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ANALYSIS OF DIFFERENTIATION CAPACITY OF CFP1 NULL EMBRYONIC STEM CELLS

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMPD	2-Amino-2-methyl-1,3-propanediol
β-ME	Beta- Mercaptoethanol
β-MHC	beta- myocin heavy chain
BMP	bone morphogenic proteins
bp	base pair
°C	Celsius
cDNA	complementary deoxyribonucleic acid
Cfp1	CXXC Finger Protein 1
cm	centimeter
CpG	cytosine phosphate guanine
CREB	cAMP response element-binding protein
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle media
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dpc	days post coitus
EB	embryoid body
EDTA	Ethylenediaminetetraacetic acid
ES	embryonic stem
FAD	flavin adenine dinucleotide
FBS	Fetal Bovine Serum
FRV	Fast Red Violet
GFP	Green Fluorescent Protein
GSK	glycogen synthase kinase
H	histone
h	hour
H1	histone protein 1
HAT	histone aceytltransferase
HDAC	histone deaceytlase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HKMT	histone lysine methyltrasferase
HMT	histone methyltrasferase
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IL-6	interleukin-6

iPS	induced pluripotent stem cells
JAK	Janus kinase
K	lysine
kb	kilobase
LIF	Leukemia Inhibitory Factor
LSD	lysine specific demethylases
LXC	leukemia-associated protein
M	molar, moles per liter
MAPK	mitogen-activated protein kinase
MBD	methyl binding domain
$MgCl_2$	magnesium chloride
µg	microgram
µm	mircometer
µl	microliter
me	methyl
min	minute
mM	millimolar
ml	milliliter
MLL	mixed lineage leukemia
mRNA	messenger ribonucleic acid
NKE	Nkx2.5 binding elements
nm	nanometer
ng	nanogram
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PHD	plant homeodomain
pmol	picomole
PI3K	phosphoinositide 3- kinase
PRMT	protein arginine methyltransferase
R	arginine
RA	retinoic acid
RAR	retinoic acid receptors
RARE	retinoic acid response element
RNA	ribonucleic acid
RT-PCR	reverse transcript polymerase chain reaction
rpm	revelations per minute
RXR	retinoid X receptor
SAM	s-adenosyl methionine
sec	second
SET	Su(var)3-9, Enhancer of Zeste, trithorax
SHP	Src homology region 2 domain- containing phosphatase
SID	Set1 interaction domain
SOCS	Suppressor of cytokine signaling 3
STAT	Signal Transducer and Activator of Transcription
TBE	Tris-Borate-EDTA

TDG	thymine DNA glycosylases
temp	temperature
TET	ten eleven translocation
TGF-β2	transforming growth factor- beta 2
U	unit
WT	wild-type
Y	tyrosine

ABSTRACT

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Epigenetics is defined as “the study of stable, often heritable, changes that influence gene expression that are not mediated by DNA sequence” (Fingerman et al., 2013). Epigenetic marks such as covalent histone modifications and DNA methylation are important for maintaining chromatin structure and epigenetic inheritance. Several proteins have been found to bind and/ or regulate epigenetic marks. One such protein, CXXC finger protein 1 (Cfp1) is an important chromatin regulator that binds to unmethylated CpG islands. It has been found to be essential for mammalian development. Mice lacking Cfp1 exhibit an embryonic-lethal phenotype. However, the function of Cfp1 can be studied using Cfp1 Null mouse ES cells, which are viable. Thus far, Cfp1 has been shown to be important for cell growth, cytosine methylation, histone modifications, subnuclear localization of Set1A histone H3K4 methyltransferase, and cellular differentiation. When Cfp1 Null ES cells are induced to differentiate by removal of Leukemia Inhibitory Factor (LIF), the cells are not able to turn off pluripotency markers such as Oct4 and alkaline phosphatase and fail to express differentiation markers

such as Gata4 and Brachyury. In this study, we used established protocols to further examine the differentiation capacity of Cfp1 Null cells. Specifically, we tested the ability of Cfp1 Null ES cells to retain stem cell properties in the absence of LIF, differentiate into cardiomyocytes in the presence of TGF-β2 and differentiate into neuron precursors in the presence of retinoic acid (RA). While the differentiation effects of RA were inconclusive, Null cells were able to start differentiating in the absence of LIF, either as individual cells or EBs, and the presence of TGF-β2 when seeded on gelatin coated tissue culture dishes. However, no difference was seen between cells treated without LIF and those treated with TGF-β2. In both conditions, only a small portion of cells were able to differentiate, while the majority of the cell population retained stem cell characteristics. Cell growth and the differentiation capacity of Cfp1 Null cells were also compromised in comparison to WT cells. Thus, further supporting the need for the correct epigenetic patterns maintained by Cfp1 during cellular differentiation.

CHAPTER 1. INTRODUCTION

1.1 Introduction

In 1942 Conrad Waddington coined the term “epigenetics” to describe a developmental complex that connected the genotype to the phenotype (Waddington, 2012). As research in the field has continued to expand, so has its concepts and definition. Most recently, epigenetics has been defined as “the study of stable, often heritable, changes that influence gene expression that are not mediated by DNA sequence” (Fingerman et al., 2013). These changes can be identified as DNA modifications, histones modifications, or noncoding RNAs. While many of these modifications are inherited via mitosis or meiosis, they can also be erased or modified through developmental or environmental cues throughout an individual’s life (Feil and Fraga, 2011; Fingerman et al., 2013; Ng and Gurdon, 2008; Teperino et al., 2013). However, no matter how these modifications are acquired, they play a critical role in regulating chromatin structure, thereby affecting several fundamental biological regulatory mechanisms such gene expression, DNA repair, recombination, and replication (Kouzarides, 2007). Without this higher ordered control, misregulated chromatin

can lead to disease, cancer, metabolic disorders and developmental defects (Dawson and Kouzarides, 2012; Feinberg, 2007; Teperino et al., 2013).

1.2 Chromatin Structure

The nucleus of higher eukaryotic cells contain about 2 meters of DNA that condense 10,000 fold into structurally organized chromosomes of about 1.5 µm in diameter. In order for this to occur, DNA is assembled into a hierarchy of chromatin through protein-DNA interactions (Lodén and van Steensel, 2005). The basic units of chromatin are known as nucleosomes. Each nucleosome is composed of about one-hundred forty-seven base pairs of DNA wrapped 1.7 turns around an octomer containing pairs of histone proteins H2A, H2B, H3, and H4. These repeating units make up the 11 nm “beads on a string” model. Small fragments of linker DNA and histone protein 1 (H1) condense this model further into a 30 nm fiber. Continual looping and nuclear scaffolding of the 30 nm fiber eventually creates the 1.5 µm chromosome (as reviewed by (Benbow, 1992; Cook, 1995).

Each level of higher-ordered chromatin is involved in gene regulation (Lodén and van Steensel, 2005). Chromatin can be broadly divided into two categories: transcriptionally active euchromatin or transcriptionally inactive heterochromatin. Euchromatin is associated with the lower-ordered, “open” 11 nm chromatin, whereas heterochromatin is defined as a region of condensed, tightly packed 30 nm chromatin (Bártová et al., 2008). Heterochromatin and

euchromatin can also be distinguished by different epigenetic modifications, histone variant composition, and recruitment of DNA binding proteins. Heterochromatin is associated with H3K9 and H3K27 trimethylation, low levels of acetylation, and cytosine methylation. It is formed by processes such as RNA interference, histone tail deacetylation, histone methylation, and DNA methylation. The nucleosomes in heterochromatin are substituted with histone variants such as H2A.X and histone 3 CENP-A. In contrast, euchromatin is associated with high levels of H3K9 acetylation, H3K4 di- and trimethylation, standard histones such as H3 and H4, and transcription machinery such as RNA polymerase (Bártová et al., 2008; Rasmussen, 2003).

Heterochromatin makes up most of the mammalian genome and can be further divided into two variations, constitutive and facultative. While both are associated with repressive chromatin, constitutive heterochromatin is associated with permanently silent regions in all cell types including telomeres, centromeres, and repetitive elements and is important for genome stability. In contrast, facultative heterochromatin is located around promoters and is important for silencing regions of euchromatin in some cell types and not in others. An example is X-chromosome inactivation (Hsieh and Fischer, 2005; Oberdoerffer and Sinclair, 2007; Rasmussen, 2003).

1.3 Histone Modification

Histones contain highly basic, unstructured amino terminal tails that extend from its globular core wrapped in DNA (Stephens et al., 2013). The tails, as well as the core, can serve as sites for several known posttranslational, covalent modifications such as methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation. Less known modifications include protein isomerization, deimination, ADP ribosylation, crotonylation, butryrlation, glycosylation, and several others have been identified as reviewed by (Hsieh and Fischer, 2005; Kouzarides, 2007; Tan et al., 2011). Together these modifications are known as the histone code and serve to control DNA mechanisms such as replication and repair as well as gene expression and chromatin assembly (Kouzarides, 2007). They do so by altering the net charge of histones or by disrupting histone-DNA and histone-histone interactions through the recruitment of nonhistone proteins or enzymes (Kouzarides, 2007; Tan et al., 2011).

Each histone modification plays a specific role in regulation and are added or removed by a higher regulation of control. Acetylation, for one, has been found to regulate transcription, chromosome structure, and DNA repair by neutralizing the basic charge on lysine residues. Marks are mainly added to lysine residues on N-terminal tails of H3 and H4 by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). Both enzymes are found associated with multi-protein complexes (Dawson and Kouzarides, 2012; Kouzarides, 2007).

Often acetylation marks are read by proteins containing a conserved bromodomain (Dawson and Kouzarides, 2012).

Methylation marks, on the other hand, do not change the charge of the residues they attach to. They can be found attached to either arginine, lysine, or histidine residues. Specifically, lysine residues can either be mono-, di-, or trimethylated. Depending on the combination of methylation marks, chromatin can be associated with the formation of heterochromatin, euchromatin, RNA polymerase II elongation (Kouzarides, 2007). For example, trimethylation of H3K9 is associated with heterochromatin and gene repression, whereas H3K9me1 has been associated with gene activation. As talked about in the previous section, methylation of H3K9, H3K27 and H3K20 are associated with heterochromatin regions, while methylation at H3K4, H3K36, and H3K79 corresponds with euchromatin (Hsieh and Fischer, 2005; Kouzarides, 2007). Methylation marks are laid down by either histone lysine methyltransferases (HKMT) containing a SET (Su(var)3-9, Enhancer of Zeste, trithorax) domain, such as SET1A, SET1B, MLL 1-5, and SUV38H1-2, or by a protein arginine methyltransferase (PRMT) including PRMT 4 and 5. Often the methyltransferases are very specific to the marks they add. For example SET1A/B and MLL 1-5 are associated with H3K4 methylation, whereas PRMT4 is associated with H4R3 methylation (Kouzarides, 2007). There are two classes of histone lysine demethylases: lysine specific demethylases (LSD1) and Jumonji demethylases. LSD1 demethylases remove methylation marks through an oxidation reaction with the cofactor FAD, but are limited to mono- and

dimethylation. Jumonji demethylases contain a JmjC domain that can demethylate all methylation states via oxidation and radical attack as reviewed by (Dawson and Kouzarides, 2012). Methylation marks serve as binding sites for proteins containing a chromo- or PHD domains.

Phosphorylation marks occur on serine, tyrosine, and threonine residues within core and variant histones and are important for chromosome condensation, DNA repair, apoptosis, replication, and transcription (Dawson and Kouzarides, 2012; Kouzarides, 2007). Like acetylation marks, phosphorylation marks alter the charge of the protein for which they are associated. They are regulated by protein kinases and phosphatases. Both are specific to the marks they add or remove, respectively. For example, histone variant H2AX is phosphorylated by a phophoinositol 3-kinase in response to a double stranded break on DNA (Kouzarides, 2007).

Collectively, modifications have been found on over 60 different histone residues and can come in different forms, much like the 3 methylation states of lysine (Kouzarides, 2007). Therefore, it is not surprising that the abundance of histone modifications allows them to communicate or crosstalk. Often it is seen that a modification on one site can change the modification on another; ultimately, this can affect either near or distant sites on histones or DNA.

1.4 DNA Methylation

DNA methylation plays an important role in the maintenance and stability of the genome as well as transcription regulation and development. Its function was first proposed independently by Riggs and Holliday and Pugh in 1975. They described DNA methylation as a heritable mark that is established on the DNA *de novo* and could later be maintained though somatic cell divisions (Holliday and Pugh, 1975; Riggs, 1975). The most common DNA methylation mark is 5-methycytosine, in which a methyl group is covalently added to the carbon 5 position on cytosine. About 70% of methylated cytosines are associated with CpG dinucleotides, or the linkage of cytosine to guanine via a phosphate group on the same DNA stand, while the other 30% of is thought to be associated with CpA, CpT or CpC regions (Ehrlich et al., 1982; Ramsahoye et al., 2000; Teperino et al., 2013).

CpG dinucleotides are found in about 1 kb long clusters within the genome and are referred to as CpG islands. According to Illingworth et al. 2010, about 50% of CpG islands are located near transcriptional start sites, while the rest are distributed between gene bodies and annotated genes. However, it is thought that the distribution of CpG dinucleotides is underrepresented due to the tendency of spontaneous or enzymatic deamination of unmethylated cytosine to uracil and 5-methylcytosine to thymine. Uracil, a RNA molecule, is easily recognized and repaired by DNA repair mechanisms, while the deamination of 5-methylcytosine can be repaired thymine DNA glycosylases (TDG) and MBD4 as

reviewed (Cortázar et al., 2007). Even though there are repair mechanisms for both, it has been shown that the rates of 5-methlycytosine deamination are about 3 times faster than that of cytosine (Cortázar et al., 2007; Shen et al., 1994). Ultimately, suggesting that the overall reduction in CpG dinucleotides is due to deamination occurring faster than the rate of repair, localization of TDG and MBD proteins or environmental stress causing damage to TDG binding (Cortázar et al., 2007). Mismatched base pairs and improperly methylated CpG sites could ultimately lead to an alteration in gene expression causing altered phenotypes and disease as reviewed by (Bird, 1987; Stephens et al., 2013).

Of the CpG islands located in the genome, most located within promoter regions of somatic cells are mainly hypomethylated. These sites are characterized by having nucleosome depleted regions, and the nucleosomes flanking these regions contain histone variants H2A.Z and H3K4me3 marks associated with active transcription (Jones, 2012). However, when CpG sites are methylated they block transcription and contribute to long term gene silencing. Genomic mapping by bisulfite treatment, enzymatic digestion with HpaII and MspI, microarray, and immunoprecipitation are a few techniques that have located DNA methylation to in active X-chromosomes, centromeres, telomeres, imprinted regions, and other repeat elements as reviewed by (Dawson and Kouzarides, 2012; Jones, 2012).

DNA methylation leads to transcriptional repression by interfering with transcription factor binding and recruiting methyl binding proteins. One example of DNA methylation controlling expression involves the role of CTCF in

imprinting. On the maternal copy of the Igf2 gene, CTCF acts as an insulator by binding to the region in between the promoter and the enhancer, which results in gene silencing. In contrast, CTCF binding on the paternal Igf2/H19 locus is inhibited by DNA methylation; thereby allowing IgF2 activation (Bell and Felsenfeld, 2000; Hark et al., 2000). The binding of other transcription factors such as E2F family proteins, involved in cell cycle control, and cAMP response element-binding protein (CREB) have also been shown to be inhibited by cytosine methylation, which again causes repression (Campanero et al., 2000; Iguchi-Ariga and Schaffner, 1989).

The second way DNA methylation leads to repression is by attracting methyl binding family proteins. MeCP2, methyl binding protein 1 (MBD1), MBD2, MBD3, and MBD4 make up a family of proteins that contain conserved methyl binding domains and are often associated with HDAC recruitment (Hendrich and Bird, 1998; Nan et al., 1998; Zhang et al., 1999). Specifically, MBD2 and MBD3 are components of the MeCP1 and Mi-2 histone deacetylase complexes (Ng et al., 1999; Wade et al., 1999). MeCP2 also enforces transcriptional repression through the recruitment of ski family proteins, Sin3a, NCoR, and c-Ski (Kokura et al., 2001), while MBD1 enforces repression through its association with SETDB1, H3K9 methylase (Sarraf and Stancheva, 2004). MBD4 also plays a role in DNA repair (Hendrich et al., 1999). Kaiso, ZBTB4, and ZBTB38 are other methyl binding proteins, but instead of a methyl binding domain, they recognize cytosine methylation through zinc finger domains (Filion et al., 2006; Prokhortchouk et al., 2001).

Unlike DNA methylation at promoters, methylation within the gene body has been found to be associated with transcriptional elongation. The thought is that this methylation serves to keep repetitive and transposable elements within the gene bodies silent while still allowing the host gene to be transcribed (Jones, 2012). Recent studies have also suggested that gene body regulation may also play a role in splicing due to the higher methylation content at exons compared to introns (Laurent et al., 2010). CpG islands located within gene bodies, known as orphan promoters, are proposed to be part of promoters during early stages of development that escaped methylation (Illingworth et al., 2010).

Methylation of DNA is regulated by enzymes known as DNA methyltransferases (DNMT) and demethyltransferases. DNMTs use the methyl donor s-adenosyl methionine (SAM) to methylate cytosine. There are five members of the DNA methyltransferase protein family: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L (Espada and Esteller, 2010) . DNMT1 was originally identified as a maintenance methyltransferase for its role in maintaining symmetrically methylated sites. When DNA replicate the parent strand retains its methylation pattern while the newly formed daughter strand is unmethylated. DNMT1 enzymes then recognize and methylate the hemimethylated sites (Gruenbaum et al., 1982; Li et al., 1992). DNMT3a and DNMT3b, in contrast, can methylate both unmethlyated and hemimethylated sites. However, these methyltransferases are very important for establishing *de novo* methylation patterns (Okano et al., 1999) pre-implantation after global cytosine methylation patterns are erased during early embryogenesis (Monk et al., 1987).

Recent studies have suggested that the mechanisms of DNMT1 and DNMT3s are not separated, instead it is believed that they are both important for maintenance and *de novo* methylation. According to Lin, I.G. et al 2002, DNMT3 enzymes favor the methylation of CpG sites flanked by pyrimidines; therefore suggesting that methylation is only introduced to one strand of DNA. The creation of hemimethylated sites would then provide appropriate substrates for DNMT1 (Jeltsch and Jurkowska, 2014). Cooperation of DNMTs has been further noted (Fatemi et al., 2002; Ramsahoye et al., 2000), suggesting that DNMT1 has *de novo* methylation activity at unmethylated sites and fixes the methylation sites set up by DNMT3 enzymes. Conversely, *de novo* DNMTs are needed for maintenance methylation. In the presence of DNMT1, the loss of DNMT3a and DNMT3b in ES cells leads to a reduction in methylation at repetitive elements and single copy genes (Chen et al., 2003). It is also suggested that DNMT3 plays a role in methylating non-CpG sites (Ramsahoye et al., 2000). No matter what the role of DNMTs, they are critical for normal development. The genetic mice knockout of any of DNMTs results in a genomic loss of methylation and lethality. Specifically, deletion of DNMT1 and DNMT3b is embryonic lethal, while mice with a DNMT3a deletion are runted and die within 4 weeks of age (Li et al., 1992; Okano et al., 1999). In contrast, overexpression of DNMT1 causes hypermethylation, loss of imprinting and embryonic lethality (Biniszkiewicz et al., 2002).

Unlike DNMT1 and DNMT3 enzymes, DNMT2 has been shown to have low levels of methylation activity (Hermann et al., 2003), while Goll and

colleagues linked DNMT2 to cytosine methylation at aspartic acid transfer RNAs (Goll et al., 2006). DNMT3L is also different from DNMT3 enzymes, in such that is does not have catalytic methyltransferase activity. Instead DNMT3L has been shown to regulate the *de novo* methyltransferases (Chen et al., 2005; Kareta et al., 2006). DNMT3L has also been shown to have roles in imprinting, germ cells development, non-coding RNA-mediated epigenetic remodeling and retrotransposon silencing as reviewed (Liao et al., 2012).

While methyl groups are added to DNA via DNMTs, they are removed by demethylation indirectly through replication or by ten eleven translocation (TET) dioxygenases, activation-induced cytisine deaminase, TDG or MBD4 as mentioned (Jeltsch and Jurkowska, 2014; Jones, 2012). In summary, it is been shown that the oxidation of 5-methycytosine by TET enzymes leads to the formation of 5-hydroxymethylcytosine which can be passively eliminated by DNA replication or actively reverted back to unmethylated cytosine by further oxidizing into 5-formylcytosine or 5-carboxylcytosine. 5-formylcytosine or 5-carboxylcytosine and 5-hydroxymethylcytosine deamination counterpart 5-hydromethyluracil can then be removed by TDG-mediated base excision repair and MBD4 proteins as reviewed (Dawson and Kouzarides, 2012; Kohli and Zhang, 2013; Moréra et al., 2012). Overall, demethylation introduces a higher level of regulation of DNA methylation that may be important for development, genome stability, and transcriptional regulation.

Through overlapping functions and molecules, histone modifications and DNA methylation reinforce chromatin structure and gene expression. Using their

role in transcriptional repression as an example, DNA methylation of CpG dinucleotides by DNMT1 recruits MBD proteins which in turn recruit HDACs to remove acetylation marks on euchromatin (Hendrich and Bird, 1998). The absence of acetylation marks activates SUV39H, a histone lysine methyltransferase, to methylate H3K9. Heterochromatin protein 1 (HP1) can then bind to H3K9 through its chromodomain, which results in the propagation of heterochromatin spreading (Bannister et al., 2001).

1.5 CXXC finger protein 1 (Cfp1)

CXXC finger protein 1 (Cfp1), unlike DNA methyl binding proteins previously discussed, has an affinity for binding to unmethylated CpG islands and contributing to transcriptional activation. This protein, encoded by the CXXC gene, was found to be expressed in a wide variety of tissues throughout the human body; suggesting its importance in maintaining unmethylated CpG and euchromatin throughout the genome (Voo et al., 2000). Over the last 14 years, several studies have classified Cfp1 as an important epigenetic regulator that links both histone modification and DNA methylation.

Cpf1 is made up of several conserved domains including two plant homeodomains (PHD), a cysteine-rich CXXC domain, basic and acidic stretches, setd1 interaction domain (SID) and a coiled-coil domain (Butler et al., 2008; Voo et al., 2000). PHD domains are highly conserved domains found in an array of eukaryotic proteins (Aasland et al., 1995). Studies have linked these domains to

chromatin regulation and their involvement in recognizing and binding to methylated H3K4, H3K36, H3K9 and unmethylated H3R2 (Bienz, 2006; Cheng et al., 2013; Li et al., 2007; Shi et al., 2006; Shi et al., 2007). In ES cells, the N-terminal PHD1 domain of Cfp1 was found to be important for plating efficiency and the C-terminal PHD2 domain was found to be important for rescuing the carboxyl fragment activity of Cfp1 (Tate et al., 2009). However, the importance of either PHD domain in Cfp1 and whether or not they are associated with H3K4 methylation and transcription are currently being explored (Mahadevan, unpublished data).

Of the domains, the CXXC domain was found to be responsible for Cfp1's interaction with unmethylated CpG motifs (Lee et al., 2001). This interaction was later confirmed through chromatin immunoprecipitation (ChIP-seq), bisulfite analysis (Thomson et al., 2010), and X-ray crystallography (Xu et al., 2011). The recruitment of Cfp1 to CpG rich sequences and nucleation of H3K4 marks were found to be independent of RNA polymerase II (Thomson et al., 2010). Crystal structures of Cpf1 CXXC domain show that the CXXC domain possesses a crescent shape that can wedge into the major grove of the DNA helix. It was also noted that the domain has a preference for binding CpGG sites through its R213 and Y216 residues (Xu et al., 2011)

Cfp1 shares the CXXC domain with other chromatin-associated proteins including mixed-lineage leukemia (MLL), DNMT1, MBD1, leukemia-associated protein (LXC) and KDM2A (Ono et al., 2002; Tsukada et al., 2006; Voo et al., 2000). Like that of Cfp1, most other CXXC domains have a binding specificity for

unmethylated CpG motifs including those from MLL, KDM2A and DNMT1. MBD1, which binds to methylated CpG dinucleotides, contains three CXXC domains; of which only one binds to unmethylated CpGs and is associated with transcriptional repression (Jørgensen et al., 2004). MLL proteins, like Cfp1, bind to unmethylated DNA in major grooves (Cierpicki et al., 2010). This family of proteins in typically associated with leukemic translocations such as MLL-RBM15, MLL-LXC and MLL-ENL (Ma et al., 2001; Ono et al., 2002; Slany et al., 1998). In the case of MLL-AF9, its binding to CpGs it has been found to contribute to leukemogenesis (Cierpicki et al., 2010). KDM2A also known as JHDM1a or FBXL11, is a demethylase that favors transcriptional activation by removing H3K36 marks (Blackledge et al., 2010; Tsukada et al., 2006). The CXXC domain of DNMT1 has been shown to act as an auto-inhibitory domain by binding and protecting unmethylated CpG motifs to ensure only hemimethylated CpGs become methylated (Song et al., 2011).

Even though Cfp1 was originally identified in humans, several homologs have been found in other species such as *Drosophila, Caenorhabditis elegans, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Mus musculus and Danio rerio (Voo et al., 2000; Young et al., 2006).* Many of which share the same conserved domains as previously described. Specifically, mice have identical PHD, CXXC, coiled-coil, and basic domains and nearly identical acidic regions in comparison to human Cfp1 proteins (Carlone et al., 2002). However, lower eukaryotes such as yeast and worms are not involved in DNA methylation and

lack a CXXC domain, thus suggesting that Cfp1 may have an additional role other than cytosine methylation (Lee and Skalnik, 2005).

Further studies revealed that Cfp1 is a component of multiprotein Set1 histone H3K4 methyltransferase complex, which is analogous to the Set1/Compass complex found in yeast (Lee and Skalnik, 2005). While yeast only contain one Set1/Compass complex, humans have six including Set1A, Set1B and MLL1-4. Each of these complexes can methylate H3K4, but have nonredundant functions (Shilatifard, 2012). It has been shown that Cfp1 only interacts with Set1A and Set1B complexes and does not associate with any of the MLL complexes. Set1A and Set1B complexes are composed of methyltransferase enzymes setd1a and setd1b, respectively, as well as Wdr5, Wdr82, Ash2L, and Rbbp5 (Lee and Skalnik, 2005; Lee et al., 2007). The Wdr82 component was found to bind to the RNA recognition motif of the complex and tether it to RNA polymerase II (Lee and Skalnik, 2008).

Along with the function of Wdr82, Cfp1 also associates with promoter and euchromatic regions. Through confocal studies, Cfp1 co-localized with Setd1A in euchromatin 4’6 diaminidino-2-phenylindone (DAPI)-dim regions of the WT ES cell nuclei. In comparison, there was a 20-30% increase of Setd1A protein and H3K4 methylation in DAPI-bright heterochromatic regions in cells lacking Cfp1. Levels of histone modifications and localization can be restored with the introduction of Cpf1 expression vectors; therefore suggesting that Cfp1 is important for tethering Setd1a to euchromatin (Tate et al., 2010). Overall creating a model in which the SID domain and CXXC domain of Cfp1 are important for

binding the Set1A complex to unmethylated CpG dinucleotides, resulting H3K4 methylation on adjacent nucleosomes (Skalnik, 2010).

The SID domain and the CXXC DNA binding domain have been found to be very important for maintaining the function of Cfp1. Tate et al. 2009 showed that neither the CXXC domain or the SID domain are required to rescue the function of Cfp1. However, one must be retained in order for the full function of Cfp1 to be rescued. In other words, cytosine methylation can still occur without the CXXC domain and H3K4me3 marks can still occur without the SID domain. This finding enforces the importance of crosstalk between DNA methylation and histone modification (Tate et al., 2009).

Mice and zebrafish have been used as models to show that Cpf1 plays a critical role in mammalian development at several developmental stages. During embryogenesis, murine embryos disrupted for Cfp1 resulted in embryonic lethality. Further experiments found that the embryos died between 3.5-6.5 days post coitus (dpc), suggesting that embryos are able to survive to the blastocyst stage but fail to gastrulate (Carlone and Skalnik, 2001). Later in development, Cpf1 was also found to play a role in murine liver development and function (Mahadevan, unpublished data) as well as hematopoiesis in zebrafish and human leukemia cell lines. Zebrafish depleted for Cfp1 through antisense morpholino oligonucleotides resulted in runting, death, increased apoptosis, decreased cytosine methylation, decreased erythroid development, cardiac edema, and incomplete vasculature formation (Young et al., 2006). Human myeloid leukemia cell line (PLB-985) was depleted of Cpf1 through short hairpin

RNA. The reduction of Cfp1 protein levels resulted in reduced proliferation, differentiation and survival (Young and Skalnik, 2007).

To continue studying the importance of Cfp1 in early development, murine ES cell lines were successfully isolated from the inner cell mass of blastocysts lacking the CXXC gene (Null) (Carlone et al., 2005). While the establishment of this cell line showed that Cfp1 is not important for stem cell viability, the cells displayed an altered phenotype as compared to those containing one or both copies the CXXC gene (wild-type, WT). Null cells displayed an increased doubling time as a result from elevated rates of apoptosis, but exhibit a normal cell cycle distribution. WT phenotypes were recovered by the transfection of Null cells with a full length Cfp1 expression vector (Carlone et al., 2005).

Null cells are also characterized by a reduction in H3K9 methylation and exhibit an overall 4-fold increase in H3K4 methylation following the induction of differentiation (Lee and Skalnik, 2005). It is unlikely that the reduction of heterochromatin is due to the miss targeting of Setd1a since H3K4me3 marks colocalize with heterochromatic regions; ultimately suggesting that H3K4 marks are insufficient for chromatin remodeling (Tate et al., 2010). Moreover, H3K4 methylation is decreased at active promoters, while they are increased at ectopic sites. In contrast, H3K4 methylation is not affected at promoters of poised genes. This suggests that basal expression of H3K4 is regulated separately by other proteins such as MLL proteins (Clouaire et al., 2012).

Additionally, Cfp1 Null cells have reduced DNA methyltransferase activity. Carlone et. al 2005 revealed a 60-80% reduction in global DNA methylation. The

same pattern was specifically seen at repetitive elements minor satellites and IAP, single copy genes Rac2 and Pgk-2, and imprinted genes H19 and Igf2r. These results were consistent with the global reduction of cytosine methylation in zebrafish depleted of Cfp1 expression (Young et al., 2006). Furthermore, cells also exhibited 60% reduction in DNA methyltransferase activity at hemimethylated sites and a 50% reduction in DNMT1 proteins while *de novo* levels were not affected (Carlone et al., 2005). Additional studies revealed that Cfp1 directly interacts with DNMT1 independently of HMT activity (Butler et al., 2008). Clouaire et al. 2012 confirmed this by showing that H3K4 depletion does not lead to an increase in DNA methylation. The overall reduction in DNMT1 resulted from increased mRNA levels, decreased protein half-life, and a global decrease in protein synthesis; therefore suggesting a dysregulation in translation (Butler et al., 2008; Butler et al., 2009). Defects were shown to be rescued by the transfection of full length Cfp1 expression vector; thus, indicating that the altered phenotype and epigenetic modifications are due to the absence of Cfp1 (Carlone et al., 2005).

Apart from aberrant H3K4 targeting and DNMT1 reduction, Cfp1 Null ES cells also fail to differentiate in the absence of leukemic inhibitory factor (LIF) (Carlone et al., 2005). LIF is an important cytokine used in ES cell media to maintain mouse ES cell pluripotency as reviewed (Trouillas et al., 2009). As expected the removal of LIF caused Cfp1 Null cells to aggregate in suspension on bacteria dishes. However, the cell aggregates, also known as embryoid bodies, remained small and failed to produce outgrowths as compared to WT

cells. Null cells were also unable to turn off stem cell markers Oct4 and alkaline phosphatase and turn on germline and lineage specific markers such as Gata4 (endoderm), c-fms (myeloid), Brachyury (mesoderm), β-MHC (cardiac), and gp-IIB (megakaryocyte). Like many other experiments previously described, cell differentiation capacity was restored with the introduction of Cfp1 expression vector (Carlone et al., 2005).

Overall, Cfp1 plays an important role in chromatin structure, mammalian development, hematopoiesis, and cellular differentiation through the regulation of cytosine and histone modifications by mediating histone and cytosine methylation.

1.6 Focus of Study

The differentiation of ES cells down different cell lineages involves the formation of heterochromatin and epigenetic regulation of gene expression. Chromatin structure is mainly regulated by covalent modifications of histone and cytosine methylation. During the initiation of cellular differentiation, histones undergo a wave of deacetylation followed by a reprograming of acetylation marks. In addition, euchromatin marks such as H3K4 are erased, while heterochromatin marks such as H3K9 are increased and cytosine methylation is slightly increased (Lee et al., 2004). Differentiation into specific lineages is restricted to a uniquely expressed set of genes (Rasmussen, 2003).

Through the knowledge of specific genes and lineage pathways, ES cells have been used to generate an array of cells including hematocytes, neuronal cells, cardiomyocytes and many more as reviewed by (Murry and Keller, 2008). While it has been previously shown that Cfp1 Null cells fail to differentiate in the absence of LIF, the cells have never been forced to differentiate down a specific lineage pathway. In this thesis we take advantage of ES cell plasticity as well as established protocols to investigate the differentiation capacity of ES cells lacking Cfp1. We hypothesize that Cfp1 Null cells will remain undifferentiated in the presence of cytokines, growth factors, or intracellular signaling molecules. Specifically, we will examine the effects of LIF in rescuing the Null phenotype, TGF-β2 in cardiomyocyte differentiation, and retinoic acid (RA) in neuronal differentiation, respectively.

CHAPTER 2. METHODS

2.1 Cell Culture

2.1.1 Maintaining

Murine ES cells WT CCE Cfp1 +/+ cells (WT) and DS1-1 Cfp1-/- cells (Null) were used for all cellular experiments. Null cells were originally isolated from inner mass cells of 3.5 dpc blastocysts derived from matings between Cfp1 +/- mice as described by Carlone et al. 2005. Both cell lines were cultured on cell culture dishes coated in 0.1% gelatin in ES cell media composed of high-glucose Dulbecco's modified Eagle media (DMEM) supplemented with 20% Fetal Bovine Serum (FBS), 100 Units/ml penicillin/streptomycin, 2 mM L-glutamine, 1% Non-Essential Amino Acids, 1% Hank's balanced salt solution, 0.025% HEPES buffer solution, 100 nM β-Mercaptoethanol (β-ME) (Sigma), and leukemia inhibitory factor (LIF) (isolated from Chinese hamster ovarian cells). All products were purchased from GIBCO unless otherwise stated. Media was changed every other day and cells were split every 3-4 days up to 35 passages. Cells were maintained at 37°C with 5% CO_2.

2.1.2 Thawing

Cell were thawed from either liquid nitrogen stocks or -80°C stocks. Vials were thawed at 37°C and diluted into 5 ml of ES cell media. Cells were centrifuged for 4 min at 1200 rpm. Media was changed to remove traces of dimethyl sulfoxide (DMSO) and plated on 0.1% gelatin coated cell culture dishes.

2.1.3 Freezing

A plate of confluent cells was dissociated with 0.05% trypsin-EDTA (GIBCO) and pipetted into a single cell suspension. Cells were then centrifuged at 1200 rpm for 3 min. The media was removed and cells were resuspended in freezing media containing 40% ES cell media, 50% FBS and 10% DMSO. Cells were put into isopropanol containers and placed in -80°C. Once frozen, the cells were then moved to liquid nitrogen.

2.2 Leukemia Inhibitory Factor Reintroduction

2.2.1 Morphological Analysis of Embryoid Bodies

To induce cell aggregation and differentiation, about 30,000 ES cells were cultured in ES cell media without LIF and plated as hanging drops (500 cells/drop) on the lids of tissue culture dishes for 2 days. Cell aggregates, known

as embryoid bodies (EBs), were then transferred to the bottom of either 10 or 15 cm bacteria culture dishes and cultured in suspension for an additional 3 to 18 days in media without LIF. Half the media was carefully changed every day so not to disturb or remove EBs. At 5, 10, or 20 days after initial plating, embryoid bodies were analyzed at 4X magnification.

2.2.2 Dissociation and Reseeding of Embryoid Bodies in the Presence or Absence of LIF

In order to assess the effects of reintroducing LIF back to the cells, EBs at either day 5, 10, and 20 from initial plating without LIF were collected and dissociated with trypsin and mild titration to create a suspension mixture of single cells and small clumps. Cells were then seeded on tissue culture dishes treated with 0.1% gelatin in either ES cell media containing or lacking LIF. These cells were then maintained for 2 more passages in the indicated media to monitor growth and morphology. Media was changed every other day. Before each passage (4 days and 7 days after initial reseeding) cells were analyzed at 4X and 20X magnification.

2.2.3 Alkaline Phosphatase Staining

Cell differentiation was assayed histochemically by analyzing alkaline phosphatase activity via an Alkaline phosphatase leukocyte detection kit (Sigma). About 150,000-200,000 cells were plated on 0.1% gelatin coated 3.5 cm gridded tissue culture dishes. Cells were allowed to recover overnight. Following the manufacturer's protocol, briefly, cells were washed twice in phosphate buffered saline (PBS) and then fixed with citrate (18 mM citric acid, 9 mM sodium citrate, 12 mM sodium chloride with surfactant, buffered at pH 3.6) for 35 sec. The cells were washed with sterile distilled H_2O for 45 sec at room temperature. For 15 min cells were rocked in an alkaline phosphatase staining solution; a diazonium salt solution. The solution was made by mixing 1 ml of 0.1 M sodium nitrate solution and 1 ml of Fast red violet (FRV)-Alkaline solution (5 mg/ml FRV LB base in 0.4 M hydrochloric acid, with stabilizer) to 45 ml of deionized water. Then after 2 min, 1 ml of Naphthol AS Bl Alkaline solution (4 mg/ml of Naphyhol AS-Bl phosphate in 2 M 2-Amino-2-methyl-1,3-propanediol [AMPD] buffer, pH 9.5) was added to the diluted diazonium salt solution. Cells were washed with sterile distilled H_2O for 2 min and analyzed. Undifferentiated, alkaline phosphatase positive cells stain red, while differentiated cells remain unstained. At least 300 cells were counted on each plate. Averages were based on a 3 to 5 independent experiments.

Embryoid bodies from 5, 10, and 20 days without LIF were moved to conical tubes. EBs were allowed to settle at the bottom. Media was removed and

separately, the EBs and their original bacteria dish were washed with PBS. EBs as well as the cells that had attached and proliferated on the dish were dissociated with trypsin. Cells reseeded in ES cell media with or without LIF were stained the day after (Days 6, 11, and 21 from original plating without LIF) and each time the reseeded cells were split (5 and 8 days after the original reseeding). For each staining, images were collected at 20X magnification.

2.2.4 Growth Curve

Growth curves were set up in order to determine the growth rate of the reseeded cells with or without LIF. Upon splitting the reseeded cells a second time, exponentially growing asynchronous cells were washed with PBS, dissociated, stained with Trypan blue (MP Biomedicals) and counted with a hemocytometer (Bright-Lite). Ten-thousand cells were plated in triplicate into 6-well tissue culture dishes treated with 0.1% gelatin. On the second, third, and fourth days after plating, cells were collected and counted in triplicate. Three independent experiments were counted and averaged. Doubling time was calculated by the equation: Doubling time= ln(2)(time in hour)/ ln(average cell count day 4/ average cell count day 3).

2.2.5 Apoptosis Analysis

To determine the rate of apoptosis in the reseeded cells with or without LIF, Vybrant Apoptosis Assay Kit #2 (Molecular probes, Invitrogen) was followed as described by the manufacturer. Briefly, cells were collected in a 15 ml conical tube, washed in cold PBS, and dissociated with trypsin. Cells were centrifuged for 2 min at 1200 rpm between each step. The supernatant was discarded and cells were resuspended to a concentration of about 1 x 10^6 cells/ml in a 1X Annexin-Binding Buffer, prepared by diluting a 5X stock solution (15 ml of 50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4) in deionized water. About 100 µl of cells were incubated for 15 min with 5 µl of Alexa Fluor 488 annexin V (250 µl of a solution in 25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, plus 0.1% bovine serum albumin (BSA)) and 1 µl of 100 µg/ml propidium iodide solution (5 µl of a 1.5 mM solution in distilled water and 45 µl of 1X Annexin-Binding Buffer). After incubation, stained cells were gently mixed with an additional 400 µl of Annexin-Binding Buffer. Cells were then analyzed by flow cytometry (FACSCalibur APC, IU Medicine). Fluorescence emission were measured at 530 nm for annexin V positive cells and >575 nm for PI positive cells. Once the data was gated, annexin V positive cells were detected in the lower right quadrant and represented apoptotic cells. In contrast, PI positive cells are detected in the Upper Left quadrant and represented necrotic cells. Dead cells emit both green and red fluorescence and are detected in the Upper Right quadrant, while viable cells are detected in the Lower Left quadrant indicating little to no fluorescence.

2.2.6 Statistical Analysis

Experimental data was collected from at least 3 independent experiments and an average was calculated for each condition. The standard error was calculated by dividing the standard deviation by the square root of the number of replicates, which is graphically denoted by error bars. A two sample t-test assuming equal variance was used to determine the statistical significance of means for the alkaline phosphatase experiments. A p-value <0.05 was interpreted as statistically significant. Graphs and analyses were performed using Microsoft Excel.

2.3 Embryonic Stem Cell Differentiation into Cardiomyocytes

2.3.1 Morphological Analysis of Embryoid Bodies

Methods were based on those previously described (Boheler et al., 2002; Kumar and Sun, 2005). To induce differentiation, ES cells were dissociated and cultured in media without LIF in hanging drops (~500 cells/ drop) on the lids of tissue culture dishes. The bottom of the dishes contained 5 ml of PBS. After 2 days in hanging drops, embryoid bodies were transferred to bacteria dishes and grown in suspension for an additional 3 days in media without LIF supplemented with either 0 or 8 ng/ml of transforming growth factor- beta 2 (TGF-β2) (GIBCO). Embryoid bodies (6-10/well) were plated on 12-well tissue culture dishes with

0.1% gelatin. Media was changed daily. EB morphology was closely analyzed for a beating/contracting phenotype. Cells were collected for mRNA expression on day 6, 12, and 18 from initial removal of LIF.

2.3.2 Alkaline Phosphatase Staining

At days 5, 11, and 17 plated EBs were dissociated, reseeded and analyzed as those previously described in 2.2.3 above. Averages reported are based on 3 to 5 separate experiments.

2.3.3 Reverse Transcript-PCR

Before detection of lineage specific mRNA expression during *in vitro* ES cell differentiation, total RNA was isolated from cells via RNeasy Kit (Qiagen) by manufacturer's protocol. Briefly, one well of a 12-well dish of EBs was collected with the addition of 350 µl of RTL buffer supplemented with 10 µl β-ME per 1 ml of RTL buffer and stored at -80°C. When all time points were collected (Day 0, 6, 12, 18 from initial removal of LIF), samples saved in RTL buffer were thawed and homogenized further by QiaShredder column (Qiagen). Cells were centrifuged for 2 min at maximum speed. One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. Each sample was then added to an RNeasy mini column and centrifuged for 30 sec at 10,000 rpm. Flow through was discarded and column was washed with 350 µl of RW1 buffer and

centrifuged for 30 sec at 10,000 rpm. DNAse I incubation mix (80 µl), prepared by adding 10 µl of DNAse I stock solution (1500 Units in 550 µl RNAse-free H_2O) to 70 µl RDD buffer (Qiagen), was directly added to the column's membrane for 15 min at room temperature. RW1 buffer (350 µl) was pipetted into the column and centrifuged for 30 sec at 10,000 rpm. Column was transferred to a new collection tube and 500 µl of RPE buffer was added and centrifuged for 15 sec at 10,000 rpm. Flow through was discarded and another 500 µl of RPE buffer was added and centrifuged for 2 min at 10,000 rpm. Flow through was discarded again and the column was centrifuged for an additional minute at 10,000 rpm. Thirty microliters of diethylpyrocarbonate (DEPC) treated H_2O was added to the column's membrane and allowed to sit for 1 min. RNA was eluted into a new collection tube at 10,000 rpm for 1 min.

Total RNA was next diluted to the same concentration and about 500 ng was reversed transcribed into cDNA via iScript cDNA synthesis kit (BioRad) by manufacturer's protocol. Briefly, RNA was combined with 5X iScript reaction mix, iScript reverse transcriptase, and DEPC treated water to a volume of 20 µl reaction. Reaction was place in a thermocycler for 5 min at 25°C, followed by 30 min at 42°C and 5 min at 85°C. Once complete, reaction was held at 4°C. cDNA (0.25-1 µl) was amplified in a 50 µl reaction mixture containing 0.2 mM of each deoxynucleoside triphosphate, 25 pmol of sense and antisense primers (synthesized by Integrated DNA Technologies), 10X PCR buffer without $MgCl_2$ (Roche), 1.5 mM $MgCl_2$ stock solution (Roche) and 1 U of *Taq* DNA polymerase (Roche). Samples were denatured at 94°C for 2 min, followed by 30 cycles: 94°C

for 30 sec, 55-60°C for 30 sec, 72°C for 30 sec, and lastly, 10 min at 72°C. Samples were held at 4°C until analyzed by electrophoresis on a 2% low- melt agarose gel (Boehringer Mannheim) run in 1X Tris-Borate-EDTA (TBE). The expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT), a known housekeeping gene, was used as a control to assess the quantity and integrity of the cDNA produced by the reverse transcriptase reaction, while Oct4 was used to test for pluripotency. Other markers including homobox protein Nkx2.5, Gata4, and beta-myosin heavy chain (β-MHC) were used to categorize endoderm formation and cardiomyocytes. For each set of primers, cycle numbers and primer amounts were optimized to ensure the signal produced was in the linear range. See Table 1 for primer sequences, annealing temperatures, and cycle numbers.

2.4 Embryonic Stem Cell Differentiation into Neural Progenitors

2.4.1 Morphological Analysis of Dissociated EBs

The protocol was modified from a previous -4/+4 day retinoic acid method in Bain et al. 1995. ES cell differentiation was induced by plating about 2 million cells in suspension in ES cell media without LIF and β-ME on bacteria dishes. Half the media was changed for the first 3 days. On day 4, EBs were moved to conical tubes and all media was removed and replaced with same media supplemented with or without 500 nM retinoic acid. Half the media was changed

every day until day 8. On the eighth day, EBs were dissociated with trypsin and mild titration and were plated onto 0.1% gelatin coated 12-well tissue culture dishes and grown in ES cell media without LIF and β-ME supplemented with 1X B27 (Invitrogen) to support the growth of neurons.

CHAPTER 3. RESULTS

3.1 Cfp1 Null cells had a developmental delay

Previous differentiation assays were performed on Cfp1 Null ES cells showing that they were incapable of differentiating. However, these studies only examined cell differentiation potential for up to 10 days (Carlone et al., 2005). Here we look at the differentiation capacity of Null cells by shortening and extending the time in which the cells are subjected to suspension without LIF.

ES cells formed EBs after two days in hanging drops. Both WT and Cfp1 Null ES cells displayed similar sized EBs. After 5 days without LIF, the majority of both cell types were still floating. However, as the length of time in suspension increased, WT colonies grew in size and eventually settled on the bottom of the bacteria culture dishes and started to form outgrowths. Null cells, on the other hand, remained small and few loosely settled on the bottom of the bacteria dish. No outgrowths were produced. By 10 days, most of the WT EBs had settled and outgrowths continued to proliferate forming more tissue-like structures. Some colonies even started expressing a beating phenotype and others showed outgrowths extending to other settled EB colonies. Null EBs typically remained floating, with few colonies loosely attached with minor outgrowths. Some of the

floating EBs started to clump together. At 20 days in suspension, very few WT EBs remained floating and the dish was overly confluent, with a majority of the dish covered in a monolayer. Several colonies started to overlap, making it hard to distinguish between two originally settled EBs. More proliferating colonies continued to display a beating phenotype. In contrast, the majority of Null EBs still remained floating either individually or in clumps of 2 or 3 EBs. Some EB's were loosely attached and very few EBs settled and formed minor outgrowths. Outgrowths did not extend and proliferate (Figure 1).

Alkaline phosphatase activity, a marker of pluripotency, is down-regulated as ES cells start to differentiate. Before WT and Cfp1 Null cells were induced to differentiate, the majority of both cell lines were alkaline phosphatase positive. It was previously shown that after 10 days without LIF about 97% of WT cells are negative for alkaline phosphatase activity, while <4% of Null cells display a differentiated phenotype (Carlone et al., 2005). As expected similar results were shown here, WT cells were about 95% alkaline phosphatase negative at 10 and 20 days, while <6% of Null cells at all-time points were differentiated. However, at day 5, only 86% of WT cells were differentiated, suggesting a longer time without LIF is needed to fully induce the remaining cells to differentiate (Figures 1B). Overall, Cfp1 Null cells were developmentally delayed when grown in suspension without LIF. They were not able to differentiate when shortening or extending the length of time in suspension.

3.2 The effect of LIF after cellular differentiation

Previous experiments had never look at Cfp1 Null cell differentiation capacity after the initial induction of differentiation. Since the time in suspension did not have an effect on cellular differentiation of Null cells, we wanted to see how the cells would behave if they continued to be cultured. Specifically, looking at whether Null cells could retain their stem cell characteristics without LIF. After the cells were grown in suspension for the 5, 10, and 20 days, EBs were dissociated and reseeded on gelatin coated tissue culture dishes in media with or without LIF.

3.2.1 Importance of LIF

LIF is a member of the IL-6 subfamily and acts as a cytokine that is important for many physiological processes from embryo implantation to immune responses to maintaining pluripotency and self-renewing properties of mouse ES cells. Its heteromer receptor contains gp190 and gp130 subparts that are important for activating three different signaling pathways: JAK-STAT3, PI3K/AKT, and SHP2/ MAPK pathways as reviewed (Hirai et al., 2011; Trouillas et al., 2009). Activation of the JAK-STAT3 or PI3K/AKT pathways are important for maintaining the self-renewing property of mouse ES cells. While STAT3 alone is sufficient for mouse ES cell pluripotency and self-renewal, PI3K/AKT procure self-renewing properties by activating STAT3 through another mechanism. The

pathway also increases self-renewing molecules Nanog and c-Myc though the inhibition of GSK3β. SOCS3 is an inhibitor of the JAK/STAT3 pathway, but normal expression does not effect it ES cell pluripotency. However, the loss of or overexpression of SOCS3 activates the SHP2/MAPK pathway which leads to the down-regulation of Nanog; thus suggesting that SOCS3 provides a balance between the self-renewing JAK/STAT3 pathway and the differentiating SHP2/MAPK pathway as reviewed (Hirai et al., 2011). Overall, the regulatory mechanisms activated by the presence of LIF provided redundant and pleiotrophic functions (Trouillas et al., 2009).

3.2.2 Morphology of cells reseeded in the absence or presence of LIF

To examine the effects of LIF on ES cells after inducing differentiation, cells were grown in the absence of LIF for either 5, 10 or 20 days followed by the reseeding of cells in either ES cell media lacking or reintroducing LIF. Initial observations for all induction time points showed that the majority of WT cells with and without LIF displayed more differentiated phenotypic features. Such features included spiny projects and spindle-like shapes mostly growing in a monolayer. In contrast, Null cells either treated with or without LIF displayed more undifferentiated phenotype including multilayered growth, little outgrowth, and no spiny projections. For the additional eight days that cells were cultured with or without LIF, some cells phenotypes altered. WT cell in the absence of LIF had a mixture of differentiated and undifferentiated phenotypes, with the majority

of them displaying a differentiated phenotype. WT cells in the presence of LIF also displayed a mixed phenotype; however, the majority of the cells looked undifferentiated. Null cells without LIF displayed a slightly mixed phenotype, with a majority of the cells appearing to be undifferentiated. All the Null cells cultured with LIF displayed an undifferentiated phenotype as originally displayed (Figures 2A, 3A, and 4A).

3.2.3 Alkaline phosphatase activity of reseeded cells

Alkaline phosphatase activity was also examined. After the initial 5, 10, 20 days of inducing cells to differentiate without LIF, EBs were collected, dissociated and reseeded on tissue culture dishes with or without LIF. Alkaline phosphatase activity was then examined 1, 5, and 8 days after the initial reseed. Cells stained for alkaline phosphates activity are darkly stained, while differentiated cells remain unstained, as represented in Figures 2B, 3B, and 4B. Conclusion for each set of alkaline phosphatase data collected are as followed:

3.2.3.1 Alkaline phosphatase activity of cells after 5 days in suspension

After being in suspension without LIF of 5 days, cells were reseeded for an additional 8 days with or without LIF. WT cells reseeded with and without LIF were 85% differentiated one day after. WT cells without LIF stayed differentiated until day 5 (about 90% of cells were negative for alkaline phosphatase staining),

but by day 8 cells displayed a decrease in differentiation and an increase in alkaline phosphatase activity. About 70% were negatively stained. WT cells cultured with LIF went from about 85% of cells to 20% of cells being differentiated, indicating that the majority of cells are undifferentiated. WT cells with LIF were not significantly different from WT cells cultured without LIF (Figure 2C; upper right). After 8 days in culture without LIF, the amount of differentiated Cfp1 Null cells detected had steadily increased and was significantly different ($p < 0.05$) from the first day after being reseeded. In contrast, Null cells cultured in the presence of LIF displayed <4% of cells alkaline phosphatase negative (Figure 2C; lower right). Even though Null cells without LIF started to differentiate, they were still significantly different ($p < 0.01$) from WT cells cultured in either condition (Figure 2C; left).

3.2.3.2 Alkaline phosphatase activity of cells after 10 days in suspension

After 10 days of being induced to differentiate in ES media lacking LIF, cells were again reseeded for 8 days. Both WT cells reseeded with and without LIF, showed a decrease in alkaline phosphatase negative cells over the 8 days. Additionally, cells in both cases tested for alkaline phosphatase activity on days 5 and 8 were significantly different ($p < 0.01$) from cells tested at day 1. About 95% of WT cells treated with and without LIF tested negative for alkaline phosphatase activity at day 1 and by day 8 the amount of differentiated cells decreased to about 55% and 35%, respectively (Figure 3C; upper right). Again, Cfp1 Null cells

cultured without LIF for 8 days had an increase in differentiated cells. These cells were significantly ($p < 0.05$) different from cells tested one day after being reseeded. However, cells did not differentiate above 9%. In contrast, Null cells cultured with LIF had a steady decline in alkaline phosphatase negative cells (Figure 3C; lower right). Null cells cultured in either condition were significantly different ($p < 0.01$) from WT cells cultured in the presence or absence of LIF (Figure 3C; left).

3.2.3.3 Alkaline phosphatase activity of cells after 20 days in suspension

After 20 days of induced cellular differentiation without LIF, cells were reseeded again for an additional 8 days. WT cells cultured in the presence and absence of LIF displayed a decrease in differentiate cells. Again, WT cells, in either condition tested at day 5 and 8 after the initial reseeding, were significantly different ($p < 0.01$) from day one. However, only WT cells cultured with LIF showed a significant difference ($p < 0.01$) between cells tested at day 5 and cells tested at day 8 (Figure 4C; upper right). Throughout the 8 days, Null cells without LIF had an increase in alkaline phosphatase negative cells, about 5% to about 9%, while Null cells with LIF showed a decrease, about 5% to about 2% (Figure 4C; lower right). Over the course of the eight days, WT cells in the presence or absence of LIF remained significantly different ($p < 0.05$) from the Null cells cultured in either condition. The only exception was eight days from the initial

reseeding, WT cells treated with and Null cells treated without LIF were not significantly different (Figure 4C; left graph).

3.2.3.4 WT and Null cells display unpredicted patterns of alkaline phosphatase activity

Looking at the alkaline phosphatase data collectively, inducing differentiation in cell lines for 5, 10, 20 days had very little effect on patterns displayed by the cells being reseeded and cultured in the presence or absence of LIF. As unexpected WT cells cultured without LIF for 8 days displayed a steady decrease in differentiated cells, >90% to about 40-70% (Figure 5A). A significant difference ($p < 0.05$) is noted at 5 and 8 days after being reseeded. Similarly, WT cells cultured in the presence of LIF showed a steady decrease in differentiation after being reseeded. However, WT cells had less differentiated cells by day 8, about 20-40% were still alkaline phosphatase negative (Figure 5B). A significant difference ($p < 0.05$) is noted 1 and 5 days after reseeding. While the amount Cfp1 Null cells differentiated in either condition was overall significantly different than WT cells cultured in either condition, they still displayed unpredicted results. Consistently, Null cells cultured without LIF show an increase in the amount of cells differentiated after being reseeded from the initial 5, 10, or 20 day of induction. From the first day of reseeding to day 8, Null cells without LIF increase from about 5% to between 8 and 15% differentiated (Figure 5C). A significant difference ($p < 0.05$) is only noted 8 days after reseeding. In contrast, Null cells

cultured in the presence of LIF consistently showed a decrease in differentiation from about 5% to 2% (Figure 5D), resembling alkaline phosphatase activity of regularly maintained Null cells (data not shown). A significant difference ($p < 0.05$) is 1 and 8 days after reseeding.

3.2.4 Growth rates of cells reseeded in the absence or presence of LIF

After reseeding cells in either ES cell media with or without LIF, there initially seemed to be a difference in the growth of differentiated cells as compared to undifferentiated cells. Specifically, undifferentiated WT cells seemed to proliferate faster than differentiated WT cells. Additionally, Null cells in the presence of LIF replated and grew better than the Null cells cultured in the absence of LIF. After 10 and 20 days without LIF, growth curves were performed on cells after being reseeded and passaged for several days in the presence or absence of LIF. While both curves showed a similar pattern, doubling times were quite different between the two. In the absence of LIF for 10 days, WT cells cultured with or without LIF had a doubling time of 13.1 h and 13.9 h, respectively. Null cells cultured with or without LIF had a doubling time of 24 h and 24.3 h, respectively (Figure 3D). In contrast, cell originally cultured for 20 days without LIF, exhibited a doubling time of 19.5 h for WT cells –LIF, 9.2 h for WT +LIF, 14 h for Null –LIF and 13.5 h for Null +LIF (Figure 4D). In both cases it took longer for WT and Null cells cultured without LIF to recover and reach their exponential growth rate.

3.2.5 Rates of apoptosis of cells reseeded in the absence or presence of LIF

Reseeded cells from the 10 day suspension without LIF were stained for Annexin V and PI to analyze apoptosis of cells treated with and without LIF. It was found that approximately 18% of WT cells cultured without LIF are either dead or undergoing apoptosis. WT and Null cells cultured with LIF had the same amount of cells either dead or undergoing apoptosis, 22% and 23% respectively. Null cells in the absence of LIF displayed the highest rate of apoptosis at 26% (Figure 3E).

3.3 Cfp1 Null cells start expressing cardiac markers in the presence of TGF-β2

3.3.1 Importance of TGF-β2

The transforming growth factor-β family is composed of 30 different growth and differentiation factors including TGF-βs, bone morphogenic proteins (BMP), activins and Nodal. Members of this pathway activate the Smad signaling cascade by promoting the binding to type 2 serine/threonine receptors on the cell membrane. Type 2 receptors then activate the formation of type 1 receptors that can phosphorylate Smad molecules. Activated Smad molecule can target gene expression in the nucleus as reviewed (Kitisin et al., 2007). Depending on the combination of family members active in ES cells, they can either contribute to self-renewing properties or help cells differentiate into any of the 3 germ layers.

For example, neural linage commitment can be achieved by down-regulating all members of the TGF-β family, whereas BMP2 and BMP4 are important for chondrogenic differentiation as reviewed (Fei and Chen, 2010).

Moreover, TGF-β2 is one of three TGF-β isoforms. All of which have been shown to play independent roles in heart development. TGF-β2 knockout mice die prenatally due to congenital heart defects. The lung, eye, spinal column, limbs, and ear development are also affected (Sanford et al., 1997). TGF-β1 knock out mice are important for hematopoiesis and vasculogenesis (Dickson et al., 1995) However, TGF-β2 is the only isoform that has been found to promote cardiomyocyte differentiation (Kumar and Sun, 2005). More recently, TGF-β2 has also been shown to be important for endothelial differentiation in iPS cells (Di Bernardini et al., 2014) and the promotion endothelial-mesenchymal transition (Medici et al., 2011).

3.3.2 Morphology of cells treated with TGF-β2

To examine the differentiation effects of TGF-β2 on Null cells, EBs were formed in hanging drops with the removal of LIF. After 2 days cells were moved to suspension in either ES cell media supplemented with or without TGF-β2. Five days from the initial induction of differentiation, WT EBs were never observed settled at the bottom of bacteria culture dishes as seen when just removing LIF. EBs, non-dissociated, were plated on tissue culture dishes coated with gelatin. In the following days, the growth of WT EBs cultured with or without TGF-β2

outgrew the Null EBs that had settled in either condition. WT EB's treated with TGF-β2 expressed a beating phenotype within 8 days from induction, while WT EBs started beating around day 10. Beating continued throughout the entire 18 days in culture. WT cells cultured without TGF-β2 also had other morphological phenotypes besides cardiomyocytes. These observations were consistent with experiments carried out with the same protocol (Kumar and Sun, 2005). In contrast, Cfp1 Null cells treated with or without TGF-β2 never expressed a beating phenotype. However, they were able to settle and started to form outgrowths the day after being plated on tissue culture dishes. This observation was dislike the Null EBs cultured without LIF on bacteria culture dishes that only loosely attached (Figure 1); thus suggesting that gelatin and cell adhesion is important for behavior and differentiation of Null cells. The observed outgrowth of Null EBs treated with or without TGF- β2 continued to slowly expand while patches of cells looked to be removed from the bodies of the settled EBs. Null cells also had a higher amount of cellular debris (Figure 6A).

3.3.3 Null cells start to express lineage specific markers

Alkaline phosphatase staining was again used to test for pluripotency. Throughout the course of 18 days of induced differentiation, WT cells in the absence and presence of TGF-β2 exhibited >90% differentiated as compared to WT cells treated with LIF (Day 0), which exhibited <2% of cells differentiate (Figure 6C; left and upper right). Cfp1 Null cells cultured in the absence of TGF-

β2 showed <6% of cells alkaline phosphatase negative and Null cells with TGF-β2 displayed <9% of cells differentiated. Differentiation of Null cells were considered significantly different than Null cells maintained and treated with LIF (<1% alkaline phosphatase negative). However, there was no significant difference between Null cells treated with and without TGF-β2 at any time point (Figure 6C; bottom right). WT cells in either condition were always significantly different ($p < 0.05$) than Null cells cultured with or without TGF-β2, showing that cells struggle to differentiate (Figure 6C; left).

Similarly, RT-PCR analysis showed that WT cells either treated with or without TGF-β2 down regulated Oct4, pluripotency marker, while up regulating cardiac markers Gata4, β-MHC, and Nkx2.5. Null cells with and without TGF-β2 failed to down regulate OCT4 and upregulate Gata4. However, both have basal expression of cardiac markers Nkx2.5 and β-MHC. Cfp1 Null cells treated with TGF-β2 have slightly higher expression of Nkx2.5 (Figure 6D).

3.4 Cfp1 Null cells are sensitive to neuronal differentiation

3.4.1 Importance of Retinoic Acid

Retinoic acid (RA), a vitamin A derivative, acts as a ligand for nuclear RA receptors (RAR). RAR receptors combine with retinoid X receptors (RXR) and form heteromic complexes. When ligands are present the RAR-RXR heterodimer recruit co-activator complexes and induce chromatin remodeling. In the absence

of RA, RAR-RXR can bind retinoic acid response element (RARE) DNA motifs and recruit co-repressors as reviewed (Rhinn and Dolle, 2012).

RA has been found important hindbrain, forebrain, forelimb, and neural tube development. Moreover, RA supports axon growth and nerve regeneration as reviewed (Maden, 2007; Rhinn and Dolle, 2012) In stem cell cultures, RA has been used in several differentiation protocols to induce and support neuron formation as well as pancreatic β cell formation, and adipocyte differentiation (Bain et al., 1995; Bost et al., 2002; Shi et al., 2005).

3.4.2 Cell growth is affected by the RA differentiation protocol

To assess the differentiation of cells in the presence of retinoic acid, two million cells were cultured in suspension for eight days. The first four days cells were cultured in ES cell media without LIF and β-ME and the second four days, cells were either cultured in the same media or in media supplemented with RA. WT and Cfp1 Null cell cultures both formed EBs within 2 days of induction. There was no difference in the size of EBs detected between cells treated with RA or without RA. Throughout the induction period, WT and Null EBs cultured in either condition were never observed settled on the bottom of the bacteria culture dishes. However, lots of cell debris was observed for all conditions. At the end of 8 days EBs were collected, dissociated with trypsin, counted with a hemocytometer, and reseeded on tissue culture dishes coated in gelatin. The counts revealed that there were significantly less Null cells treated with or without

RA and WT cells without RA as compared to WT cell cultured in RA (Figure 7). After being reseeded, all cells were cultured in ES cell media without LIF and β-ME and further supplemented with B27 to support neuron growth. Interestingly, WT cells cultured with or without RA were always able to replate, while Null cells were not able to replate consistently. The experiment was set up three times. The first time and second time only Null cells cultured without RA replated, while the third time only Null cells treated with RA replated. However, when the Null cells replated, very few were observed.

CHAPTER 4. DISCUSSION

4.1 The differentiation capacity of cells reseeded with or without LIF

4.1.1 Differentiation capacity of WT cells

It was previously shown that ES cells lacking Cfp1 fail to differentiate. We hypothesized that in presence of cytokines, growth factors, and intracellular signaling molecules, Cfp1 Null cells would remain undifferentiated. However, once cells do establish a differentiated phenotype, it is unlikely that they will spontaneously convert into a new cell type or revert back to a less differentiated state. Differentiated cells are able to stably maintain their developed state through the epigenetic inheritance of cytosine methylation patterns, covalent histone modifications, histone variants, and polycomb group complexes (Ng and Gurdon, 2008). When looking at the effects of LIF after the induction of differentiation, we used WT ES cells as a control to show that once they differentiated their phenotype could not be reverted back an ES cell phenotype. , Unexpectedly, we observed the opposite. When WT cells were reseeded either in the presence or absence of LIF >90% of cells had differentiated (alkaline phosphatase negative), but after being cultured for an additional 8 days, the cells

were reverted back to an undifferentiated phenotype (20- 70% alkaline phosphatase negative), Two theories were hypothesized to explain the observed phenomenon.

For one, alkaline phosphatase activity, though very low, was still detected in WT cultures after being reseeded for one day. WT cells cultured in LIF, as expected, probably favored the growth cells of undifferentiated cells in the population. However, this explanation does not explain the increase of alkaline phosphatase activity observed in WT cells cultured without LIF. Therefore, patterns of increased alkaline phosphatase activity in WT cells could be observed to due to the technical issues experienced with the use of trypsin to dissociate EBs as well as differentiated cells growing on bacteria and tissue culture dishes. Initial observations indicated that EBs were easier to dissociate than differentiated cells. Depending on the cell type being dissociated, trypsin can be used from 2-3 min on ES cells and monolayer endothelial cells to 8 min for the release of neuronal cells (Gimbrone et al., 1974; Meyer et al., 2004). Therefore, depending on the length of time cells were exposed to trypsin, undifferentiated cells could have been selected for during splits.

If undifferentiated cells in the population are being selected for, studies have been shown that ES cells grow faster than differentiated cells. Specifically, it has been shown that ES cells usually display a doubling time between 12-16 h when cultured on gelatin (Tamm et al., 2013), mouse embryo fibroblasts double about every 43 h (Sell et al., 1994) and neurons experience about a 24 h doubling time (Conti et al., 2005). While we initially observed similar patterns,

growth curves, set up with cells reseeded from a 10 day suspension without LIF, showed that WT cells cultured in either condition had the same doubling time (about 13.5 h); favoring an ES cell doubling time. In contrast, cells reseeded after 20 days in suspension displayed doubling times of 19.5 h and 9.2 h of WT cells cultured without or with LIF, respectively; thus indicating that undifferentiated cells grow faster than differentiated. The differences in growth rates between cultures originally grown in the absence of LIF for 10 and 20 could be due to difference in the length of time the cells were cultured and when the growth curves were set up for either time point. As seen, the more splits the cells undergo, the more undifferentiated WT cells are detected. Also, there could have been a difference in the amount of cells plated for each curve due to human error.

Secondly, cells could have been dedifferentiating or reverting back to an earlier, immature stage of development. The process of dedifferentiating mouse somatic cells into a pluripotency state was originally accomplished by introducing transcription factors Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). Further researched showed that cells could also be induced to dedifferentiate through other techniques including somatic cell nuclear transfers and fusions between embryonic germ cells or ES cells and somatic cells. However, more recent studies have shown that changes in the culture's microenvironment can also influence dedifferentiation. This could be due to the heterogeneity of mouse ES cells, growth on feeder cells, components present in the media, and oxygen tension as reviewed (Roobrouck et al., 2011). For

example, mouse spermatogonial stem cells, cultured in the presence of glial cell line-derived neurotrophic factor, fibroblast growth factor 2, epidermal growth factor, and LIF, spontaneously formed ES cells after several weeks. This subpopulation of cells, known as multipotent germ cells, were separated and cultured in traditional ES cells media. Eventually, these cells lost their spermatogonial potential (Kanatsu-Shinohara et al., 2004). Therefore, the microenvironment created during the reseeding of WT EBs in either the presence or absence of LIF could have contributed to the dedifferentiation of cells; thus, allowing the cells to present a more immature phenotype able to express alkaline phosphatase activity. Overall, WT cells cultured could be reverting back to an ES cell phenotype either due to trypsin selection or dedifferentiation. It would be curious to see if the expression of other pluripotency markers were increased and lineage specific markers were decreased after reseeding.

4.1.2 Differentiation capacity of Cfp1 Null cells

Upon the induction of differentiation though the removal of LIF, it has been repeatedly shown that Cfp1 Null cells are able to aggregate, but are not able to differentiate (Carlone et al., 2005). Instead, EBs remain floating or loosely attached to the bottom of bacteria dishes. As we show here, the length of time EBs are cultured in suspension also does not influence differentiation. It is not until the Null cells are reseeded in the absence of LIF that they are not able to fully maintain their pluripotent state and slowly start to differentiate; thus

suggesting that cell adhesion is important. Recent studies have shown that biomimetic materials that mimic surface topography are important for cell behavior. One studied showed that titanium surfaces were important for mesenchymal cell differentiation into osteogenic precursors (Park et al., 2007), while another used hydrogels, a hydrophilic network of polymer chains, to differentiate mesenchymal stem cells into myogenic or osteogenic lineage (Ayala et al., 2011). In the case of Cfp1 Null cells, gelatin and the surface of the tissue culture dish must be aiding cell differentiation. It would be interesting to see what differentiation markers are being expressed in Null cells grown without LIF.

In contrast, Null cells reseeded in the presence of LIF are not able to differentiate. The majority of the cells remain undifferentiated throughout the eight days. However, what few cells are negatively stained for alkaline phosphatase are reverted back to an ES cell phenotype. Interestingly, this suggests that Null cells might be able to dedifferentiate in the right microenvironment, as previously questioned in the differentiation and reseeding of WT cells. Furthermore, since Null cells reseeded without LIF are not able to fully differentiate and are still significantly different that WT cells, Cfp1 must still be important for establishing epigenetic patterns after cellular differentiation. It would be interesting to compare the epigenetic characteristics of cells reseeded in ES cell media without LIF to cells reseeded with LIF.

4.1.3 Apoptosis does not explain the differences in growth rate

After being cultured in suspension without LIF for 10 or 20 days, the reseed cells in the presence or absence of LIF displayed a difference in doubling time. The WT growth curve generated after 10 days in suspension showed similar doubling time between WT cells cultured with LIF and cells cultured without (about 13.5 h). Whereas WT cells reseeded after 20 days in suspension showed a difference in doubling time between cells grown with LIF, 19.5 h, and cells grown without, 9.2 h. The doubling of Cfp1 Null cells cultured with and without LIF were similar in both growth curves; however, cells after day 10 had a doubling time of about 24 h and cells after day 20 displayed a doubling time about 14 h. As mentioned before their difference could be attributed to the length of time cells were cultured before the growth curves were set up and inconsistency in the amount of cells used to step up the growth curve. However, in both cases, WT and Null cells cultured in the absence of LIF had a harder time replating. Tate and colleagues 2009 previously looked at the difference of plating efficiency between WT and Cfp1 Null cells by counting the number of colonies formed after plating 400 cells. Not only did they find that WT cells replated more efficiently than Null, but they also found that the Sid domain or CXXC DNA binding domain was sufficient to rescue plating efficiency (Tate et al., 2009). It would be interesting to determine the plating efficiency between the WT and Null cells reseeded in either condition following Tate et al. 2009 protocol.

In comparing the doubling times to the rates of apoptosis, it was concluded that apoptosis did not account for the difference in doubling times between reseeded WT and Cfp1 Null cells. Therefore, it could be possible that the difference in growth rates are due to a difference in cell cycling. PLB-985 myelolid cells, knocked-out for Cfp1 using short-hairpin RNA, were shown to have a reduced doubling time due to a defect in cell cycling, which arrested cells in the G_1 and G_2 stages (Young and Skalnik, 2007). However, both of these experiments had opposite conclusions than those presented by Carlone and colleagues, 2005. Their results suggested that the doubling time of Cfp1 Null cells was due to the 3-fold increase in apoptosis. Null cells also showed a normal cell cycle distribution (Carlone et al., 2005). Therefore, the next step would be to see if cell cycling was disrupted or altered in WT and Null cells reseeded after being induced to differentiate. If so, the epigenetic pattern of ES cells may not be fully restored in the presence of LIF after the induction of differentiation.

4.2 Differentiation capacity of Cfp1 Null cells in the presence of TGF-β2

4.2.1 The role of Cfp1 in cardiomyocyte differentiation

While cardiac markers, Nkx2.5 and β-MHC, are slightly increased by day 18, no contractile phenotype is observed, growth was considerably slower, and pluripotency markers remain highly expressed in Cfp1 Null cells subjected to cardiomyocyte differentiation. Overall, this suggest that Null cells treated with or

without TGF-β2 struggled to differentiate. However, a previously study showed that hematopoetic precursors of zebrafish treated with the zCXXC1 morpholino oligonucleotides are unable to reach complete erythroid maturation, even though they express early markers of hematopoiesis at appropriate developmental times and locations. It was concluded that hematopoiesis could not be completed due to an increase in apoptosis (Young et al., 2006). In comparison, cardiac markers in Cfp1 Null cells are not expressed at the same time as WT. Gata4, β-MHC, and Nkx2.5 are detected in WT cells at least 6 days from the initial induction and continue to be highly expressed by day 18. In contrast, the expression of these markers in Cfp1 Null cells are still very low after 18 days in culture. In addition, less than 10% of Null cells are alkaline phosphatase negative compared to WT cells (>92%) by 18 days in culture. However, Null cells do exhibit a higher amount of cell debris and contain holes in the core of the settled EB. Therefore, they might be dying before reaching cardiomyocyte maturation. I would hypothesize that the rates of apoptosis in Cfp1 Null cells would be higher to those of WT cells during cardiomyocyte differentiation.

4.2.2 Similarities between LIF and cardiomyocyte differentiation

As previously seen, Cfp1 Null EBs dissociated and reseeded on gelatin coated tissue culture dishes in the absence of LIF slowly start to differentiate. Alkaline phosphatase negative cells increased from about 5% to between 8-15%. We hypothesized that the behavior of these cells was due to adhesion.

Interestingly, the same behavior was observed of Null cells cultured in the absence and presence of TGF-β2. Like Null cells suspended in the absence of LIF, Null EBs treated with TGF-β2 also did not settle on bacteria culture dishes. Upon plating, the EBs on tissue culture dishes coated in gelatin, cells treated with and without TGF-β2 were able to start differentiating; thus further supporting our adhesion hypothesis.

In addition, only a small population of cells are differentiating when being subjected to either media without LIF and with TGF-β2; suggesting a developmental delay. When looking at the cells individually during morphology and apoptosis analysis, cells could be easily distinguished between undifferentiated and differentiated. However, when cells undergoing cardiomyocyte differentiation were analyzed by RT-PCR, the whole population of cells was being read. This is why pluripotency marker, Oct4, can still be highly expressed and lineage specific markers are able to be detected. However, high expression of pluripotency markers and low expression of differentiation markers still does not explain the reduction in growth rates and differentiation capacity. Further suggesting that Cfp1 plays an important role in cellular differentiation.

4.3 Differentiation capacity of Cfp1 Null cells cultured with Retinoic Acid

ES cells growth in the absence of LIF and β-ME were found to have an increase in cell debris. Additionally, WT EBs did not settle on the bottom of bacteria dishes and form outgrowths as observed in differentiation conditions just

removing LIF as seen above and in Carlone et al. 2005; thus, proposing that β-ME may play an important role in sustaining the health of ES cells. β-ME is an important reducing agent used in cell culture to directly affect serum components. Glutathione can be oxidized into glutathione disulfide (GSSG). In its oxidized form, GSSG has inhibitory effects on protein synthesis. The addition of β-ME in cell media reduces the disulfide bonds present in GSSG thereby preventing inhibition (Hoffeld and Oppenheim, 1980). In its reduced state, glutathione is important for protecting cells against hydrogen peroxides and oxygen radicals produced by dead cells as reviewed (Hoffeld and Oppenheim, 1980). β-ME is also found to enhance antibody formation and response (Chen and Hirsch, 1972; Hoffeld and Oppenheim, 1980).

While the reduction of ES cells during the neural differentiation could be due to the effects of β-ME, it does not explain the reduced plating ability of Cfp1 Null cells treated with or without RA. Either the cells at this point are sensitive to dissociation with trypsin or the B27 supplement, which is used to plate and support neuron growth. In the past, it has been shown that some laboratories have had trouble culturing neurons with the use of commercial supplements as briefly discussed (Chen et al., 2008). Previous studies found that cells undergoing the same protocol had better plating efficiency when they were only moderately dissociated so not to completely dissociate them into a single suspension (Meyer et al., 2006). It would be interesting to see how the EBs would replate without dissociation as done in the cardiomyocyte differentiation protocol.

As mentioned previously (Carlone et al., 2005; Young et al., 2006) Cfp1 is important for ES cell differentiation. In the absence of Cfp1, both mouse ES cells and zebrafish exhibit an increase in apoptosis. It was hypothesized that the increase rates of apoptosis in zebrafish treated with morpholino oligonucleotides was due to the failure of erythroid differentiation (Young et al., 2006). Moreover, conditional knockout mouse models displayed increased rates of apoptosis in bone marrow cells following the induction of Cre (Skalnik lab, manuscript submitted). Therefore, the increase in cell debris and the reduction in the amount of cells cultured during the neural differentiation could be due to the lack of Cfp1. It would be interesting to see how the rates of apoptosis compared between conditions. While these observations could be due to the lack of Cfp1, other variables would need to be ruled out first. Either the protocol proposed by Bain and colleagues 1995 to induce neuronal differentiation needs to be optimized for the support of Cfp1 Null cells or another method of differentiation must be considered. One alternative protocol promotes neural differentiation by culturing ES cells in an adherent monolayer with a combination of DMEM/F12 media supplemented with N2 and Neuralbasal media supplemented with B27, L-glutamine, β-ME and RA (Xu et al., 2012).

CHAPTER 5. FUTURE DIRECTION

Overall, we found that Cfp1 is still important for cellular differentiation; however, several questions were generated. First when looking at the cells reseeded and cultured in the presence or absence of LIF, we found that a portion of ES cells retain stem cell characteristics no matter what condition they were subjected to. Specifically, when LIF was added back to Cfp1 Null cells, we found that the majority of cells retained stem cell characteristics, but to what extent is unknown. It would be interesting to see how much of the phenotype is being retained by comparing reseeded cells in any condition to untreated cells maintained in LIF in terms of plating efficiency and epigenetic characteristics. Additionally, the lab has seen that the difference in cell growth between WT and Cfp1 Null cells during the differentiation experiments has either been a result of an increased rates of apoptosis or defects in cell cycle regulation (Carlone et al., 2005; Young and Skalnik, 2007). In comparison, we saw that growth rates of reseeded cells, in ES cell media containing or lacking LIF after being induced to differentiate, were not the result of increased apoptosis. Overall, these results suggest that Cfp1 is necessary for the survival and differentiation of cells. Not only do we need to determine if the reduced growth rate of reseeded cells is due to cell cycle defects, but also if Null cells undergoing differentiation into

cardiomyocytes and neural precursors have reduced growth rates due to apoptosis or cell cycle defects. Overall, to further solidify the results and verify the role of Cfp1 in differentiation covered in this thesis, we need to look at how Cfp1 Null cells, transfected with in full length Cfp1 expression vector, rescue cellular response to LIF, TGF-β2 and RA differentiation protocols.

We also found that Cfp1 Null cells reseeded in the absence of LIF, either as individual cells or EBs, were able to start differentiating after being reseeded on gelatin coated tissue culture dishes. However, only a small population showed differentiated phenotypes. To this point all of the differentiation studies performed on Cfp1 Null ES cells have been in cell culture. It would be interesting to determine in vivo differentiation capacity of Cfp1 Null cells by generating chimeric mice. First, Null ES cells would be injected into a blastocyst and then the blastocysts would be inserted into a psuedopregnant mouse. In this natural environment, cells would be exposed to all of the signaling pathways. As a result, all pups created from the experiment using WT ES cells would be mainly generated by ES cells. If chimeric mice are generated from Null ES cells, further testing would have to be performed; either organs of the mice would be harvested to test for Null allele or GFP reporter gene would have to be added to the ES cells before injection. Additionally, Cfp1 Null cells transfected with a full length Cfp1 expression vector would be used. Not only would this method eliminate the need to find and optimize an array of differentiation protocols, but this information could further explain how Cfp1 is involved in differentiation.

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TABLES

Table 1. Characteristics of markers used for analyzing development and cardiomyocyte differentiation in vitro

Marker	Forward Sequence	Reverse Sequence	bp	Annealing Temp.	Cycle Number
HPRT	CACAGGACTAGAACACCTGC	GCTGGTGAAAAGGACCTCT	248	55	30
4-Oct	GGCGTTCTCTTTGGAAAGGTGTTC	CTCGAACCACATCCTTCTCT	312	55	30
B-MHC	TGCAAAGGCTCCAGGTCTGAGGGC	GCCAACACCAACCTGTCCAAGTTC	202	55	30
Gata4	CGAGATGGGACGGGACACT	CTCACCCTCGGCATTACGAC	179	60	30
Nkx2.5	CAGAAGGCAGTGGAGCTGGACAAAGC	GCACTTGTAGCGACGGTTCTGGAACC	230	60	30

FIGURES

A.

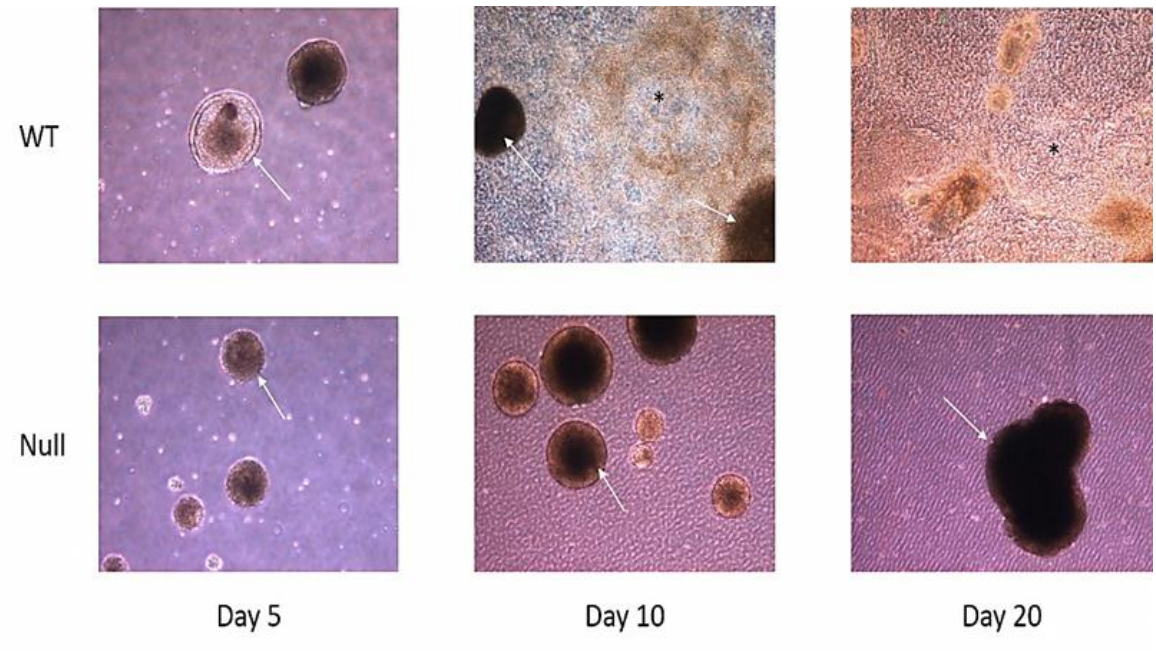

Figure 1A. In the absence of Cfp1, time does not influence EB formation during the induction of differentiation. Differentiation was induced by the hanging drop method for two days followed by cellular suspension in bacteria culture dishes in media lacking LIF. Colony morphology following induction after 5, 10, and 20 days. Most WT embyroid bodies settle and form outgrowths on the bacteria dishes by day 10, indicated by black asterisks. Null EBs remain floating throughout the induction period. Floating EBs are indicated with white arrow. Magnification, 4X.

B.

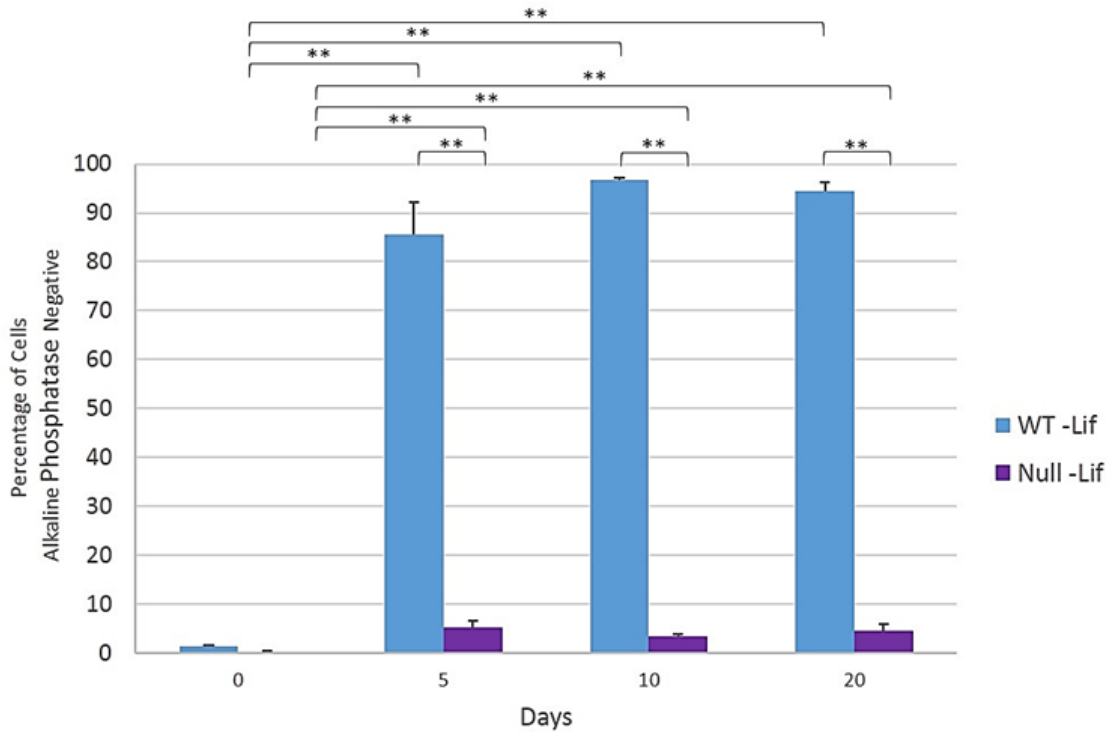

Figure 1B. In the absence of Cfp1, time does not influence alkaline phosphatase markers during the induction of differentiation. After growing the cells in suspension without LIF for either 5, 10, or 20 days, cells were collected, dissociated, and reseeded on tissue culture dishes coated in 0.01% gelatin. The next day the cells were stained for alkaline phosphatase activity. Error bars represent standard error. Day 0 represents cells that were untreated, grown, and maintained in the presence of LIF. A double asterisk denotes a significant difference ($p < 0.01$) between WT and Null cells grown in the absence of LIF.

A.

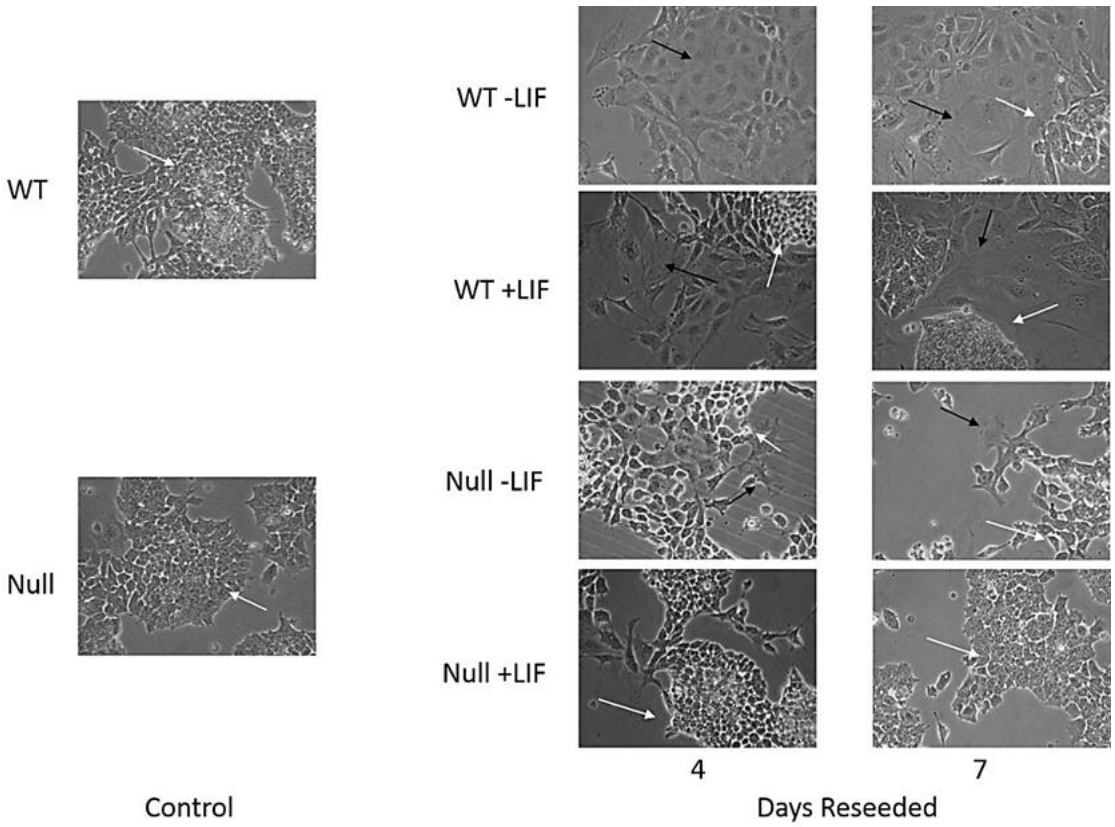

Figure 2A. After 5 days of inducing differentiation, stem cell-like morphology is retained in all cell condition. After ES cells were grown for 5 days in media without LIF, EBs were collected, disaggregated, and reseeded. Reseeded cells were either cultured in ES cell media with or without LIF for an additional 8 days. Cell morphology was captured before splitting 4 and 7 days after the initial reseed. Control represents cells being maintained in the presence of LIF before undergoing differentiation. White arrows point to ES cells, while black arrows point to differentiated cells. Magnification, 20X.

B.

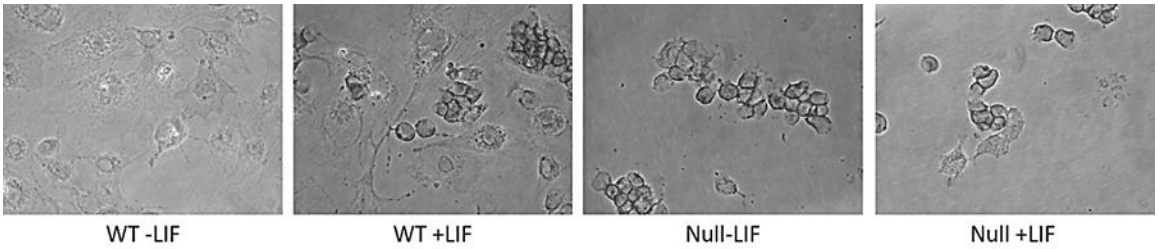

Figure 2B. Visual of alkaline phosphatase activity after 5 days without LIF, reseeded for 5. Representative morphology and staining of alkaline phosphatase activity of 5 days after initial reseeding from the 5 day differentiation induction period. Darker cells are positively stained for alkaline phosphatase. Magnification, 20X.

C.

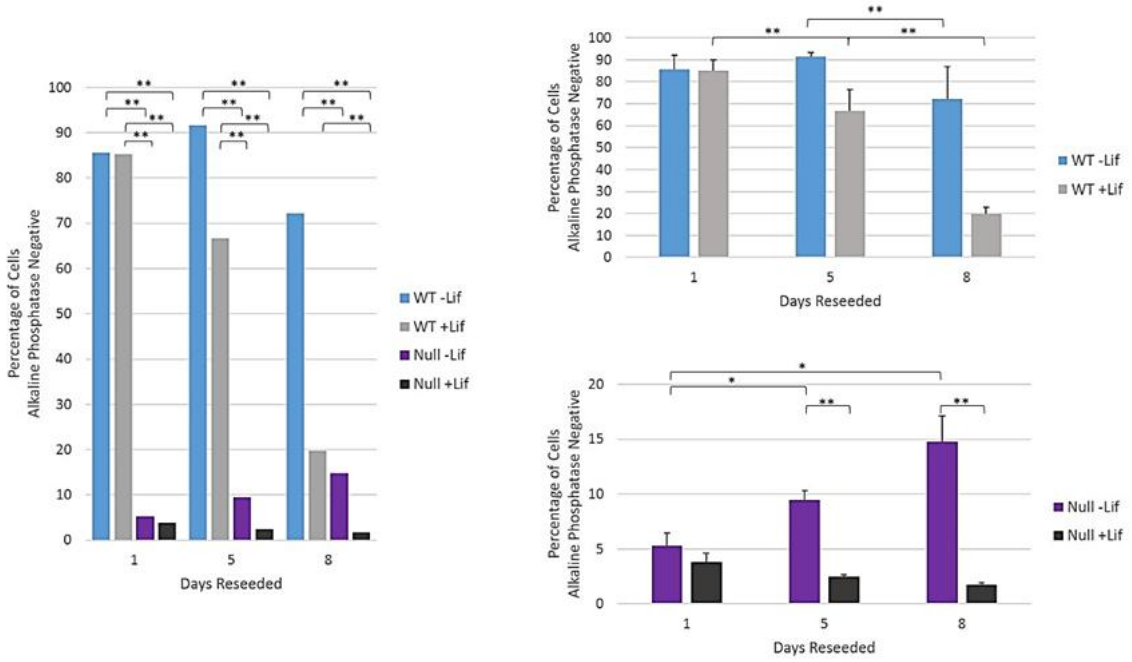

Figure 2C. After 5 days of inducing differentiation, alkaline phosphatase activity confirms that the phenotype of Null cells is recovered by LIF. ES cells grown in the absence of LIF for 5 days were collected, dissociated, reseeded and grown for an additional 8 days in ES media with or without LIF. At days 4 and 7 from the initial reseeding, cells were split and stained the next day for alkaline phosphatase activity (5 Days and 8 Days). At least 300 cells were counted and stained for each condition. Percentage of cells unstained or differentiated were graphed. The percentages were based on an average of 3-5 separate experiments. Error bars indicate standard error, while asterisks represent statistical significance difference between cell treatments and days within the same condition. A single asterisk denotes $p < 0.05$ and double asterisks denotes $p < 0.01$. The graph on the left shows all conditions collectively, whereas the graphs on the right split the data into WT (top) and Null (bottom).

A.

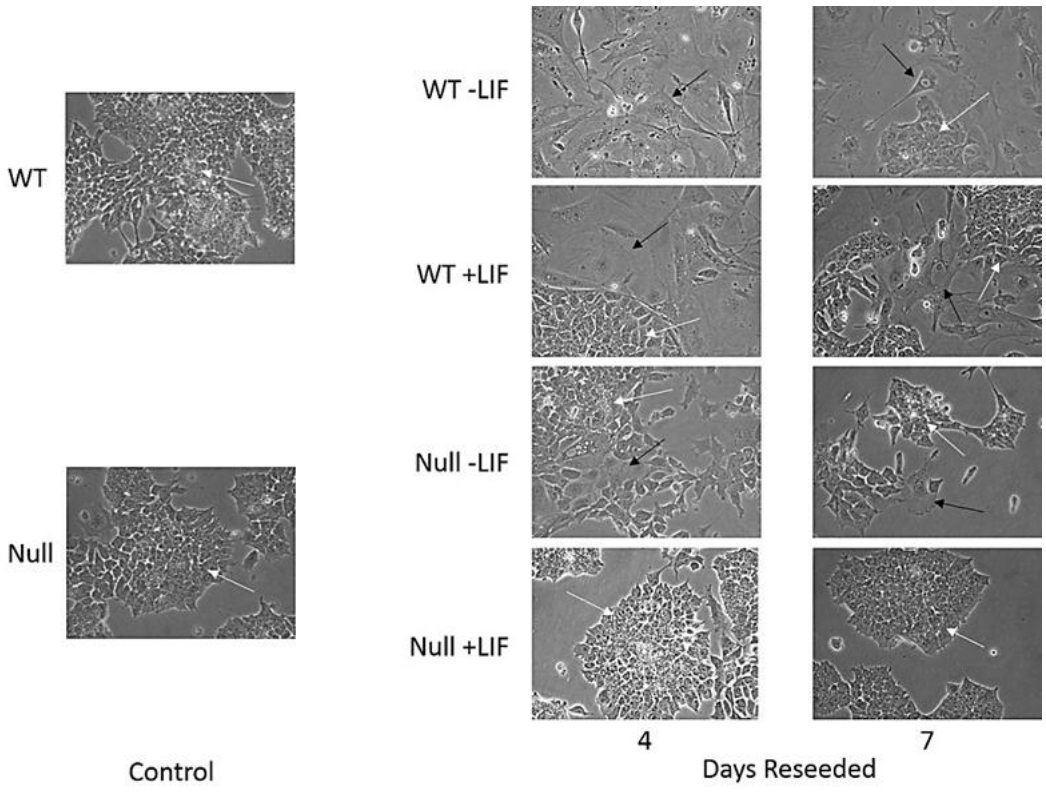

Figure 3A. After 10 days of inducing differentiation, stem cell-like morphology is retained in all cell condition. After ES cells were grown in the absence of LIF for 10 days, they were collected, disaggregated, and reseeded on tissue culture plates with 0.1% gelatin. Similar to Figure 2A, reseeded cells were cultured for an additional 8 days in ES cell media with or without LIF. Again, cells morphology was captured before cells were split 4 and 7 days after initial reseed. Control represents cells being maintained in ES cell media containing LIF. White arrows highlight ES cells, while black arrows indicate differentiated cells. Magnification, 20X**.**

B.

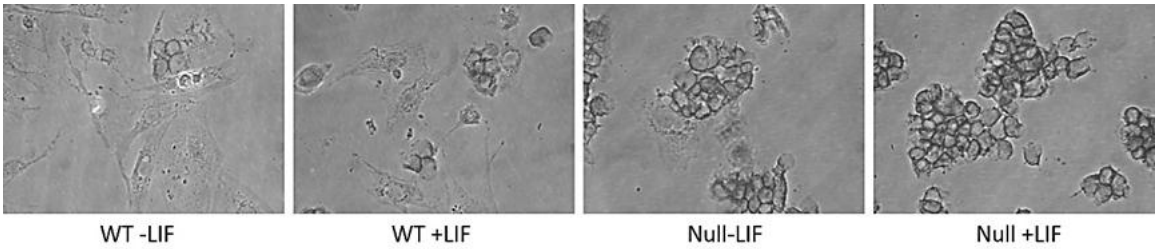

Figure 3B. Visual of alkaline phosphatase activity after 10 days without LIF, reseeded for 5. ES cells grown in the absence of LIF for 10 days were collected, dissociated, reseeded and grown for an additional 8 days in ES media with or without LIF. Representative phenotype of alkaline phosphatase staining 5 days after reseeding cells on tissue culture dishes coated in 0.1% gelatin. Darker cells are alkaline phosphatase positive. Magnification, 20X.

C.

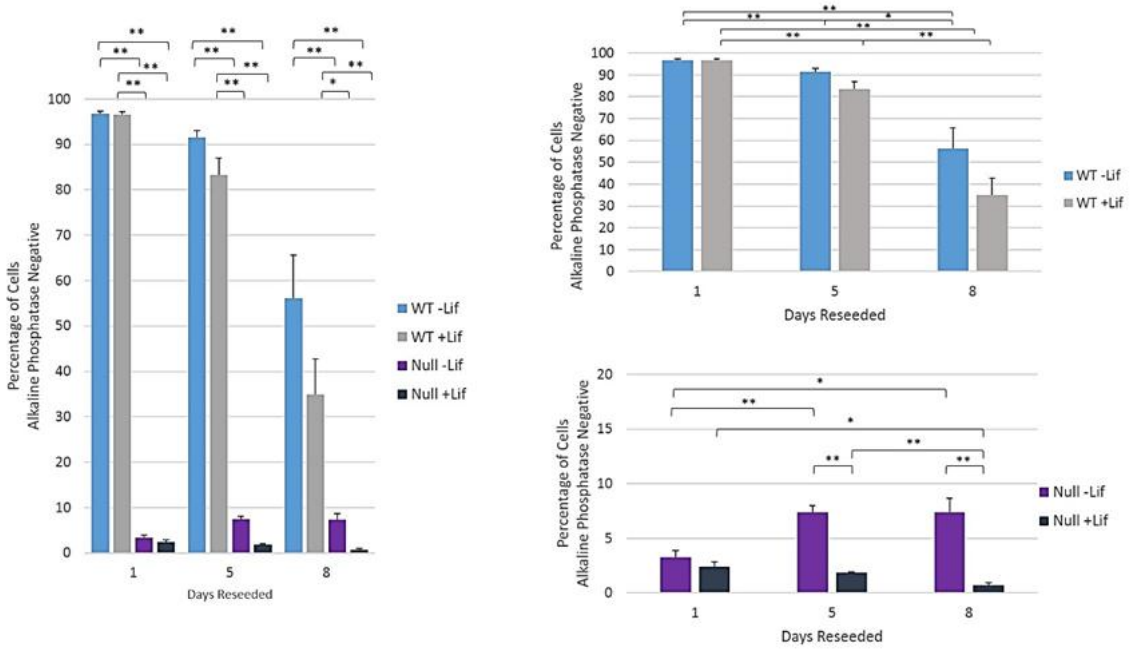

Figure 3C. After 10 days of inducing differentiation, alkaline phosphatase activity confirms that the phenotype of Null cells is recovered by LIF. ES cells grown in the absence of LIF for 10 days were collected, dissociated, reseeded and grown for an additional 8 days in ES media with or without LIF. At days 4 and 7 from the initial reseeding, cells were split and stained the next day for alkaline phosphatase activity (5 Days and 8 Days). At least 300 cells were counted and stained for each condition. Percentage of cells unstained or differentiated were graphed. The percentages were based on an average of 3-5 separate experiments. Error bars indicate standard error, while asterisks represent statistical significance difference between cell treatments and days within the same condition. A single asterisk denotes $p < 0.05$ and double asterisks denotes $p < 0.01$. The graph on the left shows all conditions collectively, whereas the graphs on the right split the data into WT (top) and Null (bottom).

D.

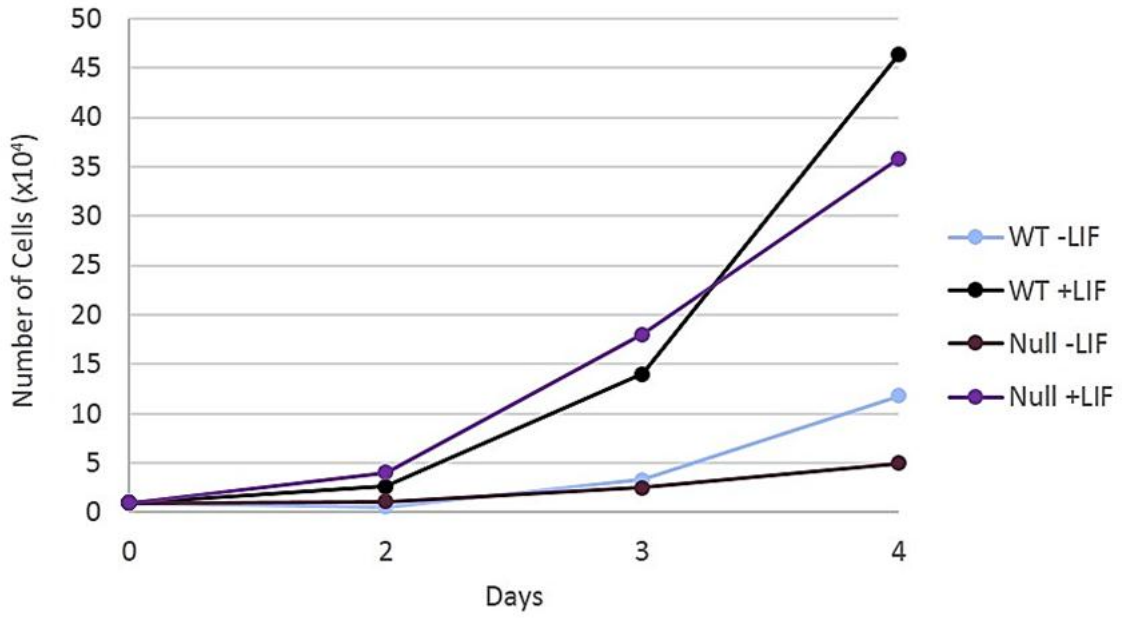

Figure 3D. After 10 days of inducing differentiation, cells treated with LIF had a faster recovery time. Cells were grown in the absence of LIF for 10 days. After cells recovered from being reseeded on tissue culture dishes coated with 0.01% gelatin in either ES cell media with or without LIF, growth rates were measured for each condition. Error bars represent standard error.

E.

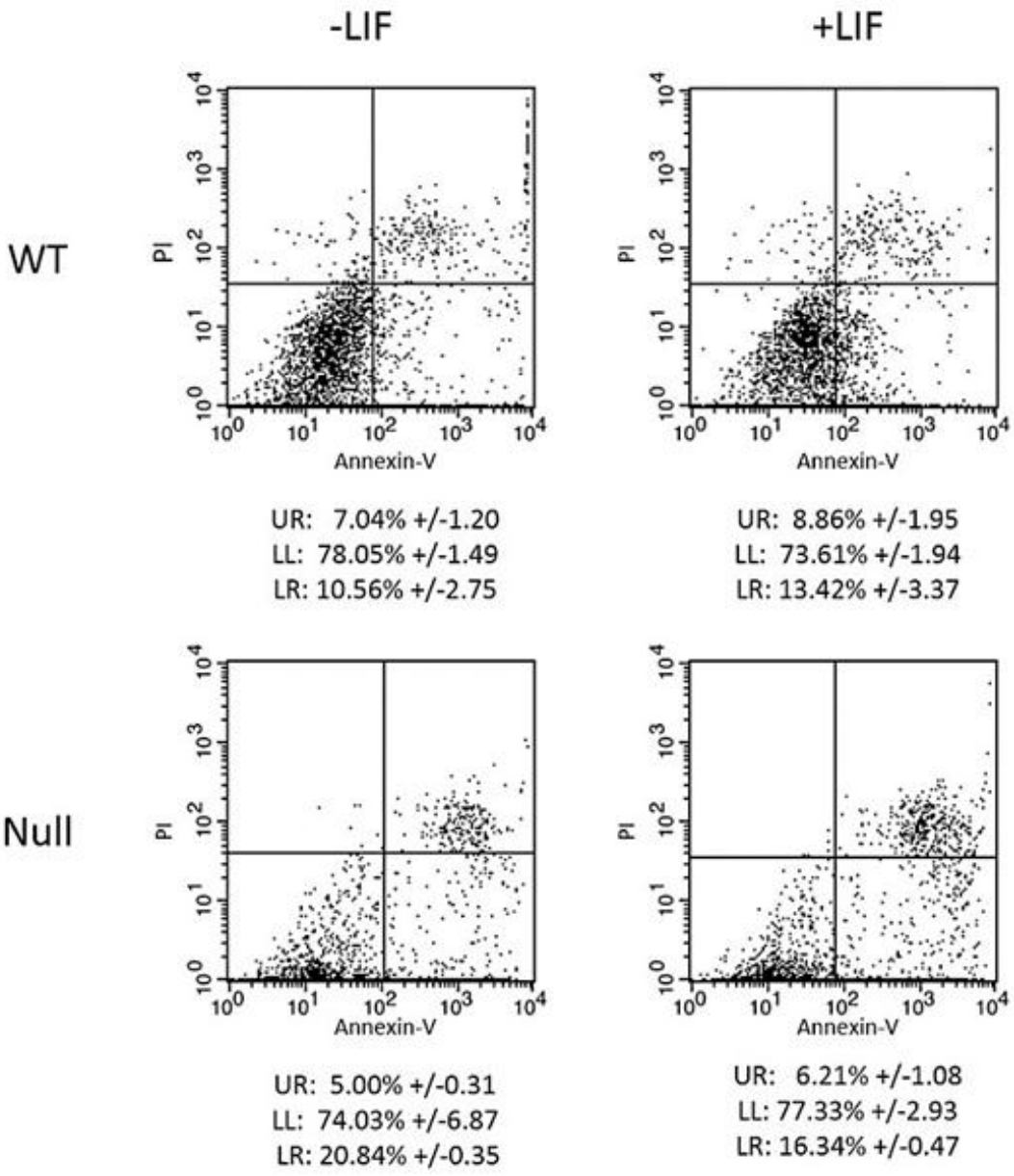

Figure 3E. After 10 days of inducing differentiation, reseeded cells displayed similar rates of apoptosis. ES cells were analyzed for apoptosis using Annexin-V and propidium iodine and flow cytometry. Upper right (UR) corresponds to dead cells, lower left (LL) corresponds to healthy living cells, lower right (LR) corresponds to apoptotic cells. Percentages (+/- standard deviation) represent an average of three experiments.

A.

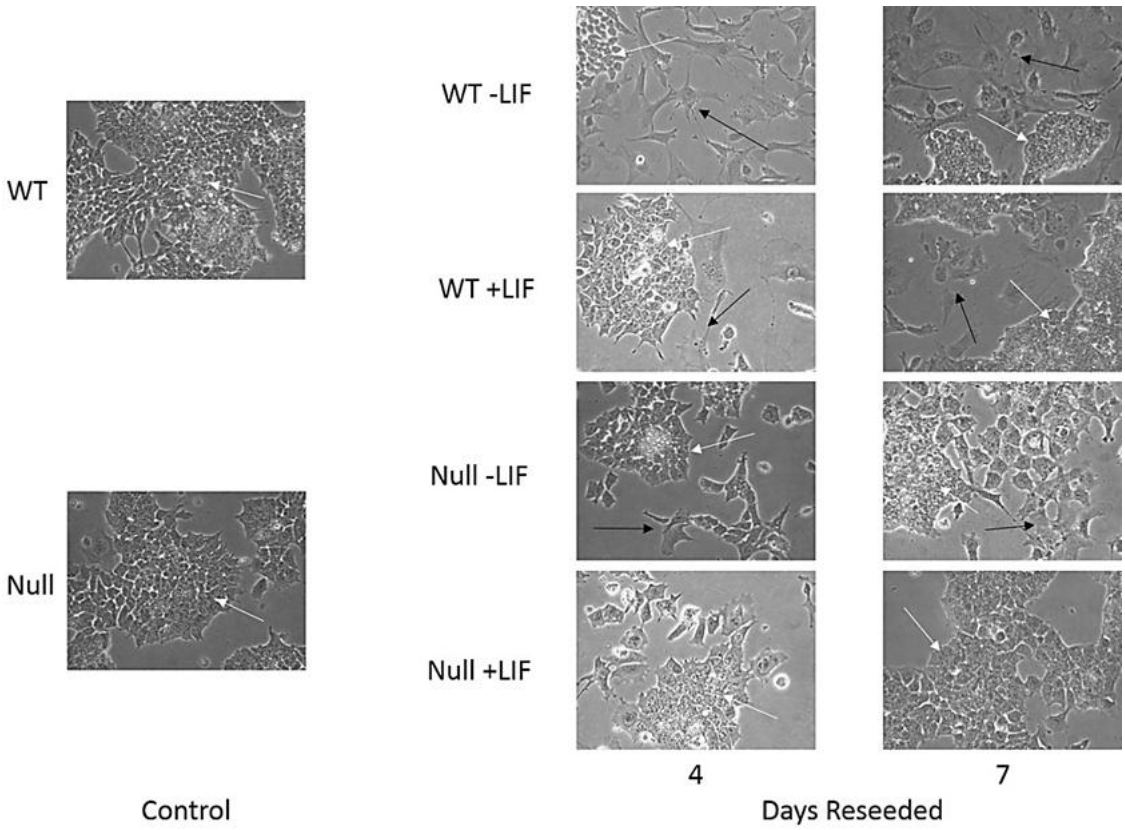

Figure 4A. After 20 days of inducing differentiation, stem cell-like morphology is retained in all cell condition. Cells remained in suspension without LIF for 20 days. EB's were then collected, dissociated, and reseeded on gelatin coated tissue culture dishes for an additional 8 days in ES cell media with or without LIF. Similar to Figures 2A and 2B, morphology was analyzed and spilt 4 and 7 days after the initial reseeding. Control represents cells being maintained in the presence of LIF. Black arrows point out differentiated cells, while white arrows indicate ES cell morphology. Magnification, 20X.

B.

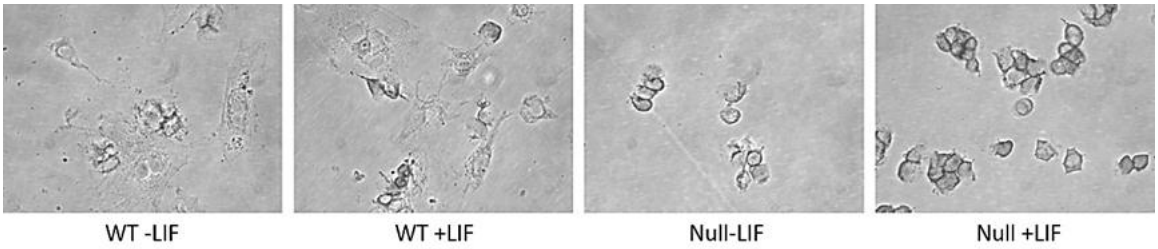

Figure 4B. Visual of alkaline phosphatase activity after 20 days without LIF, reseeded for 5. ES cells grown in ES cell media without LIF for 20 days were collected, dissociated, reseeded and grown for an additional 8 days. Representative phenotype of cells stained for alkaline phosphatase 5 days after the initial reseeding. Darker cells are alkaline phosphatase positive. Magnification, 20X.

C.

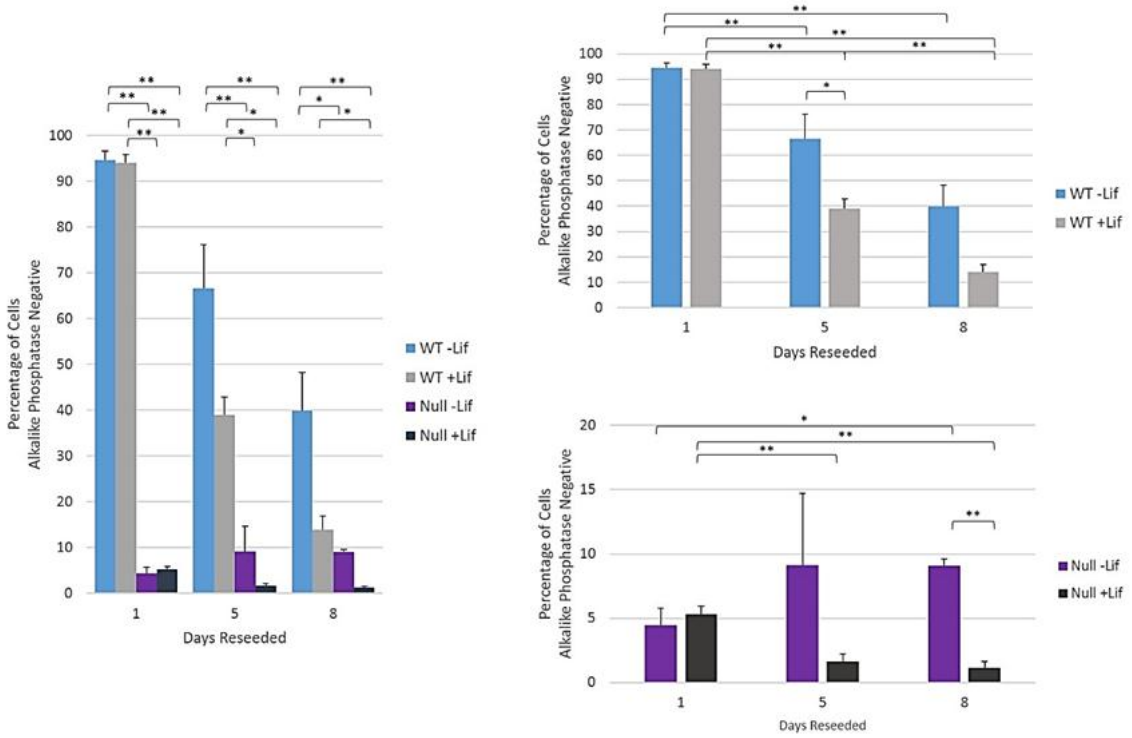

Figure 4C. After 20 days of inducing differentiation, alkaline phosphatase activity confirms that the phenotype of Null cells is recovered by LIF. Cellular differentiation was induced for 20 days in bacteria culture dishes with ES cell media absent of LIF. Cells were then reseeded on tissue culture dishes coated with gelatin. Alkaline phosphatase staining was performed on cells grown in 3.5 cm dishes 1, 5, and 8 days after initial reseeding. Percentage of cells unstained or differentiated were graphed. The percentages were based on an average of 3-5 separate experiments. As before, at least 300 cells were counted for each condition. Error bars indicate standard error. A single asterisk denotes $p < 0.05$ and double asterisks denotes $p < 0.01$. The graph on the left shows all conditions and cell lines collectively, while the right splits the data up. WT conditions are on top and Null conditions on the bottom right.

D.

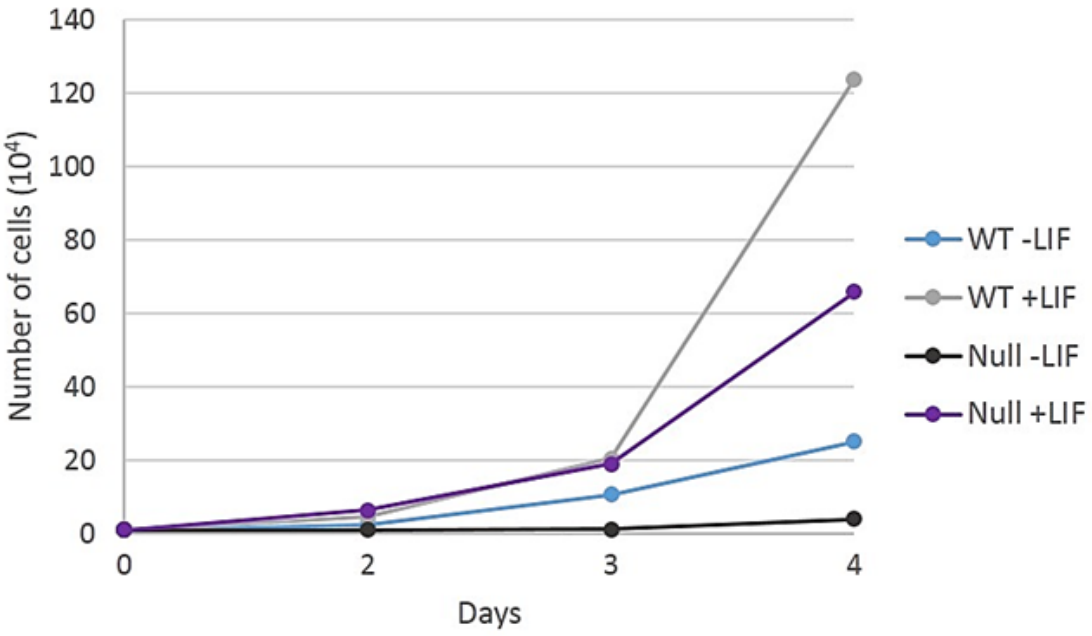

Figure 4D. After 20 days of inducing differentiation, cells treated with LIF had a faster recovery time. Cells were grown in the absence of LIF for 20 days. After cells recovered from being reseeded on tissue culture dishes coated with 0.01% gelatin in either ES cell media with or without LIF, growth rates were measured for each condition. Error bars represent standard error.

A.

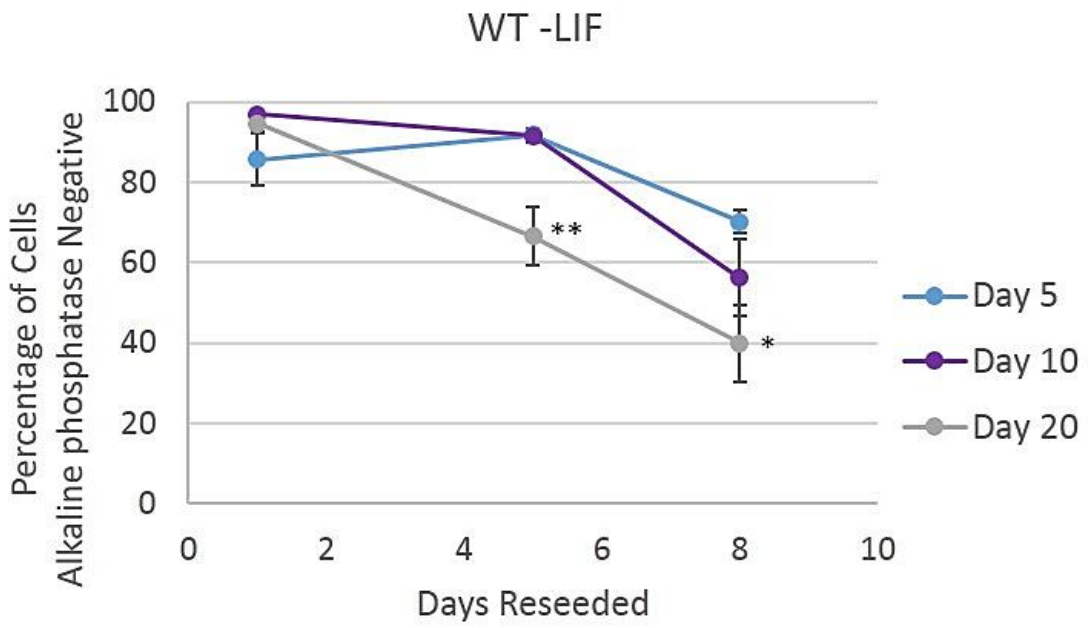

Figure 5A. Summary of alkaline phosphatase data of WT cells reseeded without LIF. In order to compare the alkaline phosphatase data of WT cells reseeded and cultured without LIF, data from previous figures 2C, 3C, and 4C was used. Days 5, 10 and 20 represent the amount of time the cells spent in suspension without LIF to induce differentiation. Error bars represent standard error. Double asterisks denotes the significant difference ($p < 0.05$) between 5 and 20 days in suspension. A single asterisk denotes the significant difference ($p < 0.05$) between 5 and 20 days and between 10 and 20 days in suspension.

B.

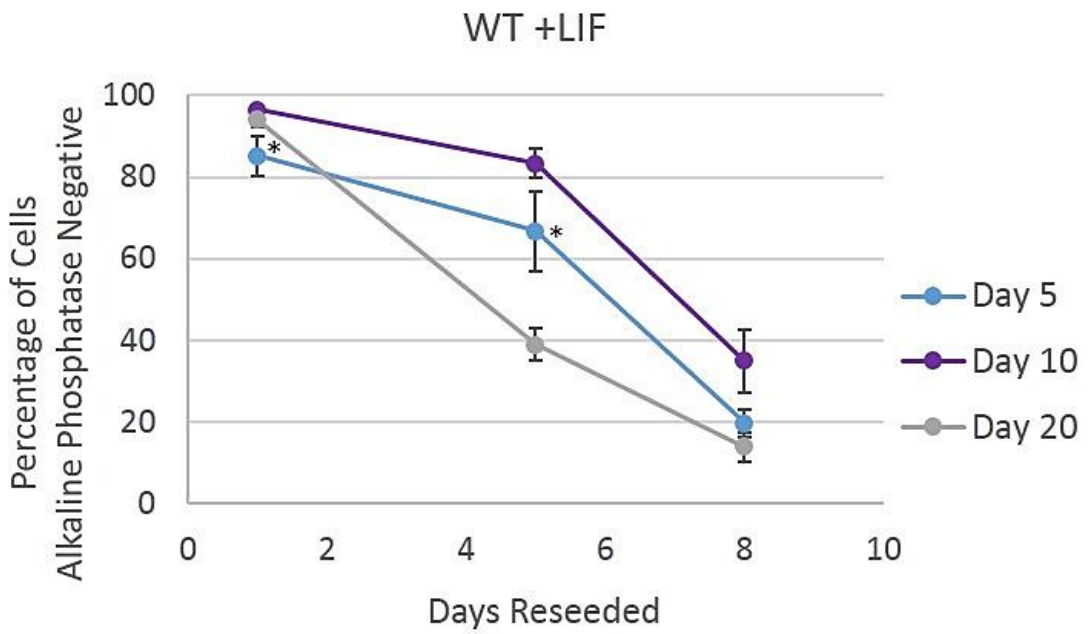

Figure 5B. Summary of alkaline phosphatase data of WT cells reseeded with LIF. In order to compare the alkaline phosphatase data of WT cells reseeded and cultured in the presence of LIF, data from previous figures 2C, 3C, and 4C was used. Days 5, 10 and 20 represent the amount of time the cells spent in suspension without LIF to induce differentiation. Error bars represent standard error. A single asterisk 1 day after reseeding denotes the significant difference ($p < 0.05$) between 5 and 20 days in suspension. A single asterisk 5 days after reseeding denotes a significant difference ($p < 0.05$) between 10 and 20 days in suspension.

C.

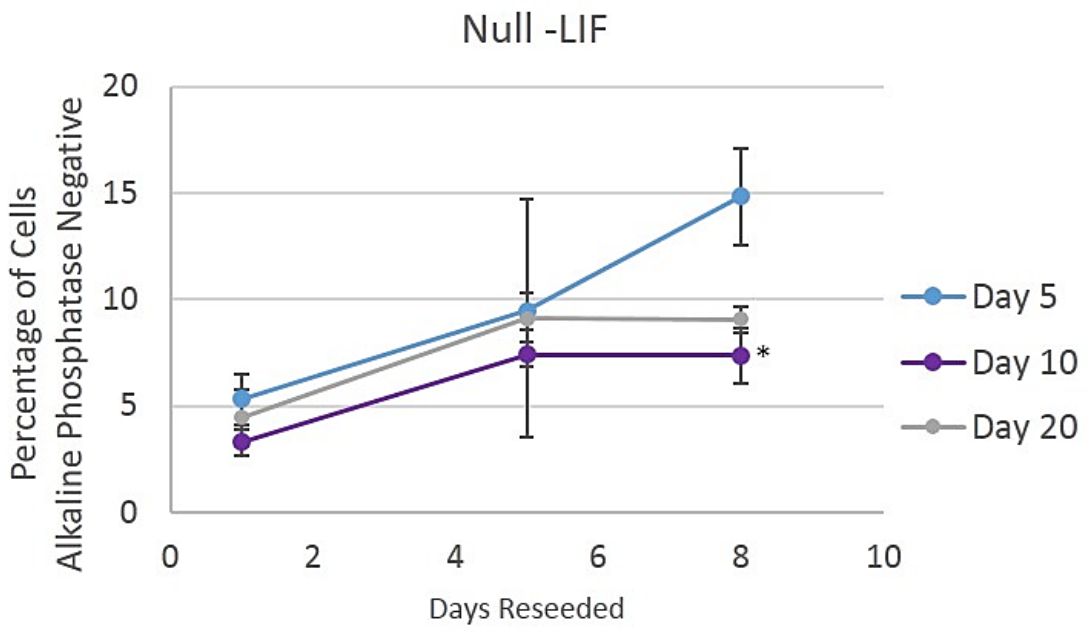

Figure 5C. Summary of alkaline phosphatase data of Cfp1 Null cells reseeded without LIF. In order to compare the alkaline phosphatase data of Null cells reseeded and cultured in the absence of LIF, data from previous figures 2C, 3C, and 4C was used. Days 5, 10 and 20 represent the amount of time the cells spent in suspension without LIF to induce differentiation. Error bars represent standard error. A single asterisk denotes a significant difference ($p < 0.05$) between 5 and 10 days in suspension.

D.

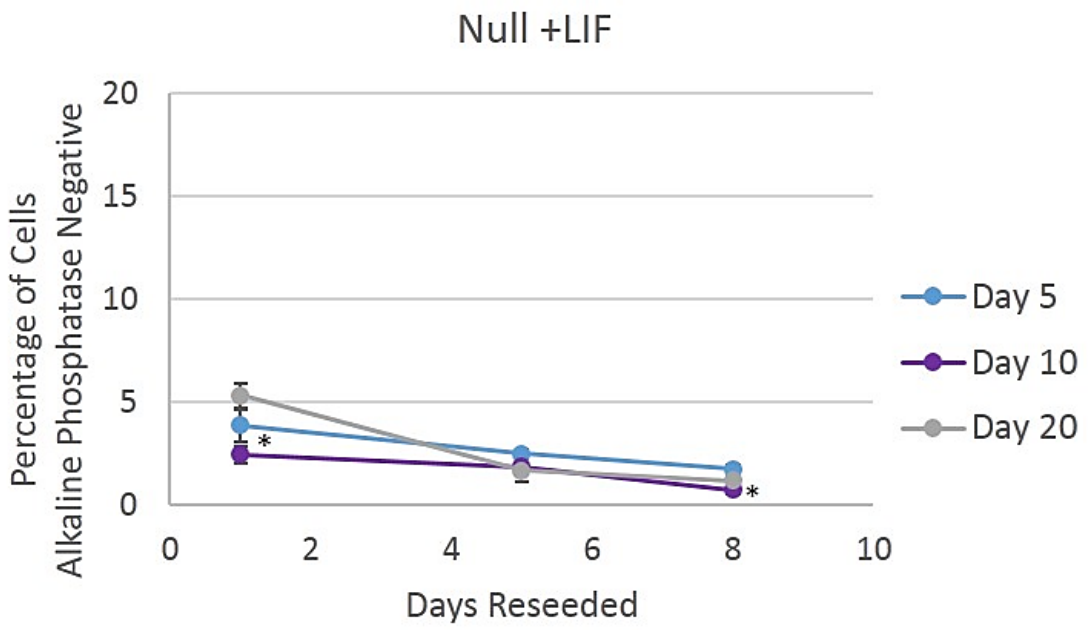

Figure 5D. Summary of alkaline phosphatase data of Cfp1 Null cells reseeded with LIF. In order to compare the alkaline phosphatase data of Null cells reseeded and cultured with LIF, data from previous figures 2C, 3C, and 4C was used. Days 5, 10 and 20 represent the amount of time the cells spent in suspension without LIF to induce differentiation. Error bars represent standard error. A single asterisk 1 day after reseeding denotes the significant difference ($p < 0.05$) between 10 and 20 days in suspension. A single asterisk 8 days after reseeding denotes a significant difference ($p < 0.05$) between 5 and 10 days in suspension.

A.

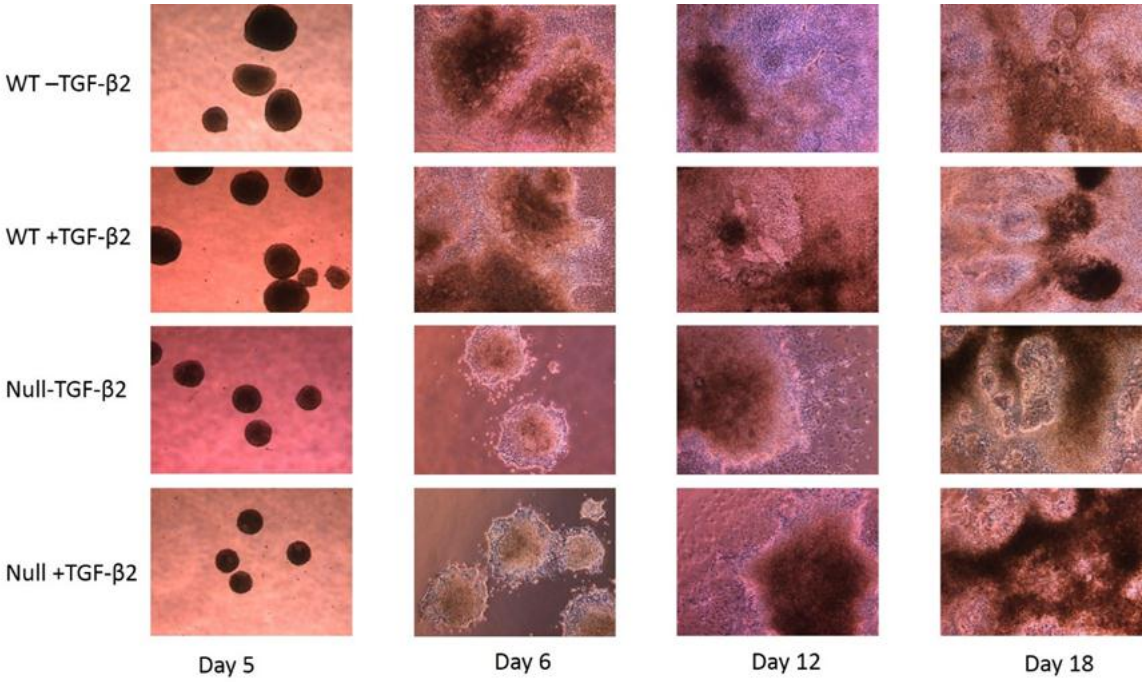

Figure 6A. Null cells form outgrowths after being reseeded on gelatin coated tissue culture dishes. Differentiation was induced for two days as hanging drops and then moved to suspension in bacteria dishes in either ES cell media supplement with or without 8 ng/ml TGF-β2. Day 5 represents floating EBs just before they were plated on tissue culture dishes for an additional 13 days. Morphology was analyzed days in which cells were collected for RNA isolation. WT EB's with TGF-β2 started beating around Day 8, whereas WT EB's without did not start beating until Day 10. Null cells either treated with or without TGF-β2 never expressed a beating phenotype. Magnification, 4X.

B.

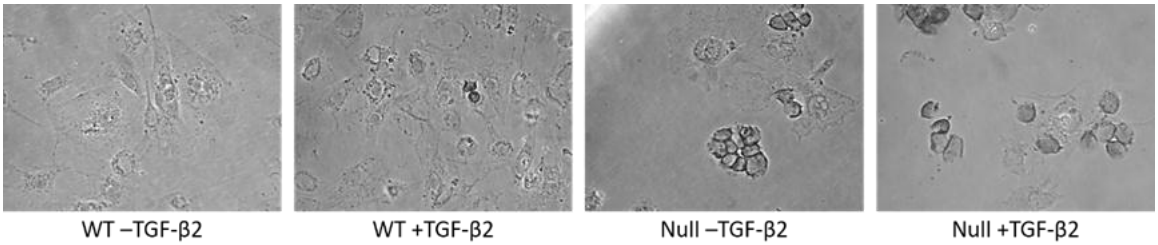

Figure 6B. Visual of alkaline phosphatase activity of cells cultured in ES cell media with or without TGF-β2. EB's were collected, dissociated with trypsin, and plated on 3.5 cm gridded dishes on days 5, 11, and 17. The day after cells were plated, they were stained for alkaline phosphatase. Above is a representative morphology of alkaline phosphatase staining of cells at 12 days. Darkly stained cells are alkaline phosphatase positive. Magnification, 20X.

C.

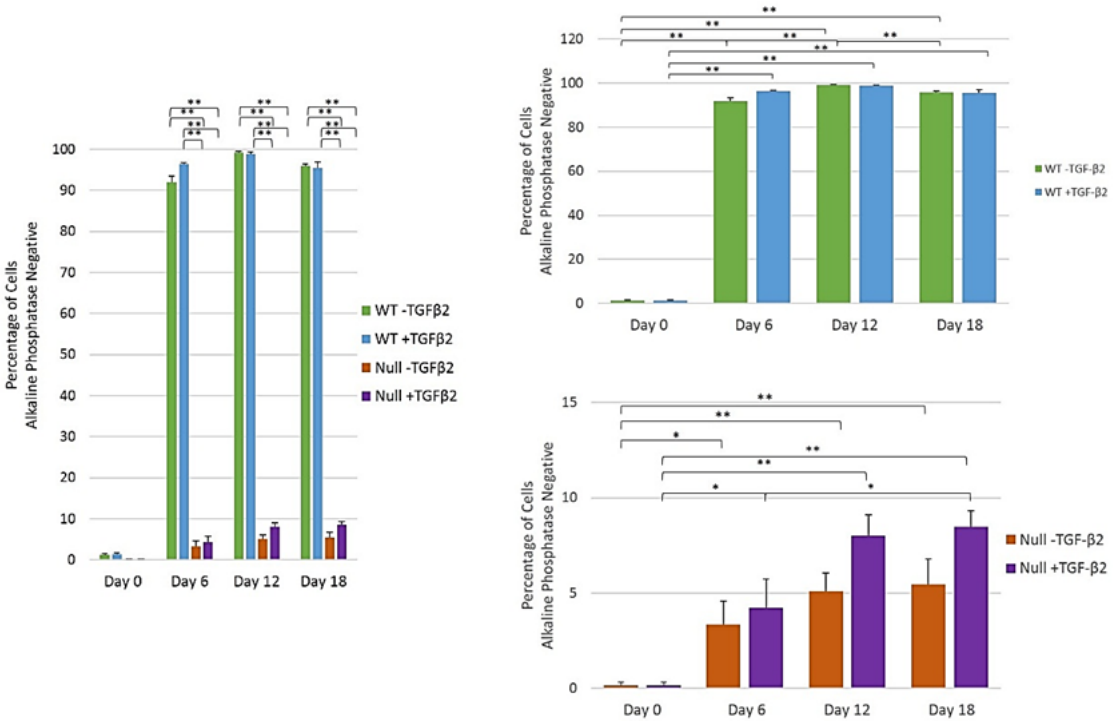

Figure 6C. Alkaline phosphatase markers slightly decrease in Cfp1 Null cells. Embryoid bodies cultured in ES cell media with or without 8 ng/ml of TGF-β2 were dissociated and reseeded on 3.5 cm gridded tissue culture dishes coated with gelatin. Cells were stained the day after reseeding 6, 12, and 18 days from the initiation of differentiation. Day 0 represents cells being maintained in the presence of LIF. Percentage of cells unstained or differentiated were graphed. The percentages were based on an average of 3-5 separate experiments. At least 300 cells were counted for each condition. Error bars indicate standard error. A single asterisk denotes $p < 0.05$ and double asterisks denotes $p < 0.01$. The graph on the left shows all conditions and cell lines collectively, while the right splits the data up. WT conditions are on top and Null conditions on the bottom right.

D.

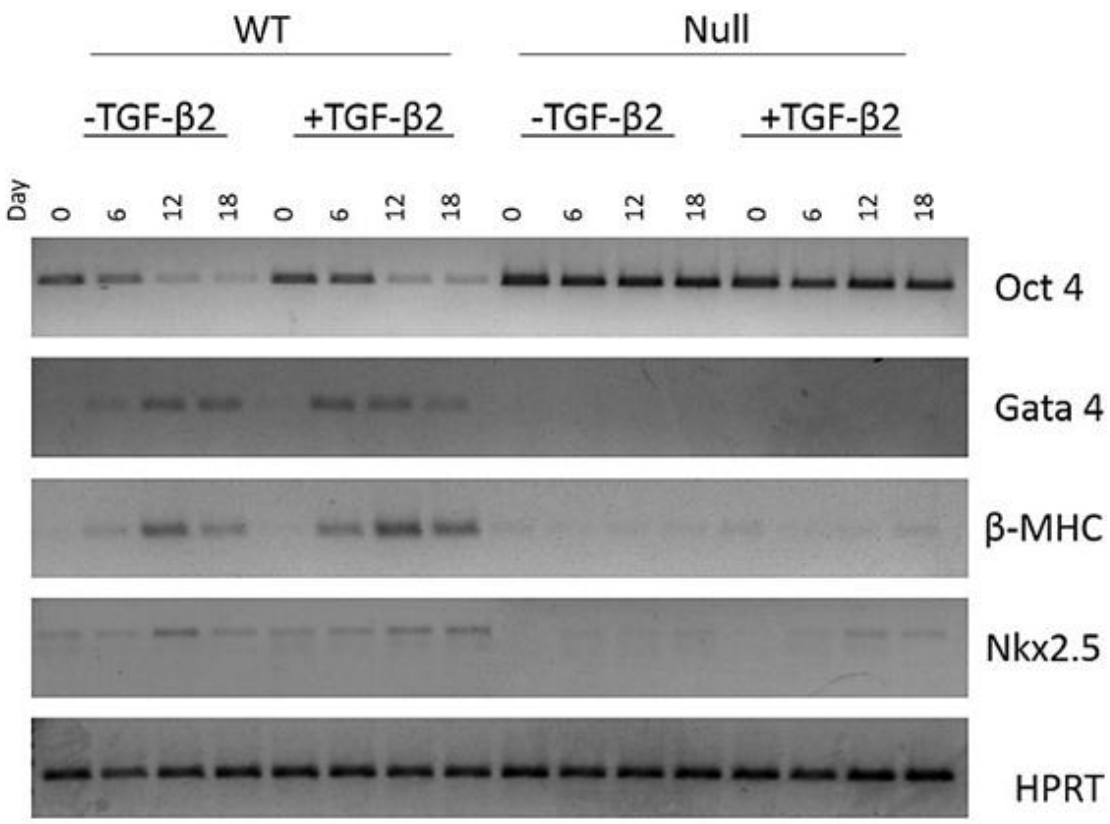

Figure 6D. Null cells start to express linage specific markers. EBs treated with or without TGF-β2 were collected at various times (0, 6. 12. And 18 days). Total RNA was isolated and reverse-transcript PCR was performed to analyze lineage and cardiac specific markers: Gata4, β-MHC, Nkx2.5. Oct4, pluripotency marker; HPRT, control for mRNA quantity and integrity. This figure is a representative of three independent experiments.

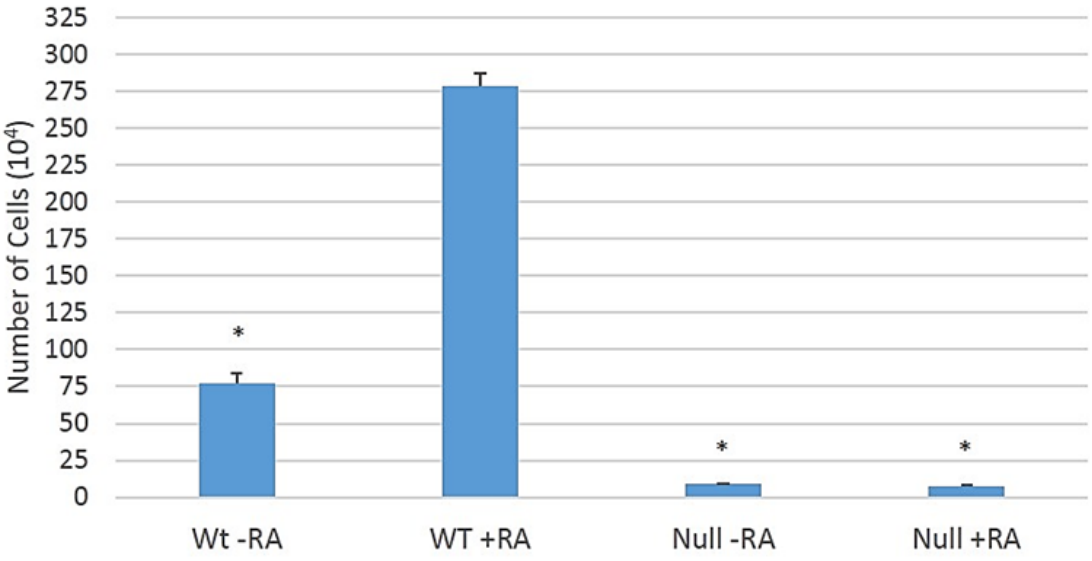

Figure 7. Few Null cells survive the neuronal differentiation protocol. After an 8 day differentiation period, with 4 days with or without RA, cells were counted before they were reseeded on tissue culture dishes coated with 0.01% gelatin. Data represented is an average of cells from 5 experiments. Error bars represent standard error. Asterisks denote a significant difference ($p < 0.05$) compared to WT cells treated with RA.