Gene Expression Changes Associated with the Differentiation of Mouse Embryonic Stem Cells to Primordial Germ Cell-Like Cells

By
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Abstract of Gene Expression Changes Associated with the Differentiation of Mouse Embryonic Stem Cells to Primordial Germ Cell-Like Cells, by Megumi Mori, Degree Sc.M., Brown University, May 2018.

Embryonic stem cells (ESCs) are found early in the developing embryo and from this initial population, all cells in the body are eventually derived. A hallmark characteristic of ESCs is their pluripotent nature, which is established to a large extent by transcriptional regulation. The core transcription factor network commonly referred to in stem cell pluripotency includes Oct3/4, Sox2 and Nanog which then contribute to a vast protein interaction network. The process by which embryonic stem cells become differentiated into cells with novel characteristics is tightly regulated and specific. In the past several decades, there has been great interest regarding how mammalian ESCs might be cultured in vitro to promote the derivation of cell types for research as well as medical endeavors. Primordial germ cells, the precursors of gametes, are an especially interesting subject for differentiation as they are linked to ESCs via their reinstated pluripotency upon fertilization to divide and constitute a new organism.

This thesis describes the theory and experimental methodology necessary to derive primordial germ cell-like cells (PGCLCs) from mouse embryonic stem cells (ESCs). Throughout this process, the optimal expansion and culture conditions of ESCs, epiblast-like cells (EpiLCs) and PGCLCs were implemented. A major initial goal was to assess the efficacy of differentiation via expected changes in gene expression throughout each differentiation step. Establishing this in vitro model system will then allow us to answer mechanistic questions about the how subunits of the core transcription factor TFIID complex may contribute to the maintenance of ESC pluripotency through the

transcriptional network and how they change to regulate the pathway by which PGCLCs are specified.

Introduction

Embryonic stem cells

Embryonic stem cells (ESCs) are defined as a unique group of cells that are derived from pre-implantation mammalian embryos. Defining characteristics of ESCs include the ability to culture them in vitro where they divide over a long period of time without differentiating and retain pluripotency in order to properly develop into many cell types and all primary three germ layers and germ cells (NIH). ESCs also have a distinct transcriptional and epigenetic profile; similar to the early embryo before implantation, ESCs exhibit high global transcription and open chromatin modeling (Morgani 2017). The discovery of a method to utilize the pluripotent nature of the early embryo and derive ESCs in vitro was crucial to work being done today. The earliest known success in this endeavor came in the early 1980's when two research groups, Martin (1981) and Evans (1981), were able to expand cell lines derived from mouse blastocysts under conditions supportive of pluripotency. In the following years, several experiments were done to confirm the characteristics that have come to define ESCs. Cell lines such as these, which can be generated for several generations while retaining standard morphology and genomic characteristics, along with the capacity to contribute to development when returned to the organism can now truly be considered embryonic stem cells (Martello 2014).

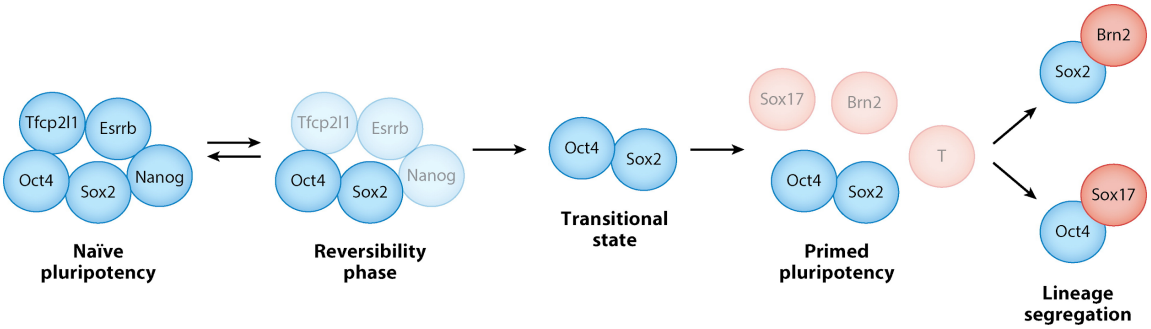
One of the most essential characteristics of ESCs is their ability to retain pluripotency, which in turn will allow the cells to differentiate into many different cell types. Naive pluripotency, a hallmark characteristic of a subset of mouse ESCs, is a highly modifiable state and is therefore an ideal system for investigating developmental

pathways and cell fate. In mice and humans, ESCs exist only in a narrow window of time- between formation of the blastocyst and implantation. From fertilization through the first several cleavage events, the embryo is totipotent and capable of differentiating into all three germ layers as well as supporting extraembryonic tissue. An additional, more stringent, requirement of a totipotent cell is the capability to develop into a complete organism.

The totipotent state transitions to the naive pluripotent state around the early to mid-blastocyst stage. At this stage, the inner cell mass (ICM) is still intact and ESCs harvested here in this preimplantation stage are known as naive pluripotent stem cells. These cells are distinct from the outer trophectoderm (TE) that support extra-embryonic placental differentiation. Following the lineage split between primitive endoderm and pluripotent epiblast, a population of epiblast-like stem cells can be identified stemming from the pluripotent epiblast (Boroviak 2014). At this post-implantation stage, the embryo may be considered to have primed pluripotency and are also known as epiblast stem cells (EpiSCs). Recent evidence suggests that although totipotency and pluripotency are controlled by similar major regulatory networks, they have significant differences such as epigenetic modification as well as differential regulation of factors for gene expression (Zhou 2015).

While both naive ESCs and primed EpiSCs exhibit pluripotency, there are clear observable differences between the two categories of naive and primed pluripotency. In vivo, these differences are thought to be a consequence of the differing environment of the developing epiblast. The preimplantation epiblast can be defined as a cluster of 10-20 cells in between the hypoblast and trophoblast which functionally can be considered the

developmental ground state (Nichols 2009). In mouse embryonic development, implantation in the uterine wall marks a distinct departure from this ground state. From this point on, the embryo is constrained within the physical environment and undergoes morphological changes in the epithelium (Nichols 2012). There are also changes at the molecular level apparent in the downregulation of naive marker transcription factors such as Nanog, Klf2/4/5, Tfcp2l1, Tbx3, Esrrb and Rex1, although master regulators such as Oct4 and Sox2 remain active at this time. Gene regulation experiments have shown that this transition is progressive and when the embryo first implants, it is the loss of naive markers that precedes the acquisition of lineage segregation factors (Kalkan 2014).



Martello G, Smith A. 2014.
Annu. Rev. Cell Dev. Biol. 30:647–75

Figure 1: Developmental progression from ground state embryonic stem cell to lineage commitment (Martello 2014).

This distinction between naive embryonic stem cells and primed embryonic stem cells is evident in the mouse system. However, in human embryonic stem cells (hESCs), there seems to be a single category of stem cell. Although hESCs are harvested from the ICM, similar to naive mouse embryonic stem cells, they exhibit traits more consistent with primed mouse EpiSCs (Duggal 2015). Aside from their comparable pluripotency capacity in the primed pluripotency state, there are significant differences between naive

pluripotent embryonic stem cells in the mouse and human embryonic stem cells. On a global scale, although mice and humans share the same pluripotency master network of Oct4, Sox2 and Nanog, recent work has shown differences in molecular and cellular mechanisms that lead to variance in measures such as growth rate, morphology and expression of cytokines and cell cycle genes (Schnerch 2010) (Ginis 2004). Downstream effects of this pluripotency network are also thought to be species-specific. Of relevance to work done in vitro, human ESC culture does not require LIF leukemia inhibitory factor (LIF), unlike mouse ESCs. Low levels of LIF signaling pathway components as well as the possibility of inhibitory regulation by cytokine signaling repressors suggests that the propagation of pluripotent ESCs in humans is due a LIF independent mechanism (Ginis 2004). These differences are important to keep in mind when determining human clinical relevance of work done with ESCs because a significant amount of what we currently know about embryonic pluripotency was discovered in the mouse. In order to sufficiently bridge the gap between model systems and human application, one must be aware of the challenges in generalization before moving forward.

Pluripotency

By its broadest definition, pluripotency describes the potential for a single cell to generate many different, specialized cell types (Morgani 2017). Early work investigating the systems that establish pluripotency in ESCs focused on transcription factors. Transcription factors influence gene control by binding to DNA and either activating or repressing transcription of designated sequences (Young 2011). The most essential of these factors in ESC pluripotency are Oct4, Sox2 and Nanog. The importance of master

regulators Oct4 and Sox2 came to the forefront of research after the success of the Yamanaka lab in generating induced pluripotent stem cells from mouse fibroblasts by transduction of candidate genes in the mid 2000's. Out of 24 candidate genes, they concluded that Oct4, Sox2 were essential to reprogram pluripotency, with the addition of tumor-related factors c-Myc and Klf4 (Takahashi 2006). Further investigation of these factors by whole genome chromatin immunoprecipitation- on chip has shown that in E14.1 mouse ESCs, these four transcription factors overlap at 58 promoters and it is hypothesized that they regulate more than a dozen developmental signaling pathways (Liu 2008). As this work related to the endogenous activity of these factors in harvested embryonic stem cells, it provides a connection between the forward expression of pluripotency in developmental time and reversal reprogramming of the pluripotency network in iPSCs.

Before Oct4 was known as a hallmark regulator of pluripotency, it was simply known to function as a transcription factor confined to early embryonic and required for germ cell development. It is also known as Oct3 and in some cases, as Oct3/4. It is encoded by the gene POU5F1 and acts by binding an octamer motif using the POU DNA binding domain. In order for Oct4 to exhibit proper expression, modulation of elements such as chromatin state, *trans*-acting regulators, miRNA and post-translational modifications must take place (Shi 2010). It is known that Oct4 also requires cooperation with other transcription factors to activate downstream targets. The regions containing promoter and enhancer elements are conserved between human, bovine and murine genomes, suggesting that the way in which Oct4 is regulated throughout mammalian embryogenesis is conserved (Jerabek 2013). The critical function of Oct4 is corroborated

by the fact that both under and overexpression of Oct4 in ESCs causes the loss of pluripotency and a shift toward differentiated cell fate (Niwa 2000). At a critical moment in embryonic development, the specification of the ICM and TE, Oct4 plays a role in not only allowing the cells that form the ICM to remain pluripotent, but also to inhibit the transcription of Cdx2, a gene required for trophectoderm development (Shi 2010). This observation has shifted the view of Oct4 from having the singular job of promoting pluripotency to also acting to directly influence the cell state transition out of the naive pluripotent state (Radzisheuskaya 2014).

Sox2 is a member of the Sry gene family and contains a high motility group DNA binding domain. It has been shown that Sox2 is essential to the developing embryo as loss of Sox2 results in a failure to form ESCs and trophoblast cells (Shimozaki 2014). Sox2 is commonly referenced in its role as a primary partner with Oct4. This cooperation was demonstrated in a 1995 study that showed that Oct4 and Sox2 bound adjacently at the FGF-4 enhancer region to promote the activity of FGF-4, which is necessary for later stage embryonic growth in mice (Yuan). Both Oct4 and Sox2, as well as many other transcription factors that play key roles in mammalian development, act within a large protein interaction network. These vast overlapping networks have the potential to have wide ranging effects and may help explain why master regulatory transcription factors must be tightly controlled within a small range and time window (Rizzino 2013). Sox2 also plays a critical role in the formation of neural stem cells (Pevny 2009) which underscores the intriguing balance between its capability to support both pluripotency and cell fate differentiation. In addition to the well characterized partnership of Sox2

with Oct4, it is also known to have some level of interdependence in regulation with the shared downstream target of Nanog (Kashyap 2009).

Nanog is the third transcription factor that is commonly thought to be one of the key controllers of the ESC state. The intertwined regulation of these factors is evidenced by overlap of mediator cofactors, binding sites at promoter regions, coactivators and shared feedback loops (Young 2011). Nanog is a homeodomain protein and when it dimerizes, it is capable of interacting with proteins such as Zfp281 and functions to maintain pluripotency (Saunders 2013). In addition to its role in pluripotency, Nanog assumes a related role in regulating the cell cycle in ESCs. Upregulation of Nanog in ESCs has been shown to allow for faster S-phase entry and hence rapid proliferation (Zhang 2016). In this way, Nanog function is of shared interest between the fields of stem cell and cancer research as the capacity to over proliferate is a hallmark of both cell types. Overexpression of Nanog allows for a high degree of self-renewal and although it is necessary and expressed in the ICM in vivo, Nanog null ESCs retain pluripotency but are much more prone to differentiation (Nichols 2012). This variance implicates the critical role of Nanog in maintaining pluripotency but may suggest that a loss or downregulation of Nanog may be compensated for in culture conditions.

It is useful to keep in mind the overlap between what is known about the environment of the source of ESCs and the current standard of their culture in vitro. One of the critical components of ESC culture in vitro is the cytokine LIF. LIF activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway which culminates in the activation of Stat3, which is critical to prevent cell differentiation (Onishi 2015). LIF also contributes to PI(3)-kinase signaling which is

shown to also be involved in maintaining ESCs (Nicola 2015). In regard to LIF pathway regulation, it is interesting to note that it is the pathways themselves that are necessary for ESC renewal, as inhibition of the PI (3)-kinase pathway even the presence of LIF does not preserve pluripotency.

Bone morphogenic protein (BMP) which normally stimulates differentiation at later time points, is a critical addition to early ESC culture along with LIF (Nicola 2015). BMP signaling was shown to complement LIF in feeder free culture, as both cytokines seem to have at least some individual gene targets. BMP4 is known to turn on Dusp9, a gene involved in repressing Erk1/2 and thereby inhibiting a key pathway in cell cycle regulation (Ohtsuka 2015). These two components comprising of activation of Stat3 by LIF and production of proteins that inhibit differentiation by BMP were the basis for early mouse ESC culture in feeder free conditions. Subsequent work has focused recapitulating the downstream functions of these factors to limit unwanted effects of cytokine signaling.

A variety of small molecule inhibitors helped address this problem, with two classes emerging as especially relevant. The first is a glycogen synthase kinase 3 (GSK3) inhibitor, the most potent and selective of which is CHIR99021 (Bain 2007). It acts through inhibition of CDK2–cyclin A and relieves the negative regulation of a variety of proteins, notably β-catenin. β-catenin is stabilized intracellularly by CHIR99021 and along with its interactor Tcf3, help to promote the undifferentiated state of ESCs (Nichols 2012). The second are a group of MAP kinase/ERK kinase (MEK) inhibitors. A commonly used molecule is PD0325901, which also has a high selectivity and potency (Nichols 2012). It acts on the FGF/ERK pathway by inhibiting MEK1 to effectively

create a block to differentiation (Van der Jeught 2013). A 2008 publication found that a combination of LIF and these two small molecule inhibitors (2i) were capable of promoting the pluripotency and propagation of mouse ESCs (Silva). This combination is now frequently referred to as LIF + 2i culture conditions and is a confirmation that ESCs recapitulate the pluripotent state seen in vivo, even in the absence of intensive external stimuli (Ying 2008).

Primordial Germ Cells

In addition to investigating the mechanisms of pluripotency, the timing and conditions of the transition of ESCs to other cell types is of great biological and clinical interest. A prime cell type target is the derivation of primordial germ cells (PGCs) from ESCs as they are linked by their separate, but similar pluripotent characteristics. It is interesting to contrast the way in which many factors work together to promote a shift to a primed pluripotency, which in vitro is capable of giving rise to germ cells that will reprogram themselves to pluripotency once again. The relationship between embryonic stem cells and primordial germ cells is tied to the shifting condition of pluripotency.

Primordial germ cells are the first specified cells that will develop into the gametes. There is a strong connection between germ cells and capacity for totipotency because they are responsible for the recapitulation of genetic and epigenetic information through generations (Saitou 2012). This acquisition of totipotency potential and commitment to primordial germ cell fate is one of the earliest events that ensure the relative immortality of an individual or species level genome and is in a sense a

mechanism that allows PGCs to “escape from the mortal somatic cell fate” (Kurimoto 2015).

The evolutionary history of germ cell specification has been the subject of intensive research for many years. Current understanding of the mechanisms by which PGCs are specified is essential to further elucidate how this process might be reconstituted in vitro as accurately as possible. The main two methods that PGCs become specified are termed preformation and epigenesis. In preformation, PGCs can be identified fairly early as they receive instruction from maternally inherited determinants in germ plasm. In brief, cells that inherit this germ plasm develop into PGCs. In epigenesis, PGCs are induced from signals sent from tissues that have already assumed a given cell fate. If the correct signals are received by cells in the pluripotent epiblast layer that are competent to develop into germ cells, they will have acquired their cell fate as PGCs (Extavour 2003). Mouse PGCs are specified by epigenesis, which fits the schema of PGC fate specification as a step-wise process that requires highly regulated signaling at many stages. The remaining discussion of primordial germ cells will focus on the mouse model system with some contrast to human PGC specification where relevant.

In normal embryonic development in the mouse, primordial germ cells are detectable at about embryonic day 7.5 (E7.5). A commonly used method to detect PGCs is alkaline phosphatase (AP) staining which is more accurate than morphological identification and allows for earlier detection (Leitch 2013). At this time point, the PGCs form in a cluster of about 40 cells at the bottom of the incipient allantois (Saitou 2012). The cells then begin to migrate through the developing hindgut to the future gonadal ridge and arrive at their final destination of the gonad by E10-10.5. Once the cells

colonize the gonadal ridge, the cells undergo a loss of motility and polarity and further germ cell development becomes sex specific (Kumar 2017). This time point marks the end of PGC development as identifiable differences in the male and female gonads are evident by E12.5 (Leitch 2013) therefore denominating the transition from primordial germ cell to germ cells.

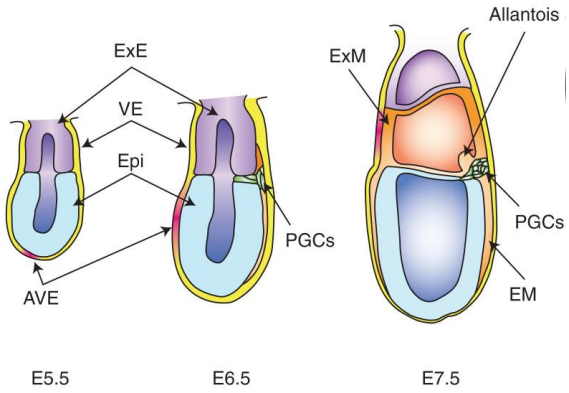


Figure 2: A schematic representation of primordial germ cell development in mice (Saitou 2012).

These events occur under the strict regulation of a vast array of transcriptional and gene level changes. Some of the earliest and most critical factors to induce PGC specification belong to a class of signaling molecules called bone morphogenic proteins (BMPs). The future PGCs located in the epiblast of the embryo receive BMP4 and BMP8 signaling from the extraembryonic ectoderm and BMP2 signaling from the visceral endoderm which are required for their specification (Leitch 2013). Studies done with knockouts and knockdowns for BMP4, BMP8 and BMP2 or their downstream signaling targets in the Smad pathway have confirmed that this pathway is necessary for PGC induction and that BMP4 and BMP8 signaling is dosage dependent (Kumar 2017).

In addition to BMP signaling, WNT signaling plays an interesting role in PGC specification and development. The WNT family is extensive and varied with at least

three distinctive pathways, including the canonical β-catenin-dependent pathway. During embryonic development, WNT signaling is required for a host of events such as body axis formation, limb patterning and stem cell proliferation (Niehrs 2012). The activation versus inhibition of specific WNTs, such as WNT3 in PGC specification have been debated (Kimura 2006) (Ohinata 2009), however there now seems to be a general consensus that WNT signaling does play a role in PGC development (Hayashi 2011) (Saitou 2012) (Günesdogan 2014). Evidence has clearly shown that WNT signaling in embryonic development and especially in primordial germ cell development is highly context specific with regard to timing and spatial regulation.

B-lymphocyte-induced maturation protein 1 [*Blimp1*], also known as PR domain-containing protein 1 [*PRDM1*] is a transcription repressor that is a core regulator of PGC specification and the first to act. Blimp1 interacts with many epigenetic regulators such as HDACs and functions as a strong repressor of somatic cell fate programs (Saitou 2012) (Kurimoto 2008). As Blimp1 can be detected in a subset of cells as early as E6.5, there is strong evidence that the epigenesis mechanism of PGC specification requires signals that will both induce germ cell fate and suppress other developmental pathways.

PR domain-containing 14 [*PRDM14*] is another regulating transcription factor. Like Blimp1 and as its name suggests, it contains a PR domain as well as five zinc fingers. Although PRDM14 is also essential for PGC specification (Saitou 2012), it may also play a role in general pluripotency. Recent work done in vitro has shown that in epiblast like cells, PRDM14 is capable of reactivating pluripotency and self-renewal genes via TET-BER mediated demethylation (Okashita 2016). PRDM14 expression in the ICM of the embryo is necessary for priming somatic cell fate, while later in the

epiblast it is necessary for PGC specification. There are also speculated differences between the role of PRDM14 in human versus that of mice (Okashita 2016), which requires significant consideration as in vitro modeling work expands.

The last transcription factor to be considered as a core PGC transcription factor is AP2γ, encoded by TCFAP2C. AP2γ is a basic helix–span–helix domain transcription factor that is maternally expressed in the blastocyst in all cells but becomes restricted to the extraembryonic ectoderm and PGCs around the time of PGC specification (Saitou 2012). Its complete function and mechanism has not been fully characterized as it has been identified as a PGC determining factor more recently than the other two transcription factors. However, it is known that AP2γ is a direct downstream target of Blimp1 and has the potential to activate PGC genes and suppress somatic genes individually and in conjunction with Blimp1 and PRDM14 (Magnúsdóttir 2014). While these three transcription factors are essential to PGC induction at a specified time in the developing embryo, they may also have separate roles at different time points. Their mutual interdependence, working in a transcriptional network, generates a novel function that is limited to a select group of cells in a tight window of developmental time. As the researchers Günesdogan et. al. note, this context dependent regulation raises further questions about the plasticity of the early embryo and how to determine cell competence (2014).

Two additional signaling factors, Activin A and basic fibroblast growth factor (b-FGF/FGF2), are known to promote the differentiation of ESCs to the primed epiblast-like cell (EpiLC) state in vitro. This is referred to as a primed state because initiating differentiation from this state results in a population enriched with PGCs. Activin A is a

member of the transforming growth factor (TGF-β) super-family and plays a role in many germ cell-related functions such as ovarian follicle development and meiotic initiation in the oocyte (Sun 2015). The link between ESCs and the EpiLC state was verified by testing epiblast marker genes, which were upregulated upon addition of Activin A (Hayashi 2011) (Sun 2015). Fibroblast growth factors play a wide variety of roles in the developing embryo (Platonova 2014). A well-studied member of this family, b-FGF, has also been found to promote the commitment of ESCs to PGC precursor state (Sugawa 2015).

As with all work done in vitro with model systems, the approximations and assumptions that are made when observing interactions removed from their environment in a living organism must constantly be acknowledged. In applying this to primordial germ cell differentiation, a number of researchers have begun to try to understand how this process might be recapitulated (Hayashi 2011) (Magnúsdóttir 2014). This process of inducing primordial germ cell-like cells from a starting population of pluripotent cells is in its early stages, and there are many opportunities to learn more about what is necessary and sufficient to create germ cells, and how to ensure that the model best mimics the in vivo counterparts to the greatest extent possible.

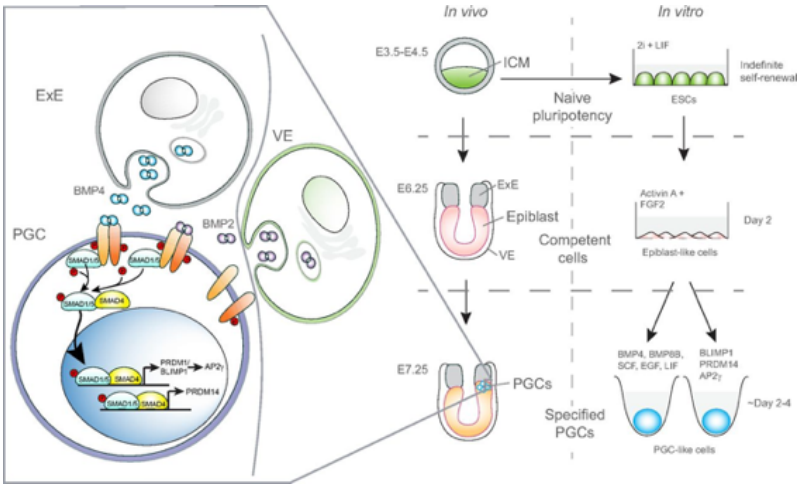


Figure 3: Specification of primordial germ cells (Magnusdottir 2014).

Project Background

This connection between embryonic stem cells and primordial germ cells is the core of ongoing work by a team of researchers based in Kyoto, Japan led by Katsuhiko Hayashi and Mitinori Saitou. Their work stems from one of the most fundamental concepts in developmental and cell biology- how the germline perpetuates the survival of a species. One of their earliest papers (Hayashi 2011) built on the foundation of previous work in many other labs which attempted to isolate and propagate primordial germ cells in vitro. In an effort to increase the yield of primordial germ cell-like cells (PGCLCs), the Saitou lab focused on a new subset of cells through which to derive them from. These cells are known as epiblast-like cells (EpiLCs), which exhibit a primed state suitable for PGC differentiation as opposed to the naive pluripotency of ESCs, which are the original starting population. This EpiLC state is thought to recapitulate the pre-gastrulating state of the epiblast in the developing embryo, although it is separate from epiblast stem cells (EpiSCs) and at a later developmental stage than the naive pluripotency of the inner cell mass (Hayashi 2011). The gold standard for gamete generation is the ability of derived

gametes that form viable offspring, and while those early trials lacked this piece of evidence (Daley 2007), there has now been success on that front.

The culmination of this step, which confirmed the capacity of these derived germ cells to re-confer totipotency upon fertilization was shown in their 2016 publication titled, “Reconstitution in vitro of the entire cycle of the mouse female germ line”. This set of experiments used the same method of deriving PGCLCs from ESCs and EpiLCs. Once the PGCLC clusters were formed, they were co-cultured with somatic cells to form a reconstituted ovary environment to support oocyte induction. From this step, a series of female germline specific factors were added to push the PGCLCs through the in vitro complement of normal oocyte maturation. Upon the oocytes’ completion of meiosis II, which were comparable in size and epigenetic markings to in vivo oocytes, these oocytes were fertilized by in vitro fertilization (IVF) and carried by pseudopregnant females. The offspring resulting from this fertilization were shown to develop normally with no notable defects and have retained fertility in both sexes. To complete the cycle of germline reconstitution shown in the figure below, preimplantation blastocysts could be reprogrammed back to an ESC state, which they termed regenerated embryonic stem cells (rESCs) (Hikabe 2016). This comprehensive demonstration of the ability for ESCs to produce competent germ cells at this level of rigor is a step toward both understanding how this process occurs in normal development as well as how in vitro techniques might be further developed in a regenerative medical context.

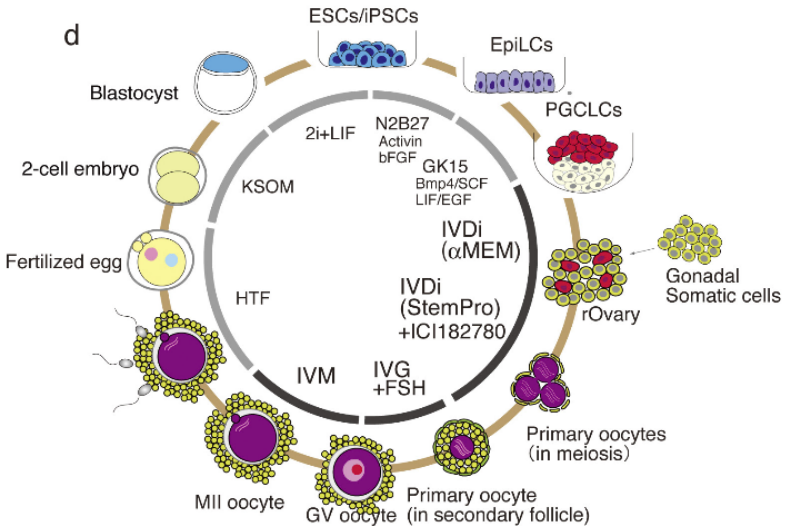


Figure 4: A schematic drawing showing the in vitro reconstitution of the entire female germline (Hikabe 2016).

Members of this lab and related researchers took many preliminary steps in order to optimize the methodology and techniques needed to accomplish this entire cycle of reprogramming. One such step was a 2013 protocol, which described the integrated process by which mouse pluripotent stem cells could be used to derive primordial germ cells and later oocytes and has been extremely helpful in the formulation of this current project (Hayashi 2013).

TFIID complex

TFIID is a general transcription factor complex that includes TATA binding protein (TBP) along with 13-14 TBP- associated factors known as TAFs (Cler 2009). Although the TFIID complex is well known to be important in transcription initiation, there are many aspects of TAF interactions that remain unknown. Emerging research suggests that the TFIID complex may play a role in maintenance of pluripotency, but modifications may then drive cells toward differentiation during development. One

family of TAFs that is of particular interest to the lab is the TAF4 family, which in mice is composed of the TAF4a and TAF4b paralogs. These molecules are similar in that they are encoded by related genes and share TAFH, a central domain (Cler 2009). Although TAF4a is considered part of the core complex which is expressed ubiquitously in mammals, TAF4b may serve as a complement component and play a role in germ cell viability.

TAF4b is gonadally-enriched and has been implicated as a crucial factor in oocyte development (Falender 2005). It was later established that TAF4b plays a role in turning on oogenesis genes and promoting the normal progression of meiosis, which is critical to the formation of a healthy ovarian reserve (Grive 2016). The germ cell/fertility functions of TAF4b raises various questions as to when it acts in the timeline, what partners it interacts with and to what extent it is necessary to mammalian germline specification. As the relation between pluripotency and primordial germ cells is strong and potentially fluid as discussed above, TAF4b along with is paralog TAF4a, are ideal candidates for study in during this differentiation pathway.

TAF4a was shown to be indispensable for normal embryogenesis in a 2016 study which showed this via the generation of Taf4a−/− embryos and visualization by in situ hybridization (Langer). Taf4a−/− embryos exhibited clear defects in body pattern and heart development, although developed through E9.5. Researchers speculate that this later embryonic lethality may suggest the ability of Taf4b to compensate for the null Taf4a at early embryonic time points. Interestingly, they reported that ESCs generated from Taf4a−/− embryos were “almost indistinguishable” from wild-type ESCs with regard to their cell physiology and expression of the network of pluripotency genes. The

concept of Taf4a as a dispensable or even antagonistic factor in the ESC state is supported by the findings of Bahat et. al. which used siRNA knockdowns of TAF4a and TAF4b to show that TAF4a may inhibit the cell growth of ESCs. Another key difference to support this theory is the interaction of TAF4b but not TAF4a with Oct4, a core pluripotency transcriptional regulation. This interaction is proposed to exist not only in ESCs, but potentially in developing germ cells as well (Bahat 2013).

However, these findings are in contrast to earlier work which showed the ability of TAF4a to act as a critical mediator for reprogramming iPSCs via reinstitution of the pluripotency transcription network (Pijnappel 2013). This work suggests that some components of the TFIID complex are directly involved in the pluripotent state and that upregulation of TAF4a specifically greatly enhanced the pluripotent state in reprogrammed fibroblasts. These contrasting views are not necessarily mutually exclusive, as working with ESC culture systems can be done under a multitude of protocols and often related but not necessarily overlapping questions are investigated. For example, the work done by Pijnappel et. al uses the system of iPSCs generated from mouse fibroblasts, while Langer et. al derived the ESCs used in their experiments from Taf4a−/− embryos. This previous work sets a foundation for learning more about TFIID and its subunits and how we can integrate work done with slightly different model systems to generate a complete understanding of how this critical complex functions.

Master's Thesis Project

The long-term goal of this project is to draw on previous research done in the Freiman Lab and others in attempting to characterize the function and contributions of the TFIID complex in relation to the pluripotency of embryonic stem cells and subsequent changes upon differentiation toward a primordial germ cell-like cell fate. Previous results implicate TFIID subunits TAF4a and TAF4b as potential contributors to the pluripotency and PGC transcriptional regulatory network, although there is not yet consensus on the timing of expression and subcellular localization of these factors.

As this work is in its beginning stages, the main goal of my project is to establish the techniques and assays specific to ESCs and their differentiation into PGCLCs. To address this, my project aims to set the groundwork for the establishment of efficient and high-quality ESC culture as well as the methodology underscoring their stepwise transition to PGCLCs. The protocol is adapted from the Saitou lab (Hayashi 2013), with significant practical modifications. This work began with the culture and expansion of C57B6 ESCs to establish the most useful culture conditions by analysis of their morphology and pluripotency gene expression at different time points. The primed epiblast-like state was then derived and analyzed for changes in pluripotency and EpiLC specific genes by q-PCR. Similar analyses were completed for the EpiLC to PGCLC stage.

Materials and Methods

Initial ESC culture and expansion

Wild-type C57Bl6 embryonic stem cells were obtained at passage 10 from the Transgenic Core Facility at Brown University in October 2017. Mouse ESC culture is traditionally supported by a feeder layer of inactive mouse embryonic fibroblasts (MEFs). The MEFs function as support cells for the ESCs by secreting various cytokines and growth factors to keep the ESCs in a pluripotent state. These factors are critical and versatile, as demonstrated by their potential to support hESCs through several generations (Desai 2015). Although these factors are now well identified and can be added to media, allowing for non-feeder layer culture, in order to generate a large, healthy working stock of ESCs, feeder layer protocols were used to support several generations of ESCs before transitioning to non-feeder layer culture. Therefore, the process of ESC culture began with the expansion of MEFs to act as a co-culture cell line.

Active MEFs were obtained in an aliquot of 1 ml active MEF media cryopreserved on May 6, 2014 from the Transgenic Core Facility at Brown University in October 2017. Media and passage protocols were also adapted from the Transgenic Core Facility.

Reagent	Volume (ml)	Source
Knockout Dulbecco's Modified Eagle Medium (DMEM)	410	Invitrogen
Penicillin-streptomycin	5	Invitrogen
L-glutamine	5	Millipore
Fetal calf serum	75	Gemini Bio
Total:	**500**	

Table 1: Active MEF media.

Active MEFs were plated in 10 ml of media in 10 cm standard Corning cell culture plates and grown to approximately 100% confluency. Media was changed every other day, not including days that the cells were passaged. Active MEFs were split following a standard cell culture protocol as follows. Active MEF media was aspirated from plates and washed with PBS. 1 ml of Trypsin- EDTA 0.25% was added and incubated at 37°C for 5 minutes to allow cells to detach from the plate. Trypsinization was quenched with an appropriate volume of media, depending on the splitting ratio. The cell suspension was then split into new plates. A set of active MEFs were frozen down in cryopreservation media (in ratio of 8:2 ml media to DMSO) and put in overnight storage in a Thermo Scientific Mr. Frosty™ Freezing Container then transferred to LN2 storage to serve as a stock. Subsequent expansion of active MEFs culminated in the formation of inactive MEFs on which ESCs would be plated.

Inactive MEFs were produced when 20 plates of active MEFs reached optimum confluency (80-100%) by treatment with Mitomycin C in order to arrest cell division (Kaur 2013). The inactive MEFs were then cryopreserved following the same protocol as the active MEFs. This stock was then used as the feeder layer for the ESCs at a ratio of 1 ml inactive MEFs: one 10 cm plate.

ESC expansion was done on 10 cm Corning plates which were pre-coated with gelatin (Millipore Ultrapure Water + 0.1% Gelatin). Before the ESCs were plated, inactive MEFs were plated and allowed to sit down on the plate for 1-2 days. The inactive MEFs were maintained with active MEF media but this was only necessary until plating of ESCs, when new ESC media was required for maintenance of the cells. The

essential component of this media is LIF, which as discussed, is a critical promoter of pluripotency in ESCs.

Reagent	Volume (ml)	Source
Knockout Dulbecco's Modified Eagle Medium (DMEM)	500	Invitrogen
1000x β- Mercaptoethanol	5	Sigma
L-glutamine	5	Millipore
Fetal calf serum	90	Gemini Bio
LIF	60 µl	Millipore
Total:	**500**	

Table 2: B6 ESC media.

After inactive MEFs adhered to the gelatinized plates, the ESCs were plated on top. ESC media was changed every day as consistently as possibly in the morning. ESCs were cultured to relative confluency and various experiments were done to test the growth conditions and cellular state of the ESCs. To facilitate ESC dissociation from the plate for collection or passage, a milder 0.05% Trypsin-EDTA was used. The ESC cell suspension was then spun down and re-suspended in either new ESC media if passaged, or in ESC cryopreservation media composed of a ratio of 1 ml DMSO: 4ml ESC media. Before freezing, this expansion step was done twice to ensure a sufficient working stock.

Non-feeder layer ESC culture was completed on 6-well Corning plates coated with poly-ornithine and laminin at varying concentrations, which were determined experimentally. At this time, a second ESC media was developed and used for culture. This media included two small molecule inhibitors, CHIR99021 and PD0325901, in addition to LIF to support the dedifferentiation and pluripotency state of ESCs in vitro.

The protocol and reagent lists were adapted from the Transgenic Core Facility and Hayashi and Saitou 2013.

Reagent	Volume (ml)	Source
Knockout Dulbecco's Modified Eagle Medium (DMEM)	500	Invitrogen
Knockout serum replacement	95	Invitrogen
Glutamax	12	Invitrogen
Penicillin-streptomycin	6	Invitrogen
2-β- Mercaptoethanol	1.2	Invitrogen
Non-essential amino acids	6	Invitrogen
Sodium pyruvate	6	Invitrogen
Insulin	300 µl	Sigma
LIF	12 µl	Millipore
PD0325901	120 µl	Stemgent
CHIR99021	600 µl	Stemgent
Total:	**600**	

Table 3: ESC 2i + LIF media.

EpiLC Derivation

The epiblast-like cell state was derived directly from a population of ESCs that had been cultured to colony formation. The ESCs were cultured in their own media until the collection step in which they were re-suspended in new EpiLC differentiation media. EpiLCs were cultured on 12-well Corning plates pre-coated with fibronectin (human plasma fibronectin; Millipore). The media components and culture techniques were also

adapted experimentally based on a combination of protocols from the Transgenic Core Facility and the Saitou lab (2013).

Reagent	Volume (ml)	Source
Knockout Dulbecco's Modified Eagle Medium (DMEM)	100	Invitrogen
Knockout serum replacement	19	Invitrogen
Glutamax	2.4	Invitrogen
Penicillin-streptomycin	1.2	Invitrogen
2-β- Mercaptoethanol	240 µl	Invitrogen
Non-essential amino acids	1.2	Invitrogen
Sodium pyruvate	1.2	Invitrogen
Insulin	60 µl	Sigma
Activin A	-	Peprotech
β-FGF	-	Peprotech
Total:	**125.3**	

Table 4: EpiLC media.

Primordial Germ Cell-Like Cell Derivation

Primordial germ cell-like cells were induced from the EpiLC state on a timeline following Hayashi and Saitou 2013. Each propagation step must be carefully timed, as the differentiation steps must be continuous to ensure the cells are given enough time to respond to the new culture conditions and differentiate. The cells were plated in low attachment 96-well U-shaped Corning plates and incubated for 3-4 days. Analysis of the PGCLC state required a departure from the adapted protocol (Hayashi 2013), as the B6

ESC line did not carry the BVSC reporter genes that would indicate successful PGCLC differentiation.

Reagent	Volume (ml)	Source
Glasgow minimal essential medium (GMEM)	80.82	Invitrogen
Knockout serum replacement	15	Invitrogen
Sodium pyruvate	1.2	Invitrogen
L-glutamine	1	Millipore
Penicillin-streptomycin	1	Invitrogen
2-β- Mercaptoethanol	180 µl	Invitrogen

Table 5: GK15- PGCLC base media.

Reagent	Volume (ml)	Source
GK15	9.778	-
BMP4	20 µl	Webb Lab
BMP8a	100 µl	R&D systems
Stem cell factor (SCF)	20 µl	R&D systems
LIF	12 µl	Millipore
Epidermal growth factor (EGF)	5 µl	Webb Lab

Table 6: PGCLC differentiation media.

RNA Extraction and Evaluation

RNA extraction was conducted for samples primarily in preparation for qPCR analysis. At the time of cell collection, the cell pellet was spun down in a centrifuge and washed in PBS before suspension in 500 µl TRIzol and stored at -80°C if the extraction was not performed immediately. The RNA protocol utilized was modified from the TRIzol protocol provided by Life Technologies. Major steps include extraction of the aqueous phase using chloroform, addition of glycogen and IPA, and washing of the RNA pellet with 75% and 100% EtOH between centrifuging at 10-minute intervals at 4°C. As the recovered RNA pellet was small, the resuspension volume of nuclease-free water was 10 µl.

RNA concentration was evaluated using a Nanodrop reading of each sample. The instrument was first blanked with 1 µl of double distilled water (ddH2O) and then loaded with 1 µl of the sample. If the RNA concentration exceeded 1000 ng/µl, subsequent dilutions in nuclease-free water were made at ratios of 1:10 or in some cases 1:5 and another Nanodrop reading was taken.

cDNA Synthesis

cDNA synthesis was completed after obtaining accurate readings of RNA concentration. The RNA sample template was normalized to 1µl by dividing the RNA concentration into 1000. Reaction components included 5x iScript Reaction Mix, iScript reverse transcriptase, nuclease-free water and normalized RNA template. The total volume of the cDNA reaction was 20 µl and incubated in a thermal cycler according to

the iScript reaction protocol. If greater amounts of cDNA were needed, two reactions with the same samples would be run simultaneously and pooled before use.

q-PCR Materials

Quantitative-PCR, or real-time PCR, is a useful and well-known assay to determine the relative gene expression of given samples compared to a reference gene as monitored by DNA amplification (Ginzinger 2002). q-PCR was used to investigate potential changes in gene expression throughout ESC culture, as well as throughout the process of differentiation of ESCs to subsequent EpiLC and PGCLC states. Samples were made up in 25 µl total volumes (12.5 µl SYBR Green dye, 7.5 µl ddH2O, 2.0 µl primer forward, 2.0 µl primer reverse; experimental sample- 1.0 µl cDNA).

Primers were chosen for genes representative of each cellular state. Primer pairs were tested in cDNA serial dilutions in a preliminary q-PCR test. Primers were diluted in ddH2O according to nmol concentration to produce a working stock. The forward and reverse sequences of each primer pair are given in Table 7.

Primer	Forward	Reverse	Source
Oct3/4	GATGCTGTGAGCCAAGGCAAG	GGCTCCTGATCAACAGCATCAC	Hayashi 2011
Sox2	CATGAGAGCAAGTACTGGCAAG	CCAACGATATCAACCTGCATGG	Hayashi 2011
Nanog	CTTTCACCTATTAAGGTGCTTGC	TGGCATCGGTTCATCATGGTAC	Hayashi 2011
Fgf5	AAAGTCAATGGCTCCCACGAA	CTTCAGTCTGTACTTCACT	Hayashi 2011
Wnt3	CAAGCACCAACAATGAAGCAGGC	TCGGGACTCACGGTGTTTCTC	Hayashi 2011
Dnmt3b	CTCGCAAGGTGTGGGCTTTTTGTAAC	CTGGGCATCTGTCATCTTTGCACC	Hayashi 2011
Prdm14	ACAGCCAAGCAATTTGCACTAC	TTACCTGGCATTTTCATTGCTC	Hayashi 2011
Blimp1	AGCATGACCTGACATTGACACC	CTCAACACTCTCATGTAAGAGGC	Hayashi 2011
Tcfap2c	GGGCTTTTCTCTCTTGGCTGGT	TCCACACGTCACCCACACAA	Hayashi 2011
TAF4a	ATCTCCACTGTGCAGGCTCC	GGTCACCTGCCGTGCAATA	Freiman lab
TAF4b	GATGTTACTAAAGGCAGCCAAGAGT	CTGCTCTGGATCTTCTTTATTGGAG	Freiman lab
18S	CCGCGGTTCTATTTTGTTGG	GGCGCTCCCTCTTAATCATG	Freiman lab
β-Actin	TCTTGGGTATGGAATCCTGTGG	CAGCACTGTGTTGGCATAGAGG	Freiman lab

Table 7: q-PCR primer sequences.

Results

ESC Culture Conditions

Following the successful expansion of ESCs on the inactive MEF feeder layer, conditions for transitioning to non-feeder layer culture were established experimentally. The two components of the non-feeder layer treatments were 0.01% poly-L-ornithine and laminin (10 ng/ml). The initial conditions consisted of 10 cm Corning plates incubated with poly-L-ornithine for 1 hour at room temperature followed by laminin for two hours at 37°C. ESCs were plated approximately 1 ml/ one 10 cm plate. The ESCs were incubated for four days and imaged each morning.

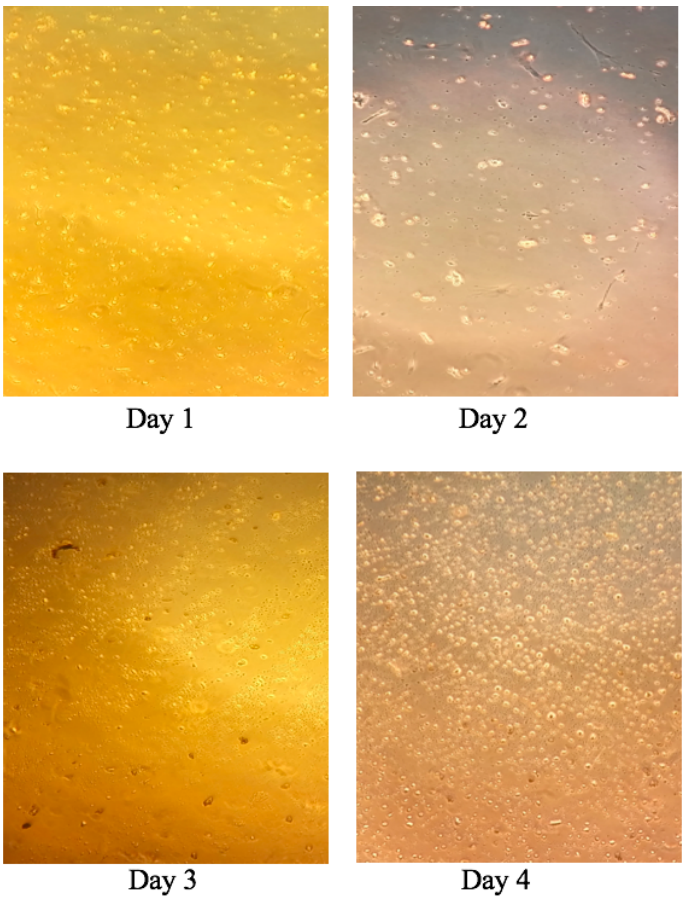


Figure 5: Embryonic stem cell culture on days 1, 2, 3 and 4 after plating.

The ESCs did not appear to thrive under these conditions. Although some ESCs adhered to the plate, they remained separated and did not effectively form the round, 3D mound shaped that is typical of ESC colonies. Furthermore, after several days cells appeared to begin to differentiate despite receiving new media every day. To address these concerns, several modifications to the protocol were made.

To increase ESC adhesion the concentration of laminin was increased from 10 ng/ml to 1.67 µg/ml. The modification of laminin was chosen over poly-L-ornithine as laminin is the top most layer of the plate treatment ensuring that the ESCs have primary contact with this component. Also, to encourage more ESCs to adhere to the plate, media was changed within 24 hours after initial plating only if a sufficient number of cells had already adhered as determined visually. In addition, 6-well plates were used instead of 10 cm plates to facilitate ESC aggregation.

Quantifying the number of ESCs for optimal growth was a second component of revisions to the ESC culture protocol. To address this, ESCs were counted before each plating with a hemocytometer. A sample 10 µl of cell suspension media was mixed with 10 µl of dye and 10 µl of this solution was pipetted into the hemocytometer. The average number of live cells from four quadrants was calculated and multiplied by $10^4$and by the total ml of cell suspension to approximate the total number of cells collected. Using this number, the appropriate volume of media was calculated to achieve a concentration of 2.5 x 10^5 cells per well of a 6-well plate. Lastly, as the ESCs appeared to differentiate over time, the ESC media was switched from B6 ESC media to ESC+ 2i LIF media. In order to assess the efficacy of these changes in maintaining ESC pluripotency and character an ESC series experiment was carried out.

ESC Series

ESCs and other pluripotent stem cells grown in culture may susceptible to altered levels of pluripotency based on passage number (Beers 2012) (Li 2007). While it is sensible that lower passages for all cell types is preferable, the possibility for high passage to affect the ability of ESCs to maintain their pluripotency state and therefore prime the cells toward a certain lineage would greatly impact the integrity of ESCs. As it took several passages to establish the working stock of ESCs for this project, evaluation of pluripotency over time became a subject of inquiry.

The ESCs were plated in 6-well Corning plates coated with poly-L-ornithine and laminin at revised concentration. On the first plate, approximately 2.5 x 10^5 cells/well were plated in two wells. The ESCs were grown to confluence spanning four days. On the fourth day, one well was collected as a sample for RNA analysis and the other was split into a new plate. This process was repeated a total of three times and three samples were collected at intervals spanning four days for a total of 12 days. This experiment was run a total of two times with no observable difference between the replicates. The results of the second trial are shown in Figure 6.

There were no significant differences in morphology or growth conditions between the three plates over time, the first (Plate 1) and last (Plate 3) of which are shown in Figure 6. Colony formation was observable by Day 1 after plating in all plates and continued to grow in round, mound-like colonies as expected in ESC culture. Additionally, this experiment also showed that the modification to the ESC protocol improved the adhesion and colony formation as it was the first experiment to be done under the revised conditions.

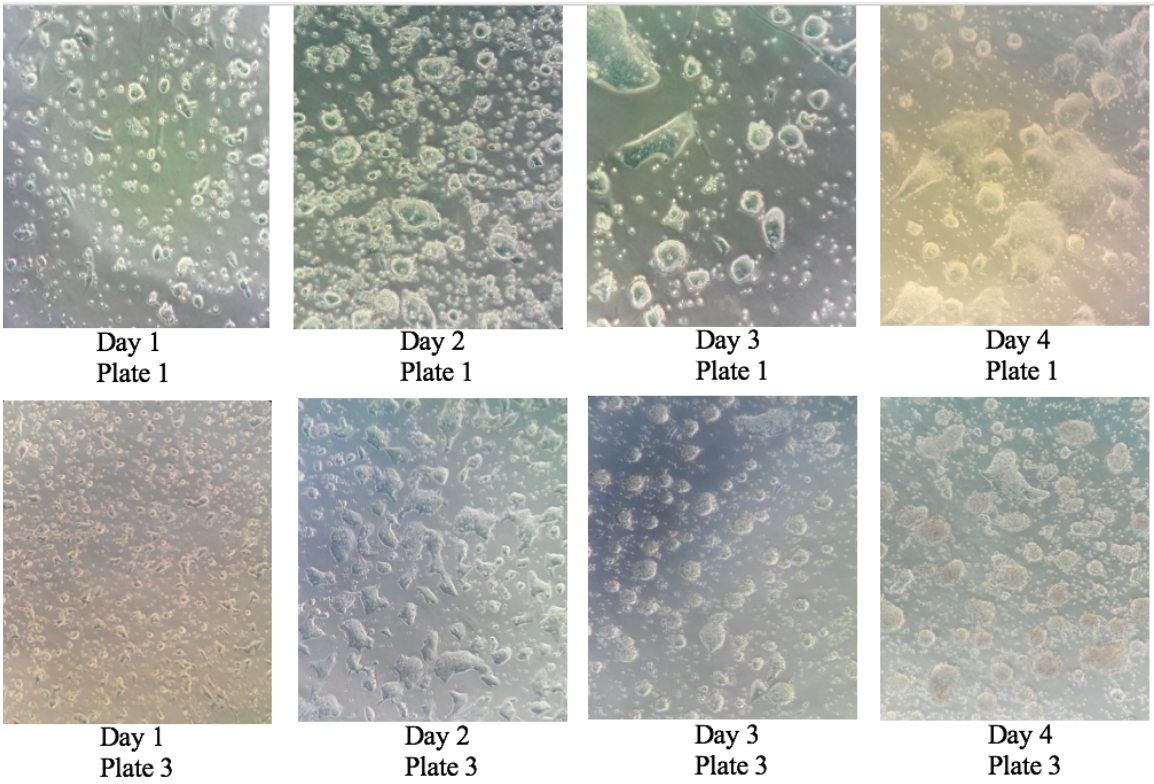


Figure 6: Embryonic stem cells cultured through three passages.

In order to quantify potential changes in pluripotency throughout the span of ESC culture, q-PCR was performed. Target genes included pluripotency marker Oct3/4, as well as a differentiation marker, Wnt3 as a negative control. Oct3/4 and Wnt3 were chosen as throughout ESC differentiation, they were shown to be constant and upregulated, respectively (Hayashi 2011). It was hypothesized that if the ESCs were significantly differentiating over time, there would be a decrease in Oct3/4 and increase in Wnt3.

Observations included a downward trend in both Oct3/4 and Wnt3. While this did not verify the hypothesis, these results suggest that there are potential changes in gene expression levels as ESCs are cultured over time. For the purposes of this project, it was

concluded that progression to the EpiLC stage should not be done on cells that had been continually passaged and splitting should be limited to stock expansion purposes only.

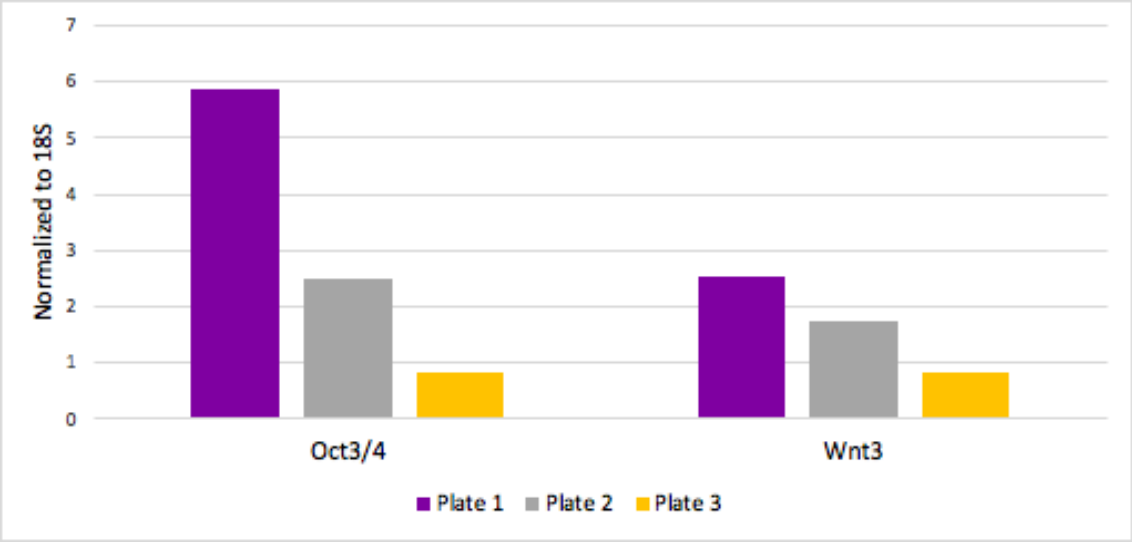


Figure 7: Relative levels of Oct3/4 and Wnt3 in ESC culture.

Hayashi 2013 Protocol

After verifying a robust protocol for ESC culture, subsequent differentiation steps were based on the Hayashi 2013 protocol for the derivation of PGCLCs through an EpiLC state. Therefore, for the purposes of this project, there was a specific focus on refining and working from the first three steps of this comprehensive protocol.

A notable change in this protocol was the base media used to culture both ESCs and EpiLCs. Hayashi et. al used N2B27 basal media, which included components such as apo-transferrin, progesterone, and sodium selenite in addition to standard supplements such as insulin, pen/strep and L-glutamine that had been used previously. To streamline the ESC culture and EpiLC differentiation process, cell culture was attempted using

media components from the Transgenic Core Facility protocol shown in Table 3 for ESCs and adapted for EpiLC derivation shown in Table 4.

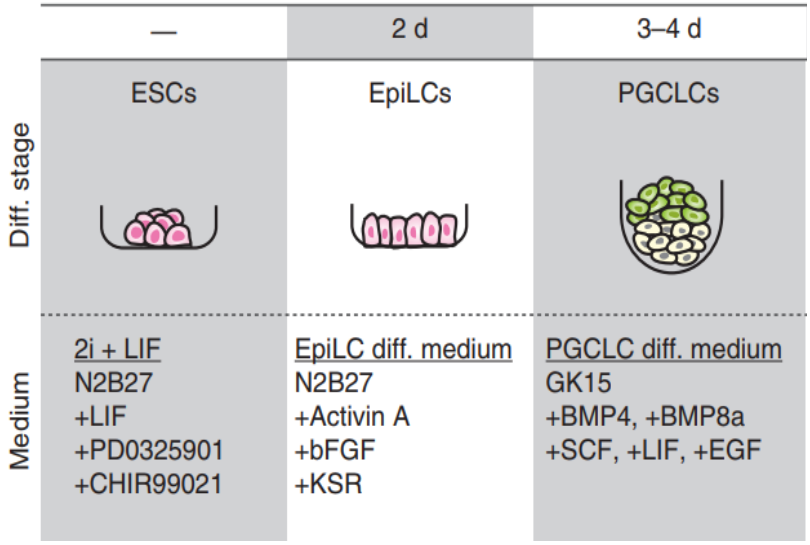


Figure 8: Embryonic stem cell to primordial germ cell like cell protocol (Adapted; Hayashi 2013).

ESC to EpiLC Culture

After 3-4 days in culture the ESC colonies achieved the appropriate morphology and were primed to begin EpiLC differentiation. EpiLCs were plated in 12-well Corning plates that had been pre-coated with fibronectin (Millipore, 1mg/1ml) diluted in PBS to 16.6 µl/ml for 1 hour at 37°C.

As previously mentioned, similar core components of base media were used for EpiLC differentiation as ESC culture. EpiLC media was made up in volumes of 5 ml and included the addition of 2µl Activin A and 6µl β-FGF. The ESCs were counted before resuspension in EpiLC media to achieve a concentration of 1 x 10^5 cells per well. After plating, the ESCs were incubated for 1-2 days in EpiLC media and differentiation was

assessed morphologically before collection. This experiment was repeated three times, with the last done in technical triplicate and results as described here.

Plating of the ESCs for this experiment first began with thawing 3 vials of ESCs, combined and allocated into 6 wells of a 6-well treated plate labeled A-F. The ESCs were imaged every day and after three days of culture they were collected and re-plated in EpiLC differentiation media. Wells A, B and C were collected to create ESC RNA samples and wells D, E and F were collected and differentiated into EpiLCs. These samples were pooled and plated into wells labeled A-F, although these labels do not correlate exactly with the ESC wells. Once plated, this set of EpiLCs grew quickly so collection of EpiLCs was done after one day, although in previous experiments the EpiLCs were incubated for two days with similar morphological results.

The morphology apparent in the transition between ESCs at Day 3 to EpiLCs at Day 1 is shown in Figure 9. This transition is noted by the flattened, more narrow morphology of the EpiLCs as well as the lack of round, distinct colonies commonly found in ESCs. This change was observed within 24 hours, which demonstrates the effectiveness of the new media components Activin A and β-FGF in affecting differentiation.

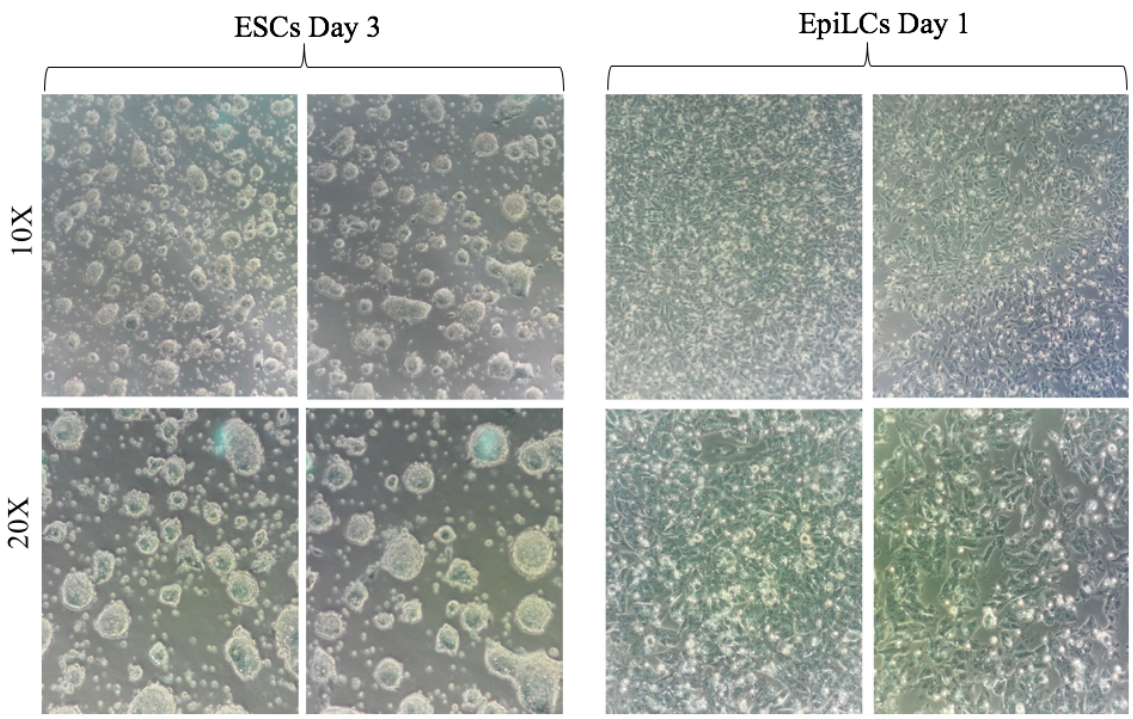


Figure 9: Morphology of embryonic stem cell colonies on Day 3 and EpiLCs on Day 1 after plating imaged at 10X and 20X magnification.

In order to investigate the changes in gene expression that take place during this transition, several genes were tested using q-PCR. The expression of a subset of genes known to play roles in either ESC or EpiLC state maintenance were previously examined by Hayashi et. al. (2011) and the predicted expression for each gene was determined and shown in Table 8. In addition to these state-specific genes, Taf4a and Taf4b were chosen as preliminary testing of potential TFIID complex contributions to this step. The ESC and EpiLC values are comprised of averages of samples in technical triplicate, run in technical triplicate and normalized to 18S.

Gene	Expected Condition upon ESC → EpiLC transition
Oct3/4	Unchanged
Sox2	Decreased
Nanog	Decreased
Fgf5	Increased
Wnt3	Increased
TAF4a	Unknown
TAF4b	Unknown

Table 8: Predicted gene expression dynamics of selected genes during ESC to EpiLC transition (Hayashi 2011).

As expected, the trends apparent in analysis of the q-PCR data showed that Oct3/4 remained relatively constant, Nanog decreased and Wnt3 as well as Fgf5 had a large relative increase in gene expression. However, Sox2 did not show notable changes in expression. While Dnmt3b was tested on the same plate as Sox2 and Fgf5, the amount of cDNA was insufficient to produce technical replicates and therefore was omitted in review. Of interest, Taf4a and Taf4b were expressed at similarly low levels and did not appear to change dramatically in expression through the ESC to EpiLC differentiation step. Overall, these mRNA changes matched expected outcomes and confirmed the successful transition from ESC to EpiLC state.

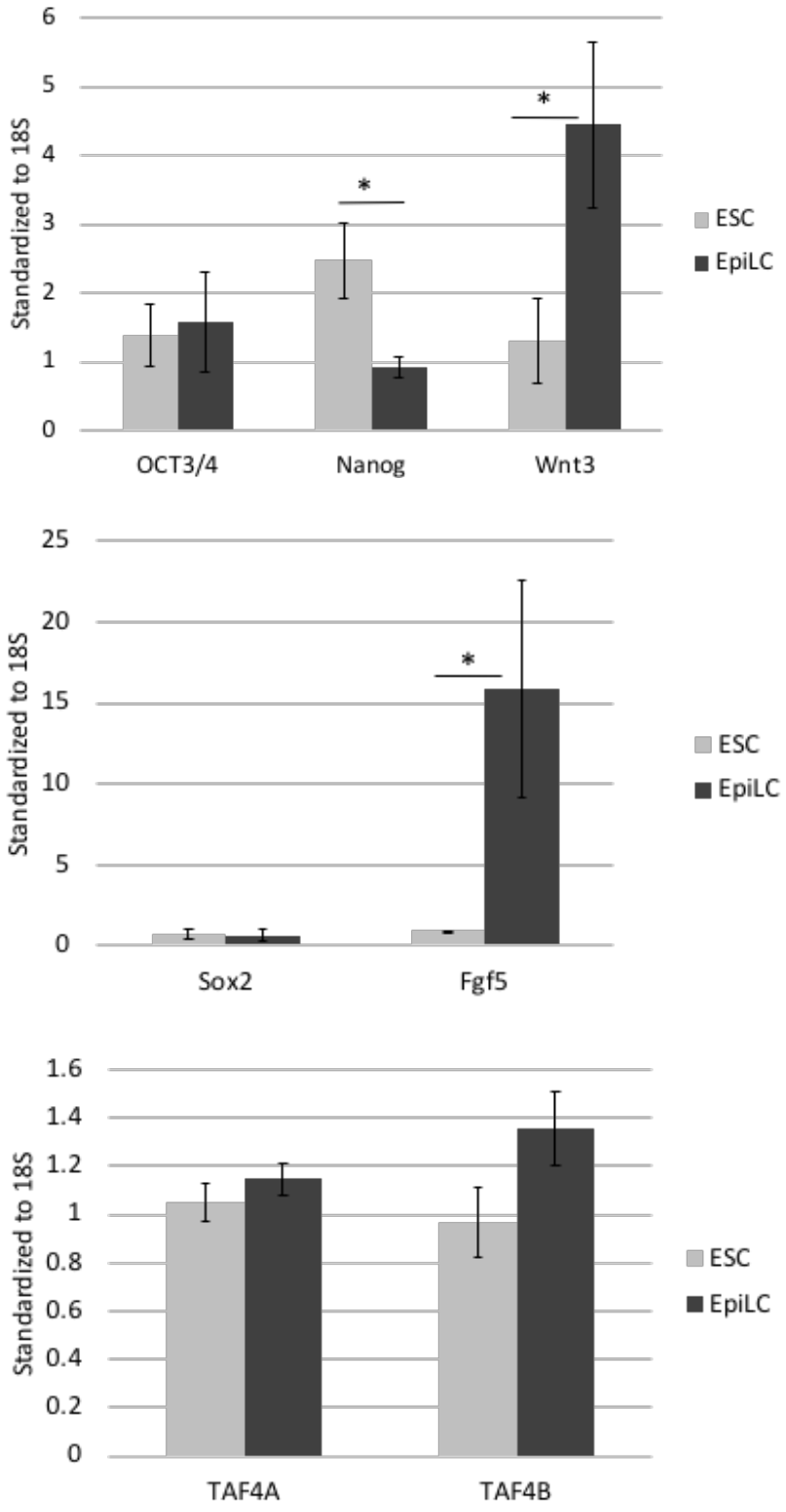


Figure 10: Relative change in gene expression during ESC to EpiLC transition as quantified by q-PCR.

EpiLC to PGCLC Culture

To prepare for PGCLC culture differentiation, ESCs were plated in 6 wells of a 6-well plate and incubated for three days and differentiated to EpiLCs which were incubated for two days in 10 wells of a 12-well plate. On day 2 of EpiLC culture, all 10 wells were collected and plated in 96 wells of 96-well round bottom ultra-low attachment plate. Collected EpiLCs were re-suspended in 1 ml GK15 media which was diluted to the appropriate volume with PGCLC differentiation media. Each well contained approximately 2 x 10^3 cells suspended in 100 µl and media was not changed throughout the PGCLC differentiation period. Cell aggregates were imaged and collected on day 3 and day 4.

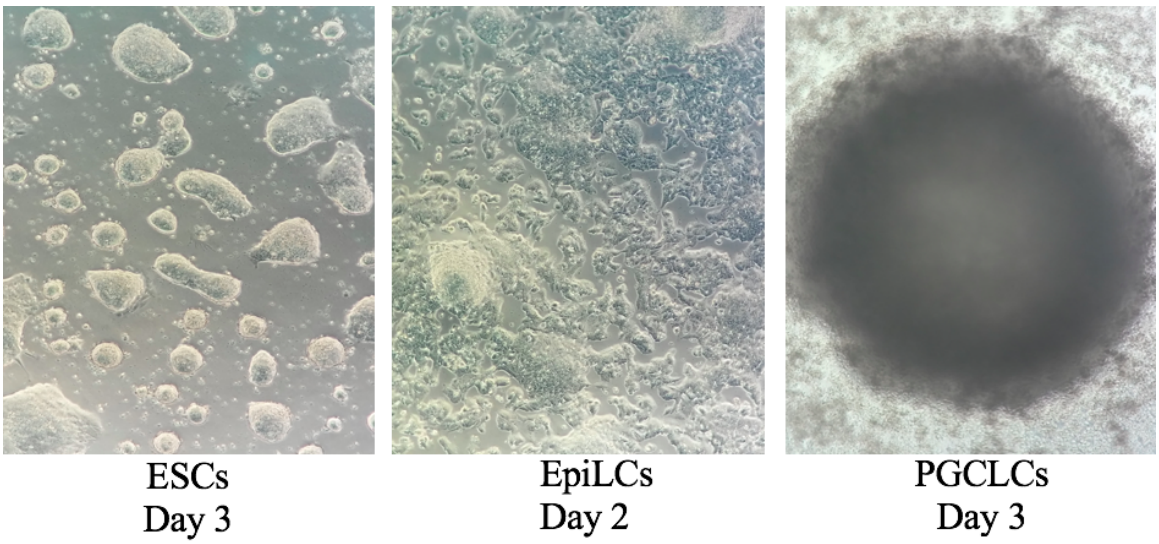


Figure 11: Morphology of ESCs, EpiLCs, and PGCLCs at representative time points post induction.

In order to prepare samples for q-PCR, each of the 8 rows (12 wells) of the 96-well plate were pooled. Rows A-D were collected on day 3 and rows E-H were collected

on day 4. One sample from each day was chosen to assay based on RNA quantity (ng/µl) and quality (A260/A280). While the ESC and EpiLC RNA was extracted with the protocol described in the Methods section, due to the anticipated low cell count, PGCLC RNA was extracted with the Direct-zol RNA MiniPrep Plus kit. cDNA was prepared using the standard protocol. To assess changes in gene expression and evaluate the efficacy of PGCLC differentiation, selected genes were then analyzed by q-PCR. The EpiLC and PGCLC values are comprised of single samples, run in technical triplicate and normalized to 18S. In addition to pluripotency genes Oct3/4, and Nanog, PGCLC specific genes Prdm14, Blimp1 and Tcfap2c were assessed as positive controls for PGCLC-specific gene expression. TAF4a and TAF4b relative expressions were assessed and Dnmt3b was included as a negative control.

The q-PCR results from this experiment suggest that the PGCLC's are most PGC-like at day 3 after induction with PGCLC differentiation media. The relative gene expression levels of PGCLC specific genes, PRDM14, BLIMP1 and Tcfap2c peaked in the sample collected on day 3 and declined in the sample collected at day 4. However, the relative levels of these genes in the PGCLCs at both time points were greater than or equal to the relative level in the EpiLCs which supports the notion that the cells progressed to a primordial germ cell-like state. While the difference in gene expression between days 3-4 of PGCLC induction merit further investigation, suggested explanations at present include the possibility that as maintenance of PGC development requires very specific timing of genes like BLIMP1/PRDM14, the gene expression in our cell line may naturally peak at day 3 and afterward, the transcriptional network is not maintained, and the aggregate begins to differentiate. It may also be possible that the

extra day caused the cells to lose viability as there is no media change throughout the protocol.

As secondary controls, the relative levels of Dnmt3b, known to be upregulated in EpiLCs, declined with PGCLC induction and Nanog levels remained constant as expected. Oct3/4 gene expression declined upon induction, which is not consistent with previously reported results and merits further investigation. Similar to the EpiLC derivation stage, the relative levels of Taf4a and Taf4b did not seem to vary significantly.

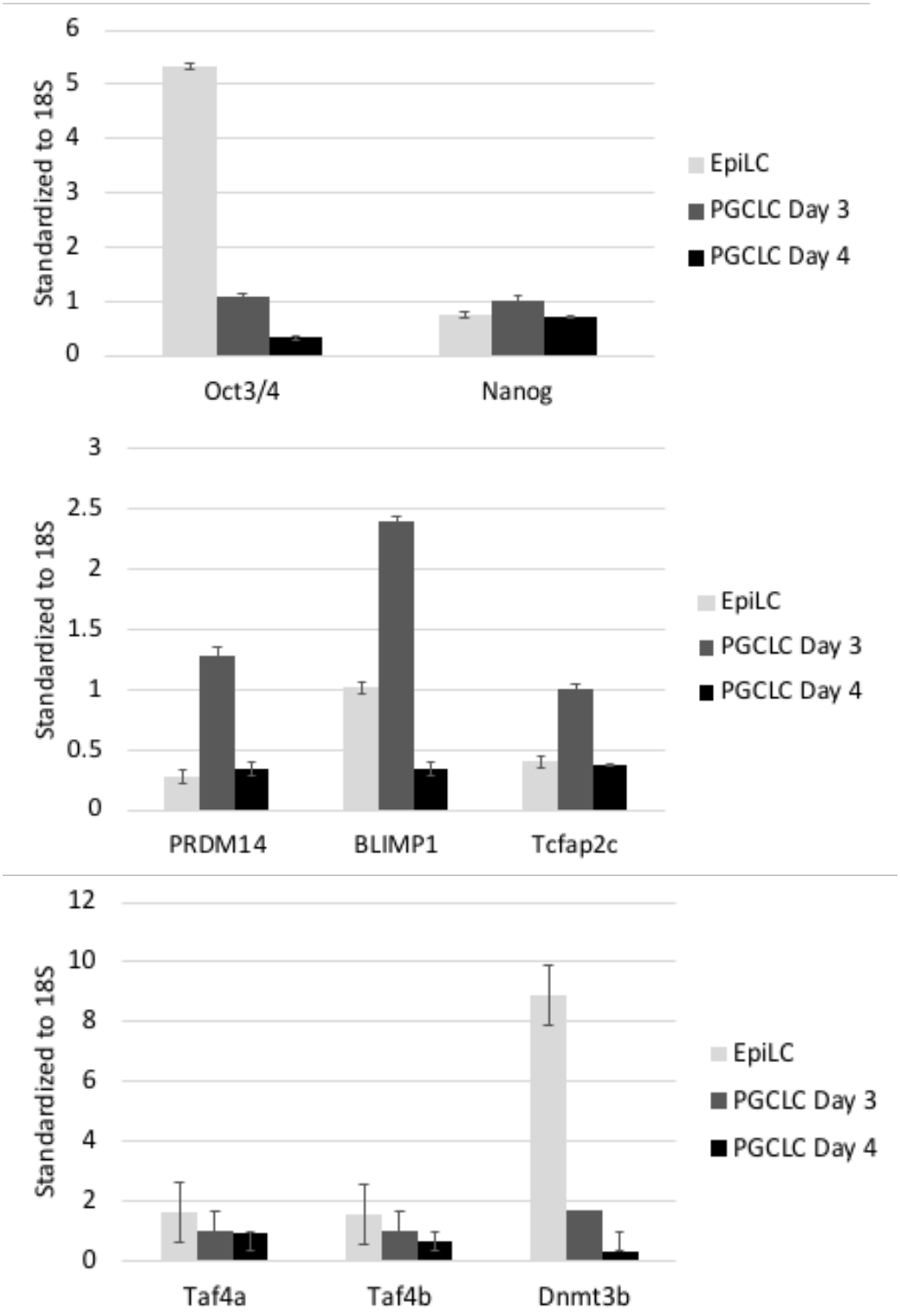


Figure 12: Relative change in gene expression during ESC to EpiLC to PGCLC transition as quantified by q-PCR.

Future Directions

Repetition of entire differentiation pathway

As the sample sizes for each differentiation step were repeated less often the further along in the pathway, it will be prudent to continue to repeat the sequence to verify the preliminary results. This will allow for more accurate statistical analysis as well as further refinement of the protocol and technique. Specifically, it will be helpful to develop formulas to aid in the plating of the ideal number of cells, as well as determination of the necessary number of cells for reliable RNA and cDNA synthesis. Designing plates in which a small sample could be taken each day during the 8-day differentiation process might be useful in determining these time points. Also, conceiving of additional ways to evaluate the cellular state of each step will be helpful in quantification and analysis.

Evaluation of TFIID Subunits

As previously mentioned, the role of TFIID complex proteins may play a role in the differentiation of ESCs to PGCLCs. While this work was started with the investigation of TAF4a and TAF4b, no definitive conclusion may be made at this time and alternative methods of investigation may prove more useful to answer this question. This would allow for a more concrete experimental design to address the question of whether a given subunit functionally contributes to the derivation steps necessary to create fully functional germ cells.

Generating an inducible knockout of TAFs in mESCs may be a useful first step in this investigation. This could be done via inserting LoxP sites flanking the appropriate

exons using CRISPR-Cas9 gene editing. Experimentally, this system would then be used to evaluate the pluripotency and differentiation potential of the inducible knockout vs. wild-type mice. The generation of a mouse colony from which the ESCs could directly be harvested would also be beneficial as the lab would be able produce a consistent supply of low passage blastocyst derived ESCs. After verifying the pluripotency capacity of the ESCs, as was done in this thesis project, in vitro culture could begin with a novel ESC line and allow for greater continuity through each step.

Beginning with evaluation of the pluripotency capacity of the inducible knockout line, TAF4a and TBP knockouts could be generated. Working under the assumption that TAF4a may play a role in maintaining or re-establishing the pluripotency network (Pijnappel 2013), it will be helpful to understand the consequences to the TFIID subunit after its loss of functionality. Similarly, this system could also be used to evaluate the effect of specific TFIID subunits on the primordial germ cell differentiation capacity of ESCs. As TAF4b is known to be a germ cell enriched factor, it is possible to test its potential role in germ cell specification. Conducting an experiment with inducible TAF4b knockout ESCs would be a more direct method of evaluating this hypothesis.

Additionally, genome editing may be done to tag individual TFIID components to investigate their interaction at the protein level. This will allow for characterization of where and when these proteins interact, and which components are necessary for maintenance of certain states. Both pluripotency and primordial germ cell differentiation states exist as the result of a large transcriptome and protein interactome. The ability to tag TFIID components will allow us to look at how these proteins interact with each other at various time points, as well as how they might fit in to the broader system.

Discussion

Embryonic stem cells and pluripotent stem cells remain the subject of intensive research. In particular, more work must be done to elucidate the ways in which signals are regulated in vivo in order to understand how this might be recapitulated in culture. Part of this process is learning how to effectively and efficiently generate cell types of interest. A prime subject for this targeted investigation are primordial germ cells, as they overlap with ESCs in their pluripotency and capacity to contribute to the complete development of an organism. PGCs and germ cells are also a fascinating subject of interest in their own right and furthering knowledge of how they can be derived will be crucial to serve as a foundation in developmental and reproductive biology as well as many associated fields.

This preliminary work with ESCs and PGCs, done over the span of one year, has shown promising results for the future of embryonic cell culture and differentiation towards a primordial germ cell-like cell fate. The deduction of optimal conditions for ESC culture on non-feeder layer plates will greatly expedite the ESC expansion process as the combination of MEF feeder culture can add significant time investments. Non-feeder layer culture also limits the number of external signals and cellular crosstalk beyond what is provided for ESC maintenance and allows for more confidence in the efficiency of the protocol used.

The early successes of differentiating epiblast-like cells and primordial germ cell-like cells contributes to the verification of the work done by the Saitou lab (Hayashi 2011, Hikabe 2016) and also provides a potential alternative method to achieve the same differentiation. In utilizing the ESC media protocols provided by the Transgenic Core

Facility and modifying the EpiLC media protocol to omit the use of N2B27 media, a less expensive and complicated alternative is proposed. This protocol has yet to be subjected to rigorous evaluation to verify whether the same levels of differentiation are achieved, however, preliminary results suggest that it may serve as a useful option.

Refinement of the protocol for each differentiation step required extensive problem solving and multiple assays were done to confirm initial observations. This allowed for more confidence in asserting, for example, that the ESCs still exhibited observable levels of pluripotency genes despite multiple passages, as supported by q-PCR data. Early culture of ESCs and the first few trials of EpiLC differentiation were not extensively reported in this body of work, however, they provided key insights that were incorporated in later experiments.

In addition to further clarifying the process by which embryonic stem cells can be induced to primordial germ cells, this research has the potential to greatly enhance related fields, such as biomedical engineering and ecological conservation. With regard to embryonic stem cells, the early hopes of scientists who believed in the extraordinary capacity of these cells to contribute to regenerative medicine are more relevant now than ever. Although this thesis focused primarily on mouse embryonic stem cells, there has been exciting progress utilizing human embryonic stem cells and induced pluripotent stem cells as well. Advances in both mouse and human systems inform one another as differences can be informative in a comparative and evolutionary context, and similarities may be useful in developing novel approaches. An example of exemplary improvement is the culture of ESCs without relying on fibroblast feeder layers and minimizing potentially incompatible serum components to increase the clinical potential of human ESCs.

Long term goals regarding primordial germ cell and gamete derivation from human embryonic stem cells are extremely relevant to reproductive medicine. The ability to generate human oocytes via a technique similar to Hayashi et. al., in which oocytes were derived and produced viable offspring, would be a novel opportunity to replenish the ovarian reserve in humans which naturally is not supported by germline stem cells (Grive 2015). In the transition from the mouse to human system, there remain many technical barriers, such as the thorough recapitulation of genetic and epigenetic data and ethical questions regarding the manipulation of inherited germline information. However, this work and others have demonstrated the unique and powerful capacity of embryonic stem cells to contribute to differentiated cell lines and in the future, safe human gamete regeneration may one day be possible. Ongoing basic science research that continues to improve the efficacy and safety of generated gametes while keeping these concerns in mind is essential to reaching this goal (Ishii 2017).

In addition to human reproduction, the reproductive capacity of other organisms also may be aided by in vitro gametogenesis as well as stem cell pluripotency. For endangered or rare animal species, it may be possible to produce new organisms through the route of induced pluripotent stem cells and then induced gametogenesis to produce viable offspring. The potential applications for this research in preserving and enriching the biological heritage of the earth is a fascinating and worthwhile endeavor. There are many potential directions for ongoing research and as this work has built on itself, it is my hope that future efforts to understand the nature of embryonic stem cells, primordial germ cells and their developmental capacity will continue to expand in the future.

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