Characterizing changes in the transcriptional networks underlying pluripotency in mouse embryonic stem cells upon the induction of differentiation

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Mouse embryonic stem cells (mESCs) are pluripotent cells capable of differentiating into all three germ layers present in the adult mouse. In this thesis, I have investigated the transcriptional changes that mESCs undergo as they are induced to differentiate towards the mesoderm lineage by 2i/LIF withdrawal and dimethyl sulfoxide (DMSO) treatment. 5 days of differentiation causes significant drops in expression of Sox2 and Oct4 primary transcript, while expression of Nanog and Kit significantly drops after only 1 day. It was determined that DMSO has no effect on the short-term changes in Nanog and Kit expression induced by 2i/LIF withdrawal. An expanded look at pluripotency-associated genes shows significant up-regulation of Oct4 and down-regulation of Klf4 and Stat3 following only 6 hours of 2i/LIF withdrawal. This data indicates that while some aspects of the transcriptional networks underlying pluripotency respond quickly to mesodermal differentiation cues, others remain unchanged for up to 5 days.
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**Introduction**

**A background on pluripotency**

While the phenomenon of pluripotency is currently associated with embryonic stem cells (ESCs), this was not always the case. Science’s first experiences with pluripotency came via research into malignant teratocarcinomas. While rare in occurrence, they attracted lots of attention due to the fact that these tumours commonly contained mixtures of cells in various stages of development from all three embryonic lineages (reviewed by Solter, 2006). In 1964, intraperitoneal injection of a single tumour cell yielded new tumours containing all cell types from the donor teratocarcinoma (Kleinsmith and Pierce, 1964), and in 1970, it was discovered that pre- and post-implantation mouse embryos grafted onto adult mouse testes yielded transplantable teratocarcinomas (Stevens, 1970). This introduced the concept of self-renewal and the notion that pluripotency arises from the embryo. The first pluripotent mouse ESC (mESC) lines were established in 1981 by culturing the inner cell mass (ICM) of delayed blastocysts (Evans and Kaufman, 1981). By definition, a pluripotent cell is capable of forming all cell types present in the adult form of an organism, as well as possessing the ability to proliferate in an undifferentiated state (Jaenisch and Young, 2008). On top of this, when injected into host blastocysts, mESCs are also able to contribute to chimera formation (Nagy et al., 1993). In their initial publication, Evans and Kaufman discussed the possibilities of using ESCs to introduce mutant alleles into the mouse genome. Thus, ESCs have been linked with genome manipulation and field-changing innovation from their inception. Currently, great efforts are being made to understand the biological underpinnings of pluripotency (Chen et al., 2008) and to therapeutically harness the vast developmental potential that pluripotent cells possess (Warren et al., 2010).

**Oct4, Sox2, Nanog and transcriptional control of the pluripotent state**

While there are many pathways and biological processes involved in the development and maintenance of pluripotency, three transcription factors, *Pou5f1* (commonly termed *Oct4*), *Sox2* and *Nanog*, comprise the core regulatory circuit underlying this phenotype (Young, 2011). Each of these genes when studied in isolation have been found to control different aspects of differentiation and pluripotency. The collective interaction of *Oct4*, *Sox2* and
Nanog serves to co-ordinate multiple networks which allows for both self-renewal and differentiation in response to intrinsic and extrinsic signals.

Oct4 is a member of the POU transcription factor (TF) family and it is only expressed in blastomeres, pluripotent early embryo cells and the germ cell lineage. Embryos lacking Oct4 will develop to the blastocyst stage, but their ICM is not pluripotent and therefore differentiates only to the extra-embryonic trophectoderm lineage (Nichols et al., 1998). Oct4 over-expression causes differentiation to mesoderm and primitive endoderm lineages, while Oct4 inactivation in mESCs causes differentiation to the trophectoderm lineage (Niwa et al., 2000). Inhibition of Oct4 in mESCs results in their inability to differentiate to the mesoderm lineage, and Oct4 siRNA injection into the ICM impairs cardiogenesis (Zeineddine et al., 2006). Deviations in Oct4 expression by 50% (in either direction) from levels in mESCs are sufficient for the induction of differentiation. Notably, in the absence of Oct4, the presence of LIF (leukemia inhibitory factor) does not maintain pluripotency (Niwa et al., 2000).

Sox2 is a part of the SRY-related high-mobility-group box family of TFs that has only 1 exon (Lefebvre et al., 2007). Unlike Oct4 and Nanog, the expression of Sox2 is widespread, including tissues such as extraembryonic ectoderm (Avilion et al., 2003) and a variety of endodermal and ectodermal tissue progenitor cells in both the developing and adult mouse (Arnold et al., 2011). Sox2-null embryos die shortly after implantation (Avilion et al., 2003). Sox2 is necessary for pluripotency in mESCs as inducible Sox2-null cell lines differentiate mainly into trophectoderm-like cells. This elimination of Sox2 also causes the expression of Oct4 and Nanog to drop by 50% 72 hours after induction (Masui et al., 2007). During the same timeframe, over-expression of Sox2 causes an increase in expression of genes associated with ectodermal and mesodermal lineages, even in the presence of LIF. At this timepoint, while Nanog expression drops, Oct4 expression is not modulated by the increase in Sox2 (Kopp et al., 2008).

Nanog is a highly divergent homeodomain-containing TF which was identified by a screen using mESCs with the LIF receptor deleted (Chambers et al., 2003). mESC cDNAs were transfected into these modified cells and cultured without LIF. In contrast to Oct4 and Sox2,
the forced expression of the Nanog cDNA was able to maintain pluripotential self-renewal of cells without any LIF signaling (Chambers et al., 2003). Nanog is expressed only in the ICM, mESCs (derived from the ICM) and in the proximal epiblast at embryonic day 6 (Hart et al., 2004). While Nanog null embryos do develop an ICM by embryonic day 3.5, the ICM does not proliferate when cultured on gelatinized plastic, instead differentiating into parietal endoderm-like cells (Mitsui et al., 2003). Interestingly, Nanog is dispensable for the maintenance of pluripotency. While conditionally-deleted Nanog mESCs are more likely to differentiate, undifferentiated cells remained after continuous passaging (Chambers et al., 2007).

**Network dynamics of Oct4, Sox2 and Nanog**

Numerous results indicate that Oct4, Sox2 and Nanog are extensively interconnected and function together in a network-like manner. OCT4, SOX2 and NANOG TFs (the proteins are collectively referred to as OSN) have been found, as a group, to bind to the promoters of the Oct4, Sox2 and Nanog genes, pointing to the existence of autoregulatory feedback loops (Kim et al., 2008). Chromatin immunoprecipitation followed by paired-end tag sequencing (ChIP-PET) has shown that around 70% of OCT4 clusters also contain an adjacent SOX2 binding site, termed sox-oct composite elements (Loh et al., 2006). In fact, motif identification of this OCT4 ChIP-PET data, revealed a perfect match to previously validated OCT4-SOX2 elements, highlighting the frequent co-localization of these transcription factor binding sites (TFBS) (Loh et al., 2006). By way of these composite elements, SOX2 has been found to regulate expression of multiple TFs which influence Oct4 expression (Masui et al., 2007), suggesting that the OCT4/SOX2 heterodimer is the functional binding unit (Chen et al., 2008). ChIP-seq (ChIP followed by deep sequencing) analysis has shown that OSN TFBS extensively co-localize, with over 600 sites in the mouse genome being bound by OSN and at least one other TF. SMAD1 and STAT3 (other TFs integrated into the signaling of pluripotency) also frequently co-bind to regions occupied by OSN (Chen et al., 2008). Multiple TF–binding loci (MTLs, sites containing more than three bound TFs) containing OSN binding sites have been shown to be bound by p300 and have robust, mESC-specific enhancer activity (Chen et al., 2008). In addition, only 2% of all genes bound by OSN are not bound by any other pluripotency-associated TFs, suggesting extended co-ordination of the
*Oct4*, *Sox2* and *Nanog* circuit with other pathways implicated in pluripotency (Young, 2011). OSN has also been shown to bind to and repress genes involved in regulation of lineage specification (Young, 2011). Interestingly, many Polycomb Group proteins, which are known to epigenetically silence genes, are bound to the same repressed lineage-specific genes as OSN, indicating a distinct linkage between chromatin remodeling and transcriptional control of pluripotency (Boyer et al., 2006). These results highlight the widespread influence on gene expression and network organization that OSN collectively exerts on pluripotent mESCs.

**Signaling pathways underlying pluripotency in vitro**

Another important development which came from embryonal carcinoma research was the use of mitotically-inactivated mouse embryonic fibroblasts (MEFs) in co-cultures to maintain pluripotency (Smith, 2001). MEF co-culture was used in early establishments of mESC lines (Evans and Kaufman, 1981). It has since been determined that MEFs support pluripotency by providing LIF and factors involved in the Wnt and Bone Morphogenic Protein (BMP) signaling pathways (Young, 2011). Interestingly, these pathways are all directly integrated into the OSN TF network which controls pluripotency.

After its discovery and isolation, LIF was used to derive and maintain mESCs without the use of MEFs (Nichols et al., 1990). LIF exerts its effect by binding to the LIF receptor, which heterodimerizes with another class I cytokine receptor, gp130 (Davis et al., 1993). This triggers phosphorylation/activation of JAK molecules (Narazaki et al., 1994), which in turn phosphorylates/activates STAT3 (Niwa et al., 1998). When STAT3 is phosphorylated, it forms a homodimer and translocates to the nucleus, acting as a TF (Ihle, 1996). As mentioned earlier, STAT3 extensively co-localizes to OSN-bound MTLs (Chen et al., 2008), illustrating a direct connection between external signal recognition and the core transcriptional circuit underpinning pluripotency.

The importance of BMP signaling for pluripotency was highlighted when attempts to culture pluripotent mESCs without MEFs in Fetal Bovine Serum (FBS)-free media supplemented with LIF failed (Ying et al., 2003a). This highlighted the notion that LIF/STAT3 signaling alone is not sufficient to maintain pluripotency, and that FBS/MEFs must be stimulating
other pathways integral for self-renewal. Another report had shown that while inhibition of endogenous BMPs does not induce differentiation, addition of BMP4 to neural differentiation media antagonized the development of neural precursors, instead generating non-neural differentiated cells (Ying et al., 2003b). The addition of BMP4 to mESC cultures lacking MEFs and FBS but containing LIF allows for long-term undifferentiated passaging, derivation of new mESC lines as well as the ability to contribute to the generation of chimeric mice (Ying et al., 2003a). In serum-free media containing LIF alone, mESCs have an impaired capacity for self-renewal at low cell density and cultures eventually differentiate into neuroectoderm. In BMP4 alone, mESCs quickly differentiate to non-neural fates. It is currently believed that LIF and BMP4 work in tandem to prevent differentiation to specific lineages, and if one of these components is removed, the resulting imbalance promotes differentiation (Wray et al., 2010). The presence of BMP4 also causes increased phosphorylation/activation of SMAD1 and increased expression of Id1 and Id3 (Inhibitor of differentiation) genes in mESCs (Ying et al., 2003a). SMAD1 is another TF which was studied by ChIP-seq and found to preferentially localized to OSN MTLs (Chen et al., 2008), representing another direct assimilation between externally-derived signals and the transcriptional regulation of mESCs.

It is interesting to note that a reduction in Oct4 (by RNA interference) causes a reduction in the binding of SMAD1 and STAT3 TFs specifically on OCT4, SMAD1 and STAT3 cobound sites, and that this reduction is not due to a drop in SMAD1 or STAT3 levels (Chen et al., 2008). Furthermore, while withdrawal of LIF reduced STAT3 binding and withdrawal of BMP4 reduced SMAD1 binding, OCT4 binding is not perturbed by either treatment (Chen et al., 2008). Collectively, these results suggest the presence of a hierarchal relationship between TFs involved in external signal transduction and TFs comprising the core regulatory circuit of pluripotency.

In more recent times, the Wnt signaling pathway has become implicated in the maintenance of pluripotency. The main components of Wnt signaling were active in human ESCs (Sato et al., 2003) and the expression of a Wnt antagonist (Sfrp2) induces the formation of neural progenitor cells in mESCs (Aubert et al., 2002). Wnt proteins bind to the Frizzled family of
receptor proteins, which results in downstream activation of Dishevelled proteins. Active Dishevelled serves to inactivate the downstream APC/Axin/GSK3β complex, and when this complex is inactive, β-catenin can accumulate in the nucleus to activate transcription. In its active form, the APC/Axin/GSK3β complex phosphorylates β-catenin which results in degradation (Okita and Yamanaka, 2006). Nuclear β-catenin has been shown to activate the LEF/Tcf group of TFs (Okita and Yamanaka, 2006), of which Tcf3 has been shown to co-occupy promoters with Oct4 and Nanog (Cole et al., 2008). Interestingly, pharmacological inhibition of GSK3β using 6-bromoindirubin-3’-oxime (BIO) results in nuclear accumulation of β-catenin, which in turn maintains the expression of pluripotency-associated genes even in the absence of LIF (Sato et al., 2004).

The last pathway to be intensively studied with regards to pluripotency is the fibroblast growth factor (FGF) signaling cascade. FGFs, through binding to FGF receptors, have been shown to activate the Ras-Raf-MEK-ERK pathway (Thisse and Thisse, 2005). Both the FGF (Thisse and Thisse, 2005) and ERK pathways (Roux and Blenis, 2004) have been implicated in development, differentiation and cell proliferation. Fgf4-null mESCs are resistant to both neural and mesodermal differentiation, as well as having greatly reduced levels of active/phosphorylated ERK1/2, effects which are restored by the presence of FGF4 (Kunath et al., 2007). ERK-null mESCs have been derived and these cells are resistant to mesoderm formation, as they continue to express Oct4, Nanog and Rex1 after 4 days of differentiation (Kunath et al., 2007). The impairments in differentiation to different lineages suggests that Fgf4-induced ERK signaling is responsible only for the exit of pluripotency and not the commitment to specific lineages (Wray et al., 2010).

The pathways described above and their widespread integration with the OSN transcription factor network suggests that pluripotency is tightly linked to external stimuli. Recently, highly specific chemical inhibitors of ERK- and GSK3-signaling have been developed (Bain et al., 2007) and present research efforts have focused on how these molecules affect pluripotency in vitro. The culture of mESCs in the presence of CHIR99021 (GSK3 inhibitor) and PD0325901 (ERK inhibitor) is known as “dual inhibition” or 2i (Ying et al., 2008). mESCs cultured in 2i media are proposed to be the closest in vitro representation of naïve
pluripotency, or precursor cells from the pluripotent epiblast (ICM) of pre-implantation embryos (Nichols and Smith, 2009). 2i media also homogenizes expression of various pluripotency factors such as Rex1 and Nanog, whereas under LIF + serum conditions, expression can be quite variable (Wray et al., 2010). Following implantation, the ICM forms two distinct lineages; the epiblast which generates the embryo, and the hypoblast which generates primitive endoderm/the yolk sac (Yamanaka et al., 2006). Culturing 8-cell stage mouse embryos in 2i prevents development of the hypoblast, with the ICM instead developing entirely into Oct4, Sox2 and Nanog-expressing epiblast (Nichols et al., 2009). Since the generation of the hypoblast seems to be dependent on FGF/ERK and Wnt signaling, it is hypothesized that in the absence of external signaling, the default developmental program for the ICM is to maintain pluripotency (Wray et al., 2010). These results collectively point towards a “ground state” of pluripotency, and it is currently believed that mESCs cultured in 2i are, for most intents and purposes, identical to pre-implantation epiblast cells (Nichols et al., 2009).

**Unique features of pluripotent mESCs**

In addition to the OSN-mediated transcriptional network controlling pluripotency, mESCs have been shown to display several distinct cellular features which play a role in both the maintenance of self-renewal and permissiveness to differentiation (Young, 2011). In most cases, these changes can be quickly induced by withdrawing LIF for 24 hours, suggesting that mESCs undergo rapid, early time-point changes in response to differentiation signals. Most relevant to the study proposed by this thesis is that pluripotent mESCs have been shown to be transcriptionally hyperactive on a genome-wide scale (Efroni et al., 2008). When compared to neuronal progenitor cells (NPCs, 7 day differentiation protocol), mESCs express higher levels of both total and messenger RNA, as well as repeat sequences and transposable and retroviral elements (Efroni et al., 2008). In addition, mESCs express low levels of lineage committed genes (Efroni et al., 2008). However, following 24 hours of LIF withdrawal from mESC cultures, the pluripotent-specific global up-regulation of transcription is lost, with significant reductions in intergenic, intronic and exonic transcription found in 9-15 of the 21 assayed chromosomes (Efroni et al., 2008). After differentiation to NPCs, reductions of the same features are found in 12-17 chromosomes,
suggesting that progression of differentiation further down-regulates global transcription (Efroni et al., 2008). When looking only at regions active in both mESCs and NPCs, 24 hours of LIF withdrawal induces a down-regulation of intergenic, intronic and exonic transcription in 14-19 chromosomes (Efroni et al., 2008). Taken together, these results signify that mESCs have higher levels of transcription from more genomic regions, and that this feature is rapidly lost following the initiation of differentiation (Efroni et al., 2008).

Supporting the findings of higher global transcription, mESCs and pluripotency in general are associated with open chromatin structure (Ahmed et al., 2010). When a mouse embryo reaches the 8-cell stage, chromatin is largely dispersed into a mesh of 10 nm fibres with many visible ribonucleoproteins (RNP) (Ahmed et al., 2010). A similar chromatin state is observed in both pre-implantation embryonic day 3.5 epiblast cells and mESCs (Ahmed et al., 2010). In contrast, post-implantation embryonic day 5.5 epiblast cells showed many areas of chromatin compaction of various sizes. 10 nm fibers were rarely observed and RNP density was reduced (Ahmed et al., 2010). This pattern was mirrored in other cells in various stages of differentiation such as in embryonic day 5.5 primitive endoderm, suggesting that differentiation is associated with less transcriptionally-permissive chromatin (Ahmed et al., 2010). In support of this, Oct4-null early epiblast cells also show widespread condensation of chromatin (Ahmed et al., 2010).

Pluripotent mESCs also exhibit an increase in the dynamics of chromatin-associated proteins when compared to mESCs cultured without LIF for 24 hours (Meshorer et al., 2006). Nucleosomes are composed of a histone octamer; four heterodimers of the core histone proteins H2A, H2B, H3 and H4. Histone octamers are linked together by H1, and the interaction of the nucleosome, H1 and DNA are what drives the formation of condensed chromatin (Sarma and Reinberg, 2005). Using Fluorescent Recovery After Photobleaching (FRAP) techniques, it was determined that recovery of HP1 and H2B was significantly faster in mESCs when compared to mESCs cultured without LIF for 24 hours (Sarma and Reinberg, 2005). This result was not obtained due to an increase in histone protein expression and suggests that pluripotent cells are quickly able to remodel chromatin in response to differentiation.
Finally, mESC possess many instances of bivalent domains. The aforementioned core histone proteins can be modified by the addition of numerous different molecules such as phosphorylation, acetylation and methylation (Jenuwein and Allis, 2001). While there are many potential histone modifications mediated by a wide variety of proteins, of particular interest are the transcriptionally-permissive marking, methylation of lysine 4 on Histone H3 (H3K4) and the transcriptionally-repressive marking, methylation of lysine 27 on Histone H3 (H3K27) (Jenuwein and Allis, 2001). Many important developmentally-associated genes are marked with both H3K4 and H3K27 in pluripotent mESCs (Bernstein et al., 2006). These genes are expressed at very low levels in mESCs, and the bivalent chromatin markings are said to repress high levels of transcription while keeping them poised for activation upon differentiation (Bernstein et al., 2006). Several genes associated with neural differentiation were determined to have bivalent markings in mESCs, but upon differentiation to neural precursor cells, the repressive H3K27 signatures were erased as these genes became expressed (Bernstein et al., 2006).

**Specification of mesoderm and hematopoiesis in the murine embryo and mESCs**

A critical event in the development of the murine embryo is the specification of the three primary germ layers; mesoderm, endoderm and ectoderm (Murry and Keller, 2008). Following the implantation of an early blastocyst to the uterine wall, the ICM begins to divide into two distinct cell populations; the extraembryonic endoderm and the epiblast (Niwa, 2007). Extraembryonic endoderm eventually gives rise to all extraembryonic tissues, while the epiblast yields the pluripotent primitive ectoderm (Niwa, 2007). Around embryonic day 6.25, the epiblast epithelializes into a cup-like structure called the egg cylinder which is composed of primitive ectoderm surrounded by primitive endoderm (Tam and Behringer, 1997). Shortly after (between embryonic day 6.5-7.5), formation of the primitive streak (PS) occurs (Tam and Behringer, 1997) and the presence of this structure marks the beginning of gastrulation (Murry and Keller, 2008). The PS is responsible for generating the anterior-posterior body axis and facilitates the differentiation of primitive ectoderm (former epiblast) to both the mesoderm and endoderm lineages (Downs, 2009). Interestingly, especially given the importance of the PS, its structure and localization have not been fully elucidated.
(Downs, 2009). It is currently known that the PS has three distinct regions (posterior, middle and anterior) and gene expression patterns are different in each of these areas (Murry and Keller, 2008). *Brachyury*, a specifier and marker of mesoderm, is expressed in all three regions of the PS (Kispert and Herrmann, 1994). Genes such as *Foxa2* and *Goosecoid* are found in anterior regions, while genes such as *HoxB1* and *Evx1* are found in posterior regions (Murry and Keller, 2008). To initiate formation of mesoderm, epiblast cells are mobilized, and migrate through the posterior PS. The first such cells which transverse the PS are responsible for forming the yolk sac (Murry and Keller, 2008), a structure composed of extraembryonic mesoderm. Initiation of primitive hematopoiesis occurs in the yolk sac, and the first detectable hematopoietic cells are found at embryonic day 7.5 (Haar and Ackerman, 1971). Primitive erythroblasts from the yolk sac are the predominant blood cells in early development and synthesize the embryonic globin genes (Palis et al., 1999). Circulation is established by embryonic day 8.5, and slightly prior to this, definitive hematopoiesis is established in the para-aortic splanchnopleura region (Ferkowicz, 2003). Definitive hematopoiesis is marked by the expression of the adult globin genes, and by embryonic day 9.5, the main site of definitive hematopoiesis is the fetal liver (Palis et al., 1999).

In order to eventually take advantage of the therapeutic potential pluripotency holds, numerous mESC differentiation protocols have been developed in order to generate mesoderm and various precursors of the hematopoietic lineage. In fact, the *in vitro* establishment of hematopoiesis is one of the most studied developmental programs by use of mESCs (Murry and Keller, 2008). The earliest differentiation protocols used chemical treatments such as dimethyl sulfoxide (DMSO) to generate mixed populations of cells from mESCs (Smith 1991). These early protocols involved a withdrawal of LIF for seven days, followed by reintroduction of LIF in conjunction with an inducer of differentiation (Smith 1991). The next generation of differentiation protocols by and large used embryoid bodies as a model of development (Keller et al., 1993). Embryoid bodies (EBs) are three-dimensional aggregations of mESCs created by the withdrawal of LIF and a transition to growth in non-adherent suspension culture (Keller, 1995). EBs are differentiated, containing cell lineages from the mesoderm, endoderm and ectoderm lineage and are reported to be the closest *in vitro* representation of mouse blastocyst development (Murry and Keller, 2008). Treatment of EBs
with 1% DMSO and withdrawal of LIF yields an 80% rate of commitment to the hematopoietic lineage, with a stepwise acquisition of markers of mesoderm as well as early and later markers of hematopoiesis (Lako et al., 2001). Current efforts to direct differentiation involve the use of conditional expression of genes which induce differentiation, addition of signaling ligands, complex cell sorting protocols as well as culture on coated tissue culture flasks (stromal cells or extra-cellular matrix proteins) (Keller, 2005).

**Transcription factories organize the nucleus in three-dimensional space**

The colocalization of genes, the chromosomes they lie on and transcription machinery offers the eukaryotic cell various opportunities for fine-tuned gene regulation. Past research has highlighted the existence of foci of hyperphosphorylated RNA polymerase II (RNApolII) within the nucleus, termed transcription factories (Iborra et al., 1996). Previous studies have shown that these transcription factories persist upon inhibition of transcription initiation and elongation and are not simply RNApolII aggregates on actively transcribed genes (Mitchell and Fraser, 2008). This finding is in stark contrast to the canonical view of transcription, in which the gene to be transcribed remains static and recruits transcriptional machinery to its promoter. Furthermore, transcription factories can recruit several genes regulated by the same TF for transcription, creating detectable interactions between genes located either close or far away from each other in cis or even on other chromosomes (trans) (Schoenfelder et al., 2010). Interestingly, occupancy of transcription factories by TFs seems to be selective. *Klf1* is a TF expressed in definitive erythrocytes, yet it only occupies between 10-20% of transcription factories (Schoenfelder et al., 2010). These findings suggest that transcription is highly organized in the three-dimensional space of the nucleus.

**Experimental Proposal**

Pluripotent mESCs clearly have unique properties related to transcription and many of these features have been shown to be affected by as little as 24 hours of differentiation. This research project therefore proposes to examine both the short-term and long-term changes in transcription and nuclear organization that pluripotent mESCs undergo as they are treated with a simple differentiation protocol. Since the Mitchell Lab is ultimately interested in identifying chromatin loops associated with pluripotency, EBs are not an applicable model
system. For this reason, the effects of 2i/LIF withdrawal and 1% DMSO treatment of pluripotent mESCs will be investigated. As introns are co-transcriptionally spliced (Singh and Padgett, 2009), both primary and processed transcripts will be quantified for a more complete understanding of the changes in transcription induced by differentiation. Markers of pluripotency will be monitored in order to determine which genes are up- or down-regulated first, while markers of mesoderm and hematopoiesis will be monitored in order to determine how differentiated mESCs become. Whereas finely mapping the early time-point changes in Oct4, Sox2 and Nanog (and other associated genes) expression represents a novel result in itself, a comprehensive elucidation of the response of mESCs to a specific differentiation protocol will serve to inform future experiments. By knowing the precise window at which a gene is either up- or down-regulated, future members of the Mitchell Lab will have critical a priori knowledge into the time at which chromatin loops sustaining pluripotency are likely disrupted.
Methods

Cell Culture
The cells used for all described experiments were E14TG2a (E14), a mouse embryonic stem cell line available from the ATCC (CRL-1821). E14s were grown in DMEM containing 15% FBS, LIF, 1mM sodium pyruvate, 1 mM non-essential amino acids, penicillin/streptomycin, GlutaMAX and 0.1 mM 2-mercaptoethanol. This media was further supplemented with 3 µM CHIR99021 (GSK3β inhibitor) and 1 µM PD0325901 (MEK inhibitor), referred to hereafter as 2i media. Frozen stocks of E14s were established with mitotically-inactive mouse embryonic fibroblast feeder cells (MEFs and media provided by Malgosia Kownacka). E14s were subcultured every other day at a ratio between 1:6-1:10 and media was changed on the days in between passaging. All cell-growing surfaces were coated in 0.1% gelatin (Bioshop GEL771.100) solubilized in endotoxin-free cell culture water. MEFs were removed from cultures before the beginning of experiments by allowing the resuspended pellet of trypsinized cells to sit in solution for 20 minutes (causing MEFs to sink to the bottom of the tube). The upper portion of media (which is enriched for smaller E14 cells) was plated and passaged at least twice before starting differentiation to ensure that MEFs are effectively removed.

Differentiation Protocol
E14 cultures (MEF-free) were plated onto 0.1% gelatin-coated growing surfaces in 2i media for 12 hours prior to the initiation of differentiation (to ensure the treatment does not affect cell attachment). Following this 12 hour period, cells were washed once with PBS (containing no divalent cations) and treated with differentiation media. The time at which cells received this media was designated as the start of treatments (0 hours/days). Differentiation media was the same as described above, only without the addition of LIF and 2i. For initial experiments, 1% DMSO was added to differentiation media. For the final early time-point experiment, 1% DMSO was not added to differentiation media.

RNA Isolation and cDNA Synthesis
Total RNA was isolated from E14 and differentiation-induced E14 cells using TRIzol (according to manufacturer’s protocol, Invitrogen). 10,000 ng (as determined by NanoDrop
spectrophotometric quantification) of total RNA was treated with DNase-I (Fermentas) in a 100 µL reaction. This was then followed by an acidic phenol-chloroform extraction in order to remove any remaining DNA and DNase-I. The resulting RNA was quantified by NanoDrop again, and used for cDNA synthesis. 600 ng of purified RNA was processed into cDNA using the iScript Select cDNA synthesis kit (Bio-Rad) in a 20 µL reaction. Random hexamer primers were used in order to amplify both primary and processed mRNA. As a control for DNA contamination, 600 ng of each RNA sample was put into a cDNA synthesis reaction with no reverse-transcriptase. The conditions for cDNA synthesis thermocycling were as follows: 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. The resulting 20 µL reaction was diluted 5x and 1 µL of diluted cDNA was used for every RT-qPCR reaction.

**Real-Time Quantitative PCR (RT-qPCR)**

Reverse-transcribed cDNA samples were quantified using a standard curve to determine absolute expression levels. For standard curve material, genomic DNA was extracted from the same E14 cells used for experiments. Cells were pelleted, then resuspended in a DNA extraction buffer containing 10 mM Tris-HCl (pH-8.0), 0.1 M EDTA (pH-8.0) and 0.5% SDS. This was followed by Proteinase K treatment (BioShop) and phenol-chloroform extraction. The concentration of resulting genomic DNA was accurately quantified using the PicoGreen assay (Invitrogen). Genomic DNA was then subject to five five-fold serial dilutions were made (25 ng/µL to .04 ng/µL). 1 µL of diluted genomic DNA was used for every RT-qPCR reaction.

RT-qPCR was performed on the Bio-Rad CFX384 thermocycler. Each RT-qPCR reaction contained 1x Bio-Rad iTaq SYBR green mastermix (no ROX), 0.3 or 0.75 pM of each primer (forward and reverse) and 1 µL of template (either genomic DNA or cDNA). cDNA samples were ran in triplicate, while genomic DNA standard curves were assayed in duplicate. Each reaction had a total volume of 10 µL. The conditions for RT-qPCR were as follows: 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds then 62°C for 30 seconds.
**RT-qPCR Data Analysis**

To standardize RT-qPCR expression levels, all data was normalized to *Gapdh* primary transcript. The 5 day differentiation study was repeated three independent times. The two 1 day differentiation experiments were each performed two independent times, but on one of these repetitions, two separate tissue culture plates were differentiated and harvested for each given time-point. The graphs shown in the Results section represent the average (mean) normalized expression value of these replicates. Error bars represent the standard error of the mean. The significance of difference between RT-qPCR values from differentiated and pluripotent cells over time were assessed by two-way ANOVA and Bonferroni post tests using Sigma Plot 12. The results from the 1 day experiment which compares the effects of DMSO at only one time-point were analyzed by one-way ANOVA using Sigma Plot 12.

**Immunofluorescence Staining and Imaging**

Fluorescence images for OCT4, SOX2, NANO4 and RNApolII were obtained by seeding pluripotent or differentiated cells onto sterile 0.1% gelatin-coated glass coverslips for between 24 and 48 hours (antibody list and dilutions in Appendix). Cells were fixed at room temperature for 20 minutes in neutral-buffered 10% formalin (Sigma-Aldrich). After washing with PBS, cells were simultaneously blocked and permeabilized at room temperature for 30 minutes in 10% Fetal Bovine Serum in 0.1% Triton X-100 in PBS. For OCT4, SOX2 and NANO4 images, cells were incubated with primary antibodies at 4°C overnight in antibody buffer (0.2% Fetal Bovine Serum in 0.1% Triton X-100 in PBS). Cells were washed with PBS + 0.1% Tween, then were incubated with the RNApolII primary antibody for 30 minutes at room temperature (in antibody buffer). Cells were washed with PBS + 0.1% Tween three times for five minutes each. After incubation in antibody buffer containing secondary antibodies for 30 minutes at room temperature, cells were washed with PBS + 0.1% Tween four times for five minutes each. Cells were then counterstained in DAPI, washed twice in PBS then washed once in distilled, molecular biology grade water (to remove remaining PBS). Coverslips were then mounted onto glass microscope slides using VECTASHIELD (Vector Labs) and edges sealed with transparent nail polish (Revlon). Images were taken using a Leica SP5 Confocal or Zeiss + Metasystems camera.
In order to determine the number of cells within a colony with nuclei positive for OCT4, SOX2 or NANOG, slide labels were covered up and placed onto the microscope (without knowledge of the treatment or timepoint). At 40x magnification with the FITC (ab5131) or Texas Red (ab4H8) filter selected, a colony was chosen and the number of cells with a strong RNApolII signal was counted. The filter was then switched to Texas Red (OCT4 and SOX2) or FITC (NANOG) and the number of cells with distinct nuclear staining was counted. Approximately 500 cells were counted from two independently prepared coverslips. Significance of results was analyzed by one-way ANOVA using SigmaPlot 12.
Results

Introduction

While changes in Oct4, Sox2 and Nanog expression have been characterized in numerous differentiation protocols, early changes in primary transcript levels have not been quantitatively measured as mESCs exit pluripotency. Additionally, the effects of 2i/LIF withdrawal and DMSO treatment of mESCs maintained in the absence of feeder cells have not been investigated. As mESCs are very sensitive to the conditions in which they are grown and since there numerous different ways of maintaining pluripotency in vitro, it is important to first characterize the differentiation process for mESCs maintained under different conditions. In this body of work, I have investigated the dynamics of Oct4, Sox2 and Nanog expression when mESCs cultured in media containing 2i, LIF and FBS are differentiated. For a comprehensive view of how differentiation proceeds, both short-term (up to 24 hours) and long-term (up to 5 days) investigations were made. The main goal of these experiments was to obtain a general idea of the timeframe and the magnitude of changes to Oct4, Sox2 and Nanog expression as mESCs exit the pluripotent state and are induced to differentiate.

Long-term differentiation induces changes to the mESC-specific gene expression profile

For preliminary insight into the changes in gene expression caused by the described differentiation protocol, a long-term study was performed. Days 1, 2 and 5 were chosen as timepoints for investigation into the progress of differentiation. Total RNA was harvested and RT-qPCR for Oct4, Sox2, Nanog, Kit, Flk1, Brachyury, CD41 and GATA1 primary and processed transcripts was performed. After 1 day of differentiation, significant reductions in Nanog (Figure 1) and Kit (Figure 2) primary and processed transcript were discovered. Following this initial drop, Nanog and Kit expression did not significantly change for the remaining 4 days of differentiation. By day 5, significant decreases in Oct4 (Figure 3a) primary transcript and Sox2 (Figure 4) were found. This was accompanied by significant increases in Brachyury primary (70-fold) and processed (200-fold) transcript (Figure 5), a marker of commitment to the mesodermal lineage. While significant decreases of Flk1 processed transcript were found in days 1 and 2 of differentiation (Figure 6b), these expression values were extremely low (under 20-fold lower than Oct4 primary transcript
expression after 5 days of differentiation). No expression of CD41 or GATA1 was detected (results not shown). Taken together, these results demonstrate that following 5 days of differentiation, mESCs express lower levels of Oct4, Sox2, Nanog and Kit while Brachyury becomes expressed at a comparatively high rate.
Figure 1. Nanog expression significantly drops after 1 day of differentiation

*Nanog* primary (1a) and processed (1b) transcript expression are found to be significantly down-regulated in differentiated mESCs after 1 and 5 days. At 2 days, \( p = 0.07 \) for *Nanog* processed transcripts, (slightly above the statistical cutoff), while primary transcripts remain significantly lower in differentiated cells. Blue boxes represent *Nanog* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal and treatment with DMSO. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. * = \( p<0.05 \), ** = \( p<0.01 \)
Figure 2. *Kit* expression significantly drops after 1 day of differentiation

*Kit* primary (2a) and processed (2b) transcripts are found to be significantly down-regulated in differentiated mESCs after 1, 2 and 5 days. Blue boxes represent *Kit* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal and treatment with DMSO. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. **=P<0.01, ***= p<0.001.
Figure 3. Oct4 primary transcript expression significantly drops after 5 days of differentiation. Oct4 primary transcript expression (3a) drops significantly after 5 days of differentiation, but Oct4 processed transcript (3b) is not similarly affected. Blue boxes represent Oct4 expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal and treatment with DMSO. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM. ** = p<0.01.
Figure 4. Sox2 expression significantly drops after 5 days of differentiation
Sox2 (which contains only one exon) expression is significantly down-regulated after 5 days of differentiation. Blue boxes represent Sox2 expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal and treatment with DMSO. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM. * = p<0.05.
Figure 5. *Brachyury* expression significantly increases after 5 days of differentiation
*Brachyury* primary (5a) and processed (5b) transcript expression is significantly up-regulated after 5 days of differentiation. Blue boxes represent *Brachyury* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal and treatment with DMSO. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. * = p<0.05.
Figure 6. *Flk1* transcription significantly drops after 1 day of differentiation

*Flk1* processed transcripts (6b) are found to be significantly down-regulated in differentiated mESCs after 1 and 2 days of differentiation. No significant differences in *Flk1* primary transcript expression were found (6a). In either treatment, *Flk1* is expressed at very low (likely biologically irrelevant) levels. Blue boxes represent *Flk1* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal and treatment with DMSO. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. * = p<0.05, **= p<0.01.
Long-term differentiation induces changes to mESC-specific cellular morphology

Brightfield microscopy images were also taken at days 1, 2 and 5 (Figure 7). After 1 day of differentiation, colonies begin to lose the hallmark raised, 3-dimensional structure associated with pluripotency (A and B from Figure 7). Individual nuclei become visible within colonies, and cells begin to form protrusions which more readily enable contact with nearby colonies. By day 2 of differentiation, the features described in day 1 become more pronounced (Figure 7C). Individual cells begin to physically separate themselves from other nearby cells. While colonies still form, they are more irregular in shape (as opposed to the tightly packed circular colonies present in pluripotent mESCs). Protrusions also become more prevalent and generally extend further away from the cell which they project from (Figure 7C). On day 5, a proportion of cells take on a very flattened, almost sunken-in morphology characteristic of mesodermal precursor cells (Figure 7D). These cells lack protrusions and are capable of growing in a monolayer sheet if plated at a high enough density. Interestingly, some cells still maintain morphology resembling that of day 2 of differentiation (Figure 7D).
Figure 7. Brightfield microscopy images of pluripotent and differentiated mESCs

Figure 7 shows brightfield images of pluripotent mESCs (A) and mESCs withdrawn from 2i/LIF and treated with DMSO for 1, 2 and 5 days (B, C and D). Raised colony structure is largely lost after 1 day of differentiation. Individual cells separate out more and have more defined projections after 2 days of differentiation. At 5 days of differentiation, select cells adopt a sunken-down morphology. Images taken at 10x magnification. Scale bar represents 100 µm.
Long-term differentiation induces changes to the localization of Oct4, Sox2 and Nanog proteins

Immunofluorescence images of cells stained for OCT4 (Figures 8-12), SOX2 (Figures 13-17) or NANOG (Figures 18-22) with RNA Polymerase II were also taken in order to determine how nuclear organization changes as cells differentiate. OCT4, SOX2 and NANOG are present in the nucleus of pluripotent mESCs. As hypothesized earlier, each transcription factor along with RNA Polymerase II displays a focal staining pattern, first visible at 40x magnification. Cells are tightly packed within a colony and have very little cytoplasm, with no OCT4, SOX2 or NANOG foci visible outside the boundary of the DAPI counterstain. Cells in the process of mitosis (metaphase and anaphase) are readily visible and do not have strong Oct4, Sox2, Nanog or RNA Polymerase II signals. Mirroring gene expression results, the proportion of cells within a colony with a positive nuclear signal for OCT4 does not significantly change until day 5 (Figure 8). Even at this later timepoint, nuclear OCT4 staining is still widespread, with an average of 83% of cells within a colony positive for OCT4 (Figures 8 and 12). Interestingly, Sox2 staining in pluripotent cells is less bright than OCT4 and NANOG, yet more focalized. While there is a slight yet significant decrease in the proportion of SOX2-positive nuclei after 1 day of differentiation (Figures 13 and 15), this ratio drops significantly on day 2 and again on day 5 (Figures 13, 16 and 17). By this time, very few cells are positive for SOX2 (1.35%). The proportion of nuclei with NANOG signals significantly drops after 1 day of differentiation and again after 2 days (Figures 18, 20 and 21). This drop is curbed by day 5 (Figures 18 and 22), where a statistically similar number of cells within a colony to day 2 have nuclear positive signals (15%). By day 5 of differentiation, cells have developed larger cytoplasms. At this time-point, SOX2 and NANOG foci are no longer localized to the nucleus, instead adopting a uniform, colony-wide weak staining pattern (Figures 16, 17, 21 and 22).
**Figure 8. Differentiation causes a loss of OCT4 staining**

Figure 8 quantifies the loss of OCT4-positive cells following 1, 2 and 5 days of differentiation. The percentage of cells within a colony positive for OCT4 significantly drops after 5 days of differentiation. Error bars represent SEM. * = p<0.01
Figure 9. OCT4 immunofluorescence in pluripotent mESCs
Figure 9 depicts OCT4 (red), RNA Polymerase II (green) and DAPI (blue) staining in pluripotent mESCs. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 10. OCT4 immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 1 day

Figure 10 depicts OCT4 (red), RNA Polymerase II (green) and DAPI (blue) staining in mESCs differentiated for 1 day. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 11. OCT4 immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 2 days
Figure 11 depicts OCT4 (red), RNA Polymerase II (green) and DAPI (blue) staining in mESCs differentiated for 2 days. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 12. Oct4 immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 5 days
Figure 12 depicts OCT4 (red), RNA Polymerase II (green) and DAPI (blue) staining in mESCs differentiated for 5 days. Colonies grow almost exclusively in monolayers at high density. OCT4 staining is finally lost in some cells, and examples of “nuclear positive” and “nuclear negative” cells are easily visible in the red section of the top panel. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 13. Differentiation causes a loss of SOX2 staining
Figure 13 quantifies the loss of Sox2-positive cells following 1, 2 and 5 days of differentiation. The percentage of cells within a colony positive for SOX2 significantly drops after 1, 2 and 5 days of differentiation. Error bars represent SEM. * = p<0.05, **=p<0.01
Figure 14. SOX2 immunofluorescence in pluripotent mESCs
Figure 14 depicts SOX2 (red), RNA Polymerase II (green) and DAPI (blue) staining in pluripotent mESCs. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 15. SOX2 immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 1 day
Figure 15 depicts SOX2 (red), RNA Polymerase II (green) and DAPI (blue) staining in mESCs differentiated for 1 day. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 16. SOX2 immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 2 days.

Figure 16 depicts SOX2 (red), RNA Polymerase II (green) and DAPI (blue) staining in mESCs differentiated for 2 days. The colony-wide weak signal is first visible at this time-point. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 17. SOX2 immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 5 days

Figure 17 depicts SOX2 (red), RNA Polymerase II (green) and DAPI (blue) staining in mESCs differentiated for 5 days. Again, a colony-wide weak signal is visible, yet since distinct SOX2-positive cells cannot be visualized, none are not counted as positive. Image was taken at 100x magnification, scale bar represents 25 µm.
**Figure 18. Differentiation causes a loss of NANOG staining**

Figure 18 quantifies the loss of NANOG-positive cells following 1, 2 and 5 days of differentiation. The percentage of cells within a colony positive for NANOG significantly drops after 1 and 2 but no further loss of NANOG signal is observed after 5 days of differentiation. Error bars represent SEM. **=p<0.001
Figure 19. NANOGL immunofluorescence in pluripotent mESCs
Figure 19 depicts NANOGL (green), RNA Polymerase II (red) and DAPI (blue) staining in pluripotent mESCs. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 20. NANOG immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 1 day

Figure 20 depicts NANOG (green), RNA Polymerase II (red) and DAPI (blue) staining in mESCs differentiated for 1 day. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 21. NANOG immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 2 days

Figure 21 depicts NANOG (green), RNA Polymerase II (red) and DAPI (blue) staining in mESCs differentiated for 2 days. The colony-wide weak signal is first visible at this time-point. This image shows 6 cells positive for PolII but only 2 positive for NANOG, again illustrating how the colony-wide weak signal does not influence the cell counting results displayed in Figure 18b. Image was taken at 100x magnification, scale bar represents 25 µm.
Figure 22. NANOG immunofluorescence in mESCs subject to 2i/LIF withdrawal and DMSO treatment for 5 days

Figure 22 depicts NANOG (green), RNA Polymerase II (red) and DAPI (blue) staining in mESCs differentiated for 5 days. Again, a colony-wide weak signal is visible, yet since distinct NANOG-positive cells cannot be visualized, none are not counted as positive. Image was taken at 100x magnification, scale bar represents 25 µm.
The presence of DMSO has no significant effect on 2i/Lif withdrawal after one day of differentiation

The results of the 5 day differentiation study indicate that the differentiation protocol causes longer-term changes in Oct4, Sox2 and Brachyury expression while Kit and Nanog expression is significantly attenuated within a 24 hour timepoint. Unfortunately, the results described above do not address the fact that the applied differentiation protocol alters two aspects of mESC culture at the same time; the withdrawal of 2i/LIF and the addition of DMSO. In order to ensure that early down-regulation of Nanog and Kit was repeatable (and therefore biologically relevant), and to distinguish between the effects of 2i/LIF withdrawal and DMSO treatment, a 1 day differentiation experiment was performed. The same genes as in the previous experiment will be quantified. mESCs were either maintained as pluripotent cells or given one of two differentiation treatments; one without DMSO, 2i or LIF (-2i/LIF, -DMSO) and one with DMSO but without 2i or LIF (-2i/LIF, +DMSO). The results of this experiment will confirm the early timepoint conclusions drawn from the long-term differentiation study while also determining the effects of DMSO on early changes in gene expression.

After 1 day of differentiation, relative to pluripotent mESCs, no significant differences in Oct4 or Sox2 expression were found. Nanog expression dropped significantly (14-fold for both primary and processed transcript, Figure 25) while Kit showed a similar decrease in expression (13-fold for primary transcript, 9-fold for processed transcript, Figure 26). This therefore confirms the results obtained from the 1 day time-point of the long-term differentiation experiment. Significant differences were found in the expression of Brachyury and Flk1 processed transcript, but it is important to note that both of these genes are expressed at extremely low levels after 1 day of differentiation.

In the same timeframe, no significant differences in expression of Oct4, Sox2, Nanog, Kit, Brachyury or Flk1 (Figures 23-28) were found between differentiation treatments lacking or containing DMSO (-2i/LIF, -DMSO compared with -2i/LIF, +DMSO). These results indicate
that the presence of DMSO does not have a significant effect on the early time-point changes in gene expression induced by 2i/LIF withdrawal.
Figure 23. Oct4 expression is not significantly affected by 1 day of differentiation

Oct4 primary transcript (23a) and processed transcript (23b) expression is not significantly affected by 1 day of 2i/LIF withdrawal, either in the presence or absence of DMSO. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM.
Figure 24. Sox2 expression is not significantly affected by 1 day of differentiation

Sox2 expression is not significantly affected by 1 day of 2i/LIF withdrawal, either in the presence or absence of DMSO. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM.
Figure 25. *Nanog* expression significantly drops after 1 day of differentiation, but the magnitude of drop is not affected by the presence of DMSO

*Nanog* primary transcript (25a) and processed transcript (25b) expression is significantly down-regulated after 1 day of 2i/LIF withdrawal, either in the presence or absence of DMSO. The presence of DMSO has no significant effect on the expression of *Nanog* when mESCs are withdrawn from 2i/LIF for 1 day. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. **=*p*<0.001
Figure 26. *Kit* expression significantly drops after 1 day of differentiation, but the magnitude of drop is not affected by the presence of DMSO

*Kit* primary transcript (26a) and processed transcript (26b) expression is significantly down-regulated by 1 day of 2i/LIF withdrawal, either in the presence or absence of DMSO. The presence of DMSO has no significant effect on the expression of *Kit* when mESCs are withdrawn from 2i/LIF for 1 day. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. **=*p<0.001
Figure 27. *Brachyury* processed transcript expression significantly drops after 1 day of differentiation, but the magnitude of drop is not affected by the presence of DMSO. *Brachyury* processed transcript (27b) expression is significantly down-regulated by 1 day of 2i/LIF withdrawal, either in the presence or absence of DMSO. No significant differences were found in *Brachyury* primary transcript expression (27a). The presence of DMSO has no significant effect on the expression of *Brachyury* when mESCs are withdrawn from 2i/LIF for 1 day. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM. **=p<0.001
Figure 28. *Flk1* processed transcript expression significantly drops after 1 day of differentiation, but the magnitude of drop is not affected by the presence of DMSO. *Flk1* processed transcript (28b) expression is significantly affected by 1 day of 2i/LIF withdrawal, either in the presence or absence of DMSO. No significant differences were found in *Flk1* primary transcript expression (28a). The presence of DMSO has no significant effect on the expression of *Flk1* when mESCs are withdrawn from 2i/LIF for 1 day. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. **=p<0.001
Fine-mapping of short-term differentiation uncovers significant changes in gene expression as early as 6 hours after treatment

The results of the two previous experiments provided justification for finely-mapping the drop in the expression of Nanog and Kit in order to determine when these genes are first down-regulated. To better characterize the changes to the mESC-specific gene expression profile occurring in the first 24 hours of differentiation, the number of genes to be quantified by RT-qPCR was expanded. In addition to Oct4, Sox2, Nanog and Kit, primary and processed transcripts for Tet1, Lefty2, Rex1, Fgf4, Tdgf1, Zic3, Dppa2, Klf4, cMyc, Sall4, Stat3, Smad1 and Tcfcp2 were quantified after 6, 12 and 24 hours of differentiation. These genes were chosen using BioGPS, a searchable online database containing microarray data from many different cell types. Using the gene correlation function, BioGPS allows a researcher to identify genes which have a similar expression profile to a query gene. For this experiment, Nanog was chosen as a query as it is expressed almost exclusively in pluripotent mESCs, thus identifying other genes which show pluripotent-specific expression. Genes of interest with a correlation value of .90 or higher were selected for quantification by RT-qPCR.

Following 6 hours of differentiation, Kit (Figure 29) and Klf4 (Figure 30) primary and processed transcript as well as Stat3 processed transcript (Figure 31b) were significantly down-regulated. These genes were expressed lower in differentiated cells for the remaining 18 hours of the experiment. Zic3 primary transcript expression was significantly higher in differentiated cells after 6 hours and stayed this way until the end of the experiment (Figure 32a). There were no significant differences found in Zic3 processed transcript expression (Figure 32b). Oct4 primary and processed transcript was significantly (1.5-fold) higher in differentiated cells after 6 hours (Figure 33). Interestingly, the expression of Oct4 primary and processed transcript dropped back to levels similar to pluripotent mESCs and stayed the same for the remainder of the experiment. At the 12 hour timepoint, a significant (3-fold) decline in the expression of Nanog primary and processed transcript is observed in differentiating cells (Figure 34). This difference remains significant throughout the rest of the experiment. A similar pattern is seen with Lefty2, with expression levels significantly
dropping first after 12 hours of differentiation and remaining low after 24 hours (Figure 35). *Tdgrf1* primary and processed transcripts are both significantly higher in differentiated cells after 24 hours of differentiation (Figure 36). While changes in *c-Myc* (Figure 37), *Dppa2* (Figure 38) and *Rex1* (Figure 39) were found, these differences were only significant at p<0.1, and it is therefore difficult to conclude that these changes have any biological significance. No significant changes were found in the expression of *Tet1* (Figure 40a and b), *Fgf4* (Figure 40c and d), *Sall4* (Figure 40 e and f), *Smad1* (Figure 41 a and b), *Tcfcp2* (Figure 41 c and d) or *Sox2* (Figure 41 e).
Figure 29. *Kit* expression significantly drops after 6 hours of 2i/LIF withdrawal

*Kit* primary (29a) and processed (29b) transcript expression is significantly down-regulated after 6 hours of differentiation. Expression remains significantly low for the remaining 18 hours of the experiment. Blue boxes represent *Kit* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. * = p<0.05, ** = p<0.01, *** = p<0.001.
Figure 30. Klf4 expression significantly drops after 6 hours of 2i/LIF withdrawal

Klf4 primary (30a) and processed (30b) transcript expression is significantly down-regulated after 6 hours of differentiation. Expression remains significantly low for the remaining 18 hours of the experiment. Blue boxes represent Klf4 expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM. * = p<0.05, ** = p<0.01, *** = p<0.001.
**Figure 31. Stat3 processed transcript expression significantly drops after 6 hours of 2i/LIF withdrawal**

Stat3 processed (31b) transcript expression is significantly down-regulated after 6 hours of differentiation. Expression remains significantly low for the remaining 18 hours of the experiment. No significant differences in Stat3 primary transcript expression (31a) were found. Blue boxes represent Stat3 expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM. * = p<0.05, ** = p<0.01, *** = p<0.001.
Figure 32. Zic3 primary transcript expression is significantly up-regulated after 6 hours of 2i/LIF withdrawal

Zic3 primary (32a) transcript expression is significantly up-regulated after 6 hours of differentiation. Expression remains significantly high for the remaining 18 hours of the experiment. No significant differences in Zic3 processed transcript expression (32b) were found. Blue boxes represent Zic3 expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM. * = p<0.05, ** = p<0.01.
Figure 33. *Oct4* expression is significantly up-regulated after 6 hours of 2i/LIF withdrawal

*Oct4* primary (33a) and processed (33b) transcript expression is significantly up-regulated after 6 hours of differentiation. *Oct4* expression in differentiated cells drops back to levels not significantly different from pluripotent mESCs at 12 and 24 hours. Blue boxes represent *Oct4* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. * = p<0.05, *** = p<0.001
**Figure 34.** *Nanog* expression significantly drops after 12 hours of 2i/LIF withdrawal

*Nanog* primary (34a) and processed (34b) transcript expression is significantly down-regulated after 12 hours of differentiation. Processed transcript expression remains significantly low for the remaining 12 hours of the experiment. *Nanog* primary transcript expression after 24 hours is higher in pluripotent mESCs, but the difference is right on the statistical cutoff (p=0.05). Blue boxes represent *Nanog* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. + = p=0.05, * = p<0.05.
Figure 35. *Lefty2* expression significantly drops after 12 hours of 2i/LIF withdrawal

*Lefty2* primary (35a) and processed (35b) transcript expression is significantly down-regulated after 12 hours of differentiation. Expression remains significantly low for the remaining 12 hours of the experiment. Blue boxes represent *Lefty2* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. ** = p<0.01, *** = p<0.001.
Figure 36. *Tdgf1* expression is significantly up-regulated after 1 day of differentiation

*Tdgf1* primary and processed transcripts are found to be significantly up-regulated in differentiated mESCs after 24 hours of differentiation. Blue boxes represent *Tdgf1* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM. ** = p<0.01.
Figure 37. *c-Myc* processed transcription is up-regulated after 1 day of differentiation

*c-Myc* processed transcript (37b) expression is found to be up-regulated in differentiated mESCs after 1 day of differentiation, but this value is not statistically significant (p = .066). No significant changes in *c-Myc* primary transcript (37a) expression were detected. Blue boxes represent *c-Myc* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM.
**Figure 38. Dppa2 processed transcription is up-regulated after 1 day of differentiation**

*Dppa2* processed transcript (38b) expression is found to be up-regulated in differentiated mESCs after 1 day of differentiation, but this value is not statistically significant (p = .083). No significant changes in *c-Myc* primary transcript (38a) expression were detected. Blue boxes represent *Dppa2* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to *GAPDH* expression. Error bars depict SEM.
**Figure 39.** *Rex1* primary transcript expression significantly drops after 1 day of differentiation

*Rex1* primary transcript (39a) expression is found to be significantly down-regulated in differentiated mESCs after 1 day. *Rex1* processed transcript (39b) expression is down-regulated in differentiated cells after 24 hours, but not significantly (p=0.057). Blue boxes represent *Rex1* expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM. * = p<0.05.
Figure 40. Tet1, Fgf4 and Sall4 expression does not significantly change after 1 day of differentiation

Following 24 hours of 2i/LIF withdrawal, Tet1 primary transcript (40a) and processed transcript (40b) as well as Fgf4 primary transcript (40c) and processed transcript (40d) and Sall4 primary transcript (40e) and processed transcript (40f) expression does not significantly change. Blue boxes represent Tet1/Fgf4/Sall4 expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal and treatment with DMSO. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM.
Figure 41. Smad1, Tcfcp2 and Sox2 expression does not significantly change after 1 day of differentiation

Following 24 hours of 2i/LIF withdrawal, Smad1 primary transcript (41a) and processed transcript (41b) as well as Tcfcp2 primary transcript (41c) and processed transcript (41d) and Sox2 (41e) expression does not significantly change. Blue boxes represent Smad1/Tcfcp2/Sox2 expression in pluripotent mESCs, while red boxes represent mESCs subject to 2i/LIF withdrawal and treatment with DMSO. Data points are an average of three independent replicates, normalized to GAPDH expression. Error bars depict SEM.
**Discussion**

**Summary of Results**

It was found that 2i/LIF withdrawal in combination with DMSO treatment causes significant down-regulation of the expression of Nanog and Kit following only one day of differentiation. Oct4 primary transcript and Sox2 expression was down-regulated after 5 days of differentiation while Brachyury was significantly up-regulated over the same timeframe. Differentiation did not cause an up-regulation of Flk1, CD41 or GATA-1. Differentiation caused changes in cellular morphology visible after only 1 day, as well as a change in the localization of OCT4, SOX2 and NANOG TFs. Further experimentation showed that DMSO does not have an effect on the early time-point changes in expression caused by 2i/LIF withdrawal. An expanded look into the early changes in gene expression associated with 2i/LIF withdrawal revealed significant differences in the expression of Kit, Klf4, Zic3, Stat3 and Oct4 following 6 hours of differentiation. Changes in the expression of Nanog and Lefty2 were found after 12 hours, while Tdgf1 expression was significantly up-regulated following 24 hours of differentiation.

**DMSO, 2i/LIF withdrawal and changes in gene expression after 5 days of differentiation**

Two major issues arise when attempting to place the drop in Oct4, Sox2 and Nanog expression from long-term differentiation into the context of relevant literature. The first problem is the relative age of DMSO differentiation protocols. DMSO was found to induce the differentiation of mouse erythroleukemia cells to hemoglobin-producing precursors of the erythroid lineage (Friend et al., 1971). A second wave of differentiation studies using DMSO followed in the 1990’s (Dani et al., 1997). However, since Nanog was not identified until 2003 (Chambers et al., 2003), all studies involving DMSO prior to 2003 do not measure Nanog expression. The second issue is the use of DMSO is most commonly applied to embryoid bodies (EBs), which contain subsets of differentiated cells such as precursors from the blood and vascular systems (Kennedy and M. Keller, 2003). Even though the study on which this thesis is based reports that DMSO contributes to the development of the mesoderm and more specifically, the hematopoietic lineage (Lako et al., 2001), this publication fails to distinguish whether DMSO promotes the differentiation of pluripotent
cells to the hematopoietic lineage, or whether DMSO responsiveness is dependent on a pre-existing commitment to the mesoderm lineage. The same problem arises even when the expression of markers of pluripotency (such as Oct4) is quantified (Adler et al., 2006). Adding another level of complexity to fully understanding published results is the fact that the removal of LIF is the first step in the generation of EBs (Keller et al., 1993). In some experiments, it is hard to discern whether “-LIF 48hrs” refers to the withdrawal of LIF from plated mESCs, or mESCs in the process of being differentiated to EBs (Trouillas et al., 2009).

Even though some modern studies have used DMSO to induce differentiation in non-EB mESCs, the results are again not easily interpreted. One study reports a 0.18 fold reduction in Nanog and a .7 fold reduction in Oct4 expression following DMSO treatment, yet this was after 4 days of growth in media containing only 1% FBS in addition to a chemically defined serum replacement (Torres et al., 2012). Another study quantifies Oct4, Sox2 and Nanog expression in mESCs treated with DMSO, albeit in the presence of LIF and only at one time-point (Adamo et al., 2009). DMSO is also often used as a vehicle for delivering treatments. The 2i chemicals are solubilized in DMSO, as are other small molecule activators, inhibitors and treatments such as retinoic acid (Santostefano et al., 2012). Some studies occasionally quantify pluripotency-associated genes in DMSO-treated mESCs, yet only at a concentration of DMSO used for other treatments in the publication (often orders of magnitude below 1% DMSO). There are some studies which have looked at the effects of DMSO treatment on human ESCs (Pal et al., 2012), but the transcriptional networks associated with pluripotency (especially with regards to Nanog) in human ESCs is marked with key differences when directly compared to mESCs. Aside from these reports, there are very few, if any, publications which measure Oct4, Sox2 and Nanog gene expression upon the withdrawal of 2i/LIF and the addition of DMSO to non-EB mESCs.

In comparison to the publication which formed the basis of this investigation (Lako et al., 2001), 2i/LIF withdrawal and DMSO treatment of pluripotent mESCs does not achieve significant progression through to the hematopoietic lineage. In the study by Lako et al. 2001, EBs (following aggregation by hanging drop for 48 hours) were treated with DMSO in
the absence of LIF for 6 days. Quantification of markers of hematopoiesis was performed on each day by reverse-transcriptase PCR (RT-PCR). Step-wise expression of hematopoietic markers was seen. Brachyury is expressed only at day 2 after down-regulation of activin β, recapitulating the transition seen in embryos from primitive ectoderm to mesoderm (Lako et al., 2001). Expression of Flk1 and Kit (markers of primitive hematopoiesis) peaked at day 3 of DMSO treatment. Finally, expression of GATA-1 and globin βH1 (markers of further commitment to hematopoiesis) is seen at days 5 and 6 (Lako et al., 2001). Whereas simply removing LIF from EBs (no DMSO treatment) for the same 6 day time period achieves expression of all mentioned genes, the process is in a less co-ordinated/step-wise manner (Lako et al., 2001). Brachyury is expressed for a total of 4 days as opposed to just one while Flk1 is expressed robustly for 3 days as opposed to one. For the experiments I performed, after 5 days of 2i/LIF withdrawal and DMSO treatment administered to pluripotent mESCs, only Brachyury is significantly upregulated. No appreciable expression of Flk1, GATA-1 or CD41 was detected. In agreement with Lako et al. 2001, expression of Kit is greatly reduced after one day of differentiation but in contrast, Kit is not up-regulated at later timepoints. The comparison of previous results with newly obtained results indicates that three-dimensional aggregation is necessary for the expression of hematopoietic markers, and that DMSO is not significantly contributing to hematopoietic commitment of pluripotent mESCs.

Another report describes changes in expression of Oct4 and Brachyury similar to the obtained long-term differentiation results. Using RT-PCR (a less quantitative assay than RT-qPCR) to assay mESCs following 5 days of LIF withdrawal without DMSO treatment, the authors found that Oct4 expression declines gradually, but is ultimately still robustly expressed (Sauter et al., 2005). The same holds true of Oct4 expression through 16 days of EB culture (Sauter et al., 2005). Brachyury expression was upregulated following 3 days of LIF withdrawal and its expression increases again over the next two days. (Sauter et al., 2005). Also in corroboration of obtained results, microarray analysis of pluripotent mESCs subject to LIF withdrawal for 1 and 2 days identified significant drops in Nanog at both indicated time-points (Trouillas et al., 2009). No significant changes in either Oct4 or Sox2 were detected. Interestingly, a change in Kit expression was not described (Trouillas et al., 2009). Additionally, one report has described the changes in Oct4, Sox2 and Nanog following
withdrawal of LIF and BMP (Thomson et al., 2011). Following 2 days of differentiation, relative to pluripotent mESCs, Oct4 expression is reduced by 26%. Sox2 expression is reduced by 71% while Nanog expression is reduced by 95% (Thomson et al., 2011). While these changes are greater than found in my experiments, it is important to note that BMP4 is still present in my differentiation protocol by way of FBS, and is likely playing a role in maintaining the expression of Oct4, Sox2 and Nanog at higher levels than Thomson et al. 2011.

Even though the observed pattern of Kit expression in differentiated mESCs is somewhat counter-intuitive, it has been previously described. Kit encodes the Kit ligand, which binds to the Stem Cell Factor receptor (Iemura et al., 1994). Kit has been shown to play roles in hematopoiesis (Bernex et al., 1996) and mast cell development (Iemura et al., 1994). Kit is expressed in mESCs (Lako et al., 2001) and in the yolk sac of embryonic day 7.5-9.5 embryos (Bernex et al., 1996). Homozygous-null mutant embryos develop to term but these mice die shortly after birth due to severe anemia (Bernex et al., 1996). Interestingly, 1 day of EB formation and LIF withdrawal (in either the presence or absence of DMSO) causes a sharp down-regulation of Kit expression which is regained after an additional day of differentiation (Lako et al., 2001). The expression of Kit and subsequent rapid down-regulation induced by differentiation could be explained by the aforementioned widespread transcription (and the early time-point down-regulation induced by LIF withdrawal) in pluripotent mESCs, although this hypothesis is speculative at best without further experimental validation.

**Comparison of cell morphology after 5 days of differentiation indicates that 2i/LIF withdrawal and addition of DMSO enables BMP4-mediated non-neural differentiation**

A survey of relevant literature reveals striking morphological similarities between mESCs subject to 5 days of my differentiation protocol and pluripotent mESCs cultured in N2B27 media supplemented with BMP4 (Ying et al., 2003b). mESCs grown in N2B27 without BMP4 or LIF develop into neural precursors expressing Sox1 and Nestin, markers of neural ectoderm (Ying et al., 2003b). If BMP4 is added, mESCs differentiate into distinctly non-neural precursors, with a mixed-flattened morphology very similar to mESCs after 5 days of
my differentiation protocol (compare figure 7d with figure 4b of Ying et al., 2003b).
Furthermore, N2B27+BMP4 can also yield confluent monolayers similar in appearance to 5 days of 2i/LIF withdrawal and DMSO treatment (compare figure 7d with the bottom right panel of figure 1a from Ying et al., 2003a). Indeed, one report has established a link between LIF withdrawal and BMP action, indicating that in the absence of LIF, BMP function switches from promoting self-renewal to inducing mesoderm and endoderm (Ying et al., 2003a). This same report concludes that LIF prevents the non-neural differentiation induced by BMP4 (Ying et al., 2003a). Since FBS (which contains BMP4) is still present in my differentiation protocol, it is likely that BMP4 is playing a significant role in the changes caused by 5 days of differentiation by 2i/LIF withdrawal and DMSO treatment.

**Immunofluorescence uncovers changes in Oct4, Sox2 and Nanog localization following 2i/LIF withdrawal and DMSO treatment**

While the publication of fluorescence images of OCT4, SOX2 and NANOG in both blastocysts (Keramari et al., 2010) and mESCs (Sustackova et al., 2012) is common, very few (if any) reports have shown large, high-magnification images of either the pluripotency factors themselves or in conjunction with RNA Polymerase II staining. These images are usually included to confirm the presence of pluripotent cells in differentiation or reprogramming experiments. In addition to the scarcity of high resolution OCT4, SOX2 and NANOG images, mESC culture in 2i/LIF has been shown to increase the proportion of cells within a pluripotent colony bi-allelically expressing Nanog (Miyanari and Torres-Padilla, 2012), meaning NANOG staining in 2i/LIF might yield different patterns than mESCs cultured in LIF alone. Therefore, it is somewhat difficult to place the staining results obtained into the context of relevant literature.

With regards to focalization of pluripotency-associated TFs, one report has published high-magnification images of a green fluorescent protein-tagged (GFP) OCT4 which are distinctly focal (Sustackova et al., 2012). From examining the localizations of OCT4, SOX2 and NANOG proteins following 5 days of 2i/LIF withdrawal and DMSO treatment (Figures 8-22), it is clear that differentiation causes a change in the nuclear-specific localization of these TF foci as seen in pluripotent mESCs. It is difficult to determine what the SOX2 and
NANOG colony-wide weak signal visible in between nuclei after 2 and 5 days of differentiation represent (Figures 16, 17, 21 and 22). Since transcription of both Sox2 and Nanog is significantly lower than mESCs following 5 days of differentiation, and since NANOG has a half-life of 120 minutes (Ramakrishna et al., 2011), it is likely that these colony-wide cytoplasmic foci are artifacts of the immunofluorescence protocol. Indeed, one report shows a similarly noisy Sox2 stain in embryonic day 5 blastocysts which is not seen in OCT4 stainings (Keramari et al., 2010). Another report, albeit at low magnification, shows a cytoplasmic staining pattern of NANOG in 3T3 mouse embryonic fibroblast cells (Chambers et al., 2007). This pattern is not observed in OCT4 stainings of 3T3 cells (Chambers et al., 2007). Interestingly, a putative nuclear export signal has been found in the human NANOG protein, and forced expression of a GFP-NANOG construct with its nuclear localization signals deleted in COS-7 cells causes a localization of NANOG to the cytoplasm (Park et al., 2012). Without further experimental examination (such as protein quantification by Western blot), it is impossible to discount the possibility (however unlikely it may be) that SOX2 and NANOG proteins remain in the cytoplasm after differentiation. It is important to note that these cytoplasmic foci do not affect the cell-counting results as a cell within a colony is only counted as positive if it possesses a strong nuclear OCT4, SOX2 or NANOG signal.

The presence of DMSO has no significant effects on the changes of gene expression induced by 1 day of 2i/LIF withdrawal

As indicated by my results (figures 23-28), the presence of DMSO has no effect on the changes in gene expression induced by 1 day of 2i/LIF withdrawal. Expression of Brachyury and Flk1 processed transcript was significantly higher in mESCs even though they are markers of differentiation. This is most likely due to the generally higher levels of transcription in mESCs as these genes are expressed at extremely low levels in mESCs (100-fold lower than Oct4 processed transcript). In contrast, Kit processed transcript expression in mESCs is only 6-fold lower than Oct4 processed transcript, indicating that Kit expression is not likely due to globally high levels of transcription in mESCs. It is tempting to speculate that DMSO treatment does not have an effect on the long-term (i.e. 2 and 5 days) changes in gene expression induced by differentiation. This must be experimentally verified before such a claim can be made. It is also important to note that the Oct4, Sox2 and Nanog gene
expression results obtained from this 1 day differentiation experiment were in agreement with the 1 day time-point of the long-term differentiation experiment.

**Changes in the expression of Kit, Oct4, Zic3, Klf4 and Stat3 are detected as early as 6 hours after withdrawal of 2i/LIF**

In line with the reports that indicate early time-point changes in the expression profile of pluripotent mESCs, changes in the expression of Kit, Klf4, Stat3, Zic3 and Oct4 are detected 6 hours after the withdrawal of 2i/LIF. Kit processed transcript expression is reduced over 4-fold when compared to mESCs and this drop is maintained for the remaining 18 hours of the experiment. As discussed earlier, the levels at which Kit is expressed is likely too high to represent the leaky transcription associated with pluripotency. The potentially counter-intuitive expression of Kit might warrant further experimental investigation. Oct4 expression is significantly up-regulated after 6 hours of differentiation, as Oct4 primary and processed transcripts are 1.5-fold higher in differentiated cells when compared to mESCs. This is likely biologically relevant as previous reports have shown a similar increase in Oct4 causes differentiation to mesoderm and endoderm lineages (Niwa et al., 2000). This transient increase (and subsequent return to levels similar to mESCs) could represent an initial transcriptional response to trigger differentiation to the mesoderm lineage. It is interesting to note that another publication which quantifies the change in Oct4 expression following 6 hours of LIF withdrawal does not report a similar spike (Zhang et al., 2010). Zhang et al. 2010 also report an upregulation of Sox2 expression following 24 hours of LIF withdrawal. These findings indicate that the response of mESCs to 2i/LIF withdrawal is different than that of mESCs cultured in and withdrawn from LIF alone.

Zic3 is a zinc finger transcription factor necessary for the maintenance of pluripotency in mESCs (Lim et al., 2007). Experimental knockdown of Zic3 causes a significant drop in the expression of Nanog, and cells consequently up-regulate markers of the endoderm lineage (Lim et al., 2007). The promoter of Zic3 is bound by both OCT4 and NANOG (Loh et al., 2006) and siRNA against Oct4, Sox2 or Nanog causes a decrease in the expression of Zic3 (Lim et al., 2007). Furthermore, ZIC3 has been shown to occupy and activate the Nanog promoter independently of OCT4 and SOX2 and Zic3 over-expression during 4 days of LIF
withdrawal maintains *Nanog* expression (Lim et al., 2010). Taken together, these results demonstrate that *Zic3* is extensively connected to the core transcriptional circuit controlling pluripotency. In light of the role *Zic3* plays in pluripotency, the fact that *Zic3* primary transcripts are significantly more highly expressed in differentiated cells was surprising. At the same time, there was no significant difference found in the expression of *Zic3* processed transcripts between pluripotent and differentiated cells. While these results indicate a potential increase in the rate of *Zic3* transcription, this finding is likely not biologically relevant due to the lack of change in the expression of *Zic3* processed transcript.

*Klf4* (a member of the Krüppel-like family) is another zinc finger transcription factor which is involved in the regulation of cell proliferation (Chen et al., 2001) and differentiation (Klaewsongkram et al., 2007). *Klf4* is necessary for pluripotency, as experimental knockdown of *Klf4* leads to a reduction in the levels of *Oct4*, *Sox2* and *Nanog* as well as differentiation (Zhang et al., 2010). *Klf4* knockdown also causes a 5-fold upregulation of *Brachyury* (Zhang et al., 2010). Over-expression of *Klf4* in N2B27 media lacking both LIF and BMP4 maintains the expression of *Oct4*, *Sox2* and *Nanog*, indicating that the pluripotency-maintaining action of *Klf4* is downstream of the LIF/Stat3 and BMP4/Smad pathways (Zhang et al., 2010). Along with *Oct4*, *Sox2* and c-Myc, *Klf4* was one of the four factors virally transduced into MEFs in the first publication reporting the reprogramming of non-stem cells back to pluripotency (Takahashi and Yamanaka, 2006). While it is not necessary for reprogramming, *Klf4* is commonly included in the combination of genes delivered to somatic cells in the generation of induced pluripotent stem cells (Gonzalez et al., 2011). ERK1 and ERK2, which are specifically inhibited by the 2i component PD0325901 (Bain et al., 2007), have been shown to inactivate Klf4 by phophorylation (Kim et al., 2012). Furthermore, the addition of LIF to mESCs cultured in 2i and cyclohexamide (an inhibitor of protein synthesis) caused an up-regulation of *Klf4* (Hall et al., 2009). Taken together, these results demonstrate a significant link between *Klf4* expression and 2i/LIF, explaining why *Klf4* levels drop (and continue to do so) after 6 hours of 2i/LIF withdrawal. A previous publication reports a 0.15-fold reduction of *Klf4* following 6 hours of LIF withdrawal (Zhang et al., 2010). My findings show that 2i/LIF withdrawal causes a 3.5-fold drop in both *Klf4* primary and processed transcript in the same timeframe. While this previous report...
corroborates my findings, the difference in the fold-change of \textit{Klf4} expression when compared to this publication again signifies that the transcriptional response of mESCs to 2i/LIF withdrawal is different than LIF withdrawal alone.

As described in previous sections, LIF binds to the LIF receptor which homodimerizes with the gp130 receptor (Davis et al., 1993). This results in the phosphorylation and activation of STAT3, which translocates to the nucleus to act as a TF (Niwa et al., 1998). STAT3 extensively colocalizes with Oct4, Sox2 and Nanog binding sites, indicating a direct connection between external signaling pathways and the core regulatory circuit of pluripotency (Chen et al., 2008). One report shows that both Stat3 and phosphorylated Stat3 proteins are less abundant in mESCs following only 15 minutes of LIF withdrawal (Mitsui et al., 2003). Trouillas et al. 2009 also show that LIF withdrawal causes a significant reduction in \textit{Stat3} transcription following 24 hours of LIF withdrawal. It is therefore not surprising that expression of \textit{Stat3} processed transcript was found to be significantly down-regulated after only 6 hours of 2i/LIF withdrawal.

\textbf{Changes in the expression of Nanog and \textit{Lefty2} are detected as early as 12 hours after withdrawal of 2i/LIF, while \textit{Tdgf1} changes are detected after 24 hours}

After a further 6 hours of differentiation by withdrawal of 2i/LIF, two additional genes, \textit{Nanog} and \textit{Lefty2} were found to be significantly down-regulated when compared to mESCs. \textit{Lefty2} is a member of the TGF-\(\beta\) superfamily and is involved in left-right patterning in the mouse embryo (Tabibzadeh and Hemmati-Brivanlou, 2006). \textit{Lefty2} is expressed in both pluripotent mESCs as well as mESCs treated with retinoic acid for 24 and 48 hours (Oulad-Abdelghani et al., 1998). While \textit{Lefty2} is commonly used as a marker for pluripotency, there has been little research into its potential targets in mESCs. It is therefore expected that 2i/LIF withdrawal would cause a significant down-regulation of \textit{Lefty2}.

One report demonstrates that the drop in \textit{Nanog} expression is likely due to the preceding down-regulation of \textit{Klf4}. \textit{Klf4} has been shown to bind two sites in the \textit{Nanog} promoter (Zhang et al., 2010). Knockdown of \textit{Klf4} has been shown to reduce the expression of \textit{Nanog} (Zhang et al., 2010). Interestingly, this result is a direct parallel to the expression profile of
cells withdrawn from 2i/LIF from 0-12 hours. After 2 hours of LIF withdrawal, *Klf4* is down-regulated more rapidly than *Nanog* (Zhang et al., 2010). Zhang et al. 2010 also looked at the effects of re-introducing LIF following 16 hours of LIF withdrawal. The subsequent up-regulation of *Klf4* precedes that of *Nanog*, and this result is most pronounced between 0-12 hours following the re-introduction of LIF (Zhang et al., 2010). The combination of these results with my results demonstrates that *Nanog* expression is at least partially reliant on the expression of *Klf4*. In contrast to my results though, Zhang et al. 2010 report only a 0.05-fold reduction in *Nanog* expression after 12 hours of LIF withdrawal. This result, as well as the findings that 2 days of LIF withdrawal results in a 2-fold up-regulation of *Sox2* (Zhang et al., 2010) and 6 hours of LIF withdrawal does not result in a spike in *Oct4* (Zhang et al., 2010) signifies considerable differences in the changes in expression of *Oct4*, *Sox2* and *Nanog* when 2i/LIF withdrawal is compared to LIF withdrawal alone.

*Tdgf1* (also known as *Cripto-1*) has been referred to as a marker of pluripotency (Zhang et al., 2010). Its promoter is bound by both OCT4 and NANOG, and it is a downstream effector in the WNT/β-catenin signaling pathway (Bianco et al., 2010). It is therefore difficult to place the up-regulation of *Tdgf1* found in differentiated cells into the context of the literature. TDGF1 has been studied in human cancer, and results often indicate that its expression is associated with enhanced tumourigenesis in human cancers (de Castro et al., 2010). It is possible that *Tdgf1*, similar to *Lefty2*, plays a role in both the maintenance of pluripotency and in lineage specification.

Changes in expression were also found in *c-Myc, Dppa2* and *Rex-1* following 24 hours of 2i/LIF withdrawal, but the P-values for the differences were all between 0.05 and 0.1. Given that this experiment was only performed three times, and also considering the fact that all of the genes discussed above had P-values well below 0.05, more repetitions should be performed before concluding that 2i/LIF withdrawal causes a significant difference in the expression of these genes.
Future Direction
The results described in this have outlined several avenues potentially worthy of future experimentation. The most logical follow-up to the expanded gene expression study would be to sequence RNA extracted from mESCs differentiated for 6, 12 and 24 hours. This would allow for a complete elucidation of genome-wide changes in expression caused by differentiation. It would also be interesting to examine the degree of pluripotency that mESCs differentiated for 1 day exhibit. While cells subject to this treatment no longer express Nanog, they continue to express Oct4 and Sox2. An embryoid body formation assay similar to the one described by Lako et al., 2001 could be performed. If mESCs withdrawn from 2i/LIF for 24 hours are still pluripotent, their ability to form embryoid bodies should not be compromised. The levels of lineage commitment achieved by any given period of 2i/LIF withdrawal could also be assessed by the re-introduction of 2i/LIF. The time at which the expression of a gene significantly changes could also be examined by the chromosome confirmation capture (3C, or one of its variants, e4C or 5C). If there is a chromatin loop responsible for the transcription of a gene, it is likely disrupted upon the down-regulation of said gene. This thesis has identified several candidates for 3C analysis within the first 24 hours of the induction of differentiation

Conclusions
Pluripotency is a unique and transient cellular characteristic which is crucial for mammalian development. While the nature of pluripotency has nearly limitless potential for personal therapeutic use, the molecular underpinnings of both self-renewal and differentiation must be better understood before this potential can be fully harnessed. This thesis describes the transcriptional changes mESCs undergo as they are induced to differentiate towards the mesoderm lineage. The above results and relevant literature search show that in the long-term, 2i/LIF withdrawal in the presence of DMSO was sufficient to cause mESCs to exit pluripotency. Nanog and Kit expression was found to be significantly down-regulated after 1 day of differentiation, while Sox2 and Oct4 primary transcript expression was significantly down-regulated after 5 days. While Brachyury expression was highly upregulated after 5 days (signifying commitment to the mesoderm lineage), expression of early markers of hematopoiesis was not achieved. When compared to the findings of Lako et al., 2001, these
results confirm the notion that 3-dimensional aggregation, as in EBs, is likely critical for the \textit{in vitro} differentiation of mESCs to the hematopoietic lineage. Differentiation as seen by 5 days was likely BMP4-mediated, given a similar morphological appearance of my cells to other reports (Ying et al. 2003b). 5 days of differentiation also alters the nuclear-specific localization of OCT4, SOX2 and NANOG. My experiments have also shown that the presence of DMSO does not significantly affect the changes in Nanog and Kit expression found after 1 day of differentiation by 2i/LIF withdrawal. Significant changes in Stat3, Klf4, Kit, Zic3 and Oct4 were found after 6 hours of 2i/LIF withdrawal. The rapid loss of expression of Stat3 and Klf4 likely causes a subsequent down-regulation of Nanog after 12 hours of differentiation, while the up-regulation of Oct4 could be playing a role in the ultimate commitment to the mesoderm lineage. This study has therefore characterized both short-term and long-term changes to the transcriptional networks underlying pluripotency in mESCs treated with a simple differentiation protocol.
References


### Appendices

#### Appendix 1. Chart of primers used for RT-qPCR

List of primers used for RT-qPCR in this thesis. All primers (except for Oct4, Sox2 and Nanog primary transcript) were used at a 3 µM concentration. Oct4, Sox2 and Nanog primary transcript primers were used at a 7.5 µM concentration.

<table>
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<th>Primer Name</th>
<th>Description</th>
<th>Sequence</th>
<th>Chromosome</th>
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<th>End</th>
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<td>Exon 2 Left</td>
<td>CCTGACGACGACCCCTCAT</td>
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Appendix 2. Gel electrophoresis of RT-qPCR primer products
Amplicons of all primers used in this thesis and outlined in Appendix 1. Ladder is 2-Log ladder (100 bp-10,000 bp), available from New England Biolabs. (Note, T is another gene name for Brachyury)

List of Primers for top gel, Lanes 1-30:
2-Log ladder, Oct4-EX5, Oct4I1E1, Sox2, Nanog-EX4, Nanog E1I1, Tet1-E3I3, Tet1-EX1, Lefty2-EX1, Lefty2-E1I1, Zic3-EX3, Zic3-E1I1, Rex1-EX4, Rex1-E4I3, Tdgf1-E5I4, Tdgf1-EX4, Fgf4-I2E3, Fgf4-EX1, Dppa2-I1E2, Dppa2-EX2, Klf4-E2I1, Klf4-EX2, cMyc-I1E2, cMycEX2a, Sall4-I2E2, Sall4-EX2, Smad1-EX2a, Stat3-I3E3, Stat3-EX21, 2-Log ladder

List of Primers for bottom gel, Lanes 31-42:
2-Log ladder, Smad1-E3I2, Tcfcp2-I13E13, Tcfcp2-EX7, Kit-E8I8, Kit-EX3, T-I2E3, T-EX2, blank, Flk1-I1E1, Flk1-EX3, GAPDH-E1I1
### Appendix 3. Antibodies used for immunofluorescence

Appendix 3a lists the primary antibodies used for immunofluorescence while Appendix 3b lists the secondary antibodies used for immunofluorescence.

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