A Study of Primary and Secondary Heart Field Populations in iPS Cell-Derived Embryoid Bodies

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Dr. Randy Daughters & Dr. Susan Keirstead

December 2013

Acknowledgements

I would like to thank Dr. Randy Daughters for guidance and support throughout this work. He served as an excellent mentor.

I would also like to thank Dr. Susan Keirstead for her suggestions and direction throughout this work, and especially during the writing process.

Additionally, I would like to thanks Dr. James Dutton for his suggestions and academic discussions.

Members of the Slack/Daughters/Dutton lab all were quite helpful in teaching techniques and holding scientific discussion, in particular Sarah Burley and Lucas Greder.

Dedication

This thesis is dedicated to my family, whose support has encouraged me to further my education in pursuit of my goals. My parents, Tom Johnson and Lisakay Smith, and grandparents, Joan and Gerald Johnson, have provided unwavering support and guidance.

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Introduction

The steps through which cardiac differentiation progresses are fairly well defined, but some mechanistic questions still remain. It is well established that *Oct4* is a key regulator of pluripotency. Emerging data supports claims that *Oct4* may have an active role in cardiac lineage commitment. To further explore this possibility, *in vitro* development modeled through embryoid bodies may provide a platform to confirm the role of *Oct4* in a setting reflective of normal cardiac differentiation.

Pluripotent Cells

Embryonic stem (ES) cells are pluripotent cells capable of differentiating into all somatic cell types, and serve as an effective *in vitro* model of development for many mammalian systems (Evans, 2005). The groundwork for ES cell work was laid by pluripotent cells isolated from teratocarcinomas of mice, referred to as embryonal carcinoma (EC) cells (Damjanov, 2005; Andrews et al., 2005, Chambers and Smith, 2004). EC cells were isolated from testicular teratocarcinomas, and were observed to differentiate successfully into all three germ layers upon injection into immune-compromised mice (Andrews et al., 2005; Damjanov, 2005). When isolated and injected into a mouse blastocyst, the EC cells contributed to a variety of cell types in the resulting mice (Chambers & Smith, 2004). When cultured in suspension, EC cells formed aggregates resembling mouse egg-cylinder stage embryos, in both morphology and differentiation capacity (Stevens, 1960).

Based on the knowledge from EC cells, in 1981, Martin, as well as Evans and Kaufman, both successfully isolated and cultured mouse ES (mES) cells from the inner cell mass (ICM) of normal mouse blastocysts for the first time (Martin, 1981; Evans and Kaufman, 1981). In contrast to EC cells, ES cells are not malignant and can regularly contribute to the germline of chimeric animals (Andrews et al., 2005; Bradley et al., 1984). They maintain pluripotency and can

be cultured indefinitely when grown on a fibroblast feeder layer in the presence of serum and leukemia inhibitory factor (LIF) (Evans, 2005; Ying et al., 2008; Smith et al., 1988). Early studies indicated that medium conditioned with buffalo rat liver cells could replace a feeder layer of fibroblasts with the purpose of maintaining ES cells in a pluripotent state (Smith and Hooper, 1987). LIF, a member of the interleukin-6 cytokine family, was determined to be the active molecule in the aforementioned medium that functioned to maintain the pluripotency of ES cells (Smith et al., 1988). Upon removal of LIF from culture conditions, ES cells begin to spontaneously differentiate (Chambers and Smith, 2004).

After further analysis of LIF and the mechanism by which it maintains ES cells in an undifferentiated state, it was determined that it functioned to activate specific genes which in turn activate or repress other transcription factors that prevent a cell from differentiating and allow for self-renewal to maintain pluripotency (Chambers and Smith, 2004). After it was determined that LIF played an active role in activating these "pluripotent" gene networks, a closer examination revealed three genes in particular that are central to pluripotency: *Sox2* (Masui et al., 2007), *Nanog* (Chambers et al., 2003), and *Oct4, which is* also known as *Pou5f1* (Niwa et al., 2000). All three of these genes are expressed in the inner cell mass (ICM) of blastocysts, epiblast cells, and ES cells (Chambers and Smith, 2004). *Oct4* has been demonstrated to be critical for the maintenance of pluripotency of ICM and ES cells (Kehler et al., 2004; Zeineddine et al., 2006).

Pluripotent cells have become increasingly more attractive for possible clinical research and treatments, including drug development, cell therapy, and *in vitro* developmental studies. However, the ethical implications associated with destroying a human blastocyst to harvest cells of the ICM for ES culture is not likely an obstacle that will be overcome for use in clinical settings (Evans, 2005; Moerkamp and Goumans, 2012). In 2006, Takahashi and Yamanaka determined a way to dedifferentiate somatic cell types into a pluripotent state. Murine

fibroblasts were transfected using retroviral vectors encoding *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (Takahashi and Yamanaka, 2006). The cells that were successfully transfected began to express genes that are specific to pluripotent cells; they also displayed ES-like morphology and growth patterns (Takahashi and Yamanaka, 2006). These reprogrammed cells were termed "induced pluripotent stem cells," or iPS cells (Takahashi and Yamanaka, 2006). High endogenous expression of *Oct4*, *Sox2*, and *Nanog* was observed in these iPS cells (Takahaski and Yamanaka, 2006; Wernig et al., 2007). Further studies to characterize these cells by the Wernig group in 2007 confirmed that the DNA methylation state and chromatin state of iPS cells were nearly analogous to ES cells (Wernig et al., 2007). However, recent studies suggest that early passages of iPS cells maintain some degree of epigenetic memory from the original somatic cell type (Ohi et al., 2011; Kim et al., 2010; Moerkamp et al., 2012). For example, the DNA methylation statuses of some genes were similar to the cell type of origin, such as hepatocytes, skin fibroblasts, and melanocytes (Ohi et al., 2011; Kim et al., 2010; Moerkamp et al., 2012). The epigenetic memory was not observed in the iPS cells after subsequent passaging, suggesting that they do eventually reach a more ES-like state (Ohi et al., 2011). iPS cells are analogous to ES cells in that they are pluripotent and have the capacity to generate chimeric mice when introduced to a normal mouse blastocyst (Takahashi and Yamanaka, 2006; Wernig et al., 2007). Both human ES (Thompson et al., 1998) and iPS (Rust et al., 2006; Yu et al., 2007) have been successfully isolated and cultured. The significance of both ES and iPS cells can be found in their unique ability to differentiate into any somatic cell of the body. To date, there is not a population capable of such plasticity that exists in the adult mammal. Through the close examination of differentiation of these pluripotent cells *in vitro*, molecular interactions during development may be better understood.

In Vitro Differentiation Methods

In early studies, cells from teratocarcinomas of testis were isolated for further characterization (Stevens, 1960; Pierce and Dixon, 1959). When injected intraperitoneally, the cells were observed to form aggregates, termed as embryoid bodies (EBs) (Pierce and Dixon, 1959). The EBs contained tissue types from all three germ layers (Pierce and Dixon, 1959; Stevens, 1960). When EBs were transplanted into the ocular cavity of mice, all three germ layers were also found in the differentiated cells (Stevens, 1960). A subset of pluripotent cells existed in the transplanted EBs with the capability of populating entirely new tumors when subsequently transplanted (Stevens, 1960). Further studies indicated that the EBs formed *in vitro* as well as *in vivo* were comparable to normal mouse embryos in morphology, differentiation capacity, and patterning (Stevens, 1960; Pierce and Dixon, 1959; Pierce and Verney, 1961). A homogenous pluripotent stem cell population was isolated from mouse teratocarcinomas and cultured *in vitro* by Martin and Evans in 1974. The cells comprising the population were termed embryonal carcinoma (EC) cells (Martin and Evans, 1974). When aggregated and allowed to differentiate *in vitro*, cystic EBs demonstrated a patterning of differentiation similar to that of ICM cells cultured *in vitro* (Martin et al., 1977).

Following the successful isolation and culture of mouse ES cells and revelation of iPS cells, it was demonstrated that both have the capacity to aggregate into EB structures (Doetschman, 1985; Keller, 1995; Takahashi, 2006; Wernig, 2007). In the study done by Doetschman in 1985, it was determined that mouse ES (mES) cells in an EB formation are most similar to normal mouse embryos between days 6 and 8 in the egg cylinder stage. EBs formed using mES cells bear a stronger resemblance of a normal mouse embryo than EBs formed with EC cells in that they develop a normal mesoderm layer between the endoderm and ectoderm layers (Doetschman, 1985). This indicates that mES

EBs resemble the morphology and differentiation capacity of normal mouse embryos, which makes them good models for *in vitro* developmental studies.

Dependent on the study and desired ending cell type, a wide variety of methods have been proposed to differentiate ES cells toward specific lineages. Typically, one of the following culture conditions are used to differentiate ES cells: embryoid body (EB) formation, monolayer culture on extracellular matrix (ECM) proteins, or co-culture with a specific somatic cell type (Murry and Keller, 2008). Cells may be allowed to differentiate spontaneously, or may be directed toward a particular lineage by manipulation of the extracellular environment (i.e. soluble factors, small molecules). EB models have been demonstrated to best recapitulate the developing embryo, and are the most common method of differentiation (Doetschman, 1985; Keller, 1995)

For the purpose of this study, EBs made from iPS cells were chosen as the means of differentiation. EBs can be formed with various methods, such as static suspension in low-adherence plates, hydrodynamic culture, centrifugation, and hanging drop. Each method offers different strengths, as well as limitations. Static culture of EBs is arguably the simplest method by which to form EBs. A given number of single ES cells are suspended in medium on a low-adherence plate, to which the cells are unlikely to form attachments with the surface (Bratt-Leal et al., 2009). The cells will spontaneously form cell-cell adhesions, resulting in EBs. However, there is no way to control how many cells aggregate in this method, and the resulting population of EBs is heterogeneous in size and shape (Bratt-Leal et al., 2009; Kurosawa, 2007). In hydrodynamic culture, single ES cells are again suspended in medium on a low-adherence surface. In contrast to the static method, the culture is constantly moving. This method can be utilized with techniques using rotary orbital culture, stirred flasks, or spinner flasks (Bratt-Leal et al., 2009). The resultant EBs are more homogeneous than static cultures, but exact sizes cannot be controlled. The culture conditions are also maintained at a higher level of uniformity, as the medium is continuously moving, distributing oxygen, nutrients, and soluble factors evenly (Kurosawa, 2007; Bratt-Leal et al.,

2009). The centrifugation, or spin, method allow for selection of the size of EB desired. A defined number of cells is suspended in medium and dispersed into the wells of a low-adherence plate. The plate is centrifuged, forcing the cells to aggregate in the center of the wells (Antonchuk, 2013). EBs resulting from this method are generally uniform in size and shape, but tend to exhibit some signs of cell death near the center of the EB (Antonchuk, 2013). The observed cell death may be attributed to the force exerted on the cells during centrifugation. When EBs are formed via the hanging drop method, a designated number of cells are suspended from the lid of petri culture dish in drops of media (Bratt-Leal et al., 2009; Kurosawa, 2007). The rounded drops facilitate the formation of EBs through gravitational pull, allowing them to aggregate. EBs generated via hanging drop method are generally homogenous, in size and shape (Keller, 1995; Bratt-Leal et al., 2009). It has been observed that EBs formed with this method develop polarity (Mogi, 2009; ten Berge et al., 2008). In order to decrease variability within individual cultures and improve reproducibility between biological replicates, the hanging drop method was chosen as the differentiation method in this work. By choosing to allow spontaneous differentiation versus introducing soluble factors to direct the differentiation, the EBs will be a more accurate recapitulation of temporal events of cardiogenesis in the developing embryo. Previous publications have demonstrated from *in vitro* studies that pluripotent cell populations can generate cells in all stages of cardiac commitment, from early precursors to specialized, mature cells (Maltsev et al., 1994; Moerkamp and Goumans, 2012).

Cardiac Lineage Commitment and *Oct4* Involvement

The heart is the first functional organ to develop in an embryo (Van Vliet et al., 2012; Rana et al., 2013). The cells of the ICM of the blastocyst express pluripotency-regulatory genes such as *Oct4*, *Sox2*, and *Nanog* (Figure 1) (Stefanovic et al., 2009). *Oct4* itself has been determined to be required for

pluripotency maintenance (Nichols et al., 1998; Niwa, 2007; Kehler et al., 2004). Knockout models cannot maintain a pluripotent state in the ICM after blastocyst development (Nichols et al., 1998), and repression of *Oct4 in vitro* results in a loss of pluripotency and dedifferentiation to trophectoderm (Niwa et al., 2000). *Oct4* expression is generally limited to oocytes, ES cells, and primordial germ cells (Scholer et al., 1989; Yeom et al., 1996). By day 8.0-8.5, the only cells still expressing *Oct4* are the primordial germ cells (Scholer et al., 1990).

Pluripotent cells have been demonstrated to be sensitive to the level of *Oct4* expression. A certain level will maintain a pluripotent phenotype, while upregulation pushes a cell toward primitive endoderm or mesoderm (Niwa et al., 2000). *Sox2* and *Oct4* have been demonstrated to coregulate certain genes tightly regulating pluripotency via binding motifs in the same enhancer regions (Figure 1A) (Wamstead et al., 2012; Stefanovic et al., 2009; Masui et al., 2007). When *Oct4* is expressed at a basal level, it forms a complex with *Sox2* binding to the enhancer and/or promoter region of many genes to maintain the pluripotent state of the cell (Stefanovic et al. 2009). Together, they tightly regulate the expression of genes directly involved in maintenance of pluripotencty, such as *Oct4*, *Sox2*, and *Nanog* (Masui et al., 2007, Bernstein et al., 2006). In *Sox2*-null mice, *Oct4* forced expression can rescue pluripotency if present at the proper level (Masui et al., 2007).

In the developing embryo, *Oct4* is upregulated during gastrulation, a process undergone by the primitive streak (Buckingham et al., 2005). Gastrulation is the process by which the three germ layers develop (Buckingham et al., 2005, Van Vliet et al., 2012). An increase in *Oct4* expression leads to stimulates *Sox17* expression (Figure 1) (Van Vliet et al., 2012; Stefanovic et al., 2009). *Sox17* competes with *Sox2* to form complexes with *Oct4* (Stefanovic et al., 2009). The *Oct4-Sox17* complex binds to DNA upstream of *Sox17*, stimulating its transcription (Stefanovic et al., 2009). With *Oct4* binding *Sox17* instead of *Sox2*, there is no complex binding to the *Sox2* promoter so it is no longer expressed. *Sox17* is a transcription factor with expression observed in

endoderm and some mesoderm. Inhibition of *Sox17* does not prevent mesoderm formation, but it does prevent induction of *mesoderm posterior 1* (*Mesp1*), suggesting that it may directly regulate expression of this gene (Van Vliet et al., 2012; Liu et al., 2007). Differential *Oct4* expression may therefore influence *Sox17* expression, which in turn regulates the expression of *Mesp1*, providing a direct link between *Oct4* expression at gastrulation and the control of genes involved in early cardiac commitment.

The cardiac mesoderm of mice originates from the anterior part of the primitive streak of the embryo, which *Mesp1* facilitates (Vincent and Buckingham, 2010). *Mesp1* expression is one of the first detectable markers of cardiac commitment, and a master regulator of cardiac fate determination (Vincent and Buckingham, 2010, Van Vliet et al., 2012).

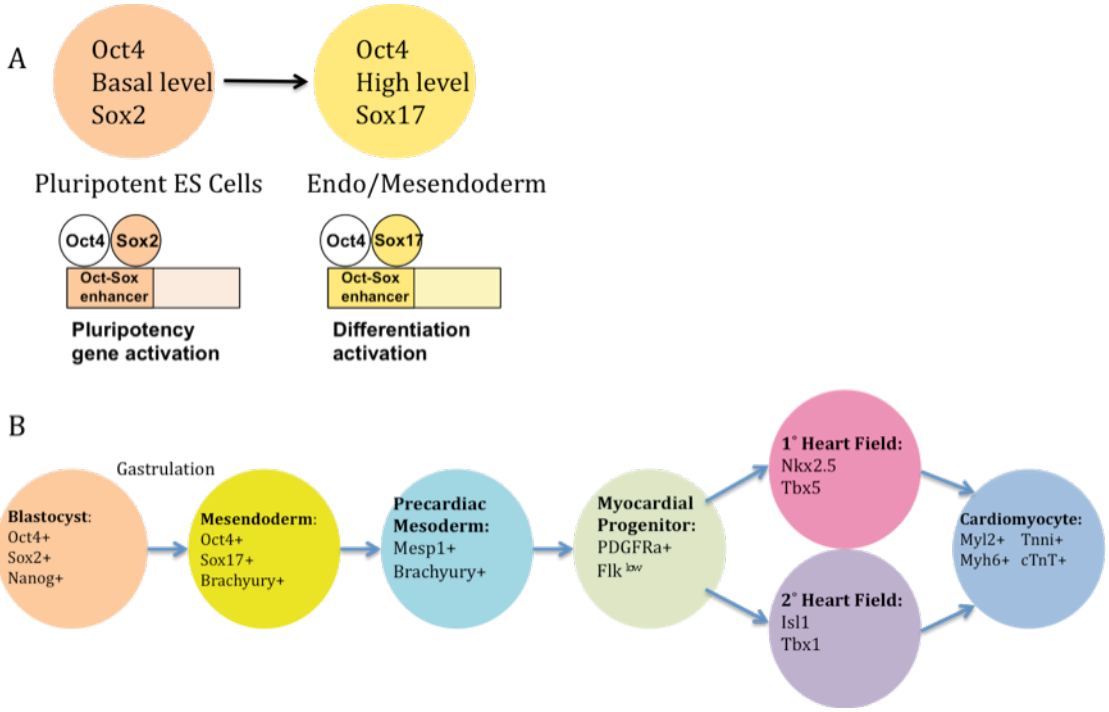


Figure 1. Cardiac Differentiation. A. *Sox2*/*Sox17*-*Oct4* loop (adapted from Stefanovic et al., 2009). B. Gene expression through cell stages along cardiac lineage commitment. (Van Vliet et al., 2012; Stefanovic et al., 2009; Rana et al., 2013; Bondue et al., 2011)

Mesp1 functions to downregulate pluripotency genes, and upregulate the expression of cardiac transcription factors (Rana et al., 2013). *Mesp1* has been demonstrated to directly regulate genes important in cardiac lineage

determination such as *Nkx2.5*, *Tbx5*, *cardiac troponin-T* (*cTnT*), *myosin heavy chain-6* (*Myh6*), and *myosin light chain-2* (*Myl2*) (Bondue et al., 2011). *Mesp1* also has a binding site in the upstream enhancer region of *Sox17*, leading to suppression of the expression of the gene (Bondue et al., 2008). Multiple binding regions for *Mesp1* were identified in the promoter of *Nkx2.5* specifically (Bondue et al., 2008). *Brachyury* (*Bry*) is also required for mesoderm specification and expressed during gastrulation into early organogenesis (Figure 1) (Lui et al., 2007; Leahy et al., 1999). From the precardiac mesoderm expressing *Mesp1* and *Bry*, myocardial progenitors capable of differentiating into cardiomyocytes (CM), smooth muscle cells (SMC), and endothelial cells arise (Bondue et al., 2008; Bondue et al., 2011). These myocardial progenitors express low levels of *fetal liver kinase-1* (*Flk1*), which is a receptor for *vascular endothelial growth factor* (*VEGF*) (Figure 1) (Murry and Keller, 2008), as well as expression *of platelet-derived growth factor receptor-alpha* (*PDGFRa*) (Bondue et al., 2011), both influential factors involved in proliferation and differentiation. *Islet1* (*Isl1*) cooperates with *Mesp1* to direct cell fate toward the cardiovascular lineage (Bondue et al., 2011). *Isl1* is also expressed in a subset of myocardial progenitor cells (Bondue et al., 2011).

In the developing heart, there are four main contributors: primary heart field (PHF), secondary heart field (SHF), cardiac neural crest (CNC), and the proepicardial organ (Vincent and Buckingham, 2010). The cells of the PHF contributes to the inflow tract, atria, and left ventricle; cells from the SHF form the outflow tract and right ventricle (Abu-Issa and Kirby, 2007; Vincent and Buckingham, 2010). Distinct gene expression patterns have been identified in cells of the PHF and SHF (Figure 1). The cells originating from the PHF express *Nkx2.5* and *Tbx5* (Van Vliet et al., 2012; Rana et al., 2013). The transcription factor *Nkx2.5* was determined to be critical for the developing heart tube and chambers (Rana et al., 2013). *Tbx5* is also a transcription factor, and it functions to regulate many other genes involved in cardiac specification (Rana et al., 2013; Vincent and Buckingham, 2010). It is predominantly expressed in inflow tract,

precardiac venous mesoderm, atrio-ventricular canal, and embryonic myocardium (Rana et al., 2013). Cells of the SHF can be identified by expression of *Isl1* and *Tbx1* (Murry and Keller, 2008; Van Vliet et al., 2012; Buckingham et al., 2005). *Tbx1* expression directly represses expression of *Tbx5* (Rana et al., 2013). It has also been linked to indirect regulation *Wnt5a*, the implications of which will be discussed below (Rana et al., 2013). *Isl1* expression is observed primarily in venous and arterial poles of the cardiac tube (Buckingham et al., 2005).

Terminally differentiated cardiomyocytes express the structural genes *myosin light chain-2* (*Myl-2*), *myosin heavy chain-6* (*Myh6*), *cardiac troponin-T* (*cTnT*), and *troponin-I* (*Tnni*) (Figure 1) (Miller-Hance et al., 1993; Murry and Keller, 2008; Liu et al., 2007). In the mature cell type, the myosin proteins play a large role in muscle contraction, which is a necessary function of cardiomyocytes (Wei and Jin, 2011). Troponin functions to allow crossbridge formation between actin and myosin (Wei and Jin, 2011). Mature cardiomyocytes must express these genes in order to generate the required proteins to successfully function.

Mesodermal differentiation is also influenced by the Wnt and beta-catenin pathway (Van Vliet et al., 2012). Wnt signaling facilitates the mechanism by which EBs become polarized (ten Berge et al., 2008). The canonical *Wnt*-signaling pathway has been linked to cardiac lineage commitment (Li et al., 2013). In the canonical *Wnt* pathway, the binding of the *Wnt* ligand to a receptor complex triggers a cascade of intracellular events leading to beta-catenin translocation to the nucleus (Rana et al., 2013). Beta-catenin binds TCF/LEF transcription factors and together regulates expression of specific genes (Rana et al., 2013). Residual *Oct4* expression after gastrulation coincides with a burst of Wnt signaling in the embryo (Lie et al., 2013). Recent evidence has demonstrated a composite binding site for *Lef1* and *Oct4* in the promoter of *Mesp1*, inducing the expression of *Mesp1* (Li et al., 2013). The joint regulation of *Mesp1* by both *Oct4* and *Lef1* provides a novel explanation of the role of *Oct4* in cardiac fate determination. With *Oct4* directly influencing the expression of

Mesp1, investigating the temporal expression of *Oct4* in conjunction with expression of cardiac-specific genes may help to further characterize the role of *Oct4* in cardiac lineage commitment.

Oct4 Reporter Cell Line (3F10)

In order to investigate the proposed interactions of *Oct4* in determining commitment to the cardiac lineage, an iPS cell line with a reporter sensitive to *Oct4* expression was utilized. The cell line was generated via reprogramming of fibroblasts from transgenic mice (Greder et al., 2012). First, pluripotent cells were genetically engineered *in vitro* via a knock-in construct which contains a sequence for a Cre-recombinase fused to a tamoxifen-activated estrogen receptor in the 3' untranslated region of the endogenous *Oct4* gene (Figure 3) (Greder et al., 2012). The sequence encoding *Oct4* is separated from the Cre sequence by an internal ribosomal entry site (IRES), allowing for a polycistronic transcript (Greder et al., 2012). Next, cells which had successfully recombined and expressed the transgene were used to engineer transgenic mice via blastocyst injection. Transgenic mice expressing the *Oct4-MerCreMer* transgene were then crossed to a double reporter transgenic mouse (Muzumdar et al., 2007). The double reporter expressed floxed membrane-targeted tdTomato fluorescent protein followed by a poly-adenylation sequence, upstream of membrane-targeted enhanced green fluorescent protein (eGFP) under the control of a chicken actin promoter from the *Rosa26* locus (Figure 3) (Muzumdar et al., 2007). In this double reporter system, prior to Cre-mediated recombination, ubiquitous expression of tdTomato will be observed in all cells; activated Cre recombinase would cause the excision of tdTomato, and subsequent expression of eGFP (Muzumdar et al., 2007). In generation of a double transgenic mouse, cells expressing *Oct4* during the period of time that tamoxifen is present to activate the Cre recombinase, will be permanently labeled with eGFP (Sajini et al., 2012; Greder et al., 2012). This system is quite sensitive

to levels of *Oct4* transcripts, allowing for visual identification of cells expressing even low levels of *Oct4* (Sajini et al., 2012, Greder et al., 2012).

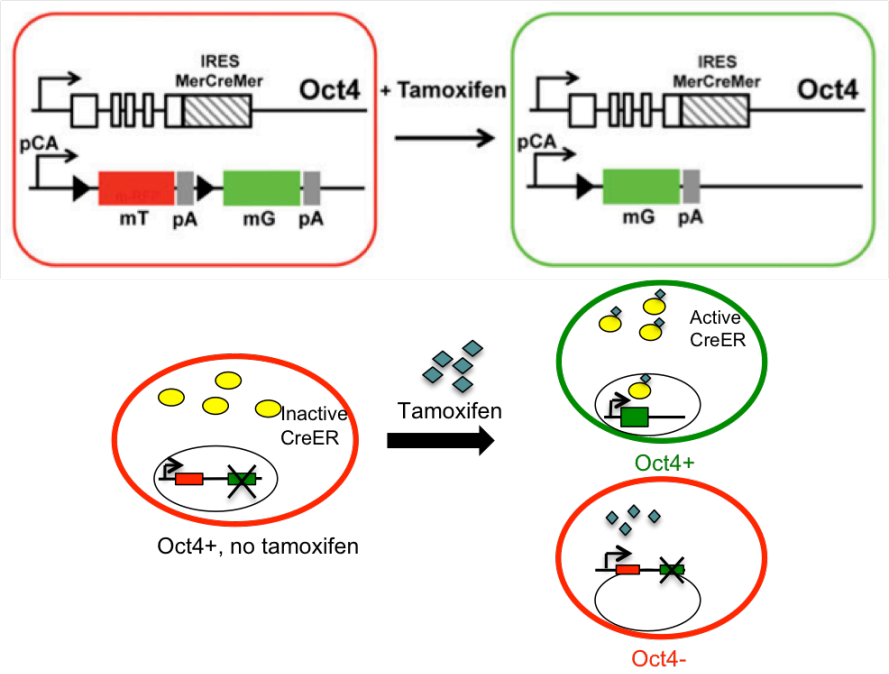


Figure 2. Reporter Construct. Construct of Oct4-mTmG system (adapted from Greder et al., 2012). Cell membranes constitutively fluoresce with tdTomato until excision by activated Cre recombinase (expressed with endogenous *Oct4*) in the presence of tamoxifen.

From the double transgenic mice, an iPS cell line was generated by conventional methods to allow for *in vitro* developmental studies of *Oct4* lineage tracing (Greder et al., 2012; Takahashi and Yamanaka, 2006). This cell line was previously used to describe the *Oct4* expression patterns in EBs (Sajini et al., 2012). In 2012, Sajini et al. described the timing of specific developmental gene expression in an EB model, confirming that EBs do in fact recapitulate normal mouse embryonic development. It was observed that cells contributing to the cardiac lineage arose from cells that stopped expressing *Oct4* between days 3 and 5 in culture (Sajini et al., 2012).

Oct4 is downregulated first in cells that are committed to a specific lineage. Cells committed to the cardiac lineage should begin to express cardiac-specific genes at the time that *Oct4* expression ceases. Based on evidence presented by Sajini et al., cells downregulating *Oct4* between days 3 and 5 were

investigated for presence of both early and late cardiac gene expression. We posit that the cells contributing to the PHF and SHF may downregulate *Oct4* expression at different times, which could help to further separate and define the individual populations. By using this double reporter system, cells can be separated on the basis of *Oct4* expression, and subsequently analyzed for expression of genes specific to each heart field. We expect to see that cells contributing to the PHF will downregulate *Oct4* expression and begin expressing cardiac markers at an early and separate time point from those cells that will contribute to the SHF.

Materials and Methods

Experimental Design

Induced pluripotent stem (iPS) cells from the *Oct4*-mTmG reporter line were cultured as embryoid bodies (EBs) to mimic embryonic development *in vitro* (Figure 3). Media was supplemented with tamoxifen for 24-hour pulses on either day 3, 4, or 5 to allow for identification of cells either expressing or not expressing Oct4 during tamoxifen administration via expression of tdTomato (*Oct4*-) or eGFP (*Oct4*+) fluorescent proteins. Following the 24-hour tamoxifen pulse (day 4, 5, or 6) or at day 10, the EBs were dissociated into single cells and processed using fluorescence-activated cell sorting (FACS). This allows separation of the cells based on *Oct4* expression at the time of tamoxifen administration. The sorted populations were immediately collected for RNA analysis using quantitative polymerase chain reaction (qPCR) to measure relative RNA expression levels. We looked for expression of specific transcripts, like *Nkx-2.5* and *Isl1,* at early stages of cardiac differentiation to determine if commitment could be determined. Transcripts for structural proteins like cardiac troponin-T (*cTnT*) and myosin heavy chain-6 (*Myh6*) indicate a more terminally differentiated cardiomyocyte and can be quantified by analysis of day 10 samples. Expression of structural proteins and transcription factors was confirmed using immunohistochemistry in adherent EBs. Far red secondary antibodies were used since the membranes of the cells were already fluorescent with either tdTomato or eGFP proteins.

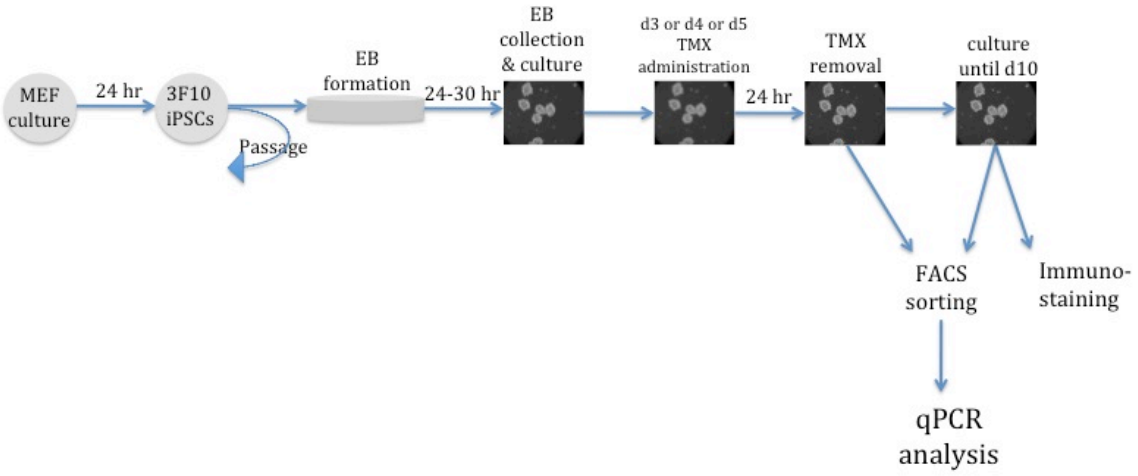


Figure 3. Experimental Design. MEFs cultured prior to introduction of iPS cells. After EB formation and collection, TMX administered to EBs for 24 hour pulse beginning on either day 3, 4, or 5. Subsequent to TMX removal, EBs either dissociated and processed via FACS sorting, or continued on in culture until day 10 before FACS sorting. Sorted populations were analyzed by qPCR.

Preparation of Embryoid Bodies

All medium described in body of text were filtered using 0.22 μl Vacuum Filtration Flasks (Denville) and/or Luer-Lok sterile syringes (BD Falcon) before use to remove bacteria and other possible contaminants.

MEF/iPS Cell Culture

Mouse embryonic fibroblast (MEF) medium was made with DMEM high glucose (Gibco). The DMEM was supplemented with 10% fetal bovine serum (FBS) (HyClone), 1% L-glutamine 200 mM (Gibco), 1% anti-anti (antibiotic-antifungal) (Gibco), and 1% MEM non-essential amino acids (NEAA) (Gibco). After MEF medium was prepared, it was stored at 4˚C and discarded within 2 weeks of the preparation date.

Embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) medium (ES media) was made with knockout DMEM (KO DMEM) (Gibco). The KO DMEM was supplemented with 10% FBS (HyClone), 10% knockout serum replacer (KSR) (Gibco), 1% L-glutamine 200 mM (Gibco), 1% anti-anti (Gibco), 1% MEM NEAA (Gibco), 0.05 mM β-mercaptoethanol (Gibco, 55mM stock), and

1000 units/mL ESGRO-mouse leukemia inhibiting factor (mLIF) (Millipore). mLIF was only added to medium as needed, in smaller aliquots (i.e. 20mL of the 250mL of media), as it expired much more quickly than basal medium. Prepared ES medium was stored at 4˚C. Prepared ES (ES-) medium was discarded within 2 weeks, and ES medium containing mLIF (ES+) was discarded after 5 days.

Cell freezing (CF) medium was prepared with 80% FBS (HyClone) and 20% dimethyl sulfoxide (DMSO) (Sigma). CF medium was prepared as needed, and remaining media was discarded the same day.

Oct4-CreER, mTmG iPS cells (line 3F10) were thawed after having been frozen in liquid nitrogen. 3F10 cells between passage 12 and 25 were used in experiments. To thaw the cells, the vials were placed in a pre-warmed water bath, set at 37˚C. Cells were resuspended in ES- medium and spun in a centrifuge at approximately 900 rmp for 5 minutes. This step is important to remove the DMSO as it is toxic to the cells. The remaining pellet was then resuspended in ES+ medium and plated onto a monolayer of MEFs on 6-well plates (Nest). MEFs were thawed and plated as above, but with MEF medium. The MEFs were plated at a density of 200,000-250,000 cells per well approximately 24 hours prior to the thaw of iPS cells to provide a stable environment in which iPS cells would retain pluripotency. All cultured cells (iPS and MEFs) were maintained in a stable environment at 37˚C and 5% CO_2. The medium was changed the day after cells were plated, to rid the wells of dead cells and debris. Once iPS cells reached near confluence (between days 2 and 4), cells were washed gently with phosphate buffered saline (PBS) (Cellgro) to remove remaining medium. Cells were then incubated with 0.25% Trypsin-EDTA (Gibco) at 37˚C for 5 minutes to break down the attachments formed between the cells and with the plate surface. To neutralize the trypsin, ES- was used to wash the cells. After collection, the cells were spun at 900 rpm for 5 minutes and resuspended in ES+. Cells (MEFs and iPS cells together) were either transferred to another well to continue culture, or frozen for storage. Cells to be frozen were resuspended with half ES medium and half CF medium, for a final concentration

of 10% DMSO. The cells were aliquoted into cryovials (Nunc) and stored at -80˚C for at least 24 hours, and then transferred to liquid nitrogen storage.

Embryoid Body Formation and Culture

Differentiation (DM) medium was made with KO DMEM supplemented with 15% FBS (HyClone), 1% L-glutamine 200 mM (Gibco), 1% MEM NEAA (Gibco), 1% anti-anti (Gibco), and 0.05 β-mercaptoethanol (Gibco, 55 mM stock). After preparation, medium was stored at 4˚C and discarded within two weeks of preparation date.

iPS cells from the 3F10 line were cultured as described above. To form embryoid bodies, cells were passaged and dissociated to a single-cell suspension as described, and then a feeder depletion step was utilized to separate the cells that would not participate in the embryoid body (EB) formation from the pluripotent cells (i.e. MEFs and differentiated iPS cells). This step consisted of culturing the cells on a gelatin-coated 6-well culture plate for between 35 and 45 minutes at 37˚C and 5% CO_2 in ES+/- medium (50-50 mixture). In this time, differentiated cells are more likely to adhere to the place surface, while pluripotent cells remain in suspension. After the allotted time, cells were gently collected and spun at 900 rpm for 5 minutes and resuspended in DM. An aliquot of the suspension was removed and counted utilizing a hemocytometer (Bright-line). Roughly 100,000 cells were used per 15 cm dish (Denville) to generate 500-cell EBs. Cells were resuspended in DM medium at a concentration that would yield 500 (+/- 10%) cells per 20 μL. The suspension was transferred to a 25 mL reagent well. Using 10 channels of a 12-channel multichannel pipette (p200, Gilson), 20 μL drops were made on the lid of 15 cm dishes. Between 150 and 190 drops were made per lid. Dishes were inverted so that the drops hung from the lid at 37˚C and 5% CO_2. EBs were collected between 24 and 30 hours after formation. The EBs were collected by washing the cells off of the lid surface with DM medium and transferred to 15 mL conical tubes. After allowing the EBs to settle for approximately 5 minutes, excess medium was removed. The EBs were resuspended gently in approximately 2 mL

of DM medium and transferred to a 6-well tissue culture plate. Between 180 and 300 EBs were cultured per well. Medium in the wells was changed every 2 days by removing half of the medium and supplying fresh medium.

Tamoxifen Administration

Z-4-hydroxytamoxifen (Sigma) was dissolved in DMSO at a concentration of 0.1 mM. This stock was diluted again using DMSO to a final concentration of 100 nM. Medium was supplemented with tamoxifen using 1μL of tamoxifen per mL of DM medium, and was filtered using a Luer-Lok syringe prior to use.

DM medium supplemented with tamoxifen (TDM), resulting in a final concentration of 0.1nM, was used to culture EBs at the indicated time points noted previously in the same manner as DM medium. TDM was only administered once and left in contact with cells for 24 hour periods, to identify the cells either expressing Oct4 or cells that no longer expressed Oct4 at some point during the 24 hour window. When TDM medium was introduced or removed, all of the medium from the well was changed. Cells were either sorted immediately after the 24 hour pulse, or continued on in culture until day ten. Cells that remained in culture were first washed with PBS to remove any trace TDM medium, and were supplied with fresh DM medium.

Fluorescence Activated Cell Sorting

Tamoxifen-treated EBs were dissociated using 0.25% Trypsin-EDTA (Gibco) to a single-cell suspension and neutralized with DM medium. The suspension was centrifuged at 900 rpm for 5 minutes, and the medium containing trypsin was removed. Cells were resuspended with PBS (Cellgro) and centrifuged at 900 rmp for 4 minutes, twice, to remove all medium from the suspension. Cells were then resuspended in FACS buffer (PBS-0.02%FBS, filtered with Luer-Lok syringe) and filtered through cell-strainer caps into 5ml polystyrene round-bottom tubes (35 μm, BD Falcon). The dissociated EBs were sorted and analyzed using FACS Aria. For fluorophore controls, 3F10 cells with

no history of tamoxifen were used to gate the tdTomato+ cells, and 3F10 cells given tamoxifen in a pluripotent state were used to set the gate for eGFP+ cells. Fluorescence activated cell sorting (FACS) was utilized to separate cells either expressing *Oct4* (eGFP+) or not expressing *Oct4* (tdTomato+/eGFP-) from a single population. RNA from the sorted populations of cells was isolated using RNeasy Mini Kit (Qiagen), and cDNA was generated from the RNA and analyzed by quantitative polymerase chain reaction (qPCR).

RNA and cDNA Preparation

Cells and EBs to be analyzed by qPCR were dissociated into single-cell suspensions as described above. After centrifugation at 900 rpm for 5 minutes, medium was removed from the cells. The pellet of a sample was resuspended in 350 μL of RLT Lysis Buffer with 1% β-mercaptoethanol and collected in a 1.5 mL eppendorf tube. Samples were immediately stored at -20˚C until processed. To extract total RNA, samples were thawed at room temperature (RT). A 31G syringe (BD Falcon) was used to homogenize the sample by drawing the sample up and expelling it between 3 and 5 times. RNA was extracted through a series of washes using RNeasy Mini Kit (Qiagen) and stored at -20˚C.

cDNA was generated from total RNA using random hexamer primers (50 ng/μL), dNTP mix (10 mM), and SuperScript II Reverse Transcriptase (Invitrogen). After preparation, samples were stored at -20˚C.

E12 Embryo Isolation

To provide a positive RNA control for qPCR, embryonic day 12 (E12) embryos were isolated from pregnant female mice. E12 was chosen as the genes of the mature structural proteins should be expressed, as should the transcription factors specific to the cells of the individual heart fields. The mice were sacrificed and dissected to extract the embryos from the womb. Embryos

were washed with PBS in two separate petri dishes to clear the field of blood for dissection. The hearts were isolated from the remainder of the embryos. The hearts were stored in RLT lysis buffer and processed in a similar fashion to cells for collection of RNA (i.e. use of insulin need to homogenize sample).

Quantitative Polymerase Chain Reaction (qPCR)

Gene expression of populations expressing and not expressing Oct4 and the time of tamoxifen administration was measured using quantitative polymerase chain reactions (qPCR). Relative gene expression measurement of the two populations included the following genes: *cardiac Troponin-T* (*cTnT*), *Islet1* (*Isl1*), *Nkx2.5*, *Flk1*, *PDGFRα*, *Troponin-I (Tnni)*, *Tbx1, Tbx5, Myosin light chain-2 (Myl2), Myosin heavy chain-6 (Myh6)*, and housekeeping gene *Gapdh*. All qPCR reactions were repeated with three technical replicates, and three biological replicates when possible. The Eppendorf RealPlex2 Mastercycler was used for all qPCR reactions with 40 cycles, with predesigned primers from IDT (Table 1) and SYBR Green Master Mix (Applied Biosystems). SYBR Green preferentially binds to double-stranded DNA and fluoresces, which is formed when the primers bind to the target transcript of cDNA. All primers were verified by melting curve analysis at least three separate times.

Relative expression (2^dct) was analyzed for all relevant data points using JMP Pro 10 software. Two datasets were compared during each analysis (i.e.*Oct4+* vs. *Oct4-* for a specific time point). Mean, standard deviation, and t-test analyses were run for each comparison to determine statistical significance. Error bars on graphs were calculated using standard error from the mean.

Agarose Gel Preparation

Samples were run on agaraose gels when threshold cycle (Ct) values were observed in qPCR reactions that differed noticeably from identical or similar

reactions. SYBR Green in the qPCR reaction recognizes and binds to double-stranded DNA formed by the IDT primer and the cDNA, as described above. Occasionally, primer dimers can form, in which the forward and reverse primers bind to each other. Primer dimers are also recognized by SYBR green, because it is not specific for primer-cDNA reactions. Primer dimers can cause fluorescence to be detected at a much earlier cycle than valid expression levels. When a PCR product is run on a gel, the size of the product can be determined. Average amplicon sizes should be approximately 100 base pairs (bp); anything significantly larger or smaller may provide evidence to discount the observed Ct value.

1.5% Ultrapure agarose (Invitrogen) gels were used to visualize the double-stranded DNA present in a sample. One gram of agarose is equivalent to 1% in 100mL of buffer. The buffer used was 1x TAE buffer (stock 50x Tris/Acetic Acid/EDTA buffer diluted) (BioRad). 1.5% agarose was added to buffer solution, and heated to dissolve the powder. Ethidium bromide (Sigma) was added to the solution to allow for visualization of the DNA bands. The solution cooled in a rack until solidified. Gels were placed in a bath of TAE buffer in the horizontal unit of the electrophoresis system (FisherBiotech). DNA ladder (100 kb, Promega) was added as a control lane. Blue-orange loading dye (Promega) was added to samples, and the mixture was loaded into lanes. The gel was run at approximately 115mv (Electrophoresis System, FisherBiotech) until the rungs of the ladder were separated enough to be distinct from one another. The gel was visualized using the GelDoc System and imager (Biorad), and images were subsequently analyzed to determine the size of the amplified transcripts and hence the validity of the Ct value of the sample.

Immunohistochemistry

Embryoid bodies (EBs) were collected from 15cm dishes and resuspended in DM medium. Approximately 35 to 50 EBs were plated on

gelatin-coated 4-chamber well slides (BD Falcon). Tamoxifen administration took place on days 3, 4, and 5, as described previously. Adherent EBs were cultured on the slides, with regular medium changes as previously described, until day 10. On day 10, EBs in the wells were first washed gently with PBS (Cellgro) and then fixed with 0.2% PFA (Sigma) for 10 minutes to preserve the structural integrity of the cells. Wells were again washed with PBS (Cellgro) three times to remove the PFA from contact with the samples. Cells were permeabilized using PBS-0.5% Triton (Sigma) (PBS-T) for 10 minutes. 5% normal donkey serum (Jackson Laboratories) in PBS-T was used to block samples for one hour. Samples were incubated overnight at 4˚ with the following primary antibodies diluted in PBS blocking buffer with 5% donkey serum: *cTnT* (CT3-s, 1:250; DSHB) and *Isl1* (40.3A4, 1:500; DSHB), both raised in mouse. Wells were washed three times with PBS to remove excess antibody, and reblocked with PBS-donkey blocking serum for one hour. Next, adherent EBs were incubated at room temperature for 2 hours with the secondary antibody diluted in PBS-donkey blocking serum. Cy5 anti-mouse antibody (A10524, 1:1000, Invitrogen) was used. Wells with no primary antibody and only secondary antibody administration were used as negative controls. Subsequently, wells were washed with PBS for 15 minutes three times before mounting. Slides were mounted with Vectashield containing DAPI (Vector Labs). After processing, slides were analyzed using the Olympus FluoView FV1000 Inverted Confocal with FLIM detector using IP Lab software.

Immunohistochemistry was performed on one round of embryoid bodies (EBs) with three separate slides for each time point. Quantification was calculated by counting the total number of cells positive for each antibody on three separate patches for each antibody for each time point. From this total, the percent that were positive for tdTomato and eGFP were recorded and used to generate an average proportion for the time point.

Results

Oct4 is downregulated during development

In the developing embryo, *Oct4* is initially expressed by all cells in the inner cell mass (ICM) and is subsequently shut off as cells commit to different lineages (Kehler et al., 2004; Niwa et al., 2000). As demonstrated by Sajini et al. in 2012, EBs made with iPS cells from the 3F10 *Oct4*-mTmG reporter line faithfully recapitulate the developing embryo in that they downregulate *Oct4* in a similar fashion. Cells expressing *Oct4* at the time of tamoxifen administration will excise tdTomato from the genome, and will express eGFP only. Cells that have stopped expressing *Oct4* when tamoxifen is administered will not have Cre-recombinase present to remove the sequence, and those cells will only express tdTomato. We expected to see a sharp down regulation of *Oct4* between days 3 and 5 in culture of EBs. When analyzed by FACS, this hypothesis was confirmed (Figure 4). The majority of cells were still expressing *Oct4* when tamoxifen was present in the media on day 3. More than half of the cells had downregulated *Oct4* at the time of tamoxifen administration on day 4, and the majority had ceased to express *Oct4* at day 5. This confirmed that cells are indeed downregulating *Oct4* in the iPS-derived EBs as would be expected in development.

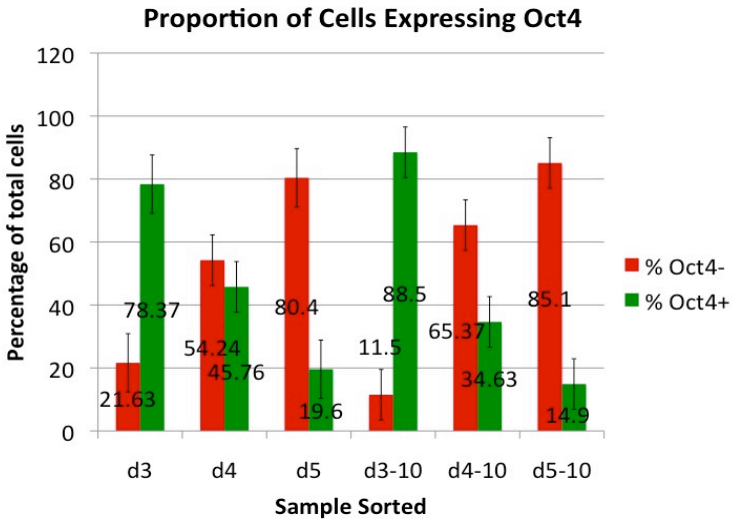


***Figure 4. Proportion of Cells Expressing* Oct4**
Average proportion of cells expressing tdTomato (*Oct4*-) or eGFP (*Oct4*+) from FACS sorting analysis. The majority of cells are still expressing *Oct4* at day 3, but *Oct4* is downregulated in the vast majority of cells by day 5. This temporal pattern was similar, regardless of whether the sort was done 24 hours later or on day 10 of culture. The values represent the mean percentage of *Oct4*+/- cells for 3 sorts for d3 and d5, and four sorts for d4, d3-10, d4-10, and d5-10.

Cells Expressing Cardiac-Specific Genes Arise from Cells that Turn Off *Oct4* Between Days 4 and 5

Based on data from Sajini et al. in 2012, we hypothesized that cells that cease to express *Oct4* between days 4 and 5 are the population from which cardiac cells originate. To test this theory, EBs were cultured on culture well slides from collection until day 10. Spontaneous beating was observed in cells by day 8 of culture (Data not shown). Day 10 was selected as an endpoint to allow further maturation of these cells. On day 10, they were fixed and stained with antibodies recognizing *Islet1* (*Isl1*), which is fairly specific to the secondary heart field in cardiac development (Van Vliet et al., 2012; Buckingham et al., 2005), and cardiac troponinT (*cTnT*), which is expressed in mature cardiomyocytes (Bondue et al., 2011).

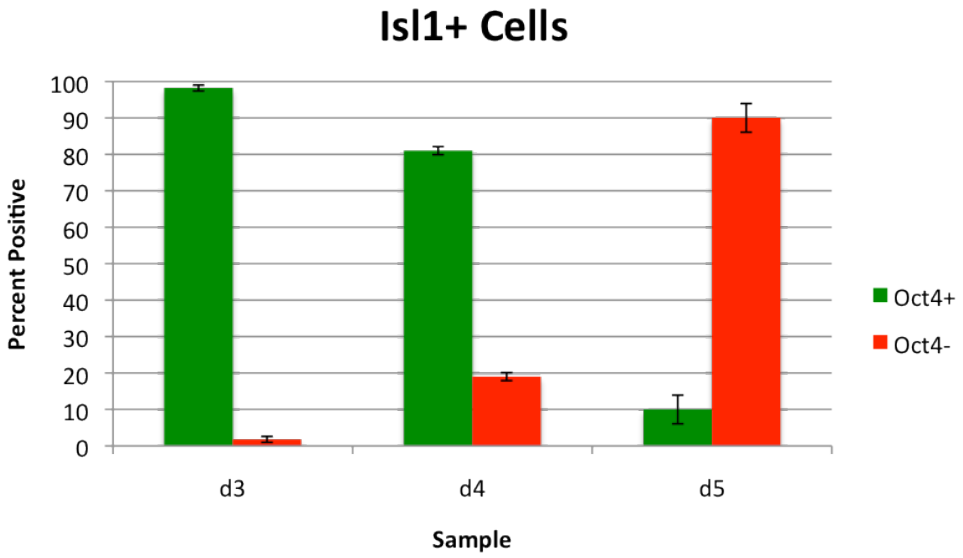


Figure 5. Quantification of Immunohistochemistry for* Isl1 *antibody
Calculated by proportion of cells positive for *Isl1* antibody and either eGFP or tdTomato for the three time points. Three separate patches positive for the antibody were counted.

Cells expressing *Isl1* on day 10 mostly originate (on average 98%, Figure 5) from cells expressing *Oct4* on day 3 (Figure 6). Cells positive for eGFP had the polycistronic *Oct4-MerCreMer* transcript present at the same time as the tamoxifen was introduced in order to cause excision of the tdTomato sequence, and induce expression of eGFP. In the image shown in Figure 6, there are no cells expressing *Isl1* that are also expressing tdTomato (*Oct4*-). These data imply that cells contributing to the secondary heart field (SHF) nearly all express *Oct4* on day 3 of culture. By day 4 of culture, cells still expressing *Oct4* give rise to the majority (approximately 81%, Figure 5) of the *Isl1*+ population on day 10 (Figure 7). This indicates that some of the *Isl1*+ cells on day 10 had stopped expressing *Oct4* by day 4. In Figure 7, it is apparent that many cells are coexpressing eGFP and tdTomato. Since the cells are expressing eGFP at all, it means that the *Oct4-MerCreMer* transcript was present at the time of tamoxifen administration. tdTomato expression would have ceased, but the resultant fluorescent protein may not have been fully degraded in the cells in Figure 7 on day 10 before fixation. However, the majority of cells expressing *Isl1* on day 10 (approximately

90%) originate from the *Oct4-* population of day 5 (Figure 5 and Figure 8). These data suggest that cells expressing *Isl1* on day 10 quickly cease to express *Oct4* between days 4 and 5. It should be noted in this system that although tamoxifen is present for a full 24 hours, most of the recombination can occur within 6 hours of tamoxifen introduction. This means that cells that are expressing eGFP on day 4 could have been expressing *Oct4* at day 4.0, but may have ceased expression by day 4.5. Since eGFP expression is permanent and irreversible, it is not possible to determine if this was the case.

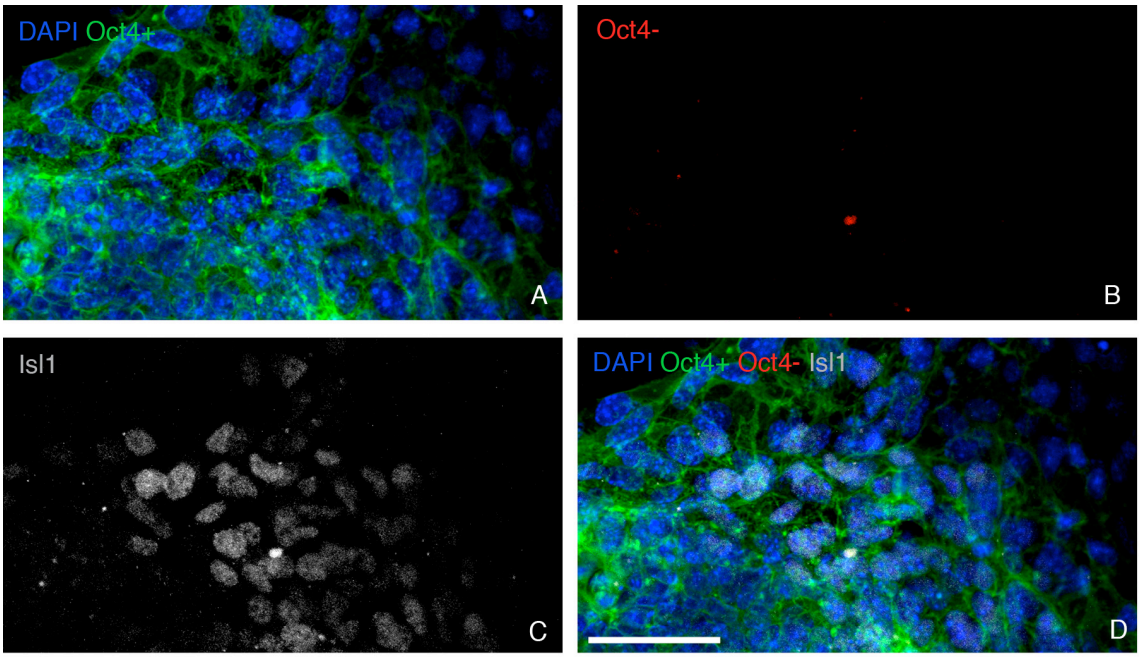


Figure 6. **Isl1*+ cells arise from cells expressing* Oct4 *on day 3***
EBs with medium supplemented with tamoxifen on day 3 of culture immunostained with *Isl1* antibody on day 10 of culture. Nearly all patches of cells expressing *Isl1* were still expressing *Oct4* during tamoxifen administration. A. DAPI with *Oct4*+ (eGFP). B. *Oct4*- (tdTomato). C. *Isl1*. D. Merge. Scale bar 50μm.

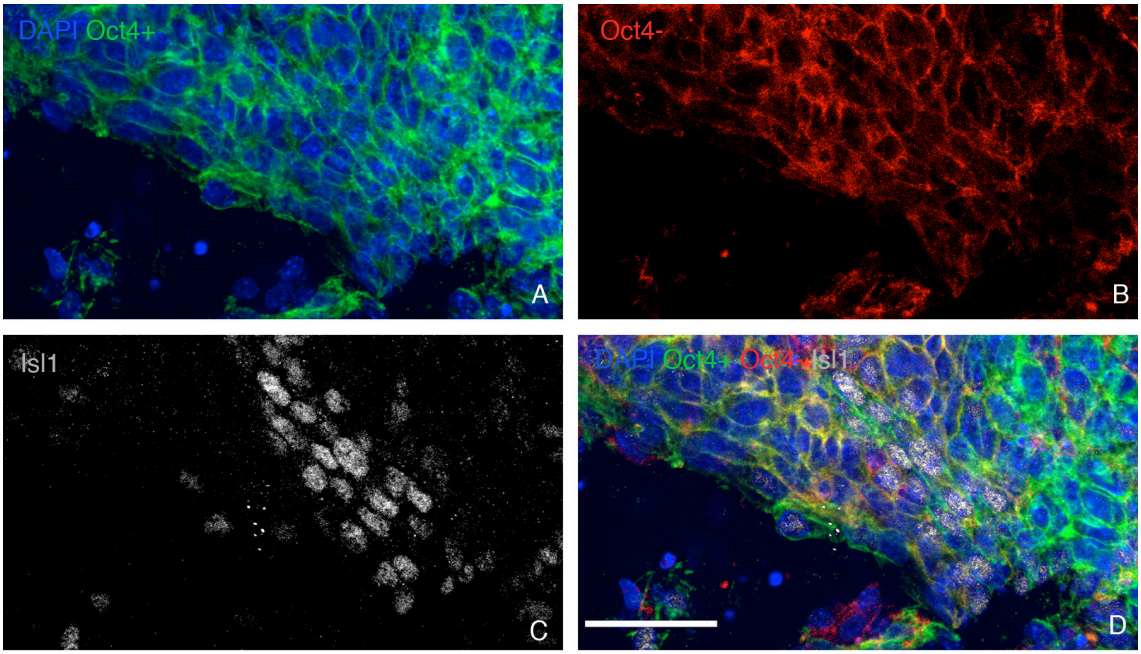


Figure 7. **Isl1*+ cells generally arise from cells still expressing* Oct4 *on day 4*** EBs with medium supplemented with tamoxifen on day 4 of culture immunostained with *Isl1* antibody on day 10 of culture. Most cells expressing *Isl1* were still expressing *Oct4* during tamoxifen administration. A. DAPI with *Oct4*+ (eGFP). B. *Oct4*- (tdTomato). C. *Isl1*. D. Merge. Scale bar 50μm.

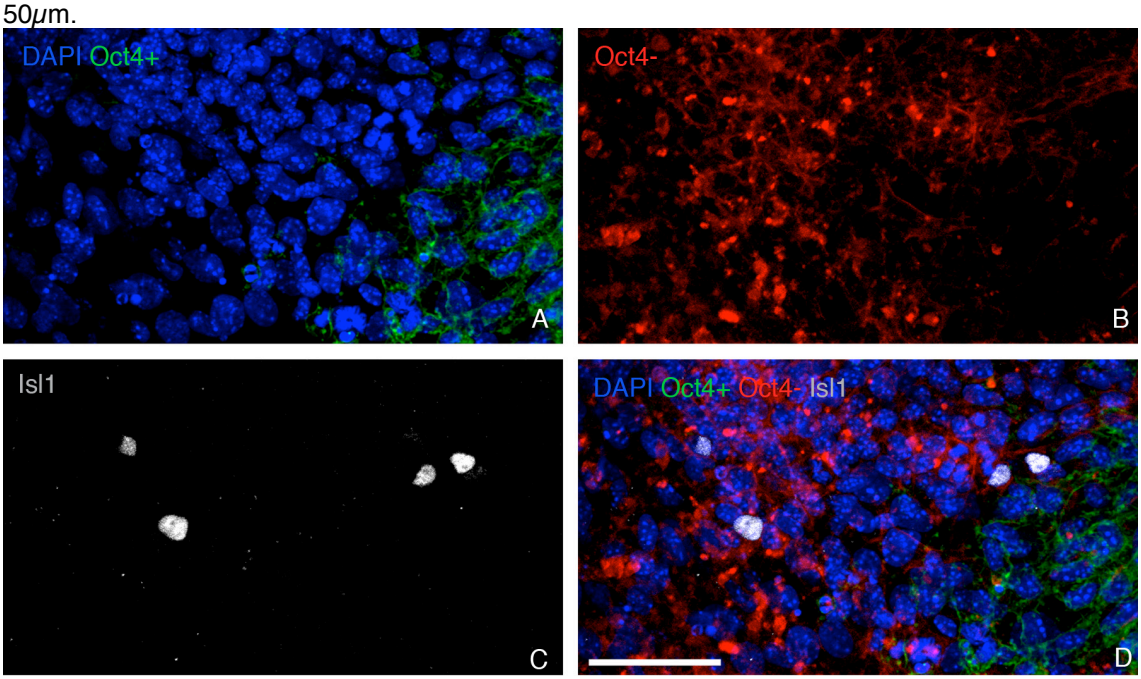


Figure 8. **Isl1*+ cells generally arise from cells not expressing* Oct4 *on day 5***
EBs with medium supplemented with tamoxifen on day 5 of culture immunostained with *Isl1* antibody on day 10 of culture. Almost all cells expressing *Isl1* were not expressing *Oct4* during tamoxifen administration. A. DAPI with *Oct4*+ (eGFP). B. *Oct4*- (tdTomato). C. *Isl1*. D. Merge. Scale bar 50μm.

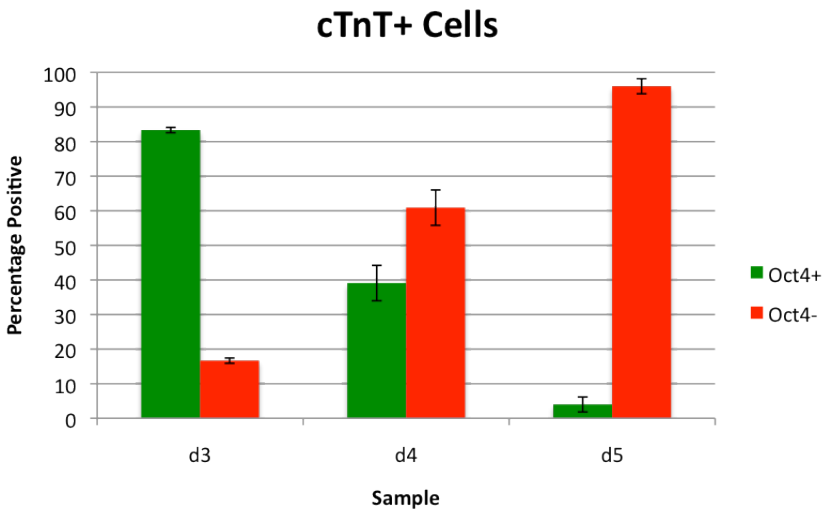


Figure 9. Quantification of Immunohistochemistry for* cTnT *antibody
Calculated by proportion of cells positive for *cTnT* antibody and either eGFP or tdTomato for the three time points. Three separate patches positive for the antibody were counted.

Similar to the observations of *Isl1* immuno-positive cells, the majority (approximately 83%, Figure 9) of cells expressing *cTnT* at day 10 were also expressing *Oct4* on day 3 of culture (Figure 10). Cells that will commit to the cardiac lineage cease *Oct4* expression at a later time point than day 3 of culture. By day 4 of culture, cells expressing *cTnT* originate from both *Oct4*- and *Oct4*+ populations, with approximately 60% being *Oct4*- and 40% *Oct4*+ (Figure 9 and Figure 11). At day 10 of culture, cells expressing *cTnT* had nearly all (96%, Figure 9) stopped expressing *Oct4* by day 5, indicated by the persistence of tdTomato proteins in the membrane (Figure 12)

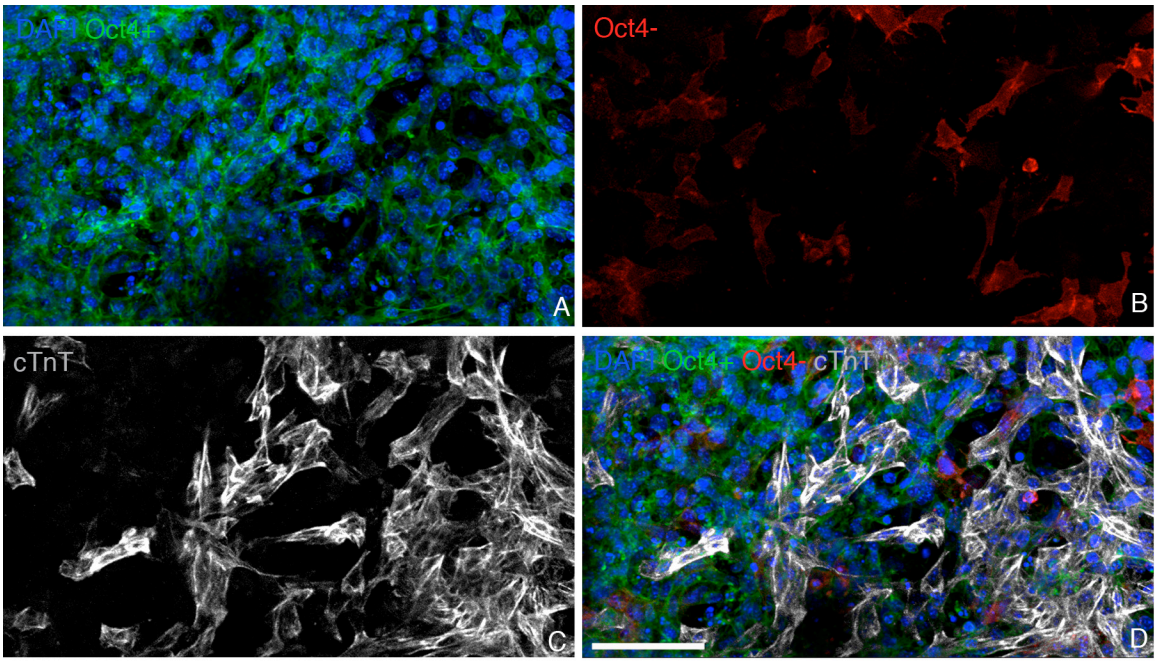


Figure 10.* cTnT*+ cells arise from cells expressing* Oct4 *on day 3

EBs with medium supplemented with tamoxifen on day 3 of culture stained with *cTnT* antibody on day 10 of culture. Nearly all of cells expressing *cTnT* were still expressing *Oct4* during tamoxifen administration. A. DAPI with *Oct4*+ (eGFP). B. *Oct4*- (tdTomato). C. *cTnT*. D. Merge. Scale bar 100μm.

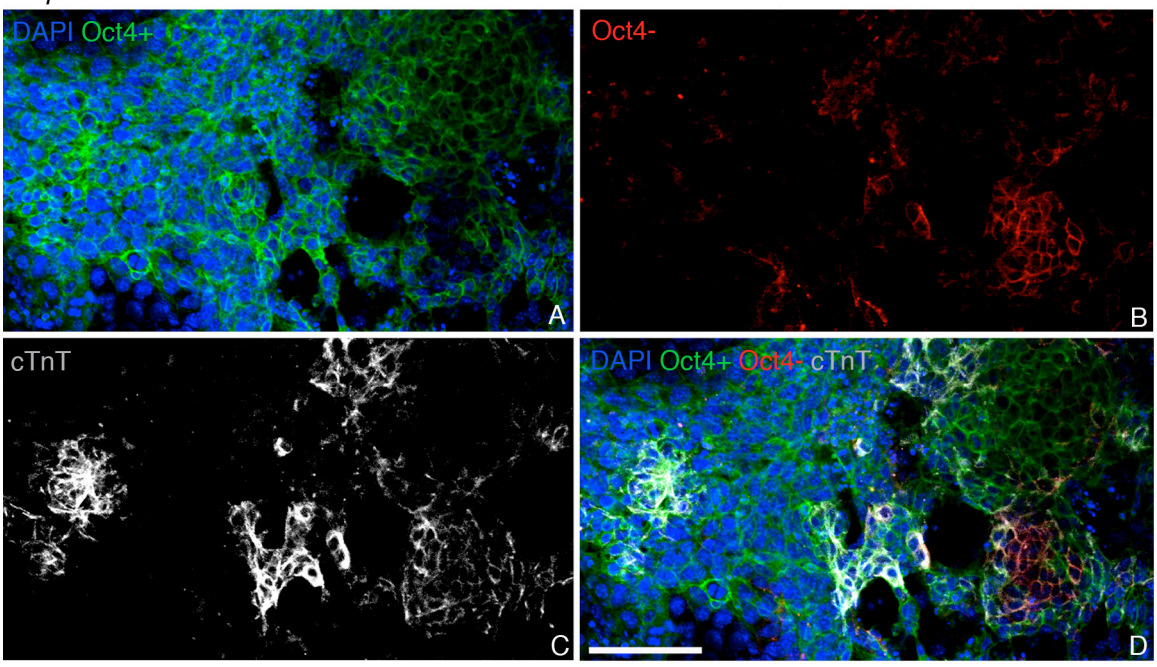


Figure 11.* cTnT*+ cells arise from cells both expressing and not expressing* Oct4 *on day 4
EBs with medium supplemented with tamoxifen on day 4 of culture stained with *cTnT* antibody on day 10 of culture. Most cells expressing *cTnT* were not expressing *Oct4* during tamoxifen administration. A. DAPI with *Oct4*+ (eGFP). B. *Oct4*- (tdTomato). C. *cTnT*. D. Merge. Scale bar 100μm.

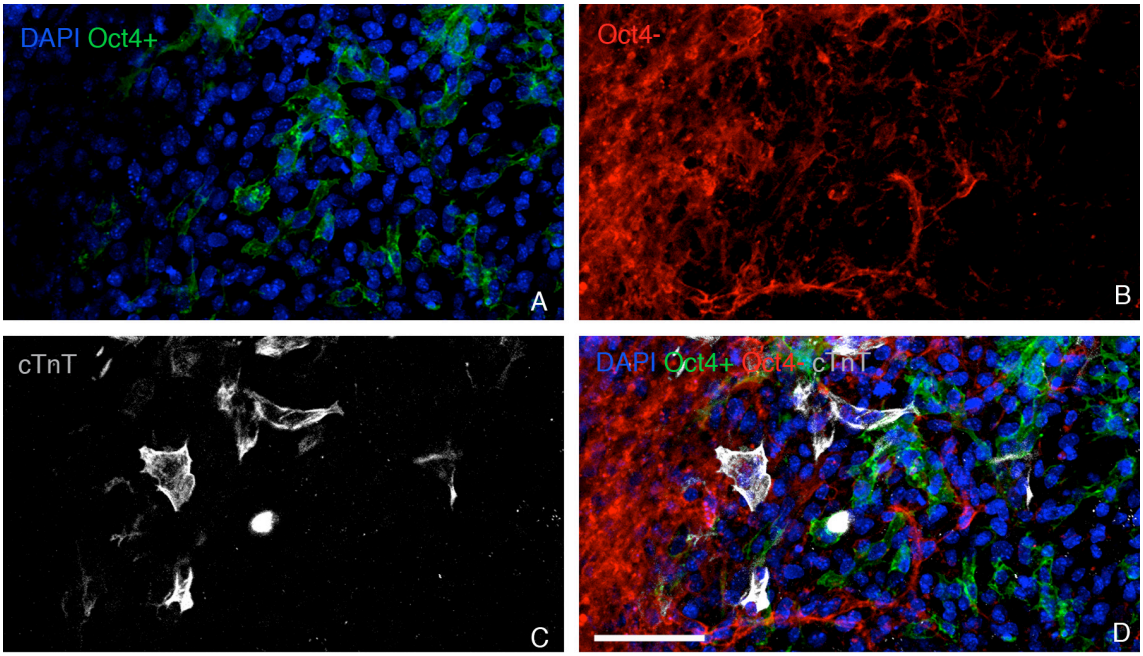


Figure 12. **cTnT*+ cells arise mostly from cells not expressing* Oct4 *on day 5***
EBs with medium supplemented with tamoxifen on day 5 of culture stained with *cTnT* antibody on day 10 of culture. Almost all cells expressing *cTnT* were not expressing *Oct4* during tamoxifen administration. A. DAPI with *Oct4*+ (eGFP). B. *Oct4*- (tdTomato). C. *cTnT*. D. Merge. Scale bar 100μm.

These data from immunostaining when taken together indicate that cells expressing *cTnT* at day 10 downregulated *Oct4* earlier than cells expressing *Isl1* at day 10. *Isl1* is generally expressed in the SHF, which contributes to heart development at a slightly later time than the primary heart field (PHF) does. The PHF progenitors contribute to the inflow tract, atria, and left ventricle and the SHF progenitors develop into the outflow tract and right ventricle (Abu-Issa and Kirby, 2007; Vincent and Buckingham, 2010). Cardiomyocytes comprising atria and ventricles are the cells responsible for contracting to expel blood from the heart, and will all express *cTnT*. Since both atria and the left ventricle will express *cTnT*, it is not surprising to see more cells that have shut off *Oct4* expression which are also *cTnT*+, as these cells may be part of the PHF. The cells from the *Isl1*+ SHF that comprise the right ventricle may still be expressing *Oct4* at this point. Immunostaining of the PHF was attempted with and *Nkx2.5* antibody, but was unsuccessfully optimized.

Preliminary qPCR Data

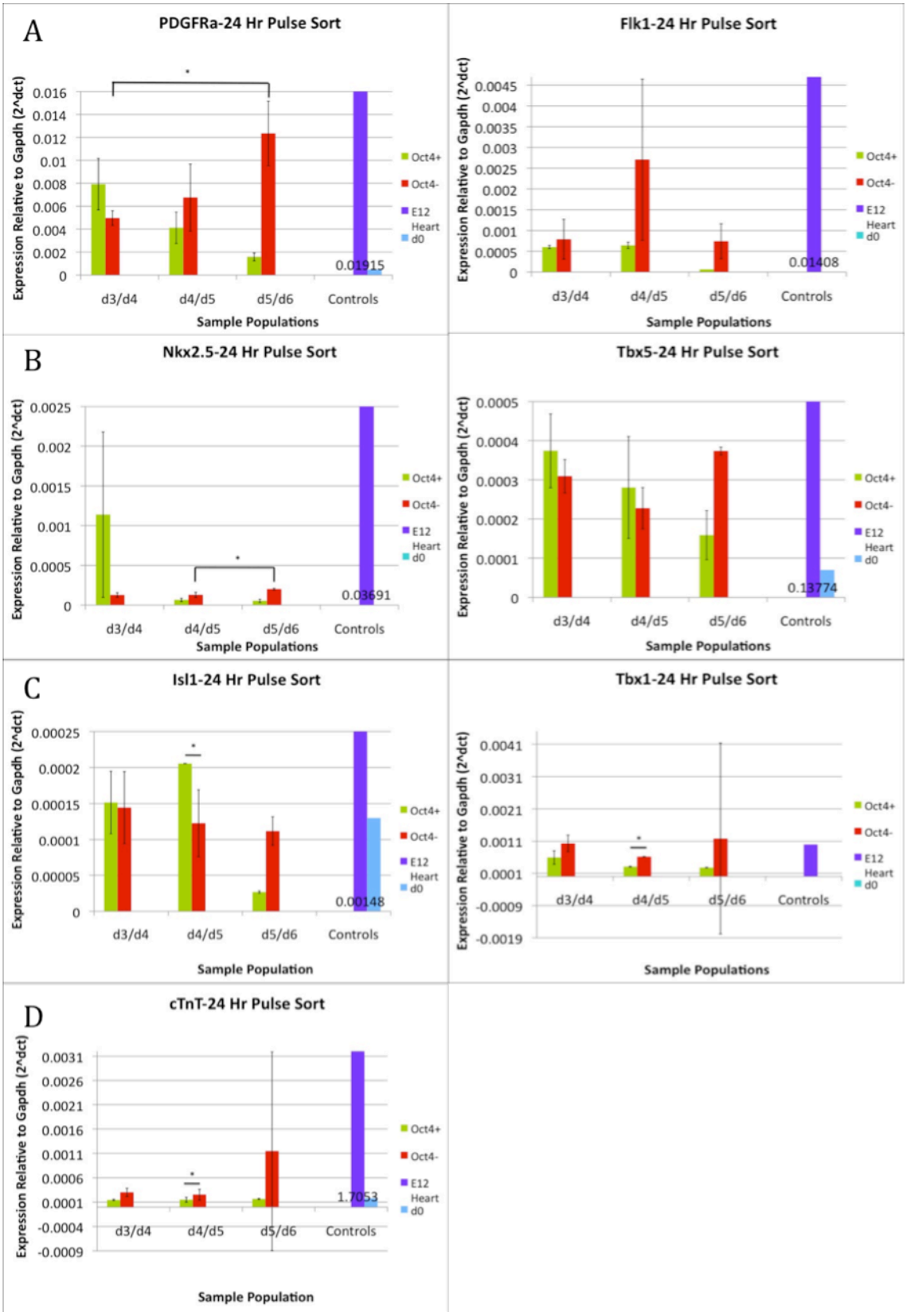
To further characterize the development of cardiac cells and the role of *Oct4* in lineage commitment, FACS was used to separate eGFP-expressing cells from tdTomato-expressing cells and these populations were analyzed using quantitative polymerase chain reactions (qPCR). Samples were sorted either immediately following the 24-hour tamoxifen window, or on day 10 of culture. Cells were processed for RNA collection immediately following sorting. Primers were used to target the following genes, which are known to play a role in cardiac differentiation (Figure 1): *PDGFRa*, *Flk1*, *Nkx2.5*, *Tbx5*, *Isl1*, *Tbx1*, *cTnT*, *Tnni*, *Myl2*, and *Myh6* (Van Vliet et al., 2012; Stefanovic et al., 2009; Rana et al., 2013; Bondue et al., 2011). In the sorts immediately following tamoxifen administration, we hypothesized that we may see differential expression between *Oct4* expressing and nonexpressing populations for genes known to be upregulated in early specification to the cardiac lineage (*Nkx2.5* & *Tbx5* in the PHF, *Isl1* &*Tbx1* in the SHF). In samples sorted on day 10, we hypothesized that a difference in expression between populations either expressing or not expressing *Oct4* may be observed in the more terminally expressed genes (*cTnT*, *Tnni*, *Myl2*, and *Myh6*), as these are structural proteins and would not be present in progenitors. For comparison, qPCR was also performed on RNA samples from iPS cells collected during passaging and from cells from murine embryonic day 12 (E12) heart. The E12 serves as a positive control for the gene expression of a terminally differentiated cell, but some of the earlier genes required for development may also be expressed in this more mature tissue. It should be noted that this data is preliminary, given the small and varied sample sizes (Table 2, Appendix). Additional replicates are necessary for more data in order to draw confident conclusions.

Figure 13 illustrates the results of the PCR performed on RNA from cells sorted after 24-hours tamoxifen administration. EBs were given tamoxifen for 24

hours on day 3, 4, or 5, and subsequently sorted on day 4, 5, or 6. The population no longer expressing *Oct4* on day 5 expressed myocardial progenitor marker *PDGFRa* at a significantly higher level than on day 3 (Figure 13A). *PDGFRa* was expressed at approximately 10-fold higher than in the day 0 iPS cells; *Flk1* was also expressed in the sorted populations and not in the day 0 iPS cells (Figure 13A). The primary heart field (PHF) can be distinguished from the SHF by expression of *Nkx2.5* and *Tbx5* (Rana et al., 2013). *Nkx2.5* expression was higher in *Oct4*- cells on day 5 than on day 4 (Figure 13B). *Nkx2.5* is also expressed in other regions of the heart, but it is more uniformly expressed by the PHF. The secondary heart field (SHF) is identified by expression of transcription factors *Isl1* and *Tbx1* (Buckingham et al., 2005). *Tbx1* was significantly upregulated in *Oct4*- cells in comparison to *Oct4*+ positive cells on day 4, indicating that the SHF may indeed originate from cells that stop *Oct4* expression on day 4 (Figure 13C). *Isl1*, on the other hand, was expressed at a higher level in the population of cells still expressing *Oct4* on day 4 (Figure 13C).

It should also be pointed out that the *Isl1* expression observed in these early time points is roughly equivalent to the expression level in the day 0 iPS cells. With that in mind, one must question whether the expression level in the sorted populations is anything more than basal expression in this cell line. Expression of other genes are also observed in the day 0 pluripotent cells (*PDGFRa*, *Tbx5*, *cTnT*, and *Isl1*). These unusual expression profiles have also been observed by others in the same pluripotent cell line (personal communication; Lucas Greder and Sarah Burley). Expression levels of *cTnT* at these early time points were low relative to an E12 heart, which is consistent with the expected expression of *cTnT* in more mature cardiomyocytes (Figure 13D). These markers for cardiac progenitors, with the exception of *Tbx1*, were expressed at a fraction of the level of expression in the E12 heart, perhaps indicating that they have not yet been upregulated (Figure 13).

Figure 13.


A
PDGFRa-24 Hr Pulse Sort
Expression Relative to Gapdh (2^dct)
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
*
0.01915
d3/d4
d4/d5
d5/d6
Controls
Sample Populations
Oct4+
Oct4-
E12 Heart d0
Flk1-24 Hr Pulse Sort
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0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0.01408
d3/d4
d4/d5
d5/d6
Controls
Sample Populations
Oct4+
Oct4-
E12 Heart d0
B
Nkx2.5-24 Hr Pulse Sort
Expression Relative to Gapdh (2^dct)
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0.002
0.0015
0.001
0.0005
0
*
0.03691
d3/d4
d4/d5
d5/d6
Controls
Sample Populations
Oct4+
Oct4-
E12 Heart d0
Tbx5-24 Hr Pulse Sort
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0.0004
0.0003
0.0002
0.0001
0
0.13774
d3/d4
d4/d5
d5/d6
Controls
Sample Populations
Oct4+
Oct4-
E12 Heart d0
C
Isl1-24 Hr Pulse Sort
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0.0002
0.00015
0.0001
0.00005
0
*
0.00148
d3/d4
d4/d5
d5/d6
Controls
Sample Population
Oct4+
Oct4-
E12 Heart d0
Tbx1-24 Hr Pulse Sort
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0.0031
0.0021
0.0011
0.0001
-0.0009
-0.0019
*
d3/d4
d4/d5
d5/d6
Controls
Sample Populations
Oct4+
Oct4-
E12 Heart d0
D
cTnT-24 Hr Pulse Sort
Expression Relative to Gapdh (2^dct)
0.0031
0.0026
0.0021
0.0016
0.0011
0.0006
0.0001
-0.0004
-0.0009
*
1.7053
d3/d4
d4/d5
d5/d6
Controls
Sample Population
Oct4+
Oct4-
E12 Heart d0

Figure 13. Gene expression analysis of 24 Hour Pulse Sorts
qPCR data from cells sorted 24 hours after tamoxifen was introduced into culture media. A. Genes expressed in myocardial progenitors. B. Genes expressed in the PHF. C. Genes expressed in the SHF. D. A gradual increase of expression of *cTnT* is observed as the cells progress through differentiation. Statistical significance ($p>0.05$) is denoted by * and error bars show standard error of the mean. Numerical values at the base of the E12 bars are the exact relative expression value, since the proportion of the graphs would be skewed if the entire bar were included.

Figure 14 illustrates the results from cells sorted on day 10 of culture, after tamoxifen was supplied for 24-hour pulses on either day 3, 4, or 5. With these sorts, there were some cell samples that did not survive the FACS sorting to be analyzed. These populations (*Oct4*- from day 3 and *Oct4*+ from day 5) were not run in triplicate (see Table 2 in appendix for sample sizes), and standard error could not be calculated. The preliminary data is still shown on the graphs in Figure 14, but given the small sample sizes it is inconclusive as of yet. In order to be fully functional, mature cardiomyocytes must express genes encoding structural proteins responsible for contraction, such as *Myl2*, *Myh6*, *Tnni*, and *cTnT* (Lui et al., 2007). These proteins were upregulated relative to iPS cells, but lower than expression in the E12 heart at day 10 (Figure 14A), suggesting that they are not yet mature. This lower expression of mature markers could also be attributed to the heterogeneous nature of the cell populations. Since qPCR calculates the average transcripts per cell based on the entire population, the cells expressing these structural proteins in Figure 14A may be expressing high levels but these cells may only represent a small fraction of the total cell population.

Genes specific to the PHF (Figure 14B) and SHF (Figure 14C) were present in both *Oct4*+ and *Oct4*- populations sorted on day 10, but there were no statistically significant differences in expression. Expression of *Isl1* increased 10-fold in comparison to the expression on days 4, 5, and 6 (Figure 13C and Figure 14C). Although there is no significant difference between the *Oct4*+ and *Oct4*- populations in the day 10 sorts, the *Isl1* expression level for all populations is relatively similar to the E12 heart expression (Figure 14C). The expression level of *Tbx1* is higher in the sorted populations in comparison to the E12 heart.

Perhaps the *Tbx1* expression is just increasing in expression in these sorted populations, before it decreases in the mature tissue.

Figure 14.

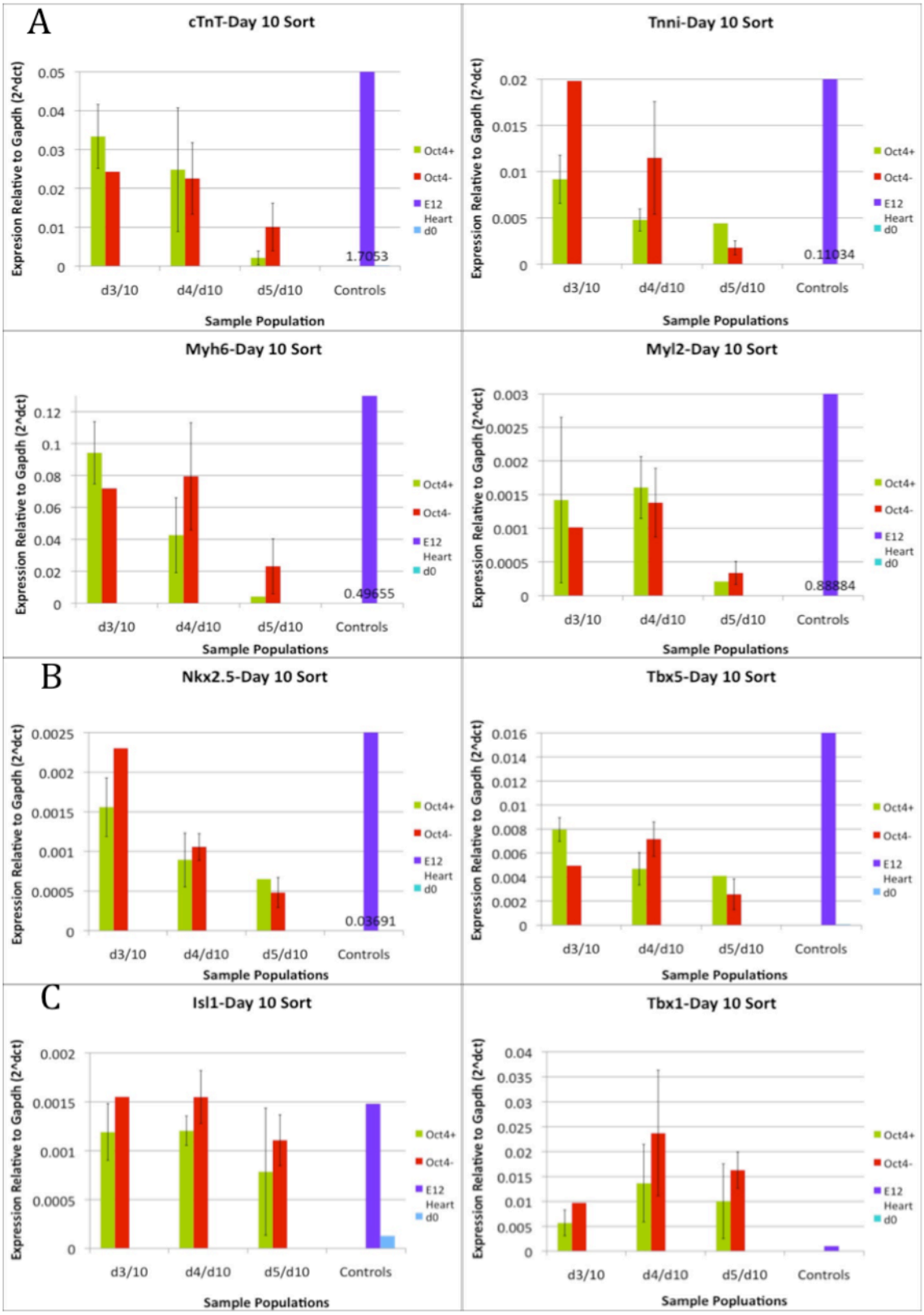
A
cTnT-Day 10 Sort
Expresion Relative to Gapdh (2^dct)
0.05
0.04
0.03
0.02
0.01
0
1.7053
d3/10
d4/d10
d5/d10
Controls
Sample Population
Oct4+
Oct4-
E12 Heart d0
Tnni-Day 10 Sort
Expression Relative to Gapdh (2^dct)
0.02
0.015
0.01
0.005
0
0.11034
Sample Populations
Myh6-Day 10 Sort
Expression Relative to Gapdh (2^dct)
0.12
0.1
0.08
0.06
0.04
0.02
0
0.49655
Myl2-Day 10 Sort
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0.88884
B
Nkx2.5-Day 10 Sort
0.0025
0.002
0.0015
0.001
0.0005
0
0.03691
Tbx5-Day 10 Sort
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
C
Isl1-Day 10 Sort
0.002
0.0015
0.001
0.0005
0
Tbx1-Day 10 Sort
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Sample Popluations

Figure 14*. *Gene Expression Analysis of Sorted Day 10 Populations

qPCR data from cells sorted on day 10 of culture when tamoxifen was administered for 24 hours on either day 3, 4, or 5. A. Genes expressed in mature cardiomyocytes. B. Genes expressed in the PHF. C. Genes expressed in the SHF. Statistical significance ($p>0.05$) is denoted by * and error bars show standard error of the mean. Numerical values at the base of the E12 bars are the exact relative expression values, since the proportion of the graphs would be skewed if the entire bars were included.

To determine the expression patterns of the genes of interest, samples were collected for RNA analysis on the same day as experimental populations were sorted, but were never supplied tamoxifen, and subsequently analyzed using qPCR. In earlier progenitors, one would not expect to see expression of structural proteins of mature cardiomyocytes. This was confirmed in Figure 15A. Expression levels of these genes were much higher at day 10 than either day 3, 4, or 5. Transcription factors demarcating the PHF and SHF from one another were also expressed at higher levels in cells sorted on day 10 (Figure 15B and Figure 15C). *Isl1* is expressed on day 10 at a level similar to the E12 heart, and *Tbx1* is expressed at a level higher than the E12 heart, which was previously stated.

It should be noted that some gene expression could be attributed to contamination from expression in other cell types in many of these markers. *Isl1* is also expressed in developing pancreatic and neural tissues (Habener et al., 2005). *Tbx1* functions in artery and gland development, as well as the cardiac development (Ryan and Chin, 2003; Scambler, 2010). *Tbx5* not only plays a role in cardiac differentiation, but also limb specification (Oberg, 2013). Day 10 in culture may also be when these transcription factors are expressed in the cardiac lineage progression in this EB system with this cell line.

Figure 15.

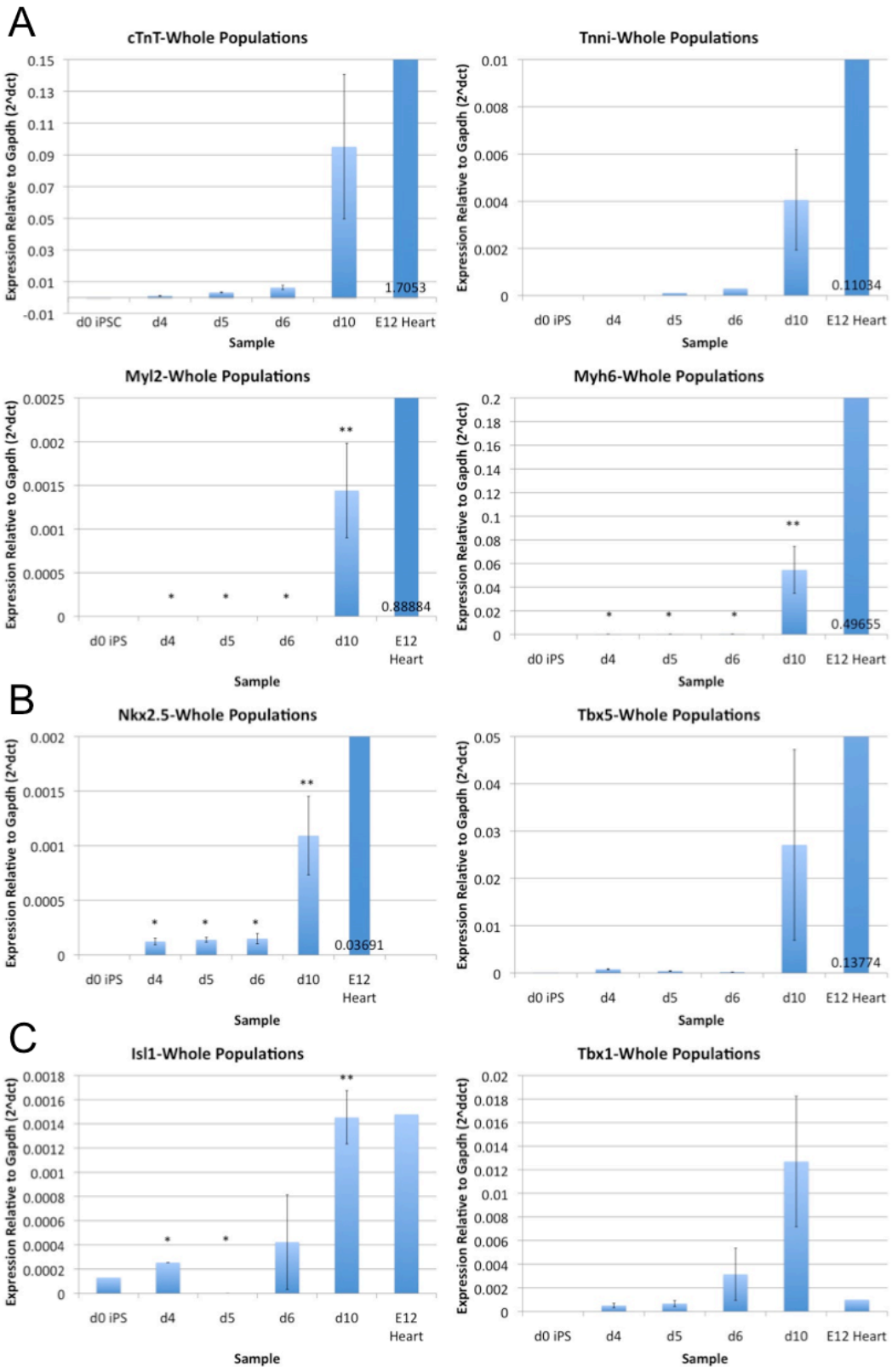
A
cTnT-Whole Populations
Expression Relative to Gapdh (2^dct)
0.15
0.13
0.11
0.09
0.07
0.05
0.03
0.01
-0.01
1.7053
d0 iPSC
d4
d5
d6
d10
E12 Heart
Sample
Tnni-Whole Populations
Expression Relative to Gapdh (2^dct)
0.01
0.008
0.006
0.004
0.002
0
0.11034
d0 iPS
d4
d5
d6
d10
E12 Heart
Sample
Myl2-Whole Populations
Expression Relative to Gapdh (2^dct)
0.0025
0.002
0.0015
0.001
0.0005
0
**
*
*
*
0.88884
d0 iPS
d4
d5
d6
d10
E12 Heart
Sample
Myh6-Whole Populations
Expression Relative to Gapdh (2^dct)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
**
*
*
*
0.49655
d0 iPS
d4
d5
d6
d10
E12 Heart
Sample
B
Nkx2.5-Whole Populations
Expression Relative to Gapdh (2^dct)
0.002
0.0015
0.001
0.0005
0
**
*
*
*
0.03691
d0 iPS
d4
d5
d6
d10
E12 Heart
Sample
Tbx5-Whole Populations
Expression Relative to Gapdh (2^dct)
0.05
0.04
0.03
0.02
0.01
0
0.13774
d0 iPS
d4
d5
d6
d10
E12 Heart
Sample
C
Isl1-Whole Populations
Expression Relative to Gapdh (2^dct)
0.0018
0.0016
0.0014
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
**
*
*
d0 iPS
d4
d5
d6
d10
E12 Heart
Sample
Tbx1-Whole Populations
Expression Relative to Gapdh (2^ddct)
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
d0 iPS
d4
d5
d6
d10
E12 Heart
Sample

Figure 15*. *Gene Expression Analysis of Unsorted Populations
qPCR data from cells collected on the same day that experimental populations were sorted, but were not given tamoxifen at any time. A. Gene expression levels of structural proteins in mature cardiomyocytes. B. Genes expressed in PHF. C. Gene expressed in SHF. Error bars show standard error of the mean. Early time points (days 4, 5, and 6) differing significantly ($p>0.05$) from the later time point (**, day 10) are demarcated by *. Numerical values at the base of the E12 bars are the exact relative expression values, since the proportion of the graphs would be skewed if the entire bars were included.

With such large standard error observed in the qPCR data, it is quite difficult to make any conclusions from the data at present with any certainty. Perhaps with more biological replicates, conclusions may be drawn with confidence. The expanded list of samples sizes is in Table 2 of the appendix.

Discussion

The intention of these experiments was to explore the possibility that the primary (PHF) and secondary heart fields (SHF) may cease to express *Oct4* at different times. We hypothesized that the PHF would shut off expression of *Oct4* before the SHF because the cells contributing to the PHF commit to the cardiac lineage before those of the SHF. Generally, cells that are differentiating do not express *Oct4*, except for in primordial germ cells and oocytes (Scholer et al., 1990).

The data gathered from immunohistochemistry suggests that cells committing to the cardiac lineage mostly cease to express *Oct4* between days 4 and 5 of culture. We found that cells that express *cTnT* at day 10 stopped expressing *Oct4* mostly between days 4 and 5 of culture, which is similar to what was observed by Sajini et al. in 2012. The methods used in this thesis were similar to Sajini et al. in nearly all aspects with two exceptions. EBs in this thesis were formed via hanging drop method instead of the spin method, and were not cultured in suspension but instead were adherent to the culture well surface. EBs grown in suspension differentiate into all lineages, while EBs grown adherent to surfaces tend to differentiate into muscle and neuronal lineages (Kehler et al., 1995). Sajini et al. only identified cardiomyocytes by *cTnT* immunostaining, whereas we used an additional marker. Our data from immunostaining using the *Isl1* antibody implies that cells contributing to the secondary heart field (SHF) stop *Oct4* expression before day 5, but sometime after tamoxifen administration on day 4. An antibody identifying a marker of the PHF (*Nkx2.5*) was also used, but the background fluorescence and nonspecific binding prevented inclusion of the resulting data.

Preliminary qPCR data demonstrates that the genes of interest do increase in expression over time in 3F10 iPS-derived EBs. However, the data gathered has large variability between replicates in the qPCR analysis, which makes it difficult to draw any conclusions of significance from this data alone. As

previously suggested, more replicates may help to decrease standard error from mean, and establish more representative expression levels for each time point. Since qPCR is a relative measure of transcript per cell, it yields an average across the entire population. When analyzing qPCR results, one must consider that if a 10-fold increase is observed, it could be attributed to a few cells expressing high levels of transcript, or many cells expressing at intermediate levels. Both of our sorted populations are comprised of cell types at different stages of differentiation toward different lineages, therefore an average measurement could obscure the expression level of cells in our specific population of interest (i.e. PHF and SHF). Taken all together, the collective data does suggest that cells committing to the cardiac lineage generally cease to express *Oct4* between days 4 and 5 of culture.

An alternative approach to answer the same question may be to use a different iPS reporter line, or multiple reporter lines. If *Oct4* expression was labeled by only a single fluorophore, like eGFP, when in the presence of tamoxifen, then a second fluorophore could be utilized to identify cells specific to the cardiac lineage. By choosing a gene expressed at a specific time in development (i.e. *Nkx2.5* in early stages or *cTnT* later in commitment) to also express tdTomato from the endogenous locus, then a more specific population may be isolated. For example, a cell line with an *Oct4*-CreER inducing expression of eGFP and tdTomato driven by the *Nkx2.5* promoter could be used to identify cells as they commit to the PHF around day 6 of culture. A cell line expressing an *Oct4*-CreER (with eGFP expressed in the presence of tamoxifen) and tdTomato driven by expression of *cTnT* could be used to separate cells as they become more mature cardiomyocytes around day 10. By default, cells not expressing eGFP but expressing tdTomato would not be expressing *Oct4* at the time of tamoxifen administration, but would be contributors to the cardiac lineage. Cells could be sorted using FACS based on *cTnT* expression, and cells with eGFP expression also would identify those expressing *Oct4* at a specific time. FACS could provide a quantitative value of the proportion of cells expressing

cTnT in relation to the entire population, and the separated populations expressing *cTnT* would more homogeneous than the sorted populations in this thesis. This way, the populations analyzed by qPCR will not be contaminated with cells from other lineages, and will permit a more representative quantification of relevant gene expression levels. This approach would better address the question of whether or not *Oct4* is turned off at different times in cells that will contribute to the PHF and SHF. Although this approach would require the generation of iPS lines from multiple lines of transgenic mice, it would allow for more specific isolation of the cell population of interest.

References

Abu-Issa, R, and M L Kirby. "Heart field: from mesoderm to heart tube." Annual review of cell and developmental biology 23(2007):45-68.

Andrews, P W, et al. "Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin." Biochemical Society transactions 33.6 (2005):1526-1530.

Antonchuk, J. "Formation of embryoid bodies from human pluripotent stem cells using AggreWell plates." Methods in molecular biology 946(2013):523-533.

Bernstein, B E, et al. "A bivalent chromatin structure marks key developmental genes in embryonic stem cells." Cell 125.2 (2006):315-326

Bradley, A, et al. "Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines." Nature 309.5965 (1984):255-256.

Bratt-Leal, A M, et al. "Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation." Biotechnology progress 25.1 (2009):43-51.

Bondue, A, et al. "Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification." Cell stem cell 3.1 (2008):69-84.

Bondue, A, et al. "Defining the earliest step of cardiovascular progenitor specification during embryonic stem cell differentiation." The Journal of cell biology 192.5 (2011):751-765.

Buckingham, M, et al. "Building the mammalian heart from two sources of myocardial cells." Nature reviews. Genetics 6.11 (2005):826-835.

Chambers, I. "The molecular basis of pluripotency in mouse embryonic stem cells." Cloning and stem cells 6.4 (2004):386-391.

Chambers, I, et al. "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells." Cell 113.5 (2003):643-655.

Chambers, I, and A Smith. "Self-renewal of teratocarcinoma and embryonic stem cells." Oncogene 23.43 (2004):7150-7160.

Chan, S S, et al. "Mesp1 patterns mesoderm into cardiac, hematopoietic, or skeletal myogenic progenitors in a context-dependent manner." Cell stem cell 12.5 (2013):587-601.

Damjanov, I. "The road from teratocarcinoma to human embryonic stem cells." Stem Cell Reviews and Reports 1.3 (2005):273-276.

Doetschman, T C, et al. "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium." Journal of embryology and experimental morphology 87(1985):27-45.

Evans, M. "Embryonic stem cells: a perspective." Novartis Found Symp 265(2005):98-103.

Evans, M J, and M H Kaufman. "Establishment in culture of pluripotential cells from mouse embryos." Nature 292.5819 (1981):154-156.

Greder, L V, et al. "Analysis of endogenous Oct4 activation during induced pluripotent stem cell reprogramming using an inducible Oct4 lineage label." Stem cells 30.11 (2012):2596-2601.
Guenet, J L, et al. "Cardiac and skeletal muscle troponin I isoforms are encoded by a dispersed gene family on mouse chromosomes 1 and 7." Mammalian genome 7.1 (1996):13-15.
Habener, J F, et al. "Minireview: transcriptional regulation in pancreatic development." Endocrinology 146.3 (2005):1025-1034.
Hidaka, K, et al. "Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells." The FASEB journal 17.6 (2003):740-742.
Kehler, J, et al. "Oct4 is required for primordial germ cell survival." EMBO reports 5.11 (2004):1078-1083.
Keller, G M. "In vitro differentiation of embryonic stem cells." Current opinion in cell biology 7.6 (1995):862-869.
Kim, K, et al. "Epigenetic memory in induced pluripotent stem cells." Nature 467.7313 (2010):285-290.
Kurosawa, H. "Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells." Journal of bioscience and bioengineering 103.5 (2007):389-398.
Leahy, A, et al. "Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation." The journal of experimental zoology 284.1 (1999):67-81.
Li, Y, et al. "Brief report: Oct4 and canonical Wnt signaling regulate the cardiac lineage factor Mesp1 through a Tcf/Lef-Oct4 composite element." Stem cells 31.6 (2013):1213-1217.
Liu, Y, et al. "Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells." Proceedings of the National Academy of Sciences of the United States of America 104.10 (2007):3859-3864.
Maltsev, V A, et al. "Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents." Circulation research 75.2 (1994):233-244.
Mantur, M, and O Koper. "[Platelet- derived growth factor--the construction, role and it's receptors]." Polski merkuriusz lekarski 24.140 (2008):173-176.
Martin, G R. "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells." Proceedings of the National Academy of Sciences of the United States of America 78.12 (1981):7634-7638.
Martin, G R, and M J Evans. "The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture." Cell 2.3 (1974):163-172.
Martin, G R, et al. "The development of cystic embryoid bodies in vitro from clonally teratocarcinoma stem cells." Developmental biology 61.2 (1977):230-244.

Masui, S, et al. "Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells." Nature Cell Biology 9.6 (2007):625-635.
Miller Hance, W C, et al. "In vitro chamber specification during embryonic stem cell cardiogenesis. Expression of the ventricular myosin light chain-2 gene is independent of heart tube formation." Journal of biological chemistry 268.33 (1993):25244-25252.
Moerkamp, A T, and MJ Goumans. "Cardiac regeneration: stem cells and beyond." Current medicinal chemistry 19.35 (2012):5993-6002.
Mogi, A, et al. "The method of mouse embryoid body establishment affects structure and developmental gene expression." Tissue and cell 41.1 (2009):79-84.
Murry, C E, and G Keller. "Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development." Cell 132.4 (2008):661-680.
Muzumdar, M D, et al. "A global double-fluorescent Cre reporter mouse." Genesis 45.9 (2007):593-605.
Nichols, J, et al. "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4." Cell 95.3 (1998):379-391.
Niwa, H. "How is pluripotency determined and maintained?." Development 134.4 (2007):635-646.
Niwa, H, et al. "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells." Nature Genetics 24.4 (2000):372-376.
Oberg, K C. "Review of the Molecular Development of the Thumb: Digit Primer." Clinical orthopedics and related research (2013)
Ohi, Y, et al. "Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells." Nature Cell Biology 13.5 (2011):541-549.
Pierce, G B, and E L Verney. "An in vitro and in vivo study of differentiation in teratocarcinomas." Cancer 14(1961):1017-1029.
Pierce, G B, and F J Dixon. "Testicular teatimes. I. Demonstration of teratogenesis by metamorphosis of multipotential cells." Cancer 12.3 (1959):573-583.
Rana, M S, et al. "A molecular and genetic outline of cardiac morphogenesis." Acta physiologica 207.4 (2013):588-615.
Rust, W L, et al. "Three-dimensional extracellular matrix stimulates gastrulation-like events in human embryoid bodies." Stem cells and development 15.6 (2006):889-904.
Ryan, K, and A J Chin. "T-box genes and cardiac development." Birth defects research. Part C, Embryo today 69.1 (2003):25-37.
Sasha, M S, et al. "Mechanisms regulating the origins of the vertebrate vascular system." Journal of cellular biochemistry 93.1 (2004):46-56.

Sajini, A A, et al. "Loss of Oct4 expression during the development of murine embryoid bodies." Developmental biology 371.2 (2012):170-179.
Scambler, P J. "22q11 deletion syndrome: a role for TBX1 in pharyngeal and cardiovascular development." Pediatric cardiology 31.3 (2010):378-390.
Scholar, H R, et al. "Octamer binding proteins confer transcriptional activity in early mouse embryogenesis." EMBO journal 8.9 (1989):2551-2557.
Scholar, H R, et al. "Oct-4: a germline-specific transcription factor mapping to the mouse t-complex." EMBO journal 9.7 (1990):2185-2195.
Smith, A G, et al. "Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides." Nature 336.6200 (1988):688-690.
Smith, A G, and M L Hooper. "Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells." Developmental biology 121.1 (1987):1-9.
Stefanovic, S, et al. "Interplay of Oct4 with Sox2 and Sox17: a molecular switch from stem cell pluripotency to specifying a cardiac fate." The Journal of cell biology 186.5 (2009):665-673.
Stevens, L C. "Embryonic potency of embryoid bodies derived from a transplantable testicular teratoma of the mouse." Developmental biology 2(1960):285-297.
Takahashi, K, and S Yamanaka. "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell 126.4 (2006):663-676.
ten Berge, D, et al. "Wnt signaling mediates self-organization and axis formation in embryoid bodies." Cell stem cell 3.5 (2008):508-518.
Thomson, J A, et al. "Embryonic stem cell lines derived from human blastocysts." Science 282.5391 (1998):1145-1147.
Van Vliet, P, et al. "Early cardiac development: a view from stem cells to embryos." Cardiovascular research 96.3 (2012):352-362.
Vincent, S D, and M E Buckingham. "How to make a heart: the origin and regulation of cardiac progenitor cells." Curr Top Dev Biol 90(2010):1-41.
Wamstad, J A, et al. "Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage." Cell 151.1 (2012):206-220.
Wei, B, and JP Jin. "Troponin T isoforms and posttranscriptional modifications: Evolution, regulation and function." Archives of biochemistry and biophysics 505.2 (2011):144-154.
Wernig, M, et al. "In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state." Nature 448.7151 (2007):318-324.
Yeom, Y I, et al. "Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells." Development 122.3 (1996):881-894.
Ying, QL, et al. "The ground state of embryonic stem cell self-renewal." Nature 453.7194 (2008):519-523.
Yu, J, et al. "Induced pluripotent stem cell lines derived from human somatic cells." Science 318.5858 (2007):1917-1920.

Zeineddine, D, et al. "Oct-3/4 dose dependently regulates specification of embryonic stem cells toward a cardiac lineage and early heart development." Developmental cell 11.4 (2006):535-546.

Appendix

Primer	Primer Sequence
Gadph	5' GTG GAG TCA TAC TGG AAC ATG TAG 3' 3' AAT GGT GAA GGT CGG TGT G 5'
Cardiac Troponin-T	5' TCA AAG TCC ACT CTC TCT CCA 3' 3' CTG ATG AAG AAG CCA AAG ATG C 5'
Isl-1	5' TCG TTC TTG CTG AAG CCT ATG 3' 3' AAT GTG CGG AGT GTA ATC AGT 5'
Nkx2.5	5' CAG CTC CAC TGC CTT CTG 3' 3' CCA AGT GCT CTC CTG CTT T 5'
Flk1 (KDR)	5' GGA TCT TGA GTT CAG ACA TGA GG 3' 3' GGA ATT GAC AAG ACA GCG ACT 5'
Troponin-I	5' TTC TTC CTC CAG TCT CCT ACC 3' 3' CTC TAA GCA CAA GGT GTC CAT 5'
Tbx1	5' ACA AAG TCC ATG AGC AGC AT 3' 3' GTG GGA CGA GTT CAA TCA GC 5'
Tbx5	5' CGT CTG CTT TCA CGA TGT GTA 3' 3' CTC AAA CTC ACC AAC AAC CAC 5'
Myl2	5' GGA AAG GCT GCG AAC ATC T 3' 3' GAC CAT TCT CAA CGC ATT CAA G 5'
Myh6	5' CTG GTC CTC CTT TAT GGT CAC 3' 3' GAG TGC TTC GTG CCT GAT 5'
PDGFRa	5' TCA CAG CCA CCT TCA TTA CAG 3' 3' GTT GCC TTA CGA CTC CAG ATG 5'

Table 1. List of qPCR Primers.

List of pre-designed primers from IDT for gene expression analysis via qPCR.

PCR conditions for these primers were as follows: 95˚ for 2 minutes; 40 cycles of 95˚ for 15 seconds, 59˚ for 15 seconds, and 68˚ for 20 seconds; and melting curve step for 95˚ for 15 seconds followed by 20 minutes beginning at 60˚ and ending at 95˚ with 15 second intervals at each temperature.

Primer	Sample	n	Primer	Sample	n	Primer	Sample	n	Primer	Sample	n
cTnT	3 Oct4+	3	**Flk1**	3 Oct4+	4	**Tbx5**	3 Oct4+	4	**PDGFRa**	3 Oct4+	4
	3 Oct4-	3		3 Oct4-	2		3 Oct4-	4		3 Oct4-	3
	3 No Sort	4		3 No Sort	4		3 No Sort	4		3 No Sort	4
	4 Oct4+	4		4 Oct4+	3		4 Oct4+	4		4 Oct4+	4
	4 Oct4-	5		4 Oct4-	3		4 Oct4-	4		4 Oct4-	4
	4 No Sort	3		4 No Sort	3		4 No Sort	3		4 No Sort	3
	5 Oct4+	3		5 Oct4+	2		5 Oct4+	3		5 Oct4+	2
	5 Oct4-	3		5 Oct4-	3		5 Oct4-	3		5 Oct4-	3
	5 No Sort	3		5 No Sort	3		5 No Sort	3		5 No Sort	3
	3-10 Oct4+	4		3-10 Oct4+	4		3-10 Oct4+	4		3-10 Oct4+	4
	3-10 Oct4-	2		3-10 Oct4-	1		3-10 Oct4-	2		3-10 Oct4-	1
	4-10 Oct4+	4		4-10 Oct4+	3		4-10 Oct4+	4		4-10 Oct4+	4
	4-10 Oct4-	4		4-10 Oct4-	4		4-10 Oct4-	4		4-10 Oct4-	4
	5-10 Oct4+	3		5-10 Oct4+	1		5-10 Oct4+	1		5-10 Oct4+	1
	5-10 Oct4-	4		5-10 Oct4-	4		5-10 Oct4-	4		5-10 Oct4-	4
	Day10 No Sort	4		Day10 No Sort	4		Day10 No Sort	4		Day10 No Sort	4
Isl1	3 Oct4+	4	**Tnni**	3 Oct4+	4	**Myl2**	3 Oct4+	4			
	3 Oct4-	4		3 Oct4-	2		3 Oct4-	2			
	3 No Sort	4		3 No Sort	4		3 No Sort	4			
	4 Oct4+	4		4 Oct4+	4		4 Oct4+	4			
	4 Oct4-	4		4 Oct4-	3		4 Oct4-	3			
	4 No Sort	3		4 No Sort	3		4 No Sort	3			
	5 Oct4+	3		5 Oct4+	3		5 Oct4+	2			
	5 Oct4-	3		5 Oct4-	3		5 Oct4-	3			
	5 No Sort	3		5 No Sort	3		5 No Sort	3			
	3-10 Oct4+	4		3-10 Oct4+	4		3-10 Oct4+	4			
	3-10 Oct4-	2		3-10 Oct4-	2		3-10 Oct4-	1			

	4-10 Oct4+	4		4-10 Oct4+	4		4-10 Oct4+	3
	4-10 Oct4-	4		4-10 Oct4-	4		4-10 Oct4-	4
	5-10 Oct4+	2		5-10 Oct4+	1		5-10 Oct4+	1
	5-10 Oct4-	4		5-10 Oct4-	4		5-10 Oct4-	4
	Day10 No Sort	4		Day10 No Sort	4		Day10 No Sort	4
Nkx2.5	3 Oct4+	4	**Tbx1**	3 Oct4+	4	**Myh6**	3 Oct4+	4
	3 Oct4-	4		3 Oct4-	4		3 Oct4-	3
	3 No Sort	4		3 No Sort	4		3 No Sort	4
	4 Oct4+	3		4 Oct4+	4		4 Oct4+	4
	4 Oct4-	4		4 Oct4-	4		4 Oct4-	3
	4 No Sort	3		4 No Sort	3		4 No Sort	3
	5 Oct4+	3		5 Oct4+	3		5 Oct4+	3
	5 Oct4-	3		5 Oct4-	3		5 Oct4-	3
	5 No Sort	3		5 No Sort	3		5 No Sort	3
	3-10 Oct4+	4		3-10 Oct4+	4		3-10 Oct4+	4
	3-10 Oct4-	2		3-10 Oct4-	2		3-10 Oct4-	2
	4-10 Oct4+	4		4-10 Oct4+	4		4-10 Oct4+	4
	4-10 Oct4-	4		4-10 Oct4-	4		4-10 Oct4-	4
	5-10 Oct4+	1		5-10 Oct4+	2		5-10 Oct4+	1
	5-10 Oct4-	4		5-10 Oct4-	1		5-10 Oct4-	4
	Day10 No Sort	4		Day10 No Sort	4		Day10 No Sort	4

Table 2: Sample size for qPCR analysis.

Sample counts for each population analyzed.