responsive stem cells. Most cell lines surveyed demonstrated little to no induction of *SP5* message following treatment with Wnt3a. Strikingly, EGR1 is regulated by Wnt signaling in a cell type that induces SP5, but not in a cell type that fails to do so. Future studies may address whether the *EGR1* promoter is negatively regulated by SP5 following Wnt pathway activation in hPSCs.

My work raises the possibility that Wnt signaling regulates the transcription of genes by mechanisms other than TCF/LEF/β-catenin signaling. This implies that Wnt pathway activation entails a much more complex transcriptional response than is generally appreciated, because many genes may be both positively and negatively regulated depending on the factors binding their promoter regions (e.g. TCF/LEF and/or SP1/5.) Because EGR1 recognizes GC-rich motifs that often overlap with SP1 binding sites (Skerka et al., 1995), its suppression by Wnt signaling represents an additional avenue by which this pathway can modulate the transcription of SP1 target genes.

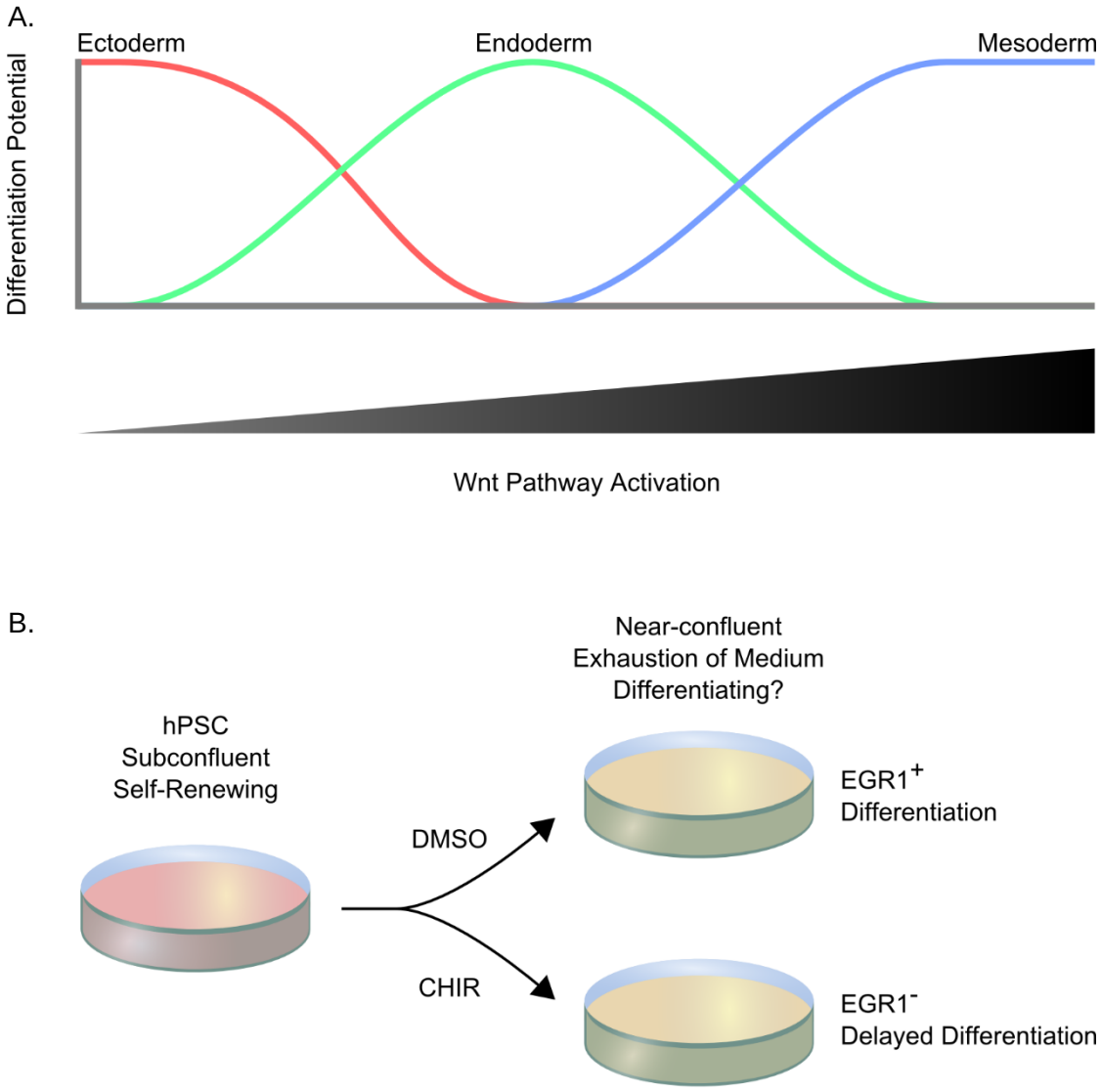

Figure 3-8: Model for roles of Wnt pathway activation in hPSCs

A. Effects of increasing Wnt pathway activation on differentiation potential of Ectoderm (red), Endoderm (green), and Mesoderm (blue). B. Effect of Wnt pathway activation on EGR1 expression in hPSCs.

In Conclusion, work in this chapter demonstrates that the effects of Wnt signaling on the transcriptome are far reaching and highly dose dependent. Future studies are needed to address the specific mechanisms behind the dose-dependent effects of Wnt signaling in hPSCs and their derivatives, especially in the formation of mesoderm and endoderm. Wnt signaling plays critical roles that span developmental timelines, tissues, and organs and must be precisely controlled in order to generate specific cell types with high specificity. Future cell therapies will rely on a detailed understanding of the molecular events required to reproducibly generate cell types that will be necessary to treat conditions including diabetes, liver failure and heart disease. Furthermore, this work has demonstrated a link between Wnt signaling and EGR1. The mechanism by which EGR1 is regulated is at this point completely unknown and may have nothing to do with SP5 protein. Future work will address whether Wnt signaling directly represses EGR1 expression, or whether EGR1 expression is a downstream consequence of another event such as a cell fate decision that can be switched by Wnt pathway activation. These studies will give us a more complete view of the events downstream of Wnt signaling in hPSCs, human development, and disease.

Acknowledgments

I would like to thank the following individuals for assistance that was instrumental to this work: Karl Willert, for help troubleshooting EGR1 immunoblots; Steven Head and his staff at the Scripps Research Institute Next Generation Sequencing Core Facility, for library preparation and sequencing of samples for RNA-

Seq; Terry Gaasterland, for her help with RNA-Seq analysis that led to our identification of EGR1 as a Wnt3a-downregulated mRNA in hPSCs. My work on Chapter 3 was supported by a training grant from the California Institute of Regenerative Medicine (TG2-01154).

Chapter 4 - Effects of Wnt signaling on gene expression in human neural stem cells

4.1 Abstract

Human neural stem cells (hNSCs) represent a powerful tool for modeling many aspects of the central nervous system, ranging from development to disorder and disease. The Wnt signaling pathway is a critical regulator of neurogenesis at several key junctures including the patterning of the neural tube and early brain regions, the expansion of cortical progenitor cells, and the terminal differentiation of cortical neurons. Here, I present studies undertaken to dissect the earliest roles of Wnt signaling in hNSCs through a combination of genetic, genomic, and biochemical studies. I demonstrate that Wnt3a supplementation can rescue diminished proliferation and reduced expression of multipotency markers in the absence of FGF2. I identify several differentially regulated genes following Wnt3a treatment using RNA-Seq and compare their expression to that in the developing mouse brain. Finally, I identify the transcription factor SP5 as a key mediator of Wnt signaling in hNSCs that delays their differentiation to neurons in tissue culture, as well as in mouse hippocampal subgranular zone stem cells *in vivo*.

4.2 Introduction

Vertebrate neural development is a complex process that starts with a sheet of cells, the neural plate ectoderm, which forms a simple tube which then goes on to form all of the complex cellular networks and architecture of the central nervous system. Much has been learned about this process from the study of lower vertebrates, especially chickens and mice, but these systems inherently lack primate- and human-specific programs and processes that ultimately give rise to the human brain. In recent years, human neural stem cells (hNSCs) (**Figure 4-1**) have arisen as

a model to study human-specific facets of neural development, homeostasis, and even disease (Jakel et al., 2004).

Human neural stem cells reside in the hippocampus and olfactory bulb of the adult brain and can be cultured from brain tissue biopsies (Pagano et al., 2000). Aborted fetal neural tissue is also a source of neural stem and progenitor cells that represent the wide array of cell types that arise during development and eventually form the adult central nervous system (CNS) (Liu et al., 2006; Vescovi et al., 1999). The advent of hESC technology has sparked interest in the generation of hNSCs in a dish (Chambers et al., 2009; Zhang et al., 2001), with the goal of studying the complexities that underlie human neurogenesis. Finally, hiPSC-derived hNSCs can be generated from individuals who suffer from neural deficits, from developmental neurological disorders such as autism (Chailangkarn et al., 2012) to neurodegenerative diseases like Alzheimer's Disease (Young and Goldstein, 2012) and Parkinson's Disease (Pu et al., 2012).

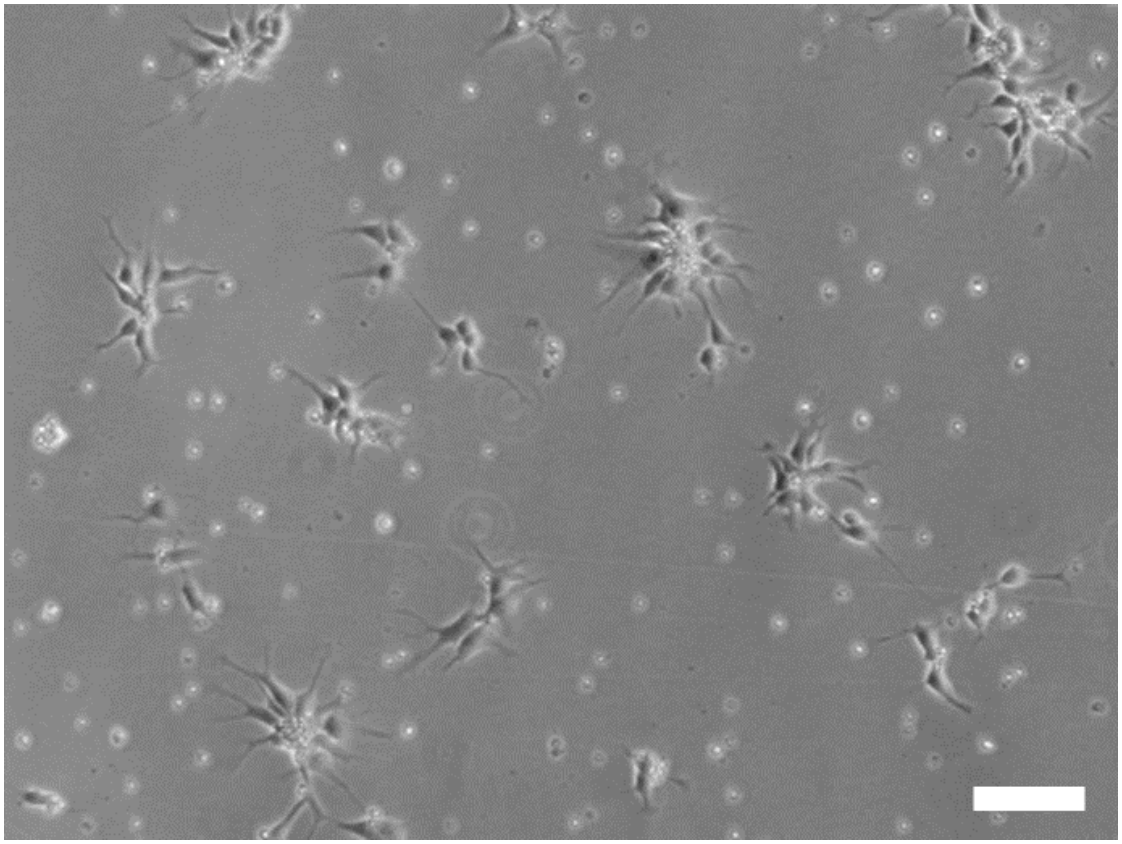

Figure 4-1: HUES7-derived hNSCs in culture

Our understanding of human neural development is still in its infancy and lacks important details, especially the roles of various growth factors and signaling pathways on stem cell decisions regarding self-renewal and differentiation. The Wnt pathway is critical for proper neural development and impacts diverse aspects of this process including patterning, proliferation, differentiation, and self-renewal of stem cells, and has been studied extensively in vertebrate models. One of the first genes knocked out in the mouse, *Wnt1*, was demonstrated to play a critical role in the generation of the cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), and *Wnt3a* for the development of the hippocampus (Lee et al., 2000). Roles for Wnt signaling throughout neurogenesis have been described at an astonishing pace in recent years. A critical early role for the Wnt signaling pathway in early development is the patterning of the neural tube, where Wnt pathway activation both posteriorizes and dorsalizes cells, antagonizing the effects of the Hedgehog pathway (Reviewed by Ulloa and Martí, 2010). Later, Wnt signaling drives alternating waves of cortical neural progenitor expansion and differentiation, and improper overexpression of Wnt pathway components can drive cortical malformations (Munji et al., 2011; Mutch et al., 2010). These discoveries made in the mouse may shed some light on the development of the human CNS, but ultimately our understanding of human neural development and the role of the Wnt pathway in that process must come from a human model system.

To shed light on the function of Wnt signaling in hNSCs, I observed the effect of treatment with recombinant purified Wnt3a protein on these cells with regard to proliferation and gene expression. To further probe the effects of Wnt treatment, I performed RNA-Seq on hNSCs treated with a timecourse of Wnt3a protein. This

analysis revealed a dynamic transcriptional response to Wnt pathway activation that included upregulation of *SP5*, several FGF family members, and the downregulation of *EGR1* (**Figure 4-3A**).

4.3 Results

4.3.1 Upregulation of PAX3 and downregulation of PAX6 following Wnt3a Treatment

Because neural stem cell identity is governed by the expression of the multipotency genes *PAX6* and *SOX2*, I sought to determine whether activation of the Wnt signaling pathway in hNSCs affects the expression of multipotency genes. Cells were seeded in hNSC medium with Wnt buffer or Wnt3a protein. Cell pellets were collected daily and stored at -80°C. Once all timepoints were harvested, RNA was extracted, reverse transcribed, and subject to qPCR analysis of gene expression. Because Wnt signaling has been shown to maintain stem cell identity in other tissues, I first sought to determine whether Wnt3a treatment of hNSCs leads to upregulation of *SOX2*, a master transcription factor regulator of the neural lineage and marker of neural stem cells (Reviewed in Ellis et al., 2004; Pevny and Nicolis, 2010). Interestingly, Wnt3a treatment had no effect on the expression of *SOX2* (**Figure 4-2A**). Because PAX6 expression is a marker of developmentally early neural stem cells and hNSCs in culture express PAX6 (Chambers et al., 2009), I next sought to determine whether Wnt3a treatment alters the expression of this marker. Surprisingly, Wnt-treated hNSCs demonstrated decreased *PAX6* expression (**Figure 4-2B**). PAX3 is also involved in patterning of the neural tube (Buxton et al., 1997). I therefore reasoned that perhaps Wnt pathway activation promotes patterning changes marked by increased *PAX3* expression. Indeed, *PAX3* was upregulated in hNSCs following Wnt3a treatment. (**Figure 4-2C**).

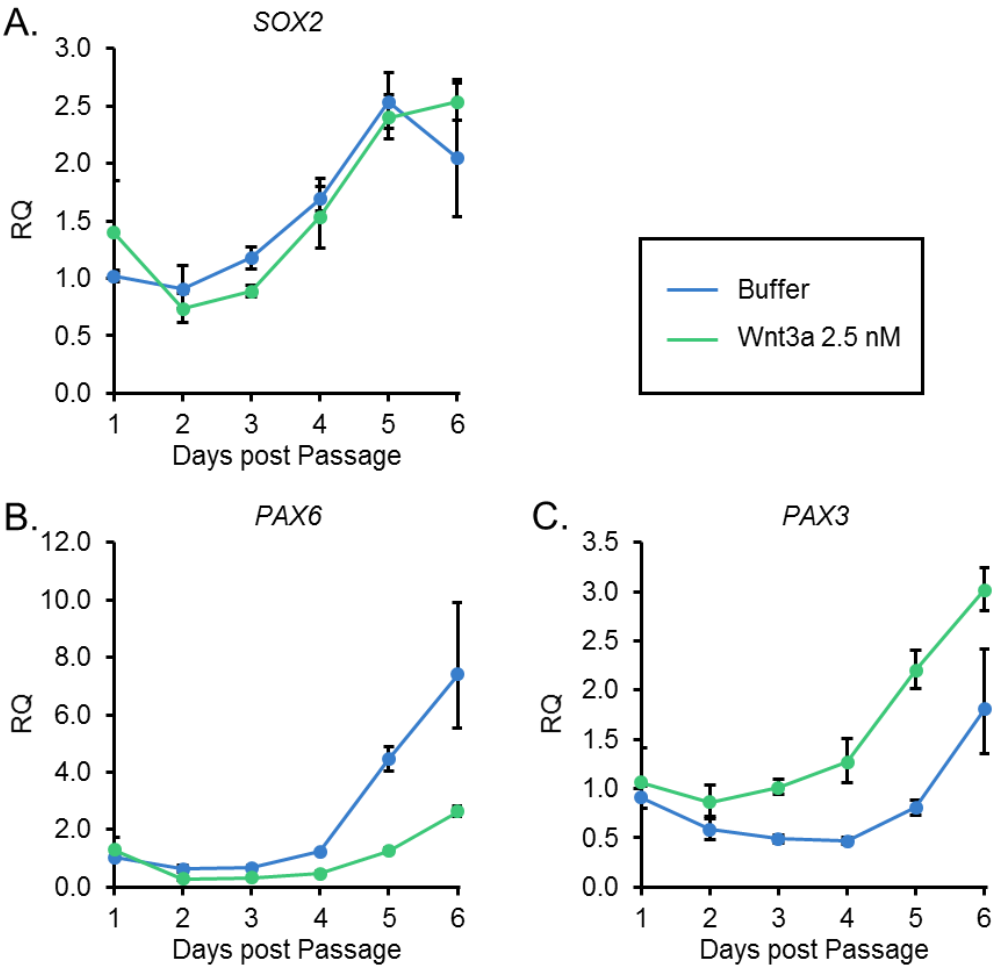

Figure 4-2: The effect of Wnt3a treatment on hNSC multipotency

A. Timecourse of *SOX2* expression in cells treated with Wnt buffer (blue) or Wnt3a 2.5 nM (green). **B.** Timecourse of *PAX6* expression. **C.** Timecourse of *PAX3* expression.

4.3.2 RNA-Seq identifies differentially expressed genes following Wnt3a treatment

The limitations of low-throughput candidate gene screening prevent the characterization of large-scale effects of Wnt pathway activation. In order to identify transcriptome-wide changes in hNSCs in response to Wnt signaling, I next performed RNA-Seq on samples prepared from hNSCs treated with a timecourse of Wnt3a (**Figure 4-3A**). Interestingly, as in hPSCs, I identified *SP5* as a potent transcriptional target of Wnt signaling in hNSCs. Other notable targets included several FGF family members (*FGF17*, *FGF18* and *FGF19*), *FZD10*, and several Wnt pathway components, especially negative feedback regulators (*AXIN2*, *DKK1*, *FRZB*, *GSK3B*, *NKD1*, *TLE3*, *WIF1* and *ZNRF3*). I next validated *SP5* and *AXIN2* as targets of Wnt signaling in hNSCs by performing a Wnt3a treatment timecourse followed by RNA isolation, reverse transcription, and qPCR (**Figure 4-3B**). Strikingly, both genes are most highly upregulated within the first few hours of treatment and then display a sharp decline. *SP5* transcript levels peak at a slightly later timepoint than those of *AXIN2.* This result contrasts with *SP5* expression kinetics in hPSCs, where levels tend to increase steadily through 48 hours of Wnt3a treatment. It is likely that recombinant Wnt protein degrades in hNSC culture and the lack of an endogenous positive feedback loop as that observed in hPSCs means that its expression is not preserved following initial stimulation.

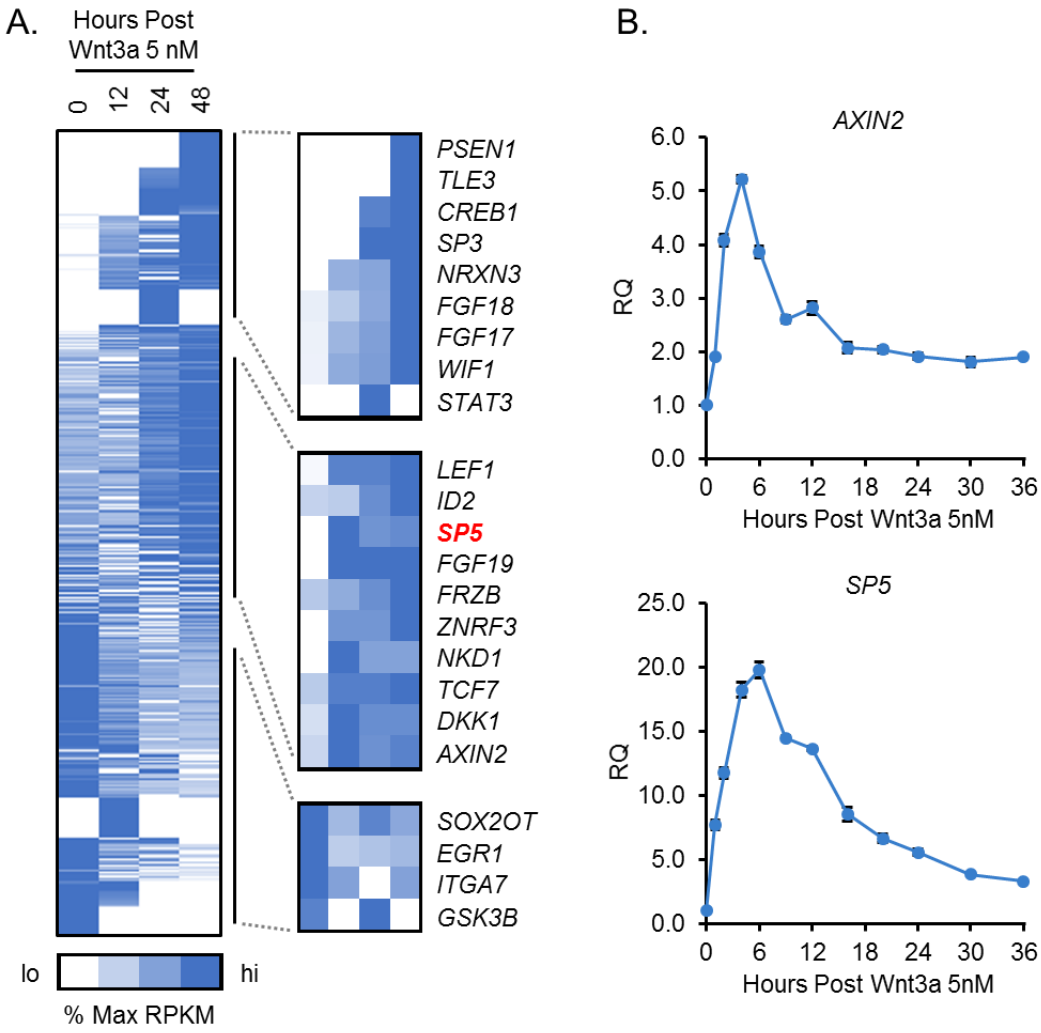

Figure 4-3: Wnt3a-driven transcriptomic changes in hNSCs

A. Heatmap of gene expression of the top 500 differentially regulated genes in hNSCs following Wnt3a (5 nM) treatment for 0, 12, 24 and 48 hours. Expression is given as percent of maximum RPKM for a given gene across treatment conditions; 0%, white; 100%, blue. Breakout boxes highlight genes of interest in three distinct clusters: Genes up late; genes up early; genes down following Wnt3a treatment. **B.** qPCR gene expression analysis of *AXIN2* and *SP5* throughout a 36 hour Wnt3a (5 nM) treatment timecourse.

4.3.3 Effect of Wnt3a treatment on proliferation and multipotency gene expression following FGF withdrawal

FGF ligands are required for the proliferation and long term maintenance of multipotent hNSC cultures (Vescovi et al., 1999). Because three FGF genes were among the most highly upregulated target genes, and FGF2 supplementation is required to maintain hNSC proliferation and multipotency, I next tested whether Wnt3a treatment could enhance proliferation. Cells were grown either in the presence of Wnt buffer or Wnt3a 6 wells of a 6-well plate per condition. Daily, one well of cells was dissociated and cell suspensions were counted to determine the impact of Wnt3a treatment on cell number until 6 timepoints had been collected. Surprisingly, Wnt3a treatment had no effect on hNSC proliferation in normal growth medium (**Figure 4-4A**). I hypothesized that the high level of FGF2 inherent in standard hNSC culture conditions precluded enhanced proliferation by the expression of additional FGF ligands. I therefore asked whether Wnt3a supplementation is sufficient to compensate for FGF2 withdrawal, treatment with a FGF receptor inhibitor PD166866, or the combination of the two. Briefly, cells were seeded in 3 wells of a 6-well plate per condition. The cells were then grown for 7 days with FGF2, without FGF, with FGF2 and PD166866, and without FGF2 but with PD166866. These four conditions were then further supplemented with either Wnt buffer, Wnt3a, or Wnt5a, a non-canonical Wnt. The cells were fed every other day. On the final day, the cells were dissociated and counted (**Figure 4-4B**). As expected, neither Wnt3a nor Wnt5a treatment increased proliferation of hNSCs in standard culture medium. Cells treated with PD166866 in the presence of Wnt buffer or Wnt5a exhibited a large decrease in cell number, but Wnt3a treatment was able to rescue this effect. Similarly, cells cultured in

the absence of FGF displayed greatly reduced cell numbers when cultured with Wnt buffer or Wnt5a, but Wnt3a provided a statistically significant boost to cell number. Finally, cells cultured without FGF and in the presence of PD166866 suffered a dramatic loss of proliferative capacity that could not be rescued by Wnt3a treatment, indicating that upregulation of FGFs likely plays a role in the proliferative effect of Wnt pathway activation on hNSCs. Wnt5a treatment never resulted in a statistically significant difference in cell count from Wnt buffer treatment only.

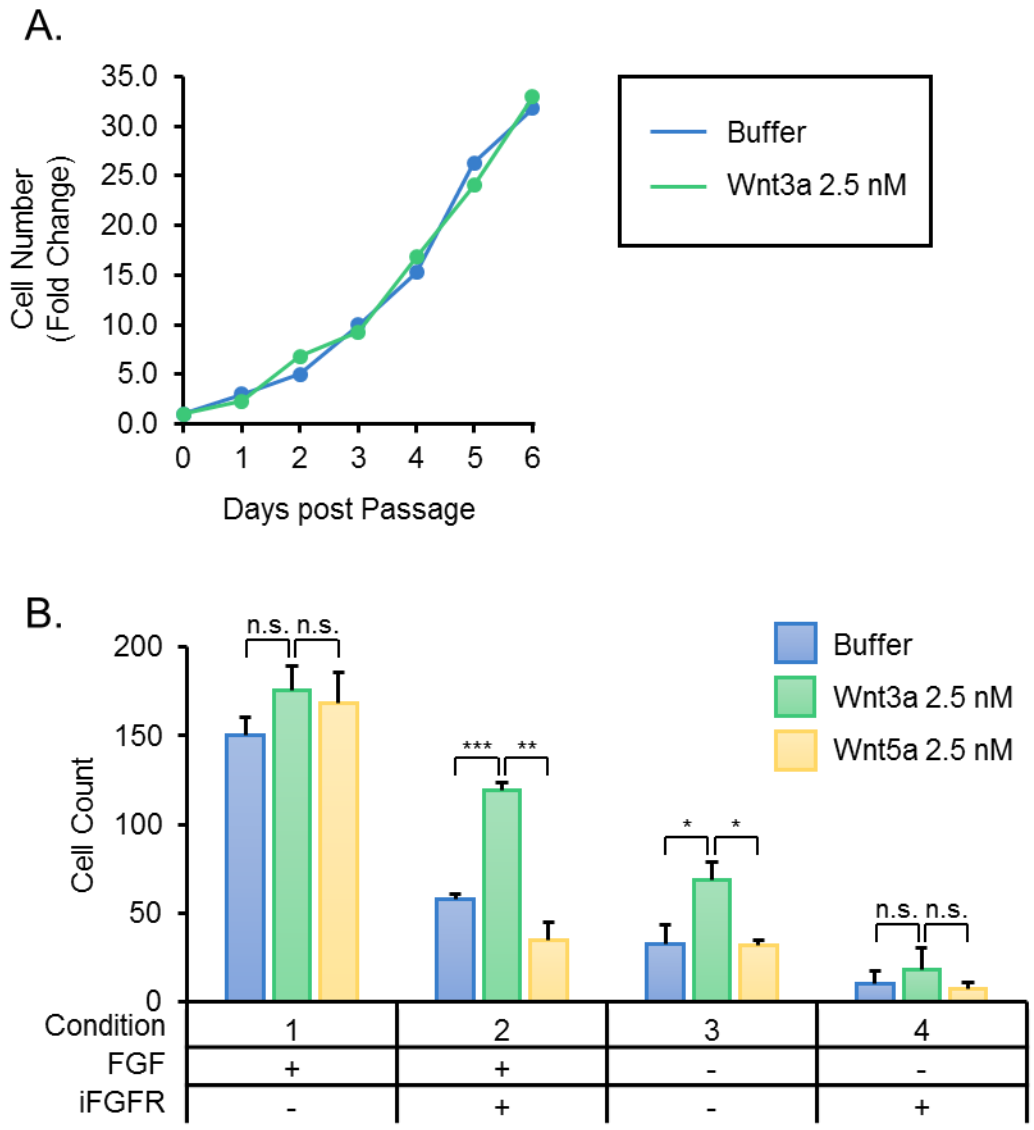

Figure 4-4: The effect of Wnt3a on hNSC proliferation

A. Growth curves of HUES7 hNSCs grown in hNSC medium in the presence of Wnt buffer (blue) or Wnt3a (2.5 nM) (green). **B.** Day 7 cell counts of HUES7 hNSCs grown in the presence of Wnt buffer (blue), Wnt3a 2.5 nM (green) and Wnt5a (2.5 nM) (yellow) under 4 conditions: 1, FGF2 20 ng/mL; 2, FGF 20 ng/mL and PD166866 1 µM; 3, FGF2 0 ng/mL; 4, FGF2 0 ng/mL and PD166866 1 µM. n.s. = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

I next sought to determine whether Wnt3a supplementation could rescue multipotency gene expression following FGF2 withdrawal. In the absence of FGF2, hNSCs not only stop proliferating, but differentiate into neurons, glia, and oligodendrocytes as well (Zhang et al., 2001). To determine whether Wnt3a treatment can preserve the expression of multipotency genes *PAX6*, *SOX1* and *SOX2*, I seeded hNSCs and removed FGF2 from the medium. The culture medium was supplemented with either Wnt buffer or Wnt3a. Every day for 6 days, one well of a 6-well plate was harvested and frozen as a pellet. After the harvest of all wells, I isolated RNA, synthesized cDNA, and performed qPCR for *PAX6*, *SOX1* and *SOX2*. I observed that in the absence of FGF2, Wnt buffer treated cells exhibited a gradual loss of multipotency gene expression (**Figure 4-5A-C**). Conversely, Wnt3a treatment was able to rescue multipotency gene expression to levels comparable to those exhibited by Day 0 cell pellets. Interestingly, levels of *AXIN2* and *SP5* rose consistently day over day in Wnt3a-treated cells, demonstrating that increased levels of Wnt signaling accompany increased expression of multipotency markers (**Figure 4-5D,E**) and suggesting that *SP5* may mediate this effect and block differentiation of hNSCs following FGF2 withdrawal.

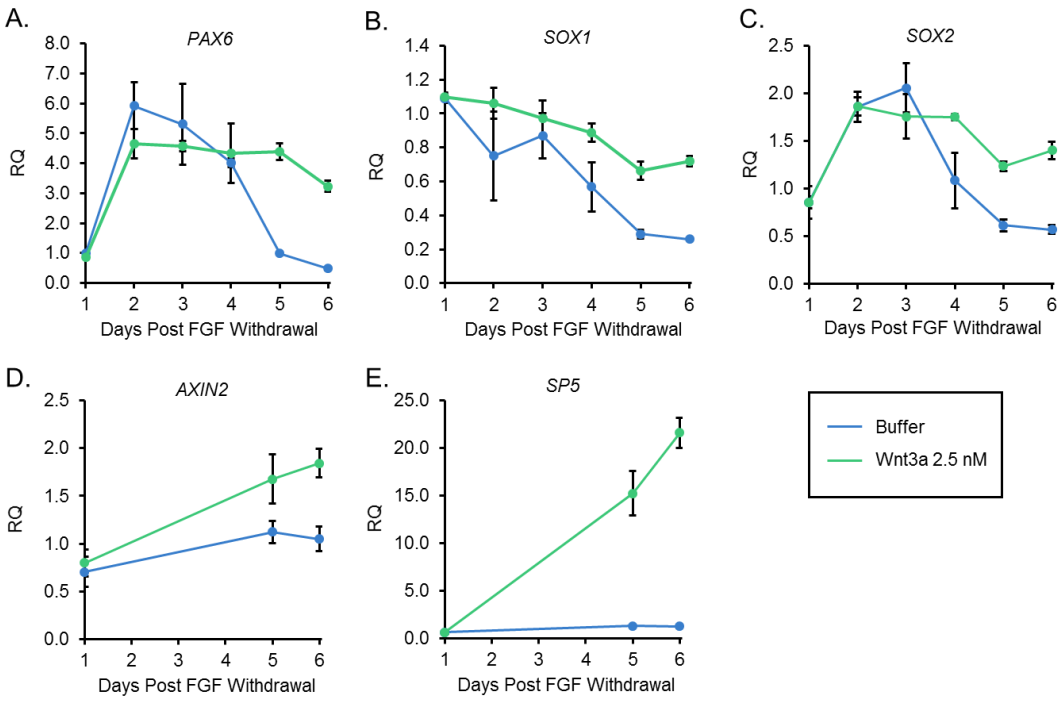

Figure 4-5: The effect of Wnt signaling on hNSC multipotency

A. *PAX6* gene expression timecourse in hNSCs following FGF2 withdrawal in the presence of Wnt buffer (blue) or Wnt3a (green). **B.** *SOX1* expression. **C.** *SOX2* expression. **D.** *AXIN2* expression. **E.** *SP5* expression. All values are given as relative quantity versus Day 0 cell pellets.

4.3.4 The role of SP5 in neural differentiation

During mouse neurogenesis, neural stem and progenitor cells must undergo a shift from proliferation to differentiation. This shift occurs over the course of embryonic days 9.5-13.5 (Hartl et al., 2008). Mining a publicly available microarray dataset containing mouse brain expression data from E9.5, E11.5 and E13.5 mouse brains, I was able to demonstrate high levels of *Wnt3a*, *Fgf15* (the homolog of human *FGF19*), and *Sp5* in E9.5 mouse brains that sharply decline over the course of development (**Figure 4-6**), indicating that these genes may play a role in the maintenance and expansion of early neural progenitor cells. These results in mouse are in agreement with the results I have generated in hNSCs and suggest that the role of Wnt signaling in the maintenance and expansion of early neural stem cells is conserved between mouse and man.

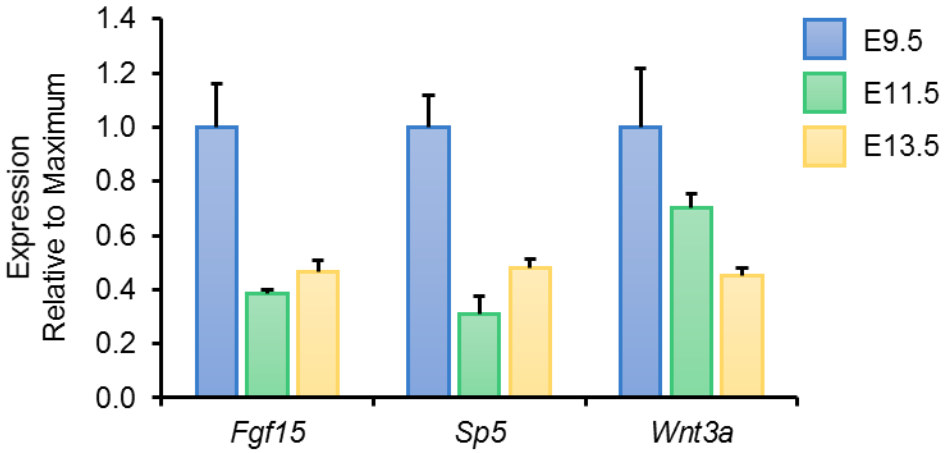

Figure 4-6: Gene expression in mouse brain development

Microarray expression analysis of *Fgf15*, *Sp5* and *Wnt3a* on embryonic days 9.5 (blue), 11.5 (green), and 13.5 (yellow). All values are given as fraction the maximum within that gene. Mined from GEO dataset GDS3442 (Hartl et al., 2008).

Little is known of *SP5* in neural development, and it has never been studied in a human system. I therefore focused my efforts on understanding the role of this gene during neural differentiation of hPSCs. To determine the long-term effects of *SP5* expression throughout neural differentiation, I designed a lentivirus bearing an inducible *SP5* cassette under the control of doxycycline (iSP5). Wild type (WT) and iSP5 hNSCs were cultured in the absence of FGF2 for 5 weeks. Each week, a well was harvested and frozen. At the conclusion of the experiment, RNA was extracted from the frozen pellets, cDNA was synthesized, and qPCR was performed for markers of neurogenesis (*DCX*, *GFAP*, *MAP2*, *and TUBB3*) and *SP5*. Interestingly, expression of differentiation genes was greatly reduced by *SP5* overexpression (**Figure 4-7A**), suggesting that this gene may function in part to delay or block differentiation. Additionally, iSP5 cells at week 5 retained a cellular morphology more reminiscent of week 1 WT cells than of week 5 (**Figure 4-7B**), strengthening the notion that *SP5* expression may retard neural differentiation or promote stem cell expansion. I next sought to test whether SP5 can block neural differentiation *in vivo* in the stem cells of the mouse hippocampus. I prepared retroviruses (MuLV) bearing *IRES-GFP* and *SP5-IRES-GFP* cassettes, which were injected in the brains of newborn mice. This class of virus is only able to transduce a dividing cell, and thus labels the progeny of cells that were dividing at the time of injection. After 6 weeks, mice were perfused, and brains were harvested, sectioned, and stained. I quantified the number of GFP+ cells in 5 regions of the hippocampus using confocal microscopy. Interestingly, *SP5* overexpression led to an increased number of GFP+ cells in both the subgranular and granular zones (**Figure 4-7C, D**), suggesting that *SP5* may promote stem cell expansion *in vivo*.

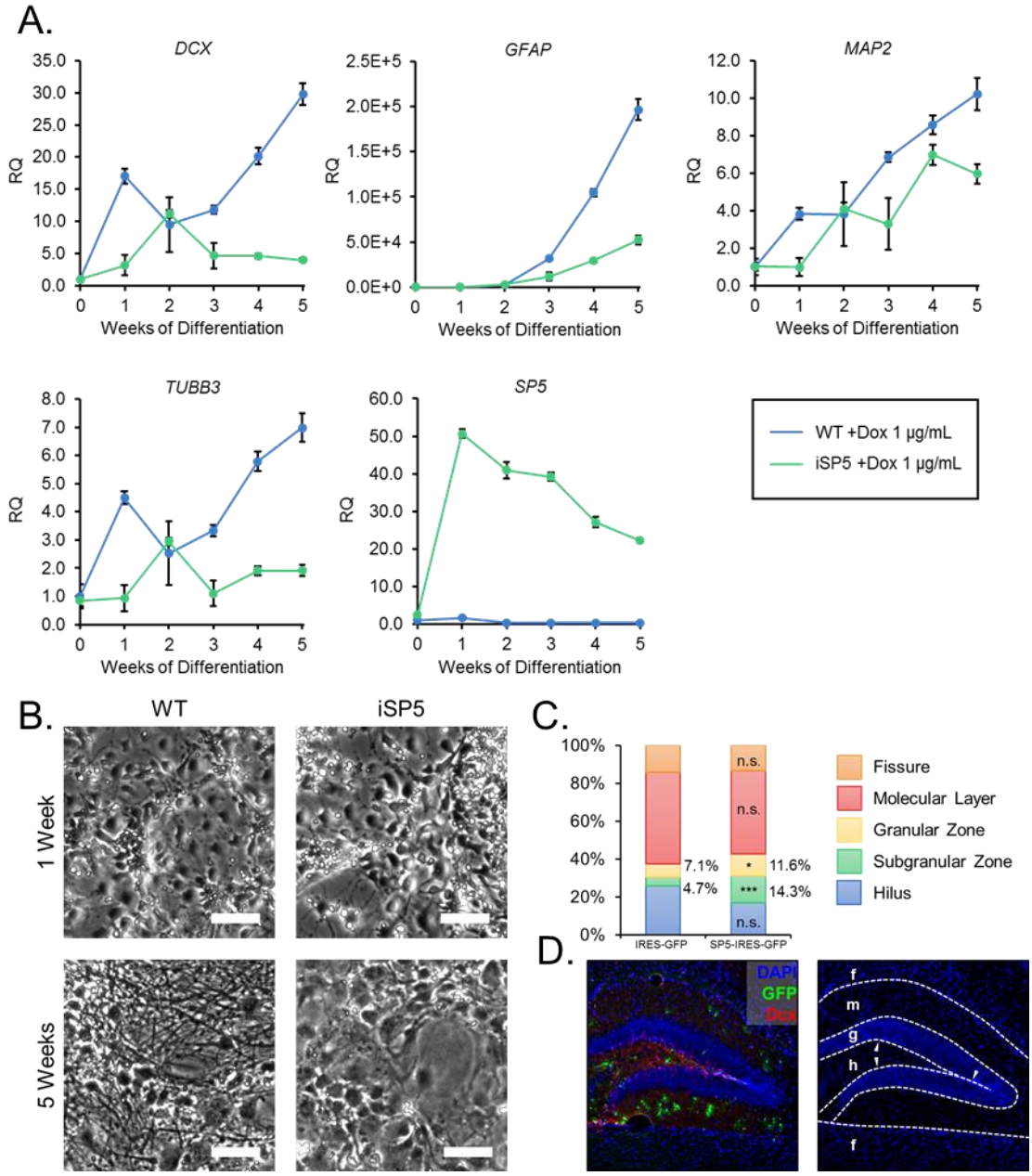

Figure 4-7: Effect of *SP5* overexpression on neural differentiation

A. Differentiation timecourse qPCR of differentiation genes and *SP5* in WT (blue) and iSP5 (green) HUES7 hNSCs in the presence of doxycycline 1 µg/mL. **B.** Phase contrast microscopy images of WT and iSP5 HUES7 hNSCs differentiated for 1 week and 5 weeks. Scale bar 50 µm. **C.** Quantification of GFP+ cell localization in hippocampi of mice injected with *IRES-GFP* or *SP5-IRES-GFP* bearing retrovirus. * = $p < 0.05$; *** = $p < 0.001$. **D.** Representative image of a stained hippocampal slice with labeled regions: f, Fissure; m, Molecular Layer; g, Granular Zone; arrowheads, Subgranular Zone; h, Hilus.

Because SP5 is known to repress SP1 target gene expression (Fujimura et al., 2007), I examined the RNA-Seq dataset for SP1 target genes involved in neurogenesis that are downregulated upon Wnt stimulation. Interestingly, several genes downregulated by Wnt3a treatment are involved in neurogenesis, and several of them have SP1 binding sites in their promoters (**Table 4-1**). Wnt treatment of hNSCs also downregulates *EGR1*, possibly through SP5-mediated repression. EGR1 has been shown to regulate neurogenesis and to synergistically bind with SP1 to coordinate gene expression (Lin and Leonard, 1997; Skerka et al., 1995), so I also sought genes with EGR1 binding sites in their promoters (**Table 4-1**). In all I was able to identify dozens of neurogenesis genes that were downregulated by Wnt3a treatment in hNSCs, several of which may be subject to direct regulation by SP1/5, or secondarily through diminished expression of *EGR1*.

The WNT, FZD, and SP/KLF gene families exhibit a wide range of expression in hNSCs (**Figure 4-8A-C**). Because *SP5* is by far the most dynamically expressed SP/KLF family member in Wnt3a-treated hNSCs **(Figure 4-8C)**, I hypothesize that *SP5* is most likely to be modulating the expression of these SP1/EGR1 target genes in hNSCs following Wnt3a treatment. This finding is significant because it points to a new mechanism whereby Wnt signaling is able to delay or block differentiation in a Wnt-responsive stem cell population.

Table 4-1: Wnt3a-downregulated neurogenesis genes

Gene	Neurogenesis	Neurons	SP1	EGR1/2	Additional Information
ABAT		x	x	x	Brain - GABA synthesis
CAMK2N1		x	x	x	neuronal cell body; dendrite
CDC42EP3		?	x	x	Increased in schizophrenia
EGR1		x	x	x	Long-term regulation of synaptic plasticity
EGR2	x	?	x	x	Axon guidance, neural development, schwann cell differentiation
JUB	?	?	x	x	Negative regulator of retinoic acid signaling
NES		x	x	x	Some mature neurons express NES
NR2F2	x		x	x	Activation of neural genes during development; neuron migration
SEMA6A	x		x	x	Axon guidance, neural development
SLC3A2		x	x	x	Cell surface marker of mature brain cells
TACC1	x		x	x	Neurogenesis
TCF7L1	x		x	x	Neurogenesis
CYP26A1	?		x	-	retinoic acid metabolism
LRRN3		x	x	-	Neuronal component
PLK2		x	x	-	dendrite component?
PMAIP1		?	x	-	neuron apoptosis?
SDC2		x	x	-	part of the neuronal cell body
CTNND2		x	x		neuron cell-cell adhesion
SEMA3A	x	x	x	-	repels axons, attracts dendrites
CAMK2D		x	?	x	calcium modulation
SOX2OT	?	?	?	-	
FZD1	x		-	x	Neuron differentiation
LPAR1	x		-	x	Neurogenesis
MAP1B	x	x	-	x	Neuronal developent
SRGAP1		x	-	x	Neuron-associated (Slit, Robo)
TFPI2	?	?	-	x	Glioma
SOX21	x		-	x	antagonizes multipotency marker SOX2
ADM		x	-	-	Neural projection regeneration
EMP1		?	-	-	Epilepsy marker
FABP7		x	-	-	Brain-specific fatty acid binding protein
ID4	?		-	-	SOX2 agonist
MAPK10		x	-	-	neuronal-specific form of c-Jun N-terminal kinases (JNKs)
MPP5	x		-	-	polarization of differentiating cells; regulates schwann cells
PCDH18	x	x	-	-	Brain development
PCSK1N		?	-	-	Symptomatic of Alzheimer's
PMP22		x	-	-	Peripheral myelin protein - mature neurons
PTX3		?	-	-	Alzheimer's marker
SLC17A8		x	-	-	Vesicular presynaptic glutamate transporter
SLC6A15		x	-	-	neuronal transporter
SLIT2	x		-	-	axon guidance
TMEM47	?	?	-	-	Found in high levels in the brain - tight junctions?

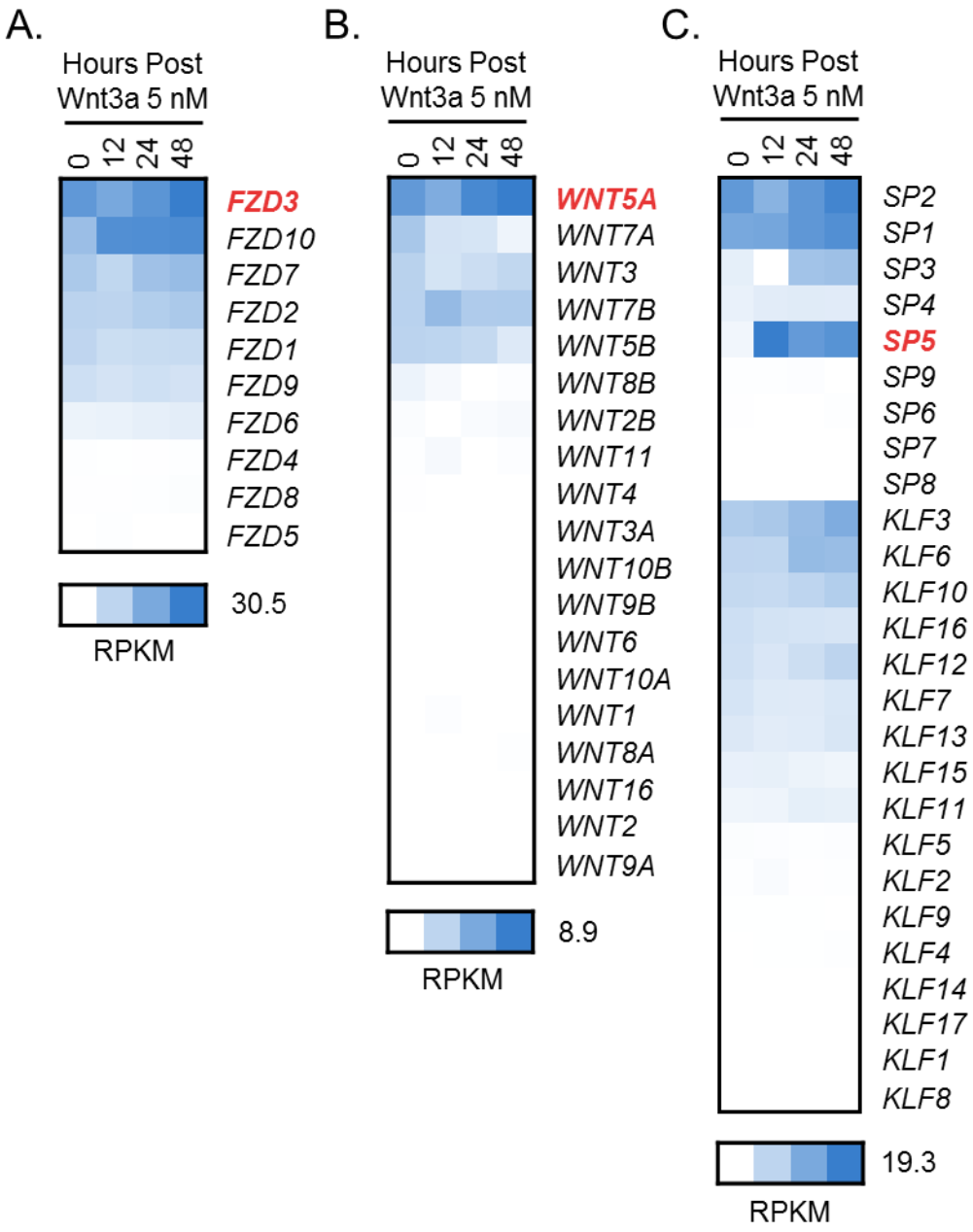

Figure 4-8: Expression of several gene families in HUES7 hNSCs

A. Expression of FZD family genes, sorted from highest to lowest RPKM in untreated hNSCs. White, RPKM = 0; blue, RPKM = 30.5. **B.** Expression of WNT family genes. **C.** Expression of SP/KLF Family genes.

4.4 Discussion

In this work, I set out to determine the effects of the Wnt signaling pathway on hNSC multipotency, proliferation, and differentiation. I demonstrated that Wnt pathway activation has no effect on the expression of *SOX2* mRNA, but that it drives simultaneous upregulation of *PAX6* and downregulation of *PAX3*. Using genome-wide transcriptome analysis, I was able to identify several genes upregulated in hNSCs in response to Wnt pathway activation, including several FGF family members and *SP5*. I further demonstrated that Wnt signaling has minimal impact on cell proliferation when hNSCs are cultured in self-renewing conditions, but that following FGF withdrawal or pharmacological FGFR inhibition, Wnt signaling is able to partially rescue the resulting decline in proliferation, likely due to upregulation of FGFs. I also demonstrated that following FGF withdrawal, Wnt pathway activation is able to rescue the expression of multipotency genes *PAX6*, *SOX1* and *SOX2*. Finally, I demonstrated that the transcription factor SP5 functions downstream of the Wnt pathway in hNSCs to delay differentiation in the absence of FGF2, and I further demonstrated its ability to expand the stem cell compartment of the subgranular zone of the hippocampus in mice. Together, these findings suggest that the Wnt signaling pathway is able to promote the multipotency and proliferation of hNSCs by modulating the expression of FGFs and the transcription factor SP5 (**Figure 4-9**).

The roles of Wnt signaling throughout neural development are wide ranging. Wnt signaling is known to play a role in the patterning of the neural tube and the specification of the neural crest, which involves upregulation of *PAX3* (Bang et al., 1999), suggesting that Wnt treatment of primitive hNSCs may be promoting the emergence of neural crest fates while suppressing neural tube fates. But this

explanation falls short of explaining the phenomena I have observed in Wnt3a-treated hNSCs. For example, Wnt3a-treated hNSCs do not adopt strikingly different morphologies or undergo epithelial to mesenchymal transition. *PAX3* is also a marker of the dorsal neural tube, along with *PAX7* (Pituello et al., 1995). Because dorsal neural tube patterning is regulated by Wnt signaling (Reviewed by Ulloa and Martí, 2010), it is more likely that Wnt treatment of hNSCs in culture is promoting dorsal neural tube fates. *PAX6* is among the earliest known markers of neural ectoderm, but is also involved in several downstream patterning events including dorsoventral patterning of the telencephalon (Stoykova et al., 2000), hindbrain (Kayam et al., 2013) and spinal cord (Augustine et al., 1995). It has also long been known as a master transcriptional regulator of eye formation (Jordan et al., 1992; Li et al., 1994). Because *PAX6* assumes multiple developmental roles, its expression alone is not a good marker of neural stem cell multipotency. *SOX1* and *SOX2* have been suggested as better markers, although their expression is also retained in the radial glial-like stem cells of the adult hippocampus (Hodge and Hevner, 2011).

These concerns highlight a major drawback to working with hNSCs derived from hPSC lines in culture, that is, the lack of defined anatomical landmarks by which to determine the positional identity of stem cells and their progeny. While it has been demonstrated that endogenous Wnt signals play a role in patterning hPSC-derived hNSCs (Moya et al., 2014), it is still very difficult to generate uniformly patterned hNSCs with high purity. Furthering the difficulty of deriving these cells is the variability imparted on each hPSC line by factors such as genetic background, derivation, and culture conditions. The challenges in deriving specific populations need to be

overcome if hPSC-derived hNSCs are ever to meet their potential as a source of cells for disease modeling, drug screening and eventually cell transplantation therapies.

I demonstrated through RNA-Seq that several well-known developmental signaling molecules are differentially regulated in hNSCs following Wnt treatment, including FGFs. I further demonstrated that the effects of FGF2 withdrawal or FGFR blockade could be partially overcome through increased Wnt signaling, and that Wnt3a supplementation rescued the effect of FGF2 withdrawal on the expression of multipotency genes. While FGF stimulation is simply required to maintain hNSC multipotency and proliferation in culture, the roles of FGF family members in patterning and other events are also likely at play downstream of Wnt signaling. Further experimentation will be necessary to identify the other roles of FGF signaling in hNSCs.

The upregulation of *SP5* in response to Wnt pathway activation was an interesting discovery of this work. It has been known for many years that *SP5* is a target of the Wnt pathway that is expressed in dynamic fashion throughout embryogenesis (Weidinger et al., 2005), and that it acts to repress SP1 target genes in neural stem cell cultures (Fujimura et al., 2007), but its role in promoting hNSC stem cell identity or blocking differentiation had not been examined. Here, I demonstrated that induced expression of *SP5* delays or prevents the onset of genes involved in neurogenesis, along with the accompanying morphological changes characteristic of spontaneously differentiated neural stem cells (e.g., axons and dendrites). I further demonstrated that *SP5* delivered to the mouse hippocampus promotes expansion of the stem cell compartment, possibly by promoting stem cell proliferation, but also likely by blocking differentiation. It has been suggested that *Sp5*

functions in the mouse in concert with *Sp8* to promote the self-renewal of a population of neuromesodermal stem cells that supply the growing tail with mesenchymal cells throughout development (Dunty et al., 2014). Perhaps *SP5* functions in many Wnt-responsive stem cell populations in just this same fashion, as a fine tuning mechanism that ensures the proper balance of stem cell maintenance and differentiation.

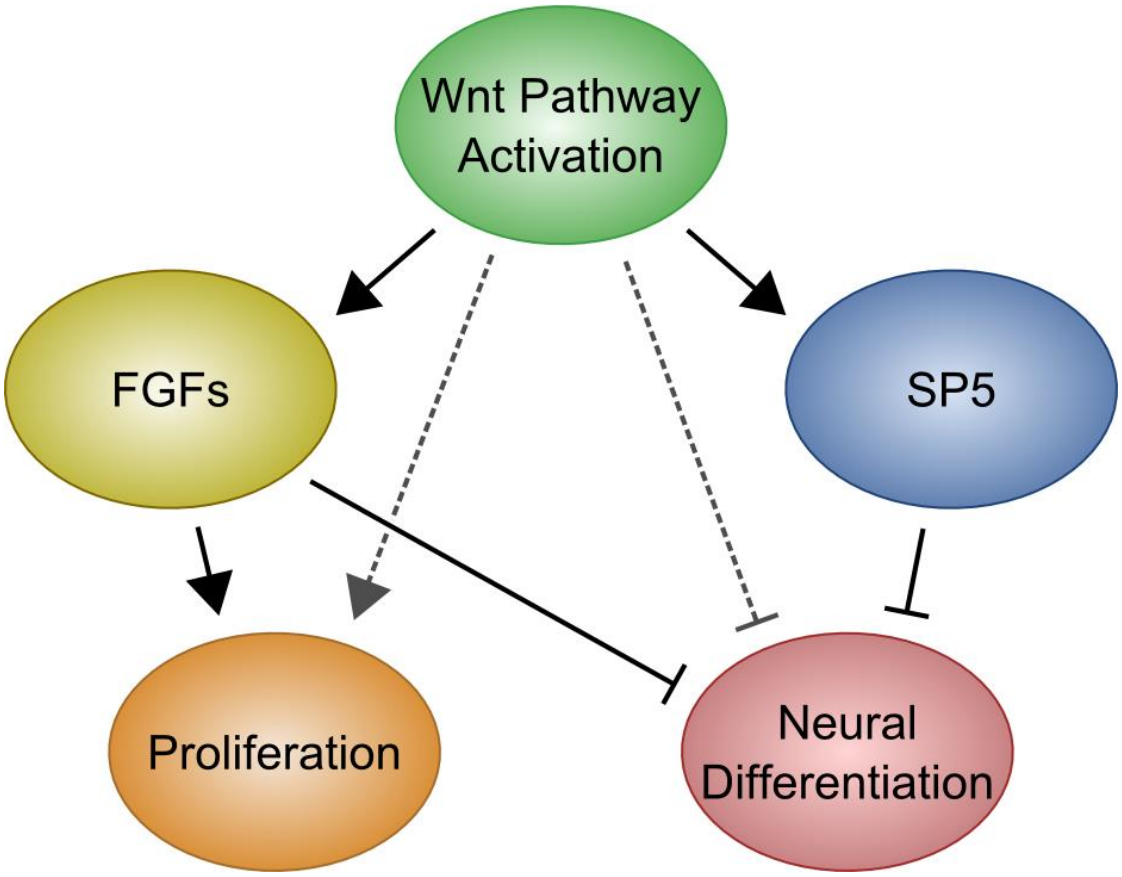

Figure 4-9: Model for the role of the Wnt signaling pathway in hNSCs.

Following Wnt pathway activation, hNSCs upregulate FGF family members *FGF17*, *FGF18* and *FGF19* as well as *SP5*. These genes promote hNSC proliferation and block neural differentiation. It is possible that Wnt signaling acts on proliferation and neural differentiation by other means as well (grey dashed lines).

In conclusion, hNSCs represent a powerful tool for modeling early human developmental processes and dissecting the complex molecular underpinnings of neurogenesis. Our work demonstrates that modulation of the Wnt signaling pathway in these cells produces biologically relevant effects that will help clarify the role of this vital signaling pathway in the proliferation, patterning, and differentiation of the cells of the developing human nervous system. Future work will focus on further understanding of the transcriptional events following Wnt pathway activation in these cells and translating those findings to other models of development and disease.

Acknowledgments

I would like to thank the following individuals for assistance that was instrumental to this work: Steven Head and his staff at the Scripps Research Institute Next Generation Sequencing Core Facility, for library preparation and sequencing of samples for RNA-Seq; Terry Gaasterland, for her help with RNA-Seq analysis that led to our identification of *SP5* as a Wnt3a-upregulated mRNA in hNSCs; Cleber Trujillo, for performing retroviral transduction of mouse brains as well as cryosectioning and immunostaining; Alexandra Stanley, for her assistance with numerous cell counts; Brett Henninger, for his work to help develop, purify and test the inducible *SP5* overexpression lentivirus. My work on Chapter 4 was supported by a training grant from the California Institute of Regenerative Medicine (TG2-01154).

Chapter 5 - Conclusions and future directions

Human pluripotent stem cells and their derivatives promise to revolutionize the study of human development, the modeling of disease, therapeutic development, and the practice of medicine. Before this promise can be realized however, much work remains to be done to further our understanding of these cells and the molecular processes that govern their behavior. In my work, I have studied the effects of the Wnt signaling pathway in hPSCs and hNSCs. This pathway is a critical modulator and master regulator of stem cell behavior throughout development and in mature tissues. Its importance in these myriad processes is surpassed perhaps only by the magnitude of our ignorance regarding the precise understanding of its effects. With that deficit in mind, I have used a combination of genetic, genomic, and biochemical approaches to scratch the surface the effects of the Wnt pathway in a human developmental context.

An important recurring theme in all of my work has been the induction of the transcription factor SP5 by Wnt pathway activation in stem cells. This transcription factor is often overlooked by others, perhaps largely because it appears dispensable for normal mouse development (Harrison et al., 2000; Treichel et al., 2001). Importantly, SP5 orthologs are required for proper development in other model organisms including *Danio rerio* (Thorpe et al., 2005) and *Xenopus laevis* (Park et al., 2013). Its high degree of conservation further indicates that SP5 confers a selective advantage in terms of organismal fitness, and must therefore play a critical but possibly subtle role in other mammals such as *Homo sapiens*. At the very least, *SP5* is a very sensitive marker of Wnt pathway activation status in both hPSCs and hNSCs and is superior to the more well-known *AXIN2* in that regard.

Another recurring theme of critical interest has been the downregulation of *EGR1* in response to Wnt pathway activation in both hPSCs and hNSCs. Though EGR1 has long been thought of as a master regulator of early growth, it is also involved in the differentiation of various cell types including hematopoietic (Nguyen et al., 1993), neural and cardiac (Sukhatme et al., 1988). Furthermore, EGR1 has been shown to block cellular reprogramming to pluripotency (Worringer et al., 2014), strengthening the notion that it often plays a role in promoting the differentiated state. That Wnt signaling regulates the expression of this well-known and potent differentiation factor invites further study. Tantalizingly, EGR1 has SP1 regulatory sequences in its promoter region and is possibly regulated by SP1/5. This raises the question of whether Wnt-induced SP5 is a driving force behind EGR1 downregulation. Indeed, in my work I observed that EGR1 is downregulated following Wnt pathway activation in two Wnt-responsive stem cell lines that induce *SP5* expression (hPSC and hNSC), but not in a Wnt-responsive cancer cell line which incidentally fails to induce *SP5* in response to Wnt pathway activation. Fortunately, the *SP5dZF* cell lines I have derived offer a perfect system in which to study this potentially intriguing connection and must be pursued in the near future.

This brings us to the topic of future studies. Future studies of the role of the Wnt signaling pathway in hPSCs and their derivatives will necessarily employ genetic tools to selectively manipulate this pathway in a manner specific to cell type. Such conditional genetic tools have proven to be powerful in other model systems, especially the mouse. The phenomenal gains in genetic engineering technologies over the past 5 years now allow for the rapid and customizable genetic engineering of hPSCs on a scale unimaginable even when I began my graduate studies. Of critical

importance to this will be the generation of Cre-ER hPSCs as platform lines into which further genetic modification can be built. Cre-ER should be placed under the control of endogenous promoters for markers of each germ layer: *SOX1*, ectoderm; *T*, mesoderm; *SOX17*, endoderm. These Cre-ER lines will then be further engineered to produce cell-type specific gene knockouts using flanking lox sites (floxed alleles). Ideally, we will generate conditional alleles for components involved in the secretion of active Wnt protein (*WLS*, *PORCN*), Wnt receptors (*FZD7*, *FZD5*, *FZD3*, etc.), negative regulators (*AXIN1/2*, *NKD1*, *KREMEN1/2*, etc.) and of course, *SP5*. Priority should be determined by preliminary experiments that determine the levels of expression and likely relative importance of these various genes in the cell types of interest, but all have the potential to produce interesting data that addresses the role of Wnt signaling in hPSCs and their early derivatives.

Another useful genetic tool for the study of the role of SP5 in hPSCs is the *SP5-YFP* fluorescent reporter cell line. These cells were generated by combining Adeno-associated Virus (AAV) mediated gene targeting with CRISPR/Cas9 genome editing and express SP5-YFP fusion protein under the control of the endogenous *SP5* promoter. Using these cells it will be possible to isolate $SP5^+$ cells from pools of cells during differentiation. Gene expression analysis of $SP5^+$ cells relative to $SP5^-$ will shed light on the biological processes regulated by this gene throughout human development.

In my studies, I identified the transcription factor SP5 as a key mediator of the effects of Wnt signaling in hPSCs, hNSCs, and in the neural stem cells of the mouse hippocampus. Further studies of the role of SP5 downstream of Wnt signaling must take advantage of the powerful genetic tools available in the mouse system. While

the mouse *Sp5* double mutant lacks an obvious phenotype (Harrison et al., 2000), to my knowledge a meticulous analysis of Wnt-responsive stem cells has not yet been performed. First, the localization of *Sp5* message in wild type (WT) tissues should be ascertained by sensitive mRNA *in situ* hybridization. I hypothesize that *Sp5* message will be elevated in the Wnt-responsive stem cells of various tissues including intestine, brain, skin, muscle and potentially blood. Once *Sp5*+ cells are identified, they will be characterized in WT and *Sp5*$^{-/-}$ tissues. The rate of proliferation will be characterized by BrdU uptake and Ki67 staining. Fate mapping could be undertaken using *Sp5-Cre* animals and would indicate whether Wnt-responsive stem cells from *Sp5* mutant animals display deficits or alterations in differentiation potential. Together, these studies will be necessary to demonstrate the important functional roles of Sp5 that have driven its high level of evolutionary conservation across vertebrate species and especially in mammals, where important roles have yet to be fully demonstrated.

Recently, a requirement for Wnt signaling in reprogramming of human fibroblasts to pluripotency was demonstrated (Ross et al., 2014). Because SP5 functions downstream of Wnt signaling in part by repressing Wnt antagonists, it is possible that SP5 expression enhances cellular reprogramming. To that end, future studies will address the role of SP5 in this process. Both knockdown and overexpression of SP5 will be tested in the presence of the 4 reprogramming factors *KLF4*, *c-Myc*, *OCT4* and *SOX2* (KMOS) and 3 reprogramming factors without *c-Myc* (KOS). As mentioned above, SP5 may also play a role in Wnt-mediated repression of EGR1 induction. Interestingly, EGR1 was recently demonstrated to be inhibitory toward cellular reprogramming (Worringer et al., 2014). It therefore stands to reason

that Wnt signaling may facilitate reprogramming at least in part by SP5-mediated EGR1 repression.

Last, I will discuss the implications of my findings for the understanding of the role of Wnt signaling in human disease. While the bulk of my studies have focused on the role of the Wnt signaling pathway in models of early human development, my findings may have special relevance to diseases that result from deregulation of the pathway, especially colon cancer. Colon cancer is often initiated by disruption of proper Wnt signaling, a relationship that has been understood for more than 20 years as of the writing of this work (Rubinfeld et al., 1993; Su et al., 1993). Importantly, *SP5* is a frequently upregulated gene in many colon cancers (Takahashi et al., 2005). My research has demonstrated that SP5 functions in part to enhance Wnt signals by repressing Wnt antagonists. While many Wnt antagonists are upregulated in colon cancer, it is possible that excess SP5 expression prevents adequate negative feedback regulation of aberrant Wnt signaling within tumor cells. Therapeutics targeting SP5 may therefore provide an ideal way to slow or halt the progression of colon cancers. SP5 deletion has little effect on the development and fertility of mice. While it may play an important role in the maintenance of Wnt-responsive stem cells, it may also be that it is not necessary for human survival. If colon cancers become addicted to SP5-mediated repression of Wnt antagonists, inhibition or siRNA-mediated knockdown may represent an attractive means of keeping this disease in check. Because SP5 acts as a transcriptional repressor and recruits mSin3a repressive machinery to chromatin (Fujimura et al., 2007), drugs that specifically block this interaction may be particularly well-suited to enhancing cells' own ability to tamp down excess Wnt pathway activation. Indeed, several human cancers exhibit

enhanced expression of *SP5* mRNA (Chen et al., 2006) and may be subject to therapeutic intervention in this fashion.

In conclusion, my studies have helped to elucidate the roles of the Wnt signaling pathway in cell-based models of human development. This work lays a foundation for future studies that will further improve our understanding of the complex processes that guide human development from a single cell to healthy adulthood and inform the creation of new therapeutics that rely on the regenerative capacity of stem cells and the molecular know-how accrued by nature over billions of years of evolution. Finally, my studies of SP5 have revealed that this transcription factor plays an important role in healthy development, tissues homeostasis and possibly cancer. An important goal of biomedical research is to develop novel chemotherapeutics that eradicate cancer while causing minimal harm to healthy tissues. An improved understanding of Wnt signaling and SP5 will be a key piece in the puzzle as we move toward that goal.

Materials and Methods

Human Pluripotent Stem Cell Lines and Culture Conditions

Human pluripotent stem cell lines H1 (WiCell) and H9 (WiCell) were cultured in E8 culture medium (Chen et al., 2011) on Matrigel® (BD Biosciences). Upon passage, cells were dissociated with Accutase® (Innovative Cell Technologies) to single cells and seeded at 2,000 cells / cm^2 in the presence of the Rock inhibitor Y-27632, 5 µM (Enzo Life Sciences). Cells were fed daily. For the formation of embryoid bodies, cells were dissociated to single cells and seeded in ultra-low-attachment culture dishes at 3.75E5 cells / mL with Y-27632 5 µM in E6 medium (E8 minus TGF-β1 and FGF2). Fresh E6 medium without Rock inhibitor was added daily. Endodermal differentiation of hPSCs was performed essentially as described previously (D'Amour et al., 2005). SOX17-GFP H9hESCs were a kind gift from Dr. Seung Kim.

Human Neural Stem Cell Lines and Culture Conditions

Human neural stem cells (HUES7) were a gift from Dr. Martin Marsala and derived as described previously (Chambers et al., 2009) and cultured in DMEM/F12 medium (Gibco) with Glutamax® (Life Technologies), N2 supplement (Life Technologies), B-27 supplement (Life Technologies), and recombinant FGF2 (20 ng/mL) (Peprotech). Cells were grown on plates coated with Poly-L-Ornithine (Sigma) and mouse Laminin (Sigma). Culture medium was replaced every other day. Upon passage, cells were dissociated with Accutase and split 1:3 onto fresh Poly-L-Ornithine / Laminin coated plates. Neural differentiation was initiated by the removal of FGF2 from the culture medium. FGFR inhibition was accomplished with PD166866 1 µM (Tocris).

Other Cell Lines and Culture Conditions

All other cell lines were cultured on uncoated tissue culture treated plastic dishes in the culture medium specified by ATCC (http://www.atcc.org).

Preparation of Retrovirus and Lentivirus

Retrovirus was prepared by transfection of HEK293T cells with the appropriate viral plasmids along with pCMVGP and pVSVG. Lentivirus was prepared by transfection of HEK293T cells with the appropriate viral plasmids along with psPAX2 and pVSVG. Supernatants were collected and filtered through 0.45 µm filter and virus was concentrated by ultracentrifugation in an Optima L-80 XP Ultracentrifuge (Beckton-Dickinson) for 2 hours at 20,000 RPM in a SW-40 Ti rotor. Pellets were resuspended overnight at 4°C in DMEM (Gibco) and frozen at -80°C until transduction.

RNA Expression Analysis

RNA expression was measured by quantitative real-time polymerase chain reaction (qPCR) on a CFX384 thermocycler (Bio-Rad). RNA was collected by TRIzol® Reagent (Life Technologies) or by column purification with RNeasy PLUS (Qiagen) or NucleoSpin RNA II (CloneTech) kits according to manufacturer's recommendation. Total RNA (50 µg/µL final concentration) was used to generate first strand cDNA using qScript cDNA Supermix (Quanta). For amplification, cDNA (0.5 µL per well), primers (0.4 µM final concentration), and SensiFAST Hi-ROX qPCR Master Mix (Bioline) were mixed and dispensed 5 µL per replicate, 4 replicates in a 384-well qPCR plate. Thermal cycler parameters: Initial denaturation, 95°C for 3 minutes;

Annealing and extension, 60°C for 5 seconds; Denaturation, 95°C for 15 seconds. 40 cycles. Primers were validated by melt curve analysis (60°C-95°C, 5 minutes) and gel electrophoresis of products. Data were analyzed using CFX Manager (Bio-Rad) and Microsoft Excel. All gene expression was normalized to the expression of an appropriate internal control gene (*18S*, *GAPDH*, *RPL13A*, *RPL37A* and *TBP*).

qPCR Primers

Table A: Primers for quantitative real-time PCR

Target	Forward Primer 5'→3'	Reverse Primer 5'→3'
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
APCDD1	GCAGAATGCCAAGAACCACG	ATGGTCAGGTCTGCCTTTGG
AXIN1	GCAGAGGTATGTGCAGGAGG	TGCGATCTTGTCTCTGTCTCG
AXIN2	TATCCAGTGATGCGCTGACG	CGGTGGGTTCTCGGGAAATG
CTNNBIP1	GACCAGCGACATAGGGACG	CGGACTCGGCCACTCACTA
DCX	TTCAAGGGGATTGTGTACGCT	GTCAGACAGAGATCGCGTCAG
FZD7	TTCTCGGACGATGGCTACC	GAACCAAGTGAGAGACAGAATGACC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
GFAP	CTGCGGCTCGATCAACTCA	TCCAGCGACTCAATCTTCCTC
KREMEN1	TGCACGTCACATTCAAATCCC	CAAGTCCTAGGCACAGTGGG
KREMEN2	GCACAACTTCTGCCGTAACC	ACAAAGCATCCCAGGTAGCC
MAP2	CTCAGCACCGCTAACAGAGG	CATTGGCGCTTCGGACAAG
NANOG	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
NKD1	CCAATACGCCTCCTGGACAA	CATCAAAGGCAAGGCTGGTT
PAX3	AAGCCCAAGCAGGTGACAAC	TGATGGAACTCACTGACGGC
PAX6	TTGAAAAGGGAACCGTGGCT	TCACTGGCCCATTAGCGAAG
POU5F1	CTTGAATCCCGAATGGAAAGGG	GTGTATATCCCAGGGTGATCCTC
RPL13A	CCTTCCTCCATTGTTGCCCT	TGCACAATTCTCCGAGTGCT
RPL37A	ATTGAAATCAGCCAGCACGC	GATGGCGGACTTTACCGTGA
SOX1	GGCTTTTGTACAGACGTTCCC	AACCCAAGTCTGGTGTCAGC
SOX17	GTGGACCGCACGGAATTTG	GGAGATTCACACCGGAGTCA
SOX2	GGGGAAAGTAGTTTGCTGCC	CGCCGCCGATGATTGTTATT
SP5	TCGGACATAGGGACCCAGTT	CTGACGGTGGGAACGGTTTA
T	CTATTCTGACAACTCACCTGCAT	ACAGGCTGGGGTACTGACT
TBP	CAGGGGTTCAGTGAGGTCG	CCCTGGGTCACTGCAAAGAT
TUBB3	GGCCAAGGGTCACTACACG	GCAGTCGCAGTTTTCACACTC
ZNRF3	ATTGATGGAGAGGAGCTGCG	TGATGTTGTGCCGACAGTGG

Protein Expression Analysis

For immunoblot analysis of protein expression, cells were washed once with dPBS, scraped and pelleted at 10,000 G, 4°C for 15 seconds. Cell pellets were lysed on ice in 1% Triton X-100, NaCl 150 mM, and Tris-Cl pH 7.5. Lysates centrifuged at 20,000 G, 4°C for 15 minutes and supernatants were collected and quantified by Bradford protein assay. 20 µg of total protein per lane was loaded and run on SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate primary antibodies overnight at 4°C. Protein was detected following incubation with secondary antibodies for 1 hour at 22°C, incubation with Western Lighting ECL Reagent (Perkin-Elmer), and exposure to autoradiography film. Primary antibodies: Rabbit anti-SP5 1:1000 (Epitomics H2748); Rabbit anti-EGR1 1:1000 (Abcam ab133695); Mouse anti-β-Actin 1:1000 (Sigma A2228). Secondary antibodies: Goat anti-Rabbit-IgG-HRP 1:5000 (Santa Cruz Biotechnology sc-2004); Goat anti-Mouse-IgG-HRP 1:5000 (Santa Cruz Biotechnology sc-2005).

For flow cytometric analysis, cells were fixed with BD Cytofix™ (BD Biosciences), permeabilized by BD Phosflow™ III Perm Buffer (BD Biosciences), blocked with FACS Buffer (dPBS, EDTA 0.5 mM, BSA 1%), and stained with fluorophore-conjugated primary antibodies according to manufacturer recommendations. Fluorescence was measured on BD FACSCanto™ II and BD LSRFortessa™ instruments and analysis was performed with Flowing Software flow cytometry analysis suite (http://www.flowingsoftware.com/). Primary antibodies: APC-Mouse anti-CD184 1:200 (BD Biosciences BD555976).

Phase Contrast Microscopy

Phase contrast microscopy was performed using Leica, Zeiss, and EVOS (Life Technologies) inverted fluorescence microscopes. Image analysis and manipulation was performed with ImageJ and GIMP.

Immunofluorescence

Cells were fixed with BD Cytofix (BD Bioscience) for 10 minutes at 22°C, washed twice with dPBS (Cellgro) and permeabilized in dPBS + Triton-X100 0.2% for 1 hour at 22°C followed by two additional dPBS washes. For antigen recovery, required for SP5 staining, cells were incubated with dPBS + 1% Sodium Dodecyl Sulfate for 10 minutes at 22°C and washed 3 times with dPBS. Cells were blocked by treatment with FACS buffer for 30 minutes at 22°C. Primary antibodies were diluted in FACS buffer and incubated with cells overnight at 4°C, followed by 2 washes with FACS buffer. Secondary antibodies were diluted in FACS buffer and incubated with cells for 1 hour at 22°C, followed by 2 washes with FACS buffer. Nuclei were labeled with HOECHST 33342 (Roche) diluted 1:10,000 in FACS buffer followed by 2 washes with FACS buffer. Cells were imaged on a Leica SPE confocal microscope. Histological sections were imaged on an Olympus FV1200 confocal microscope. Primary antibodies: Rabbit anti-SP5 1:50 (Epitomics H2748); Rabbit anti-Doublecortin 1:1000 (Abcam ab77450); Chicken anti-GFP 1:1000 (Abcam ab13970). Secondary Antibodies: Alexa Fluor® 647-Goat anti-Rabbit 1:200 (Life Technologies A-21244); Alexa Fluor 546® Goat-anti-Chicken 1:200 (Life Technologies A-11040).

Stereotactic Brain Injections of neonatal mice

Neonatal mouse hippocampi were injected as described previously (Pilpel et al., 2009) with minor modification. A Hamilton microinjection syringe and 34 gauge needle were used in place of an automated pump to deliver 1 µL of purified virus at a rate of 0.5 µL min^{-1}.

Mouse Brain Tissue Preparation

Performed as described previously (Muotri et al., 2005). See above under "Immunofluorescence" for antibodies and dilutions.

RNA-Seq

Total RNA was prepared from cells using the RNeasy Plus column purification kit (Qiagen) and depleted of ribosomal RNA. After ligating adaptors, fragmented RNA was converted to first strand cDNA using ArrayScript Reverse Transcriptase (Ambion), size selected (100–200 bp) by gel electrophoresis, and PCR amplified using adaptor-specific primers. Sequencing was executed on an Illumina HiSeq 2000. TopHat and Cufflinks were used to perform differential gene expression analysis of RNA-seq experiments as previously described (Trapnell et al., 2010, 2012). Briefly, sequencing reads were quality filtered, mapped, and aligned to the reference human genome (hg19) with TopHat and Cuffdiff was used to calculate gene expression levels as reads per thousand transcript bases per million reads mapped (RPKM). Statistically significant changes in gene expression were obtained from RPKM values. Genes were clustered by expression pattern (Genesis).

ChIP-Seq

Libraries from immunoprecipitated chromatin were prepared by the nucleic acid core of The Scripps Research Institute and sequenced on a HiSeq instrument (Illumina, San Diego, CA) by following the manufacturer's instruction. Reads were filtered by quality and trimmed and mapped to the genome (Bowtie 2). Random down sampling was used to normalize all tracks to have equal read number. Peaks were called (GEM) and visualized (IGV). Motif and average coverage analyses were performed (HOMER).

Single Cell Gene Expression

Cells were dissociated with Accutase® and captured using the Fluidigm C1 automated microfluidic platform according to manufacturer recommendations. Cells were lysed and cDNA was prepared on the system using the Cells-to-Ct reverse transcription kit (CloneTech) and qPCR was performed on the Fluidigm Biomark HD microfluidic quantitative real-time PCR instrument according to manufacturer recommendations. Relative gene expression was calculated using the delta-Ct method, and Pearson correlation was calculated. Genes were sorted from highest to lowest correlation with *SP5* expression patterns.

Plasmids

pCMMP IRES-GFP (modified pCMMP-EnvA/RG-IRES-GFP from Callaway Lab)

pCMMP SP5-IRES-GFP (modified pCMMP-EnvA/RG-IRES-GFP from Callaway Lab)

pGIPZ shSP5-GFP #3 (GE Dharmacon, Clone ID: V3LHS_372625)

pGIPZ shControl-GFP (GE Dharmacon, Catalog #: RHS4346)

pTRIPZ iSP5 (modified pTRIPZ from GE Dharmacon)

psPAX2 (Addgene)

pVSVG (Addgene)

pCMVGP (Addgene)

pCas9-EGFP (Cowan Lab)

pGRNA SP5dZF 3' (modified pGRNA from Cowan Lab)

pGRNA SP5dZF 5' (modified pGRNA from Cowan Lab)

References

Abdelrahim, M., Smith, R., Burghardt, R., and Safe, S. (2004). Role of Sp proteins in regulation of vascular endothelial growth factor expression and proliferation of pancreatic cancer cells. Cancer Res. *64*, 6740–6749.

Alagappan, D., Balan, M., Jiang, Y., Cohen, R.B., Kotenko, S.V., and Levison, S.W. (2013). Egr-1 is a critical regulator of EGF-receptor-mediated expansion of subventricular zone neural stem cells and progenitors during recovery from hypoxia-hypoglycemia. ASN Neuro *5*, 183–193.

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.P., Harris, C.P., Waknitz, M.A., Itskovitz-Eldor, J., and Thomson, J.A. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev. Biol. *227*, 271–278.

D'Amour, K.A., Agulnick, A.D., Eliazer, S., Kelly, O.G., Kroon, E., and Baetge, E.E. (2005). Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat. Biotechnol. *23*, 1534–1541.

Augustine, K.A., Liu, E.T., and Sadler, T.W. (1995). Interactions of Wnt-1 and Wnt-3a are essential for neural tube patterning. Teratology *51*, 107–119.

Bang, A.G., Papalopulu, N., Goulding, M.D., and Kintner, C. (1999). Expression of Pax-3 in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm. Dev. Biol. *212*, 366–380.

Barker, N., Huch, M., Kujala, P., van de Wetering, M., Snippert, H.J., van Es, J.H., Sato, T., Stange, D.E., Begthel, H., van den Born, M., et al. (2010). Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell Stem Cell *6*, 25–36.

Barker, R.A., Drouin-Ouellet, J., and Parmar, M. (2015). Cell-based therapies for Parkinson disease-past insights and future potential. Nat. Rev. Neurol.

Beishline, K., and Azizkhan-Clifford, J. (2015). Sp1 and the "hallmarks of cancer." FEBS J. *282*, 224–258.

Berge, D. ten, Brugmann, S.A., Helms, J.A., and Nusse, R. (2008a). Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development. Dev. Camb. Engl. *135*, 3247–3257.

Berge, D. ten, Koole, W., Fuerer, C., Fish, M., Eroglu, E., and Nusse, R. (2008b). Wnt signaling mediates self-organization and axis formation in embryoid bodies. Cell Stem Cell *3*, 508–518.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature *382*, 225–230.

Biechele, S., Cox, B.J., and Rossant, J. (2011). Porcupine homolog is required for canonical Wnt signaling and gastrulation in mouse embryos. Dev. Biol. *355*, 275–285.

Blauwkamp, T.A., Nigam, S., Ardehali, R., Weissman, I.L., and Nusse, R. (2012). Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. Nat. Commun. *3*, 1070.

Boam, D.S., Davidson, I., and Chambon, P. (1995). A TATA-less promoter containing binding sites for ubiquitous transcription factors mediates cell type-specific regulation of the gene for transcription enhancer factor-1 (TEF-1). J. Biol. Chem. *270*, 19487–19494.

Bureau, C., Hanoun, N., Torrisani, J., Vinel, J.-P., Buscail, L., and Cordelier, P. (2009). Expression and Function of Kruppel Like-Factors (KLF) in Carcinogenesis. Curr. Genomics *10*, 353–360.

Buxton, P., Hunt, P., Ferretti, P., and Thorogood, P. (1997). A role for midline closure in the reestablishment of dorsoventral pattern following dorsal hindbrain ablation. Dev. Biol. *183*, 150–165.

Cadigan, K.M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Genes Dev. *11*, 3286–3305.

Cadigan, K.M., and Peifer, M. (2009). Wnt signaling from development to disease: insights from model systems. Cold Spring Harb. Perspect. Biol. *1*, a002881.

Chailangkarn, T., Acab, A., and Muotri, A.R. (2012). Modeling neurodevelopmental disorders using human neurons. Curr. Opin. Neurobiol. *22*, 785–790.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. *27*, 275–280.

Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R., and Charnay, P. (1988). A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. EMBO J. *7*, 29–35.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E., et al. (2011). Chemically defined conditions for human iPSC derivation and culture. Nat. Methods *8*, 424–429.

Chen, Y., Guo, Y., Ge, X., Itoh, H., Watanabe, A., Fujiwara, T., Kodama, T., and Aburatani, H. (2006). Elevated expression and potential roles of human Sp5, a member of Sp transcription factor family, in human cancers. Biochem. Biophys. Res. Commun. *340*, 758–766.

Chin, M.H., Mason, M.J., Xie, W., Volinia, S., Singer, M., Peterson, C., Ambartsumyan, G., Aimiuwu, O., Richter, L., Zhang, J., et al. (2009). Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell *5*, 111–123.

Christy, B.A., Lau, L.F., and Nathans, D. (1988). A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with “zinc finger” sequences. Proc. Natl. Acad. Sci. U. S. A. *85*, 7857–7861.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell *127*, 469–480.

Coudreuse, D.Y.M., Roël, G., Betist, M.C., Destrée, O., and Korswagen, H.C. (2006). Wnt gradient formation requires retromer function in Wnt-producing cells. Science *312*, 921–924.

Davidson, K.C., Adams, A.M., Goodson, J.M., McDonald, C.E., Potter, J.C., Berndt, J.D., Biechele, T.L., Taylor, R.J., and Moon, R.T. (2012). Wnt/β-catenin signaling promotes differentiation, not self-renewal, of human embryonic stem cells and is repressed by Oct4. Proc. Natl. Acad. Sci. U. S. A. *109*, 4485–4490.

Diamandis, T., and Borlongan, C.V. (2015). One, two, three steps toward cell therapy for stroke. Stroke J. Cereb. Circ. *46*, 588–591.

Dittmer, J., Gégonne, A., Gitlin, S.D., Ghysdael, J., and Brady, J.N. (1994). Regulation of parathyroid hormone-related protein (PTHrP) gene expression. Sp1 binds through an inverted CACCC motif and regulates promoter activity in cooperation with Ets1. J. Biol. Chem. *269*, 21428–21434.

Dragunow, M. (1996). A role for immediate-early transcription factors in learning and memory. Behav. Genet. *26*, 293–299.

Dunty, W.C., Kennedy, M.W.L., Chalamalasetty, R.B., Campbell, K., and Yamaguchi, T.P. (2014). Transcriptional profiling of Wnt3a mutants identifies Sp transcription factors as essential effectors of the Wnt/β-catenin pathway in neuromesodermal stem cells. PloS One *9*, e87018.

Dynan, W.S., and Tjian, R. (1983). Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. Cell *32*, 669–680.

Edwards, S.A., Darland, T., Sosnowski, R., Samuels, M., and Adamson, E.D. (1991). The transcription factor, Egr-1, is rapidly modulated in response to retinoic acid in P19 embryonal carcinoma cells. Dev. Biol. *148*, 165–173.

Ellis, P., Fagan, B.M., Magness, S.T., Hutton, S., Taranova, O., Hayashi, S., McMahon, A., Rao, M., and Pevny, L. (2004). SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. Dev. Neurosci. *26*, 148–165.

Fancy, S.P.J., Harrington, E.P., Baranzini, S.E., Silbereis, J.C., Shiow, L.R., Yuen, T.J., Huang, E.J., Lomvardas, S., and Rowitch, D.H. (2014). Parallel states of pathological Wnt signaling in neonatal brain injury and colon cancer. Nat. Neurosci. *17*, 506–512.

Fernandez, A., Huggins, I.J., Perna, L., Brafman, D., Lu, D., Yao, S., Gaasterland, T., Carson, D.A., and Willert, K. (2014). The WNT receptor FZD7 is required for maintenance of the pluripotent state in human embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. *111*, 1409–1414.

Fernandez-Zapico, M.E., Lomberk, G.A., Tsuji, S., DeMars, C.J., Bardsley, M.R., Lin, Y.-H., Almada, L.L., Han, J.-J., Mukhopadhyay, D., Ordog, T., et al. (2011). A functional family-wide screening of SP/KLF proteins identifies a subset of suppressors of KRAS-mediated cell growth. Biochem. J. *435*, 529–537.

Flandez, M., Guilmeau, S., Blache, P., and Augenlicht, L.H. (2008). KLF4 regulation in intestinal epithelial cell maturation. Exp. Cell Res. *314*, 3712–3723.

Fujimura, N., Vacik, T., Machon, O., Vlcek, C., Scalabrin, S., Speth, M., Diep, D., Krauss, S., and Kozmik, Z. (2007). Wnt-mediated down-regulation of Sp1 target genes by a transcriptional repressor Sp5. J. Biol. Chem. *282*, 1225–1237.

Fung, Y.K., Shackleford, G.M., Brown, A.M., Sanders, G.S., and Varmus, H.E. (1985). Nucleotide sequence and expression in vitro of cDNA derived from mRNA of int-1, a provirally activated mouse mammary oncogene. Mol. Cell. Biol. *5*, 3337–3344.

Gao, B., Song, H., Bishop, K., Elliot, G., Garrett, L., English, M.A., Andre, P., Robinson, J., Sood, R., Minami, Y., et al. (2011). Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. Dev. Cell *20*, 163–176.

Garcia-Rostan, G., Camp, R.L., Herrero, A., Carcangiu, M.L., Rimm, D.L., and Tallini, G. (2001). Beta-catenin dysregulation in thyroid neoplasms: down-regulation, aberrant nuclear expression, and CTNNB1 exon 3 mutations are markers for aggressive tumor phenotypes and poor prognosis. Am. J. Pathol. *158*, 987–996.

Ghaleb, A.M., Nandan, M.O., Chanchevalap, S., Dalton, W.B., Hisamuddin, I.M., and Yang, V.W. (2005). Krüppel-like factors 4 and 5: the yin and yang regulators of cellular proliferation. Cell Res. *15*, 92–96.

Gidoni, D., Dynan, W.S., and Tjian, R. (1984). Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature *312*, 409–413.

Guo, K.J., Yamaguchi, K., and Enjoji, M. (1988). Undifferentiated carcinoma of the gallbladder. A clinicopathologic, histochemical, and immunohistochemical study of 21 patients with a poor prognosis. Cancer *61*, 1872–1879.

Hannan, N.R.., Segeritz, C.-P., Touboul, T., and Vallier, L. (2013). Production of hepatocyte like cells from human pluripotent stem cells. Nat. Protoc. *8*, 430–437.

Harrison, S.M., Houzelstein, D., Dunwoodie, S.L., and Beddington, R.S. (2000). Sp5, a new member of the Sp1 family, is dynamically expressed during development and genetically interacts with Brachyury. Dev. Biol. *227*, 358–372.

Hartl, D., Irmler, M., Römer, I., Mader, M.T., Mao, L., Zabel, C., de Angelis, M.H., Beckers, J., and Klose, J. (2008). Transcriptome and proteome analysis of early embryonic mouse brain development. Proteomics *8*, 1257–1265.

Hartman, M.E., Dai, D.-F., and Laflamme, M.A. (2015). Human pluripotent stem cells: Prospects and challenges as a source of cardiomyocytes for in vitro modeling and cell-based cardiac repair. Adv. Drug Deliv. Rev.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509–1512.

Hodge, R.D., and Hevner, R.F. (2011). Expression and actions of transcription factors in adult hippocampal neurogenesis. Dev. Neurobiol. *71*, 680–689.

Hosoi, Y., Watanabe, T., Nakagawa, K., Matsumoto, Y., Enomoto, A., Morita, A., Nagawa, H., and Suzuki, N. (2004). Up-regulation of DNA-dependent protein kinase activity and Sp1 in colorectal cancer. Int. J. Oncol. *25*, 461–468.

Huang, R.P., Darland, T., Okamura, D., Mercola, D., and Adamson, E.D. (1994). Suppression of v-sis-dependent transformation by the transcription factor, Egr-1. Oncogene *9*, 1367–1377.

Huang, R.P., Liu, C., Fan, Y., Mercola, D., and Adamson, E.D. (1995). Egr-1 negatively regulates human tumor cell growth via the DNA-binding domain. Cancer Res. *55*, 5054–5062.

Huang, S.X.L., Islam, M.N., O'Neill, J., Hu, Z., Yang, Y.-G., Chen, Y.-W., Mumau, M., Green, M.D., Vunjak-Novakovic, G., Bhattacharya, J., et al. (2014). Efficient generation of lung and airway epithelial cells from human pluripotent stem cells. Nat. Biotechnol. *32*, 84–91.

International Stem Cell Initiative, Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P.W., Beighton, G., Bello, P.A., Benvenisty, N., Berry, L.S., et al. (2007). Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat. Biotechnol. *25*, 803–816.

Jakel, R.J., Schneider, B.L., and Svendsen, C.N. (2004). Using human neural stem cells to model neurological disease. Nat. Rev. Genet. *5*, 136–144.

Janda, C.Y., Waghray, D., Levin, A.M., Thomas, C., and Garcia, K.C. (2012). Structural basis of Wnt recognition by Frizzled. Science *337*, 59–64.

Jordan, T., Hanson, I., Zaletayev, D., Hodgson, S., Prosser, J., Seawright, A., Hastie, N., and van Heyningen, V. (1992). The human PAX6 gene is mutated in two patients with aniridia. Nat. Genet. *1*, 328–332.

Kayam, G., Kohl, A., Magen, Z., Peretz, Y., Weisinger, K., Bar, A., Novikov, O., Brodski, C., and Sela-Donenfeld, D. (2013). A novel role for Pax6 in the segmental organization of the hindbrain. Dev. Camb. Engl. *140*, 2190–2202.

Kiecker, C., and Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. Dev. Camb. Engl. *128*, 4189–4201.

Kim, K.-H., Park, J.-H., Lee, W.-R., Park, J.-S., Kim, H.-C., and Park, K.-K. (2013). The inhibitory effect of chimeric decoy oligodeoxynucleotide against NF-κB and Sp1 in renal interstitial fibrosis. J. Mol. Med. Berl. Ger. *91*, 573–586.

Kim, S.J., Onwuta, U.S., Lee, Y.I., Li, R., Botchan, M.R., and Robbins, P.D. (1992). The retinoblastoma gene product regulates Sp1-mediated transcription. Mol. Cell. Biol. *12*, 2455–2463.

Klaus, A., and Birchmeier, W. (2008). Wnt signalling and its impact on development and cancer. Nat. Rev. Cancer *8*, 387–398.

Koo, B.-K., and Clevers, H. (2014). Stem cells marked by the R-spondin receptor LGR5. Gastroenterology *147*, 289–302.

Kouskoff, V., Lacaud, G., Schwantz, S., Fehling, H.J., and Keller, G. (2005). Sequential development of hematopoietic and cardiac mesoderm during embryonic stem cell differentiation. Proc. Natl. Acad. Sci. U. S. A. *102*, 13170–13175.

Krasnow, R.E., Wong, L.L., and Adler, P.N. (1995). Dishevelled is a component of the frizzled signaling pathway in Drosophila. Dev. Camb. Engl. *121*, 4095–4102.

Lee, S.M., Tole, S., Grove, E., and McMahon, A.P. (2000). A local Wnt-3a signal is required for development of the mammalian hippocampus. Dev. Camb. Engl. *127*, 457–467.

Li, H.S., Yang, J.M., Jacobson, R.D., Pasko, D., and Sundin, O. (1994). Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. Dev. Biol. *162*, 181–194.

Lian, X., Hsiao, C., Wilson, G., Zhu, K., Hazeltine, L.B., Azarin, S.M., Raval, K.K., Zhang, J., Kamp, T.J., and Palecek, S.P. (2012). Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc. Natl. Acad. Sci. U. S. A. *109*, E1848–E1857.

Lie, D.-C., Colamarino, S.A., Song, H.-J., Désiré, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R., et al. (2005). Wnt signalling regulates adult hippocampal neurogenesis. Nature *437*, 1370–1375.

Lin, J.X., and Leonard, W.J. (1997). The immediate-early gene product Egr-1 regulates the human interleukin-2 receptor beta-chain promoter through noncanonical Egr and Sp1 binding sites. Mol. Cell. Biol. *17*, 3714–3722.

Lister, R., Pelizzola, M., Kida, Y.S., Hawkins, R.D., Nery, J.R., Hon, G., Antosiewicz-Bourget, J., O'Malley, R., Castanon, R., Klugman, S., et al. (2011). Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature *471*, 68–73.

Liu, C., Adamson, E., and Mercola, D. (1996). Transcription factor EGR-1 suppresses the growth and transformation of human HT-1080 fibrosarcoma cells by induction of transforming growth factor beta 1. Proc. Natl. Acad. Sci. U. S. A. *93*, 11831–11836.

Liu, C., Yao, J., de Belle, I., Huang, R.P., Adamson, E., and Mercola, D. (1999a). The transcription factor EGR-1 suppresses transformation of human fibrosarcoma HT1080 cells by coordinated induction of transforming growth factor-beta1, fibronectin, and plasminogen activator inhibitor-1. J. Biol. Chem. *274*, 4400–4411.

Liu, P., Wakamiya, M., Shea, M.J., Albrecht, U., Behringer, R.R., and Bradley, A. (1999b). Requirement for Wnt3 in vertebrate axis formation. Nat. Genet. *22*, 361–365.

Liu, X., Zhu, Y., and Gao, W. (2006). Isolation of neural stem cells from the spinal cords of low temperature preserved abortuses. J. Neurosci. Methods *157*, 64–70.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.

Ludwig, T.E., Levenstein, M.E., Jones, J.M., Berggren, W.T., Mitchen, E.R., Frane, J.L., Crandall, L.J., Daigh, C.A., Conard, K.R., Piekarczyk, M.S., et al. (2006). Derivation of human embryonic stem cells in defined conditions. Nat. Biotechnol. *24*, 185–187.

Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P.M., Birchmeier, W., et al. (2002). Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol. Cell. Biol. *22*, 1184–1193.

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

Marin, M., Karis, A., Visser, P., Grosveld, F., and Philipsen, S. (1997). Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell *89*, 619–628.

Martin, B.L., and Kimelman, D. (2008). Regulation of canonical Wnt signaling by Brachyury is essential for posterior mesoderm formation. Dev. Cell *15*, 121–133.

Matveyenko, A.V., Georgia, S., Bhushan, A., and Butler, P.C. (2010). Inconsistent formation and nonfunction of insulin-positive cells from pancreatic endoderm derived from human embryonic stem cells in athymic nude rats. Am. J. Physiol. Endocrinol. Metab. *299*, E713–E720.

McMahon, A.P., and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell *62*, 1073–1085.

Miller, I.J., and Bieker, J.J. (1993). A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Krüppel family of nuclear proteins. Mol. Cell. Biol. *13*, 2776–2786.

Min, I.M., Pietramaggiori, G., Kim, F.S., Passegué, E., Stevenson, K.E., and Wagers, A.J. (2008). The transcription factor EGR1 controls both the proliferation and localization of hematopoietic stem cells. Cell Stem Cell *2*, 380–391.

Morkel, M., Huelsken, J., Wakamiya, M., Ding, J., van de Wetering, M., Clevers, H., Taketo, M.M., Behringer, R.R., Shen, M.M., and Birchmeier, W. (2003). Beta-catenin regulates Cripto- and Wnt3-dependent gene expression programs in mouse axis and mesoderm formation. Dev. Camb. Engl. *130*, 6283–6294.

Moya, N., Cutts, J., Gaasterland, T., Willert, K., and Brafman, D.A. (2014). Endogenous WNT signaling regulates hPSC-derived neural progenitor cell heterogeneity and specifies their regional identity. Stem Cell Rep. *3*, 1015–1028.

Munji, R.N., Choe, Y., Li, G., Siegenthaler, J.A., and Pleasure, S.J. (2011). Wnt signaling regulates neuronal differentiation of cortical intermediate progenitors. J. Neurosci. Off. J. Soc. Neurosci. *31*, 1676–1687.

Muotri, A.R., Chu, V.T., Marchetto, M.C.N., Deng, W., Moran, J.V., and Gage, F.H. (2005). Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. Nature *435*, 903–910.

Mutch, C.A., Schulte, J.D., Olson, E., and Chenn, A. (2010). Beta-catenin signaling negatively regulates intermediate progenitor population numbers in the developing cortex. PloS One *5*, e12376.

Nakanishi, M., Kurisaki, A., Hayashi, Y., Warashina, M., Ishiura, S., Kusuda-Furue, M., and Asashima, M. (2009). Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *23*, 114–122.

Naujok, O., Diekmann, U., and Lenzen, S. (2014). The generation of definitive endoderm from human embryonic stem cells is initially independent from activin A but requires canonical Wnt-signaling. Stem Cell Rev. *10*, 480–493.

Nguyen, H., Merrill, B.J., Polak, L., Nikolova, M., Rendl, M., Shaver, T.M., Pasolli, H.A., and Fuchs, E. (2009). Tcf3 and Tcf4 are essential for long-term homeostasis of skin epithelia. Nat. Genet. *41*, 1068–1075.

Nguyen, H.Q., Hoffman-Liebermann, B., and Liebermann, D.A. (1993). The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. Cell *72*, 197–209.

Nusse, R. (2001). An ancient cluster of Wnt paralogues. Trends Genet. TIG *17*, 443.

Nusse, R. (2005). Wnt signaling in disease and in development. Cell Res. *15*, 28–32.

Nusse, R., and Varmus, H.E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell *31*, 99–109.

Nusse, R., Brown, A., Papkoff, J., Scambler, P., Shackleford, G., McMahon, A., Moon, R., and Varmus, H. (1991). A new nomenclature for int-1 and related genes: the Wnt gene family. Cell *64*, 231.

van Ooyen, A., and Nusse, R. (1984). Structure and nucleotide sequence of the putative mammary oncogene int-1; proviral insertions leave the protein-encoding domain intact. Cell *39*, 233–240.

Ossipova, O., Stick, R., and Pieler, T. (2002). XSPR-1 and XSPR-2, novel Sp1 related zinc finger containing genes, are dynamically expressed during Xenopus embryogenesis. Mech. Dev. *115*, 117–122.

Owen, G.I., Richer, J.K., Tung, L., Takimoto, G., and Horwitz, K.B. (1998). Progesterone regulates transcription of the p21(WAF1) cyclin- dependent kinase inhibitor gene through Sp1 and CBP/p300. J. Biol. Chem. *273*, 10696–10701.

Pagano, S.F., Impagnatiello, F., Girelli, M., Cova, L., Grioni, E., Onofri, M., Cavallaro, M., Etteri, S., Vitello, F., Giombini, S., et al. (2000). Isolation and characterization of neural stem cells from the adult human olfactory bulb. Stem Cells Dayt. Ohio *18*, 295–300.

Pagliuca, F.W., Millman, J.R., Gürtler, M., Segel, M., Van Dervort, A., Ryu, J.H., Peterson, Q.P., Greiner, D., and Melton, D.A. (2014). Generation of functional human pancreatic β cells in vitro. Cell *159*, 428–439.

Panáková, D., Sprong, H., Marois, E., Thiele, C., and Eaton, S. (2005). Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature *435*, 58–65.

Park, D.-S., Seo, J.-H., Hong, M., Bang, W., Han, J.-K., and Choi, S.-C. (2013). Role of Sp5 as an essential early regulator of neural crest specification in xenopus. Dev. Dyn. Off. Publ. Am. Assoc. Anat. *242*, 1382–1394.

Pevny, L.H., and Nicolis, S.K. (2010). Sox2 roles in neural stem cells. Int. J. Biochem. Cell Biol. *42*, 421–424.

Pilpel, N., Landeck, N., Klugmann, M., Seeburg, P.H., and Schwarz, M.K. (2009). Rapid, reproducible transduction of select forebrain regions by targeted recombinant virus injection into the neonatal mouse brain. J. Neurosci. Methods *182*, 55–63.

Pinto, D., Gregorieff, A., Begthel, H., and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev. *17*, 1709–1713.

Pituello, F., Yamada, G., and Gruss, P. (1995). Activin A inhibits Pax-6 expression and perturbs cell differentiation in the developing spinal cord in vitro. Proc. Natl. Acad. Sci. U. S. A. *92*, 6952–6956.

Polakis, P. (2000). Wnt signaling and cancer. Genes Dev. *14*, 1837–1851.

Polakis, P. (2012). Wnt Signaling in Cancer. Cold Spring Harb. Perspect. Biol. *4*.

Prosperi, J.R., and Goss, K.H. (2010). A Wnt-ow of opportunity: targeting the Wnt/beta-catenin pathway in breast cancer. Curr. Drug Targets *11*, 1074–1088.

Pu, J., Jiang, H., Zhang, B., and Feng, J. (2012). Redefining Parkinson's disease research using induced pluripotent stem cells. Curr. Neurol. Neurosci. Rep. *12*, 392–398.

Ramer, L.M., Ramer, M.S., and Bradbury, E.J. (2014). Restoring function after spinal cord injury: towards clinical translation of experimental strategies. Lancet Neurol. *13*, 1241–1256.

Redmond, D.E., Bjugstad, K.B., Teng, Y.D., Ourednik, V., Ourednik, J., Wakeman, D.R., Parsons, X.H., Gonzalez, R., Blanchard, B.C., Kim, S.U., et al. (2007). Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. Proc. Natl. Acad. Sci. U. S. A. *104*, 12175–12180.

Reeves, H.L., Narla, G., Ogunbiyi, O., Haq, A.I., Katz, A., Benzeno, S., Hod, E., Harpaz, N., Goldberg, S., Tal-Kremer, S., et al. (2004). Kruppel-like factor 6 (KLF6) is a tumor-suppressor gene frequently inactivated in colorectal cancer. Gastroenterology *126*, 1090–1103.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409–414.

Rheinbay, E., Suvà, M.L., Gillespie, S.M., Wakimoto, H., Patel, A.P., Shahid, M., Oksuz, O., Rabkin, S.D., Martuza, R.L., Rivera, M.N., et al. (2013). An aberrant transcription factor network essential for Wnt signaling and stem cell maintenance in glioblastoma. Cell Rep. *3*, 1567–1579.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987). The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell *50*, 649–657.

Rivera-Pérez, J.A., and Magnuson, T. (2005). Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. Dev. Biol. *288*, 363–371.

Roelink, H., and Nusse, R. (1991). Expression of two members of the Wnt family during mouse development--restricted temporal and spatial patterns in the developing neural tube. Genes Dev. *5*, 381–388.

Ross, J., Busch, J., Mintz, E., Ng, D., Stanley, A., Brafman, D., Sutton, V.R., Van den Veyver, I., and Willert, K. (2014). A rare human syndrome provides genetic evidence that WNT signaling is required for reprogramming of fibroblasts to induced pluripotent stem cells. Cell Rep. *9*, 1770–1780.

Rubinfeld, B., Souza, B., Albert, I., Müller, O., Chamberlain, S.H., Masiarz, F.R., Munemitsu, S., and Polakis, P. (1993). Association of the APC gene product with beta-catenin. Science *262*, 1731–1734.

Saffer, J.D., Jackson, S.P., and Annarella, M.B. (1991). Developmental expression of Sp1 in the mouse. Mol. Cell. Biol. *11*, 2189–2199.

Schaeper, N.D., Prpic, N.-M., and Wimmer, E.A. (2010). A clustered set of three Sp-family genes is ancestral in the Metazoa: evidence from sequence analysis, protein domain structure, developmental expression patterns and chromosomal location. BMC Evol. Biol. *10*, 88.

Schäfer, D., Hamm-Künzelmann, B., and Brand, K. (1997). Glucose regulates the promoter activity of aldolase A and pyruvate kinase M2 via dephosphorylation of Sp1. FEBS Lett. *417*, 325–328.

Sebio, A., Kahn, M., and Lenz, H.-J. (2014). The potential of targeting Wnt/β-catenin in colon cancer. Expert Opin. Ther. Targets *18*, 611–615.

Sharma, R.P., and Chopra, V.L. (1976). Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. Dev. Biol. *48*, 461–465.

Shi, Q., Le, X., Abbruzzese, J.L., Peng, Z., Qian, C.N., Tang, H., Xiong, Q., Wang, B., Li, X.C., and Xie, K. (2001). Constitutive Sp1 activity is essential for differential constitutive expression of vascular endothelial growth factor in human pancreatic adenocarcinoma. Cancer Res. *61*, 4143–4154.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. U. S. A. *96*, 5522–5527.

Skerka, C., Decker, E.L., and Zipfel, P.F. (1995). A regulatory element in the human interleukin 2 gene promoter is a binding site for the zinc finger proteins Sp1 and EGR-1. J. Biol. Chem. *270*, 22500–22506.

Snippert, H.J., Haegebarth, A., Kasper, M., Jaks, V., van Es, J.H., Barker, N., van de Wetering, M., van den Born, M., Begthel, H., Vries, R.G., et al. (2010). Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science *327*, 1385–1389.

Spence, J.R., Mayhew, C.N., Rankin, S.A., Kuhar, M.F., Vallance, J.E., Tolle, K., Hoskins, E.E., Kalinichenko, V.V., Wells, S.I., Zorn, A.M., et al. (2011). Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature *470*, 105–109.

Stewart, D.J. (2014). Wnt signaling pathway in non-small cell lung cancer. J. Natl. Cancer Inst. *106*, djt356.

Stoykova, A., Treichel, D., Hallonet, M., and Gruss, P. (2000). Pax6 modulates the dorsoventral patterning of the mammalian telencephalon. J. Neurosci. Off. J. Soc. Neurosci. *20*, 8042–8050.

Strigini, M., and Cohen, S.M. (2000). Wingless gradient formation in the Drosophila wing. Curr. Biol. CB *10*, 293–300.

Su, L.K., Vogelstein, B., and Kinzler, K.W. (1993). Association of the APC tumor suppressor protein with catenins. Science *262*, 1734–1737.

Sukhatme, V.P. (1990). Early transcriptional events in cell growth: the Egr family. J. Am. Soc. Nephrol. JASN *1*, 859–866.

Sukhatme, V.P., Kartha, S., Toback, F.G., Taub, R., Hoover, R.G., and Tsai-Morris, C.H. (1987). A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. Oncogene Res. *1*, 343–355.

Sukhatme, V.P., Cao, X.M., Chang, L.C., Tsai-Morris, C.H., Stamenkovich, D., Ferreira, P.C., Cohen, D.R., Edwards, S.A., Shows, T.B., and Curran, T. (1988). A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell *53*, 37–43.

Suske, G., Bruford, E., and Philipsen, S. (2005). Mammalian SP/KLF transcription factors: bring in the family. Genomics *85*, 551–556.

Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T., and Takada, S. (2006). Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. Dev. Cell *11*, 791–801.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.

Takahashi, M., Nakamura, Y., Obama, K., and Furukawa, Y. (2005). Identification of SP5 as a downstream gene of the beta-catenin/Tcf pathway and its enhanced expression in human colon cancer. Int. J. Oncol. *27*, 1483–1487.

Tallafuss, A., Wilm, T.P., Crozatier, M., Pfeffer, P., Wassef, M., and Bally-Cuif, L. (2001). The zebrafish buttonhead-like factor Bts1 is an early regulator of pax2.1 expression during mid-hindbrain development. Dev. Camb. Engl. *128*, 4021–4034.

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422–426.

Thomas, K.R., and Capecchi, M.R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature *346*, 847–850.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145–1147.

Thorpe, C.J., Weidinger, G., and Moon, R.T. (2005). Wnt/beta-catenin regulation of the Sp1-related transcription factor sp5l promotes tail development in zebrafish. Dev. Camb. Engl. *132*, 1763–1772.

Tong, L.M., Fong, H., and Huang, Y. (2015). Stem cell therapy for Alzheimer's disease and related disorders: current status and future perspectives. Exp. Mol. Med. *47*, e151.

Ton-That, H., Kaestner, K.H., Shields, J.M., Mahatanankoon, C.S., and Yang, V.W. (1997). Expression of the gut-enriched Krüppel-like factor gene during development and intestinal tumorigenesis. FEBS Lett. *419*, 239–243.

Tran, T.H., Wang, X., Browne, C., Zhang, Y., Schinke, M., Izumo, S., and Burcin, M. (2009). Wnt3a-induced mesoderm formation and cardiomyogenesis in human embryonic stem cells. Stem Cells Dayt. Ohio *27*, 1869–1878.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. *28*, 511–515.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. *7*, 562–578.

Treichel, D., Becker, M.B., and Gruss, P. (2001). The novel transcription factor gene Sp5 exhibits a dynamic and highly restricted expression pattern during mouse embryogenesis. Mech. Dev. *101*, 175–179.

Treichel, D., Schöck, F., Jäckle, H., Gruss, P., and Mansouri, A. (2003). mBtd is required to maintain signaling during murine limb development. Genes Dev. *17*, 2630–2635.

Ulloa, F., and Martí, E. (2010). Wnt won the war: antagonistic role of Wnt over Shh controls dorso-ventral patterning of the vertebrate neural tube. Dev. Dyn. Off. Publ. Am. Assoc. Anat. *239*, 69–76.

Umbas, R., Isaacs, W.B., Bringuier, P.P., Schaafsma, H.E., Karthaus, H.F., Oosterhof, G.O., Debruyne, F.M., and Schalken, J.A. (1994). Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res. *54*, 3929–3933.

Vescovi, A.L., Parati, E.A., Gritti, A., Poulin, P., Ferrario, M., Wanke, E., Frölichsthal-Schoeller, P., Cova, L., Arcellana-Panlilio, M., Colombo, A., et al. (1999). Isolation and cloning

of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp. Neurol. *156*, 71–83.

Wakeman, D.R., Redmond, D.E., Dodiya, H.B., Sladek, J.R., Leranth, C., Teng, Y.D., Samulski, R.J., and Snyder, E.Y. (2014). Human neural stem cells survive long term in the midbrain of dopamine-depleted monkeys after GDNF overexpression and project neurites toward an appropriate target. Stem Cells Transl. Med. *3*, 692–701.

Wang, L., Wei, D., Huang, S., Peng, Z., Le, X., Wu, T.T., Yao, J., Ajani, J., and Xie, K. (2003). Transcription factor Sp1 expression is a significant predictor of survival in human gastric cancer. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *9*, 6371–6380.

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J.B., Nishikawa, S., Nishikawa, S., Muguruma, K., et al. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol. *25*, 681–686.

Weidinger, G., Thorpe, C.J., Wuennenberg-Stapleton, K., Ngai, J., and Moon, R.T. (2005). The Sp1-related transcription factors sp5 and sp5-like act downstream of Wnt/beta-catenin signaling in mesoderm and neuroectoderm patterning. Curr. Biol. CB *15*, 489–500.

Wikramanayake, A.H., Hong, M., Lee, P.N., Pang, K., Byrum, C.A., Bince, J.M., Xu, R., and Martindale, M.Q. (2003). An ancient role for nuclear beta-catenin in the evolution of axial polarity and germ layer segregation. Nature *426*, 446–450.

Willert, K., Brink, M., Wodarz, A., Varmus, H., and Nusse, R. (1997). Casein kinase 2 associates with and phosphorylates dishevelled. EMBO J. *16*, 3089–3096.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448–452.

Woodhead, G.J., Mutch, C.A., Olson, E.C., and Chenn, A. (2006). Cell-autonomous beta-catenin signaling regulates cortical precursor proliferation. J. Neurosci. Off. J. Soc. Neurosci. *26*, 12620–12630.

Worringer, K.A., Rand, T.A., Hayashi, Y., Sami, S., Takahashi, K., Tanabe, K., Narita, M., Srivastava, D., and Yamanaka, S. (2014). The let-7/LIN-41 pathway regulates reprogramming to human induced pluripotent stem cells by controlling expression of prodifferentiation genes. Cell Stem Cell *14*, 40–52.

Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., and Carpenter, M.K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat. Biotechnol. *19*, 971–974.

Xu, J., Lü, B., Xu, F., Gu, H., Fang, Y., Huang, Q., and Lai, M. (2008). Dynamic down-regulation of Krüppel-like factor 4 in colorectal adenoma-carcinoma sequence. J. Cancer Res. Clin. Oncol. *134*, 891–898.

Yang, C., Al-Aama, J., Stojkovic, M., Keavney, B., Trafford, A., Lako, M., and Armstrong, L. (2015). Concise Reviews: Cardiac Disease Modeling Using Induced Pluripotent Stem Cells. Stem Cells Dayt. Ohio.

Yao, J.C., Wang, L., Wei, D., Gong, W., Hassan, M., Wu, T.-T., Mansfield, P., Ajani, J., and Xie, K. (2004). Association between expression of transcription factor Sp1 and increased vascular endothelial growth factor expression, advanced stage, and poor survival in patients with resected gastric cancer. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *10*, 4109–4117.

Yoon, Y., Huang, T., Tortelote, G.G., Wakamiya, M., Hadjantonakis, A.-K., Behringer, R.R., and Rivera-Pérez, J.A. (2015). Extra-embryonic Wnt3 regulates the establishment of the primitive streak in mice. Dev. Biol.

Yoshioka, S., King, M.L., Ran, S., Okuda, H., MacLean, J.A., McAsey, M.E., Sugino, N., Brard, L., Watabe, K., and Hayashi, K. (2012). WNT7A regulates tumor growth and progression in ovarian cancer through the WNT/β-catenin pathway. Mol. Cancer Res. MCR *10*, 469–482.

Young, J.E., and Goldstein, L.S.B. (2012). Alzheimer's disease in a dish: promises and challenges of human stem cell models. Hum. Mol. Genet. *21*, R82–R89.

Zannetti, A., Del Vecchio, S., Carriero, M.V., Fonti, R., Franco, P., Botti, G., D'Aiuto, G., Stoppelli, M.P., and Salvatore, M. (2000). Coordinate up-regulation of Sp1 DNA-binding activity and urokinase receptor expression in breast carcinoma. Cancer Res. *60*, 1546–1551.

Zecca, M., Basler, K., and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. Cell *87*, 833–844.

Zeng, Y.A., and Nusse, R. (2010). Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. Cell Stem Cell *6*, 568–577.

Zhang, Y., and Chen, H. (2011). Genistein attenuates WNT signaling by up-regulating sFRP2 in a human colon cancer cell line. Exp. Biol. Med. Maywood NJ *236*, 714–722.

Zhang, S.C., Wernig, M., Duncan, I.D., Brüstle, O., and Thomson, J.A. (2001). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat. Biotechnol. *19*, 1129–1133.

Zhu, C., Wang, Q., Zhou, J., Liu, H., Hua, F., Yang, H., and Hu, Z. (2012). The mineralocorticoid receptor-p38MAPK-NFκB or ERK-Sp1 signal pathways mediate aldosterone-stimulated inflammatory and profibrotic responses in rat vascular smooth muscle cells. Acta Pharmacol. Sin. *33*, 873–878.