DIFFERENTIAL PLURIPOTENT REGULATION IN HUMAN INDUCED PLURIPOTENT STEM CELLS

DIFFERENTIAL PLURIPOTENT REGULATION DEPENDENT UPON DEFINED FACTORS IN HUMAN INDUCED PLURIPOTENT STEM CELLS

By

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ABSTRACT

Human pluripotent stem cells (hPSCs) exist as a heterogeneous population within a dynamic niche, which governs their ability to self-renew and differentiate. Evidence modeled after mouse embryonic stem cells (mESCs) reveals the existence of a developmentally primitive, or homogeneous, state through chemically defined culture methods that is modulated by NANOG, a core pluripotent regulator. However, the differentiation potential and transcription factor control of the homogeneous state in human pluripotent stem cells remains elusive. Previous work suggests that bFGF/ACTIVIN extrinsic regulation provides the heterogeneous nature of hiPSCs with ability to differentiate into several multilineage lineage progenitors. Here, we illustrate that altering the extrinsic environment of hiPSCs with LIF and inhibitors of GSK3β and MAPK/ERK1/2 pathways (LIF/2i), rewires the intrinsic pluripotent regulation of OCT4 and NANOG, which ultimately prevents the *in vitro* hematopoietic differentiation potential. Upon conversion of hiPSCs to a primitive state of pluripotency with LIF/2i, this study reveals that prolonged culture of hiPSCs with LIF/2i erases the hematopoietic differentiation potential through retained expression of the POU domain pluripotent transcription factor, OCT4. Interestingly, shRNA mediated knockdown of *OCT4* recovers the restricted differentiation potential in LIF/2i cultured hiPSCs, while knockdown of *NANOG*, does not. This study identifies a distorted differentiation potential of hPSCs cultured in mouse ESC conditions, despite comparable gene expression profiles and signaling pathway dependence. In efforts to simplify culture methods of human pluripotent stem cells, we identify that alteration of the extrinsic environment highlights explicit differences between human and mouse intrinsic pluripotent regulation, which ultimately controls differentiation efficiency.

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LIST OF ABBREVIATIONS

7AAD 7-amino actinomycin
ActR-II Activin receptor type 2
AKT a.k.a Protein kinase B
ALK Activin like kinase – type 1 receptor
APC Adenomatous Polyposis Coli
BIO 6-Bromoindirubin-3′-oxime
BMP Bone morphogenic protein
bFGF basic Fibroblast growth factor a.k.a FGF2
CB Cord blood
CDM Chemically defined media
CK1 Casein Kinase 1
DMEM Dulbecco's Modified Eagle Medium
Dvl Dishevelled
EB Embryoid body
EpiSC Epiblast stem cell
ERK Extracellular-Signal-Regulated Kinases
ESC embryonic stem cell
FACS Fluorescence activated cell sorting
FBS neonatal bovine serum
Fzd Frizzled
FGFR Fibroblast growth factor receptor
Flt3L Fms-like tyrosine kinase receptor-3 ligand
Gata4 or 6 GATA binding protein 4 or 6
G-CSF Granulocyte - colony stimulating factor
GM-CSF Granulocyte macrophage - colony stimulating factor
GDF Growth and differentiation factor
gp130 Glycoprotein 130
GVHD Graft vs host disease
GSK3 Glycogen synthase kinase 3
HEK Human embryonic kidney cells
hESC Human embryonic stem cell
hPSC Human pluripotent stem cell
hiPSC Human induced pluripotent stem cell
HMG High Mobility Group
HSC Hematopoietic stem cell
HSPG Heparin sulfate proteoglycan
ICM Inner cell mass
IGF-II Insulin growth factor 2
IGFR Insulin growth factor receptor
ICC Immunocytochemistry
ID Inhibitor of differentiation
IL-3 Interleukin 3

IL-6 Interleukin 6
iPSC Induced pluripotent stem cell
JAK Janus kinase
KLF Krüpple-like factor
LIF Leukemia inhibitory factor
LIF/2i Leukemia inhibitory factor plus inhibitors CHIR99021 & PD
LIFR-ß Leukemia inhibitory factor receptor beta
LRP5/6 Low-Density Lipoprotein Receptor-Related Protein 5/6
MAPK Mitogen-Activated Protein Kinase
MEF Mouse embryonic fibroblast
MEF-CM Mouse embryonic fibroblast – conditioned medium
MEK Mitogen-activated protein kinase kinase
mESC Mouse embryonic stem cells
NOD SCID Nonobese diabetic severe combined immunode_cency
OCT4 a.k.a OCT-3/4 encoded by the *POU5F1* gene
OSMK OCT4, SOX2 , c-MYC, KLF4
OSNL OCT4, SOX2, NANOG, LIN28
PBS Phosphate buffered saline
PSC Pluripotent stem cell
RNAi RNA interference
RTK Receptor tyrosine kinase
SB SB431542 Activin Smad2/3 inhibitor
SCF Stem cell factor
shRNA Short-hairpin RNA
SMAD Sma mothers against decapentaplegic
SSEA3 Stage-specific embryonic antigen-3
SOX2 SRY (sex determining region Y)-box 2,
SR Serum replacement
STAT3 Signal transducer and activator of transcription 3
TCF/LEF T-Cell Factor/Lymphoid Enhancer Factor
TRA-1-60 Tumor rejection antigen 1-60
TRA-1-81 Tumor rejection antigen 1-81
TGFβ Transforming Growth Factor β
UM Unconditioned medium

DECLARATION OF ACADEMIC ACHIEVEMENT:

This thesis was completed mainly by the work of Sarah Laronde with the following contributions from individuals in Dr. Mick Bhatia's lab. Dr. Jong Hee Lee generated and performed the initial cell line characterization of bFGF-hiPS and LIF/2i-hiPS cell lines and assisted with hematopoietic differentiation experiments. Kennedy Makondo assisted with FACS sorting and the interpretation of flow cytometry data.

Introduction

1.1 Stem Cells – The Basics

The seminal work by James Till and Ernest McCulloch in the 1960's paved the way for stem cell research by demonstrating first the physical existence of a stem cell[1,2]. Essentially, the biophysicist and the hematologist/oncologist aspired to create an assay to read-out a functional comparison between normal and cancerous radiosensitivity. Upon inoculating irradiated mice with syngeneic bone marrow cells, the spleens developed bumps, which later proved to be colonies of a unicellular origin that had the ability to self-replicate and generate multipotent myeloerythroid cells[2]. This was the first evidence of a multipotent clonal progenitor (or stem cell), referred to as the hematopoietic stem cell (HSC). Stem cells are defined by the ability to self-renew and differentiate into a specialized cell type and exist in either somatic or embryonic tissues (Figure 1.1).

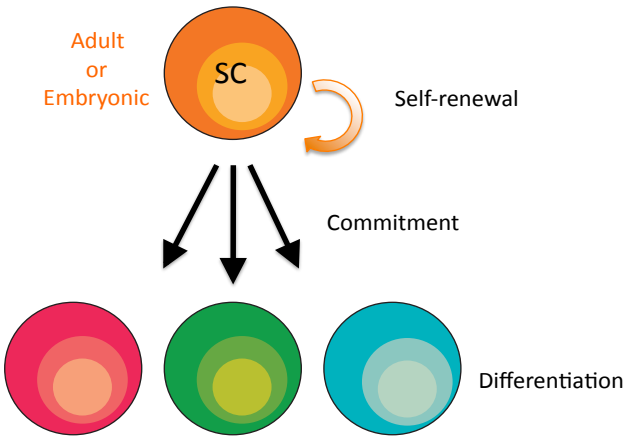


Figure 1.1 Stem Cells Self-Renew or Differentiate
A stem cell has the inherent abilities to either self-renewal indefinitely or commit to a specific lineage where it can terminally differentiate into a specialized cell type. Stem cells reside in adults or embryonic tissues of the body.

The process of differentiation initiates a hierarchy that begins with the self-renewing stem cell at the apex, producing multilineage stem and progenitor cells, followed by the generation of several terminally differentiated cell types that form a living organism. Several types of stem cells exist in vertebrates such as those from hematopoietic[1,2], mesenchymal[3,4], epithelial[5,6], neural[7,8], and intestinal[9,10] origins. However, stem cells are also distinguished by their potency (Figure 1.2). A totipotent stem cell includes germ cells within the gonads and those that arise upon fertilization and formation of the zygote. This stem cell is unique such that it solely possesses the ability to produce all cell types including the extraembryonic and placental tissues, from which pluripotent stem cells are derived. Pluripotent stem cells reside in the inner cell mass of the blastocyst and epiblast, also known as embryonic stem cells (ESC), eventually differentiate to the three germ layers of the embryo proper, the ectoderm, mesoderm and endoderm. Adult stem cells are multipotent and develop further upon germ layer specification, although, the progeny producing potency is limited to a specific tissue.

Successful clinical application of adult stem cells largely surrounds the use of the HSC for allogenic transplantation of patients with inherited and acquired malignant (leukemias, lymphomas, myelomas) or nonmalignant (anemias, thalassemia, severe combined immunodeficiency) hematopoietic disorders since the 1960's. Although less has been reported for embryonic stem cells, the in vitro derivation and propagation of embryonic stem cells has ignited the field of regenerative medicine and developmental biology.

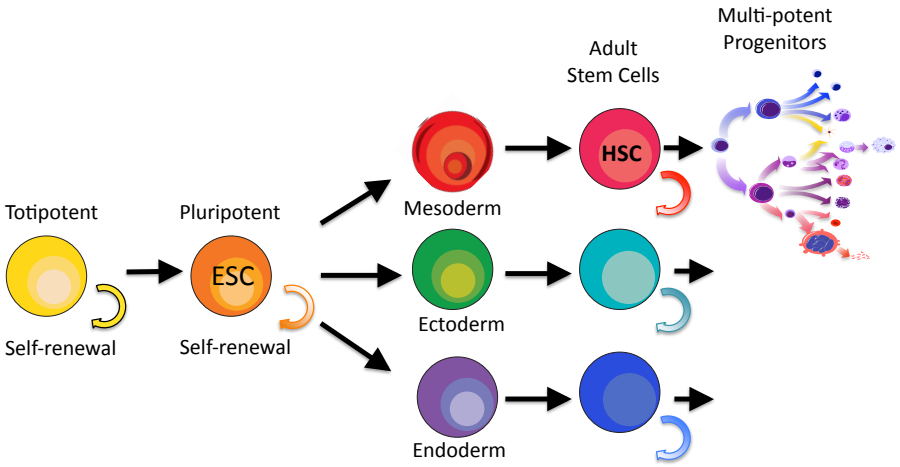


Figure 1.2. Stem Cell Potency is Defined by Hierarchical Stages of Differentiation. Stem cells exist that vary in their ability for multilineage differentiation. Totipotent and pluripotent stem cells are embryonic while multipotent stem cells generate progenitors of a particular germ layer yielding unipotent cells types, as in the hematopoietic system.

1.1.2 Embryonic & Induced Pluripotent Stem Cells

The 1981 breakthrough in developmental biology where Martin[11] and Evans[12] individually explanted the inner cell mass (ICM) of the murine pre-implantation blastocyst from the 129 mouse strain, initiated a wave of research that continues to resonate in science today. This discovery was critical for the successful generation of the first and subsequent transgenic mice[13,14], this discovery became instrumental for studying disease etiology and developmental biology. Mouse embryonic stem cells (mESC), which have the unique properties of unlimited self-renewal and pluripotency, enabled the identification of several cellular parameters such as the signaling networks necessary for self-renewal[15], phenotypic markers and transcriptional regulation of the pluripotent state[16-18]. Ultimately this enabled the discovery of human embryonic stem cells (hESC) derived and explanted from the ICM of several human blastocysts[19] by Jamie Thomson in

1998 (Figure 1.3A). Human ESC lines expressed several extracellular pluripotent markers such the stage-specific embryonic antigens 3 (SSEA3) and 4 (SSEA4), and the keratin sulfate antigens TRA-1-60 and TRA-1-81 and demonstrate pluripotency through teratocarcinoma formation (the *in vivo* differentiation assay, represented by formation of the 3 embryonic germ layers) after intra-testicular injections in mice, features similar to mESC. However, the necessary conditions to maintain hESC lines were quite different from mESCs. Mouse ESCs were initially derived on mitotically inactivated mouse embryonic fibroblasts (MEFs, which represent the feeder layer) with fetal bovine serum (FBS), both of which secrete and contain undefined factors. In serum free culture of mESCs, myeloid leukemia inhibitory factor (LIF)[20] and bone morphogenic protein 4 (BMP4)[15] support undifferentiated growth/self-renewal, however do not support undifferentiated self-renewal of hESCs. Although hESCs do express LIF receptors and demonstrate functional activation of the pathway upon exogenous addition of LIF ligand, LIF signaling does not maintain the self-renewing state of hESCs[21] and BMP4 induces trophectoderm[22] and mesoderm[23] differentiation. Alternatively, fibroblast growth factor 2 (FGF2) and Activin/Nodal produced from the feeder layer, from MEF conditioned media (MEF-CM), maintain hESC in a self-renewing state, conditions that cannot maintain mESCs[24-26 27-29].

Deciphering the essential extrinsic requirements allows us to gain critical understanding of the molecular mechanisms governing the transcriptional regulation of the pluripotent state, and ultimately the ability to generate functional cell types[30]. In addition to several transcriptional regulators, OCT4, NANOG and SOX2 make up the

core transcription factors of the regulatory circuit maintaining the pluripotent compartment in both human and mouse ESCs[17,31,32]. A dramatic change in the view that somatic cell fates are terminal occurred due to the identification of transcription factors specific to the pluripotent ICM, and the observation that the nucleus of a somatic cell, when injected into the enucleated oocyte, could de-differentiate to a pluripotent state[33,34]. This finding and the ethical concerns surrounding the use of human blastocysts to derive hESCs, inspired the discovery of induced pluripotent stem cells (iPSCs) technology. Kazutoshi Takahashi and Shinya Yamanka made the Nobel prize winning discovery that a somatic cell (such as a fibroblast), from either murine[35] or human[36,37] sources, can be reprogrammed to a pluripotent state (Figure 1.3B). They ambitiously screened 18 combinations of transcription factors using lentiviral transduction of somatic fibroblasts, and discovered that only four factors are necessary to achieve a pluripotent state (OCT4, SOX2, c-MYC and KLF4 or OSMK)[35]. This demonstrated an equivalence in the signaling requirements between hiPSCs and hESCs since hiPSCs relied on the same culture conditions defined by hESCs[36,37].

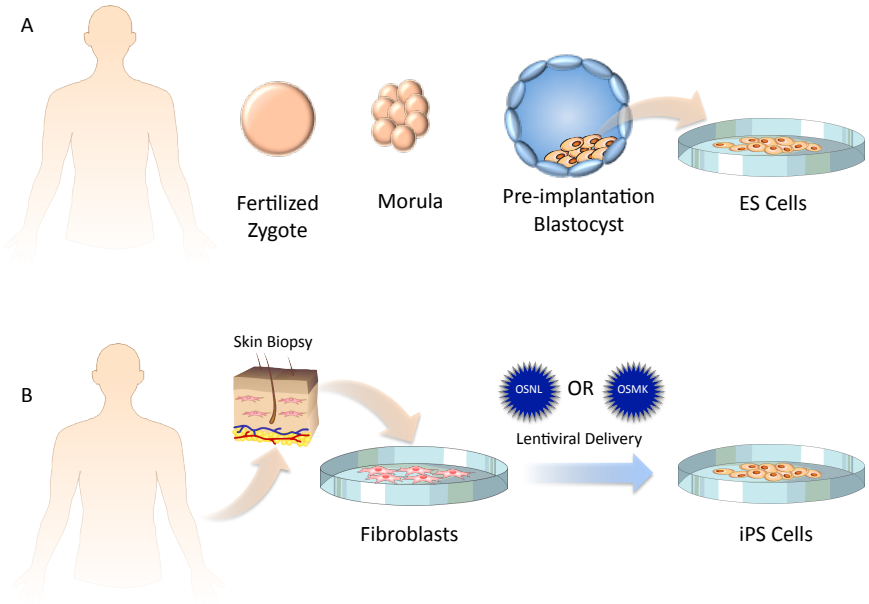


Figure 1.3. Sources of hPSCs: Human PSCs can be obtained from A) pre-implantation blastocysts, or B) through the ectopic expression of pluripotent transcription factors (OSMK or OSNL) in somatic cells, such as fibroblasts.

1.2 Signaling Pathways Regulating the Undifferentiated State

Several signal-transducing pathways modulate both self-renewal and pluripotency in PSCs. However, as noted earlier, the extrinsic factors and intrinsic pathways that maintain human and mouse PSCs are quite different, however a core set of embryonic signaling cascades regulate PSCs which include FGF, Activin/Nodal, BMP4 and Wnt pathways. This section will describe the essential pathways required to maintain the undifferentiated state in human and murine PSCs.

1.2.1 FGF

Fibroblast growth factors (FGFs) signal through receptor tyrosine kinases (RTKs) to elicit pleiotropic effects upon the biology of the cell, including self-renewal, inducing or suppressing differentiation, enhancing survival and proliferation, migration, response to injury and angiogenesis [38]. FGFs have also shown to be essential during embryonic development in mice and humans[39,40]. The FGF family of RTKs consists of four single pass transmembrane receptors, FGFR1 through FGFR4, which have intrinsic tyrosine kinase activity. However, alternative mRNA splicing events produce "b" and "c" isoforms, totaling to seven FGFRs[41]. FGFRs have an extracellular domain made up of three immunoglobulin domains (I-III) that bind FGF ligands (consisting of 22 members) in conjunction with heparin sulfate proteoglycans (HSPG), to stabilize the affinity of the receptor to its ligand. This complex of FGFR, FGF ligand and HSPG activates the dimerization and autophosphorylation of the intracellular tyrosine kinase domain. The signal is transmitted through multiple intrinsic pathways such as phosphatidylinositol 3-kinase (PI3K)/AKT, MEK and JAK/STAT and have high redundancy on FGFR binding.

Basic FGF (bFGF a.k.a FGF2) is one such ligand which elicits its signal through MEK/ERK1/2 via FGFR1, and is essential to maintain hESCs in an undifferentiated state[24-26,28] (Figure 3). FGF2 maintains the *in vitro* stem cell phenotype by preventing the spontaneous induction of trophectoderm, primitive endoderm and neuronal cell types in hPSCs and mouse epiblast stem cells (EpiSCs)[42]. The mechanisms for maintaining the self-renewing state and preventing lineage induction appear to act through routes that remain unclear and contrary to the independence of FGF within the ICM of the human

blastocyst[39,40]. An indirect effect on the supportive feeder layer (inactivated mouse embryonic fibroblasts, MEF) commonly used for hPSC culture has been reported where exogenous FGF2 induces the production of supportive factors while inhibiting negative regulators. Greber *et al.*[43] analyzed different concentrations of FGF2 ligand during the production of MEF conditioned medium (MEF-CM)* used for culturing hESCs. Human ESCs were plated in feeder-free conditions (on Matrigel®) and cultured with MEF-CM (produced with either 0ng/ml or 4ng/ml FGF2), followed by re-stimulation with either 8ng/ml and 4ng/ml FGF2, respectively. MEF-CM produced with 4ng/ml FGF2 generated higher concentrations of Activin A and TGFß1 proteins and express these genes at higher levels than MEF-CM produced without FGF2, which supported hESC self-renewal in unconditioned medium[43]. In addition, whole transcriptome expression analysis upon withdrawal and re-stimulation of FGF2, revealed up-regulation of supportive factors and the concomitant downregulation of negative regulators, such as *BMP4*. A study from our laboratory proposed a similar indirect role for the supportive effect of FGF2 ligand, but in the context of the supportive niche generated from the hESC colonies (hESC-derived fibroblasts, hDFs) cultured in feeder-free conditions. Bendall *et al.*[44] identified that FGF2 acts primarily on the hDF subfraction of hESC cultures, which produce insulin-like growth factor II (IGF-II) ligand, another RTK member. Through functional insulin-like growth factor 1 receptor (IGF1R) antibody blocking, self-renewal and apoptosis assays, IGF-II in turn elicits a direct stimulation through IGF1R on undifferentiated cells that is

* Briefly, MEF-CM is produced by culturing high density inactivated MEFs with hESC medium (KO-DMEM, Serum replacement (SR), NEAA, L-Glutamine, ß-mercaptoethanol) supplemented with 4ng/ml for 8 days which is collected and replaced with fresh hESC medium daily.

pro-survival. In addition, Bendall *et al.* were able to illustrate that receptors of these ligands, IGF1R and FGFR1, localize to the hESC colonies and the supportive niche cells, respectively. It was also seen that the niche cells produce the supportive factor, TGFß1, suggesting it functions to prevents differentiation, in support of the observations by Greber *et al*[43].

Alternatively, but in support of both the direct and indirect model, high concentrations of FGF2 has been reported to maintain hESCs in the absence of feeders for several passages, a methodology which has been routinely used in commercially available chemically defined media (CDM). However, this can also be interpreted as an indirect effect, where high concentrations of FGF2 prevent the activity of SMAD1/5/8 in BMP4/TGFß signaling, ultimately preventing spontaneous differentiation induced by components of the CDM[24] (Figure 1.4). Although the necessity of FGF2 has been clearly demonstrated for *in vitro* hESCs, the exact mechanism of maintaining the self-renewing state has not been conclusive, and remains controversial in the field. Additionally, the requirement of FGF2 is not required for the *in vivo* human blastocyst stage, and FGF2 inhibition does not effect the pluripotent ICM as it does *in vitro* [39,40].

In mice, *in vivo* FGF signaling is necessary for initial lineage segregations in the early embryo. During early blastocyst progression, autocrine production of FGF4 initiates the second wave of differentiation from the inner cells mass, demarcating the GATA6 positive primitive endoderm, or hypoblast, and the NANOG positive epiblast cells within the pre-implantation embryo[45]. FGF4 then becomes restricted to the epiblast cells[46,47] and *Fgf4*-/- null mutation within the ICM is embryonic lethal. In addition, FGF5

expression within the epiblast indicates the transition to the post-implantation embryo and onset of gastrulation[48], and is similarly expressed in EpiSCs cultured in vitro[49,50]. Although FGF4 is expressed within *in vitro* derived mESCs, inhibition of FGF signaling through MEK/ERK1/2 with small molecule inhibitors, such as PD0325901, maintains the stem cell phenotype while blocking lineage commitment[51-53]. This is in complete contrast to the *in vivo* observations and the requirement of FGF signaling in hESCs to maintain the undifferentiated state.

1.2.2 TGFß

Similar to FGF signaling, the TGFß superfamily of cytokines contributes to an array of cellular functions ranging from adhesion, apoptosis and proliferation to embryo development and differentiation in normal and diseased states. TGFß signaling is also an essential regulator of pluripotent self-renewal and differentiation in both mouse and human pluripotent stem cells, and consists of two primary branches: Activin/Nodal or BMP4/growth and differentiation factors (GDFs)[54,55]. TGFß cytokines are dimers that signal through two types of serine/threonine kinase receptors, type I and type II, both of which are essential for signal transduction[54]. However, type II receptors are constitutively active and initiate phosphorylation of the intracellular serine/threonine domains of type I receptors upon ligand stimulation, resulting in a hetero-tetrameric complex. The upstream TGFß type II receptor is also pathway specific; Activin/Nodal ligands bind ActR-IIA and/or ActR-IIB receptors while BMP ligands bind BMPR-II receptors. Type I receptors are activin-receptor-like kinases (ALK) and are less specific than type II receptors. Activins and Nodal bind ALK-4 and/or ALK-7, most BMPs bind

ALK-3 and ALK-6 while other BMPs (such as BMP6 and BMP7) bind structurally similar ALK-1 and ALK-2 receptors. Upon receptor activation, the signaling cascade is orchestrated through SMAD (small body size-mothers-against-decapentaplegic) proteins that include R-SMADs (receptor regulated), Co-SMADs (common-partner), and I-SMADs (Inhibitory). R-SMADs are divided amongst the two branches of TGFß signaling into Activin/ TGFß R-SMADs (AR-SMADs) and BMP4/ TGFß R-SMADs (BR-SMADs). AR-Smads include SMAD2 and SMAD3 (SMAD2/3) and BR-SMADs include SMAD1, SMAD5, and SMAD8 (SMAD1/5/8). The Co-SMAD, SMAD4, is common between the two branches of TGFß signaling and functions as a toggle switch, modulating the cascade of through either SMAD2/3 or SMAD1/5/8, depending on the availability of ligand or inhibition of one of the pathways. SMAD4 binds with one of the R-SMADs to form a hetero-oligomer, and upon translocation to the nucleus, is able to facilitate transcriptional regulation. Inhibitory SMAD, I-SMADs or Inhibins, as the name implies, prevent the intracellular activity of R-SMAD proteins through association with the type I receptor and prevents the association of the R-SMAD with SMAD4. I-SMADs include, SMAD6, which inhibits SMAD1/5 of the BMP4/GDF signaling and SMAD7 inhibits R-SMADs of both TGFß branches.

Although TGFß ligands employ similar molecular mechanisms for signal transduction, they have profoundly contrasting cellular effects in the modulation of PSCs. Activin/Nodal are essential components for the maintenance of hPSCs[56,57], are expressed in both mouse and human ESCs and are required to maintain the epiblast in the developing embryo[58]. As discussed previously, Activin/TGFß proteins are produced from

MEFs during conditioning of hESC medium (MEF-CM) or directly from feeders upon FGF2 stimulation[43], and the phosphorylated SMAD2/3 is detected within OCT4 positive nuclei of hESCs[28]. Inhibition using 10μM of the small molecule inhibitor, SB431542 (which specifically targets only Activin/Nodal/ TGFß receptors and endogenous effectors[59]), induces a differentiated morphology, reduces OCT4 and NANOG protein expression and spares the active BMP effector, SMAD1/5, in hESCs[29]. Using the same potent and specific small molecule inhibitor during embryoid body differentiation results in the induction of neuroectoderm fates[56]. Obliteration of SMAD4 restricts both Activin/Nodal and BMP4 but does not result in the loss of the pluripotent state, confirming the role of Activin/Nodal in stabilizing hESCs rather than promoting pluripotency by occupying SMAD4 from BMP4 ligand[60]. BMP4, which is present in the serum replacement (SR) component of hESC growth media, fails to maintain hESCs and induces trophoblast differentiation in unconditioned medium (UM)[22]; however, the addition of FGF2, recombinant Noggin (a BMP4 antagonist) or Activin A stabilizes undifferentiated proliferation by restraining the activity of SMAD1/5/8[24,61]. It appears the preservation of the hESC identity depends on the reciprocal synergy of FGF and Activin/Nodal signaling to create a blockade, preventing the lineage-inducing cue of BMP4/ TGFß signaling. The molecular basis of this mechanism is suggested to depend on the convergence and up-regulation of the pluripotent transcription factor, NANOG, through binding of the SMAD2/3 and SMAD4 complex to its proximal promoter[62]. Mouse ESCs, conversely, do not lose pluripotency upon inhibition of Activin/Nodal, in contrast to the *in vivo* observations, and are easily sustained in the presence of BMP4[15].

BMP4 induces the expression of inhibitors of differentiation (*ID*) genes, which coordinate with NANOG to impede neuroectodermal fates, similar to the function of FGF and Activin/Nodal in hESCs. Despite the equally opposite signaling requirements between the human and mouse pluripotent identity described thus far, Activin/Nodal and BMP4 do have similar roles in both species during primitive streak formation in the induction of endoderm and mesoderm[30]. Recombinant Activin A is commonly used to derive definitive endoderm in step-wise directed differentiation assays from mESCs[63] and hPSCs[64] which mimics the requirement of Nodal for primitive streak formation and patterning. Similarly, BMP4 is a necessary component in mesoderm specification, and in combination with various concentration of Activin A, efficiently produce cardiac-mesoderm progenitors from both mPSCs and hPSCs[65]. These studies underscore that signaling regulation in mouse development holds translational potential in the generation of mature cells types from hPSCs, although pluripotent maintenance factors contrast and do not mimic *in vivo* requirements.

1.2.3 LIF/STAT3

Leukemia Inhibitory Factor (LIF) is a member of the IL6 family of cytokines that typically function to stimulate macrophage differentiation in leukemic cell lines. Surprisingly, LIF has been discovered as an essential cytokine for the successful implantation of the murine blastocyst into the endometrial lining, but is dispensable in the maintenance of the inner cell mass prior to implantation[66]. Similar to the function of fibroblast feeders in the culture of hPSCs for the secretion of supportive factors, feeders secrete LIF to support the undifferentiated propagation of mESCs[20,67].

LIF and its related cytokines bind gp130 (glycoprotein 130) which heterodimerizes with LIFRß[68]. Upon stimulation with LIF, activation of the tyrosine residues of the gp130 intracellular domain recruits JAK1 (Janus kinase 1) and further activates the transcription factor STAT3 (signal transducer and activator of transcription 3). Exogenous addition of LIF[20,67] (in synergy with BMP4) or the conditional ectopic expression of *Stat3* alone[69], has proven to sustain mESCs in the absence of feeders. During reprogramming of mouse epiblast stem cells (EpiSC)[49,50] to an ES-like pluripotent state, the activation of STAT3 is essential[70,71]. Furthermore, somatic reprogramming of murine fibroblasts to a pluripotent state requires the up-regulation of STAT3, and overexpression of STAT3 in the absence of supportive growth factors permits the formation of miPSCs that are germ line competent[72]. Hall *et al.*[73] have suggested that in mESCs, LIF functions along side transcription factors, such as OCT4, to instruct the expression of other pluripotent transcription factors such as the krüpple-like factors (Klfs), KLF2 and KLF4. This study demonstrated that Klfs are necessary to sustain mESCs and resist lineage commitment in EpiSCs. Similar to the species-specific trend of differential signaling requirements between mice and men, LIF/STAT3 does not sustain hESCs[21,74]. A study by Daheron *et al.*[21] revealed that hESCs express LIFRß and gp130 LIF receptors that are capable of activating the critical phosphorylation site (Y705) of STAT3 through addition of human LIF ligand. LIF stimulation also enabled the nuclear translocation of phosphorylated STAT3, confirming the pathway is functional in hESCs. However, in conventional conditions for hESCs (on feeders or in feeder-free conditions with MEF-CM), only phosphorylation site S727 was active, suggesting active

LIF/STAT3 signaling is not associated with standard hESC requirements. The addition of LIF to standard hESC conditions appeared to impair proliferation and induced loss of pluripotency through reduction of the percentage of TRA-1-60 positive cells. Although LIF stimulated STAT3 can be activated, this pathway does not elicit a beneficial response nor does LIF synergize with hESC supportive factors such as FGF2 and Activin/Nodal.

1.2.4 Canonical Wnt: β-Catenin & GSK3 Inhibition

Wnt signaling modulates several cellular processes in mammals including growth, adhesion, motility and is most notable for body patterning during embryonic development[75]. Wnt signaling consists of two signaling cascades: Canonical and non-canonical pathways. The canonical pathway is the best described and most applicable to the pluripotent state in mouse and human PSCs[38]. Canonical Wnt strictly functions to regulate cellular localization of β-catenin. Absence of Wnt ligand activates the destruction complex (consisting of Axin, APC, and protein kinases CK-1 and GSK3), which targets cytoplasmic β-catenin for proteasomal degradation. When present, Wnt ligands stimulate the Fzd (Frizzled) receptor and the co-receptor LRP5/6 (low-density lipid-related protein 5 & 6). The activated receptors recruit the scaffolding protein, Disheveled, to the Fzd receptor and facilitates the phosphorylation of the LRP5/6 co-receptor by CK-1α (casein kinase 1 alpha). This action relocates Axin near the membrane, preventing the function of the destruction complex, permitting the accumulation of β-catenin within the cytoplasm. β-catenin then translocates to the nucleus, binds the N-terminus of TCF/LEF proteins that convert to transcriptional activators to induce the physiological actions of downstream target genes[75].

The role of the canonical Wnt pathway in maintenance of PSCs, both mouse and human, has been a subject of debate. Prior to the gastrulation of the mouse blastocyst, no convincing evidence has sufficiently demonstrated that canonical Wnt signaling governs the transient pluripotent state of the inner cell mass or epiblast. However, the use of small molecule inhibitors of GSK3 (glycogen synthase kinase-3) such as BIO (bromoindirubin-3'-oxime) and CHIR99021, which prevent the phosphorylation event that targets primed β-catenin for degradation, have demonstrated a positive response in the pluripotent self-renewal of both mESCs and hESCs in vitro[74]. The supplementation of mESCs growth conditions with GSK3 (and MEK/ERK, PD0325901) inhibitors has become routine in combination with LIF and replaces the need of undefined serum or BMP4[52]. Wray *et al.*[76] have suggested the mechanistic role of CHIR99021 to abolish the repressive effect TCF3 has on the binding of pluripotent genes (*Oct4*, *Sox2* and *Nanog*). This action is facilitated through the production of cytoplasmic β-catenin and its nuclear binding to TCF3, sequestering its activity. Others contend that in the absence or inhibition of canonical Wnt, mESCs move towards a differentiated EpiSC-like state whereas the stimulation with Wnt ligand (Wnt3a) preserves full pluripotency[77]. However, opposing claims have stated that mESCs harboring β-catenin knockout or inhibition retain full pluripotent self-renewal[78]. In hESCs, stimulated canonical Wnt signaling increases proliferation but does not recover long-term pluripotent self-renewal in the absence of feeders or supportive factors, while Wnt inhibition does not reveal a negative response[79]. Evidently, a consensus surrounding the role of canonical Wnt in these two species has not been met, and canonical Wnt does not appear to have an opposing position in the balance

between self-renewal and differentiation in mouse and human PSCs as in the aforementioned pathways.

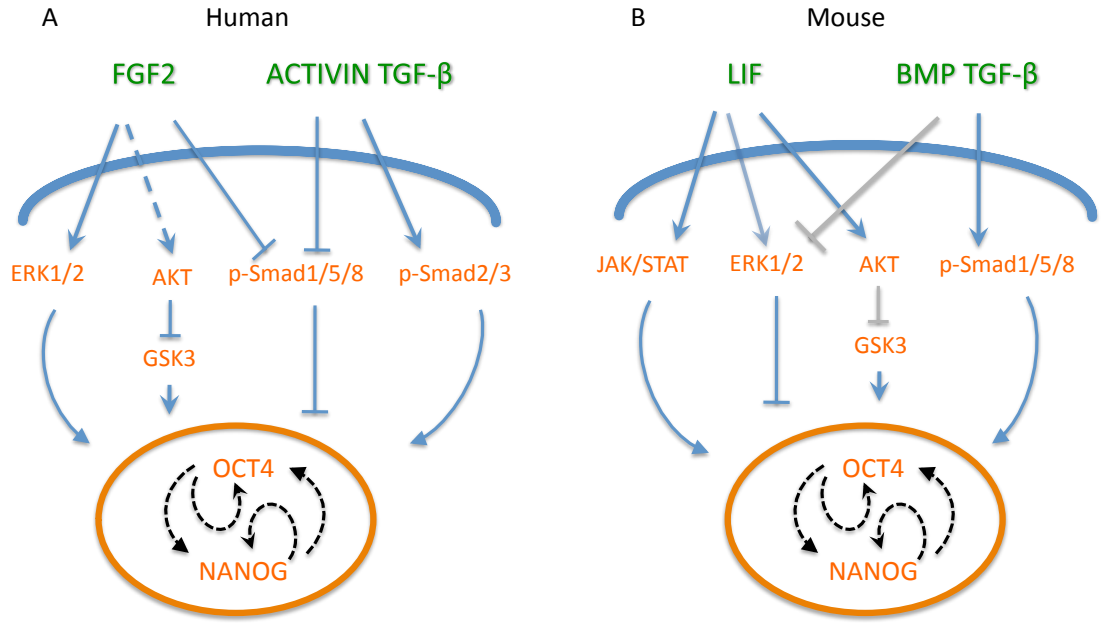


Figure 1.4: Players of the Signal Transduction Pathways in Human and Mouse PSCs are not fully conserved. The signaling networks required to maintain the pluripotent self-renewing state are in constant balance with differentiation inducing cues. However, the requirements are contrasting, although they similarly converge on pluripotent transcription factors OCT4 and NANOG. Human ES and iPS cells rely on the stimulation of MEK/ERK1/2 and PI3K/AKT via exogenous FGF2, and Smad2/3 through the ACTIVIN/TGFβ pathway. The pro-self-renewal affects of FGF2 and ACTIVIN stimulation also manifest through the simultaneous inhibition of BMP4/TGFβ pathway effector, SMAD1/5/8, which would otherwise induce a trophectoderm or mesoderm differentiation program. In mES and iPS cells, BMP4 is a positive regulator of the pluripotent state, which prevents the inhibitory effects of MEK/ERK1/2 on pluripotent transcription factors, OCT4 and NANOG, in contrast to hPSCs. Self-renewal of mESCs is dependent on LIF/JAK/STAT3 signaling and would spontaneously differentiate its the absence. LIF in combination with 2i (GSK3 and MEK/ERK inhibitors, grey arrows) has recently replaced the need for BMP4. In hPSCs, LIF/JAK/STAT pathway is inactive; no stimulatory or inhibitory implications have been uncovered for LIF signaling in hPSCs.

1.3 The Transcriptional Pluripotent Network

During the development of the ICM of the early embryo, transcription factors, in addition to paracrine and exogenous factors, dictate the intrinsic mechanisms involved in cell fate decisions. Specifically, OCT4, NANOG and SOX2 are the core set of transcription factors that modulate pluripotency in the developing mammalian embryo and within *in vitro* derived PSCs[16-18,31,32]. Each are highly expressed in the ICM and epiblast, distinguishing them from the trophectoderm and primitive endoderm (hypoblast), respectively. Upon implantation and gastrulation, the pluripotent transcription factors become downregluated[40,80]. Combined, they function in a feedforward, autoregulatory loop controlling the expression of several downstream targets and are essential for maintaining the pluripotent state in human and mouse models[31,32].

1.3.1: OCT4

OCT4 (octamer binding transcription factor) is a POU (PIT/OCT/UNC) family transcription factor containing a homeodomain, and is encoded by *Pou5f1* gene. OCT4 is expressed in totipotent cells of the dividing zygote and within all cells of the pluripotent ICM of the developing blastocyst, but not in the surrounding trophectoderm cells. Functional knockdown studies in murine embryos demonstrate that OCT4 is necessary in the formation of the pluripotent ICM and the subsequent formation of the hypoblast at the epiblast stage[16]. Overexpression of OCT4 results in differentiation to primitive endoderm and mesoderm fates[81,82], demonstrating the level of OCT4 in PSCs requires a critical balance.

1.3.2: NANOG:

NANOG is a homeodomain containing transcription factor discovered through functional expression screening of cDNA libraries, and is able to sustain undifferentiated mESCs harboring a LIF knockout mutation[18,83]. The examination of *Nanog* null embryos revealed its necessity to establish the epiblast *in vivo* and is required for explantation of the mouse blastocyst[83]. The expression pattern of NANOG, however, is not identical to that of OCT4. Nanog is confined to the epiblast, and is distinguished from the GATA4 (a marker of primitive endoderm) positive cells of the hypoblast, which retain OCT4 expression[80]. NANOG and OCT4 within the nuclei of *in vitro* mESCs also show heterogeneous expression, and *Nanog* null mESCs retain the expression of OCT4 and self-renewal capacity, but are prone to differentiate[84]. Therefore, NANOG and OCT4 seem to play different roles in regulating pluripotency, where OCT4 is essential for pluripotency acquisition while NANOG appears to sustain and prevent loss of the pluripotent state.

1.3.3: SOX2:

SOX2 (sex determining region Y-box 2) is an HMG (high mobility group) transcription factor required in addition to OCT4 and NANOG[85] to maintain pluripotent stem cells of the developing embryo and *in vitro*. Additionally, SOX2 facilitates neuronal development, marking neural progenitors[86]. SOX2 was originally thought to function as a co-factor with OCT4 to facilitate pluripotent regulation by targeting the OCT4-SOX2

enhancer elements within pluripotency associated genes[87], including NANOG[17]. However, targeted knockdown studies of *SOX2* in mESCs resulted in the reduction of OCT4 while OCT4-SOX2 enhancer elements remained active. *OCT4* overexpression recovered the undifferentiated state in *SOX2* deficient mESCs, suggesting its dispensability for activating OCT4-SOX2 enhancers and a more precise role for maintaining the expression of OCT4[88].

1.3.4: Regulating OCT4 & NANOG in Mice and Men:

OCT4 and NANOG are the best described factors in both human and mouse PSC lines[16-18,32,62,80,83,87,89-91]. Although the core set of transcription factors controlling pluripotency are conserved between these species, upstream regulators and downstream targets are not well conserved, likely due to species-specific differences in early embryo development[32,45]. This is evident through the differences in optimal culture conditions for human and mouse PSCs in order to prevent precocious differentiation described earlier. Briefly, FGF2[24,42-44] and Activin/Nodal[28,29,56] maintain the undifferentiated state of hPSCs by preventing neuroectodermal and BMP4 induced trophoblast differentiation by converging upon and up-regulating *NANOG*[62,92]. Addition of BMP4 induces differentiation in hPSCs through the up-regulation of SMAD1/5/8 with the concomitant downregulation of SMAD2/3 signaling[22,24] (Figure 1.4A). This leads to the reduction in *NANOG* as well as other pluripotency regulators, such as *OCT4* expression, and ultimately the loss of the undifferentiated state[62]. This is in contrast to the requirements for mESCs, which are maintained through the convergence of BMP4 and LIF/STAT3

signaling pathways on *OCT4* and *NANOG* to positively regulate the undifferentiated state[15,52]. Stimulation with FGF and Activin signaling induces differentiation, but this response is suppressed by BMP4 signaling by inhibiting ERK1/2 and SMAD2/3 phosphorylation[15,93,94] (Figure 1.4B). NANOG is also described as the gatekeeper of ground state[18,80,84], while OCT4, when highly expressed, is described as an impediment to the ground state and establishes a bias towards mesendoderm[82]. Overexpression of *NANOG* with the concomitant downregulation of *OCT4*, through either small molecule inhibitors or transgenic expression, stabilizes the ground state in differentiation prone EpiSCs and partially reprogrammed iPSCs[80,90,95].

The molecular regulation in hPSCs also reveals a dynamic modulation, however, a consistent lineage-specific role in hPSCs has not yet been observed for OCT4 or NANOG. A recent RNA interference study in several hESC lines reveals a modular regulation among the three core pluripotent regulators that is cell line dependent[96]. The deviation in steady-state *OCT4* expression induces differentiation, while BMP4 levels can modulate lineage-specific fates. Individual knockdown of *OCT4* or *NANOG* results in the differentiation of hESCs evident through their reciprocal down-regulation, with the exception *SOX2*. The lack of hESC differentiation following downregulation of *SOX2* reveals its dispensability in pluripotent self-renewal, which appears dependent on the co-regulation of only OCT4 and NANOG. Interestingly, and synonymous with mESCs, *SOX2* overexpression relative to *OCT4* results in neuroectoderm differentiation, consistent with its secondary role as a neural progenitor marker[82,86]. These characteristics place SOX2 further down the hierarchy of human pluripotent regulators demonstrating

the expendability of this transcription factor in comparison to OCT4 and NANOG[96]. Although the signaling dependencies are not conserved between murine and human *in vitro* pluripotency, they likewise positively regulate the core transcriptional machinery, and underscore OCT4 and NANOG as critical regulators.

1.4 Mouse & Human Pluripotent Stem Cells - The Controversy:

The pioneering explantation of mESCs[11,12] and hESCs[19] has ignited an abundance of information describing the potential of PSCs for regenerative medicine. With the advent of iPSCs, the potential has been magnified to disease modeling[97] and drug screening endeavors[98,99]. Most interestingly, iPSC technology has opened the gateway for patient and tissue-specific directed differentiation, holding great promise for the future of regenerative therapies. Evidence to date describes either the mechanisms characterizing a singular self-renewing pluripotent state or how exogenous factors manipulate the intrinsic signaling networks toward differentiation in a unidirectional fashion[100]. Specifically, these models consider differentiation to occur from a singular undifferentiated state to a terminally differentiated cell type of choice. The derivation of EpiSCs from the explanted epiblast of the post-implantation murine embryo[49,50], or from somatic cell sources[101], challenges the concept that only one *in vitro* state of pluripotency exists.

1.4.1 Early Embryo Development

The ability to isolate an *in vitro* epiblast-like state is fitting with *in vivo* embryonic development (Figure 1.5), where two states of pluripotency are distinguished by the pre-implantation and post-implantation embryo; namely, the inner cell mass (ICM) of the blastocyst and the epiblast[45]. The formation of the ICM represents the first stage of differentiation where cells of the morula compact and bifurcate into cells of the trophectoderm and those of the pluripotent ICM. The cells of the ICM are a relatively primitive state compared to the epiblast, which is derived from the ICM. The epiblast forms as a result of a second round of differentiation, where the pluripotent ICM produces the hypoblast, or primitive endoderm, which contributes to the formation of the yolk sac. The epiblast arises upon implantation of the embryo into the endometrial lining of the uterus where it differentiates into the three embryonic germ layers of the embryo proper: ectoderm, endoderm, and mesoderm[102]. Thus, the epiblast represents a more differentiated state compared to the primitive blastocyst, but represents a necessary transitional state towards the differentiated embryo.

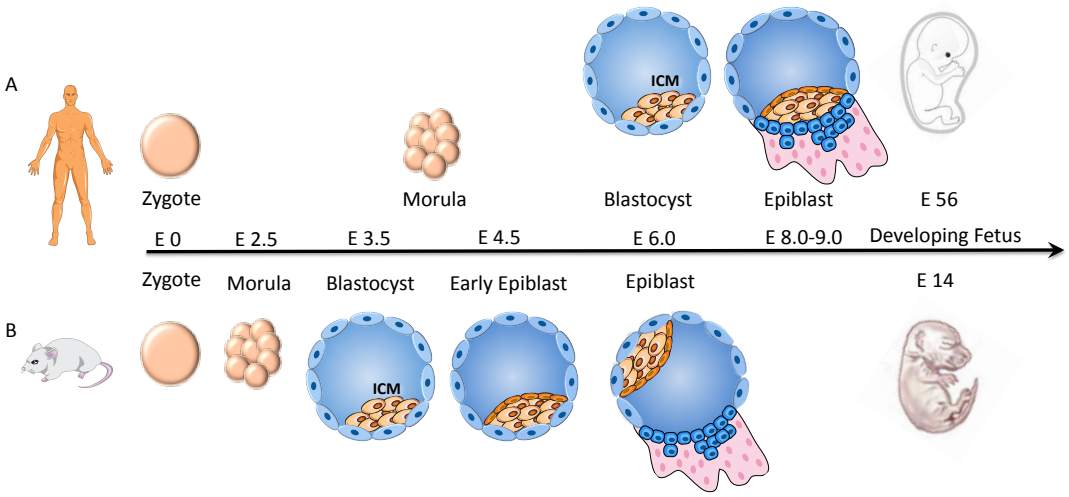


Figure 1.5. Timing of Early Embryo Development in Humans and Mice, are Different:
A) Human embryo development progresses from a zygote (fertilized oocyte) into a maturing fetus through a series of symmetric divisions leading to the morula. At the 16 cell stages, the morula begins a series of compaction stages where the inner and outer cells polarize and bifurcate into cells of the trophectoderm and the pluripotent cells of the inner cell mass (ICM). This stage is finalizes on embryonic (E) day 6.0 and is referred to as the blastocyst. Upon further maturation and implantation into the endometrial lining of the uterus, the cells of the ICM further segregates and produces a layer of differentiated cells known as the hypoblast. At this stage, the ICM is referred to as the epiblast, which differentiates to all of the cells types that contribute to the embryo proper. **B)** Early development of the mouse embryo proceeds similarly to human development, however, the timing of symmetric divisions and compactions occurs earlier than in humans. Compaction into the blastocyst occurs at the 8-cell stage and the formation of the hypoblast and epiblast occurs prior to implantation and the localization of the epiblast relative implantation is unlike that of the human epiblast.

1.4.2 Epiblast Stem Cells & Human PSCs:

Mouse EpiSCs have been derived and sustained in growth conditions described for hPSCs and share several features[42,49,50,101], suggesting that hPSCs better represent the epiblast, rather than the preimplantation blastocyst from which they were derived. EpiSCs and hESCs form flat, two-dimensional colonies refractory to clonal expansion, unlike mESCs. EpiSCs express markers associated with the epiblast such as FGF8 and Nodal[42,49]. Their self-renewal also depends on the cooperation of MEK/ERK[24] and Activin/Nodal signaling with the pluripotent network[62]. Differentiation similarly occurs

in the presence of BMP4[92], and addition of LIF is not sufficiently supportive for propagation, in direct contrast to mESCs. Nonetheless, hESCs retain qualities of the pre-implantation blastocyst such as the expression of REX1, a marker associated with the early inner cell mass that is distinctly expressed in mESCs. FGF5 and FGF8, which are associated with the post-implantation epiblast, is neither expressed in hESCs nor mESCs, but is uniquely expressed in EpiSCs[42,103]. However, markers such as OCT4, SOX2, NANOG, REX1, STELLA, TBX3, and KLF4 show enhanced expression in mESCs relative to both hESCs and EpiSCs[11,12,49,50] (Figure 1.6), making the case for a clearly defined *in vitro* developmental state confusing.

Figure 1.6: Comparison of Human and Mouse Pluripotent Stem Cell Properties.

		Signaling Requirements					*Pluripotency-related Genes*										*Tests of Pluripotency*		
Pluripotent Stem Cell	Ref.	Activin /Nodal	BMP4	bFGF	MEK/ ERKi	GSK3i	OCT4	NANOG	SOX2	KLF4	REX1	STELLA	OTX2	FGF8	FGF5	XCI Status	EB Differentiation	Teratoma	Chimerism Contribution
N-Human PSCs	107, 108 120	✗	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✗	?	?	XaXa	Yes	Yes	N/D
Human PSCs	19, 21, 22. 24, 26, 42	✓	✗	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗	Varied	Yes	Yes	N/D
Mouse PSCs	11, 12, 15, 20, 51, 52,53, 67	✗	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗	✗	XaXa	Yes	Yes	High
Mouse EpiSC	42, 49, 50,101	✓	✗	✓	✗	N/D	✓	✓	✓	✗	✗	✗	✓	✓	✓	XaXi	Yes	Yes	low

N - naïve, XCI - X chromosome inactivation, EB - embryoid body
Ref. – References
N/D – not determined

Evidence established in the mouse continues to assert that mESCs represent the early development of the pre-implantation blastocyst and hPSCs represent the developmentally advanced post-implantation epiblast[42,50,104,105]. The *in vitro* distinction of two states of pluripotency have since been termed the "naïve" and "primed" states of pluripotency, respectively[93]. However, this concept remains controversial because distinct states are seemingly attainable only in specific rodent strains with permissive genetic backgrounds that allow the derivation under specific growth conditions[106-108]. However, the modified cocktail of chemically defined small molecule inhibitors plus LIF referred as LIF/2i (dual inhibition of MEK/ERK and GSK3) was found to sustain both permissive and non-permissive rodents strains alike[52,106,108]. This suggests that extrinsic manipulation of the culture environment alone, not the permissiveness of genetic background, influences the ability to make this division[104,105,109,110]. Furthermore, it has been suggested that the similarity between hESCs and EpiSCs may be an *ex vivo* artifact; where hESCs have transitioned to an epiblast-like state during conventional *in vitro* derivation methods that are not representative of the physiological environment[110].

Comparative phenotypic evaluation of hESCs (and EpiSCs) to mESCs suggests hESCs retain features of the pre-implantation embryo, from which they were derived. However, the outlined features such as the marked contrast in extrinsic requirements, signaling cascades and "markerology" make the extrapolation of mouse developmental biology to the human system vague. Ethical limitations prevent the derivation of human EpiSCs from the post-implantation embryo, making the direct comparison of human

EpiSCs to hESCs impossible to confirm whether naïve and primed states exist in hESCs, as defined in the mouse.

1.4.3 Generation of Naïve Human Pluripotent Stem Cells:

Alternatively, deriving hPSCs in mESC culture conditions (LIF/2i) appears to be a sufficient route to explore whether hPSCs can resolve a ground state defined in the mouse. The potential of mouse-like hPSC cell lines lies in the amenability of mESCs to genetic manipulation, a property that has been difficult to achieve in hPSCs[111,112]. Through replacement of FGF2/Activin dependent conditions with LIF/2i, several groups[113-115] have claimed the existence of mouse-like hPSCs.

Li *et al.*[113] attempted to derive mouse-like human iPSCs through lentiviral overexpression of *OCT4, SOX2, NANOG and LIN28* in human fibroblasts, in the presence of LIF and 3i (MEK, GSK3β and TGFβ inhibitors). These LIF/3i-dependent hiPSCs maintained pluripotency verified by teratoma formation and pluripotent marker expression, in addition to tolerance of clonal expansion. Multilineage differentiation potential was assessed through the standard Embryoid Body (EB) assay. Differentiated LIF/3i-dependent hiPSCs immunostained positive for several lineages, however, this method was purely qualitative. Further comparative analysis to mESCs and hPSCs such as gene expression profiling and quantitative marker expression was lacking, making the conclusion of definitive mouse-like hiPSCs meager. Buecker *et al.*[114] approached the task differently and generated hiPSCs using only LIF. However, LIF alone was not able to achieve full pluripotency verified by the lack of teratoma formation, and failed to

activate endogenous expression of the core pluripotent genes, suggestive of a partially reprogrammed state. Furthermore, Buecker and colleagues attempted gene targeting through selection of homologous recombination events at the hypoxanthine phosphoribosyltransferase (*HPRT*) locus hoping that LIF derived hiPSCs would be useful for transgenic manipulation, similar to mESCs. Contrasting a previous report targeting the same *HPRT* locus in standard hESCs[111], the transgenic efficiency reported by Buecker and colleagues was significantly lower, making the gene targeting claim unsatisfactory.

Most recently, Hanna et al.[115] established LIF/2i-dependent hiPSC and hESC lines. Using a doxycycline-inducible lentiviral system for *OCT4, SOX2* and *KLF4,* combined with LIF/2i, resulted in the reprogramming of human fibroblast to mouse-like pluripotent state achieving teratoma formation, pluripotent marker expression, X chromosome reactivation, mESC signaling dependency, and mouse-like gene expression and epigenetic profiles. This approach, however, depended on constitutive expression of the lentiviral transgenes, an undesirable side effect in clinical applications of hiPSCs[97]. To circumvent this issue, Hanna and colleagues screened a panel of small molecules by systematically removing one factor at a time to determine those that would allow transgene-independent propagation in LIF/2i. The addition of forskolin briefly displaced the need for transgenic expression; however, mouse-like hESCs underwent spontaneous differentiation and death. This suggests that the stability and utility of these cells was compromised. In addition, standard EB differentiation assays demonstrated multilineage potential of LIF/2i hPSCs but the differentiation potential was not quantified and

compared to standard hES or hiPS cell lines to determine whether LIF/2i hPSCs are better, or at least functionally equivalent.

These studies highlight the significant influence of culture conditions on the qualitative analysis of hPSCs. The ability to isolate hPSC colonies in "naïve" conditions suggests the existence of heterogeneity within hPSC cultures, containing a population that can be propagated in mouse conditions, similar to the ability to revert mouse EpiSCs to a primitive state with LIF/2i[116]. However, these studies do not necessarily close the gap in understanding the discrepancies between human and mouse developmental biology. It would be necessary to demonstrate the ability of LIF/2i-dependent hPSCs to transition through an epiblast-like state during differentiation, similar to the observations in mESCs.

1.5 Rationale:

Current assessments of pluripotential in LIF/2i-dependent hPSCs remain qualitative and lack functional and quantitative comparisons of pluripotent self-renewal and multilineage differentiation. Such evaluations are necessary to determine the functional utility of new hPSC cell lines for the future of regenerative medicine. Most intriguingly, the effect of the extrinsic environment on the molecular mechanisms that modulate pluripotency is largely undefined for hPSCs cultured in mouse conditions. Whether LIF/2i influences the molecular regulators of pluripotency, such as OCT4 and NANOG, ultimately changing the balance between self-renewal and differentiation is unknown. Studies in our laboratory have established LIF/2i-dependent hiPSCs that share

features of *bona fide* mESCs. The results discussed in this document surround the experimental testing of the following hypothesis:

1.6 Hypothesis:

The use of functional and quantitative assays for pluripotent self-renewal and directed differentiation in LIF/2i-hiPSCs will demonstrate signaling dependency, self-renewal and differentiation propensities that contrast with hPSCs dependent on bFGF. I predict that altering the extrinsic conditions with LIF/2i will enhanced pluripotent self-renewal, as reported for LIF/2i-hiPSCs[107,114,115,117] (and explored in our lab), while drawing at the expense of efficient differentiation.

1.7 Experimental Objectives:

Therefore, this thesis aims to:

1. Evaluate the signaling dependency of LIF/2i-hiPSCs while quantitatively monitoring the variation in expression of pluripotent factors, OCT4 and NANOG, to distinguish between LIF/2i- and bFGF-dependent hiPSCs.

2. Employing lentiviral based RNA interference to assess the functional requirement of *OCT4* and *NANOG* in LIF/2i-hiPSCs in pluripotent self-renewal and differentiation.

Chapter 2

Methods & Materials:

2.1 Human iPSC Cell culture:

Human iPSC lines were cultured on Matrigel® (BD Biosciences) in mouse embryonic fibroblast (MEF) conditioned medium (MEF-CM) supplemented with 8 ng/ml human recombinant basic fibroblast growth factor (bFGF, Life Technologies) as previously described[23]. Alternatively, hiPSCs were also cultured on irradiated MEFs with iPS medium: DMEM/F12 base (Gibco) with 20% Knockout Serum Replacement (Gibco), 100 μM β-mercaptoethanol, 100 μM nonessential amino acid (Gibco), and 1 mM L-glutamine (Gibco) supplemented with 10 ng/ml bFGF (Life Technologies). LIF/2i-dependent hiPSCs were cultured on Matrigel® (BD Biosciences) also with iPSC medium but supplemented with 10ng/ml leukemia inhibitory factor (LIF), 3μM CHIR99021 (specific GSK3 inhibitor, Stemgent) and 1μM PD0325901 (specific MEK/ERK1/2 kinase inhibitor, Stemgent). Cells were fed with fresh medium daily and confluent cultures were mechanically or enzymatically passaged every 5-7 days using 100 Units/ml of Collagenase IV (Invitrogen). One clone each of LIF/2i-hiPSC and bFGF-hiPSC were analyzed for this study.

2.2 Preparation of Lentiviral Vectors for iPS Generation and RNAi of *OCT4* and *NANOG*:

For iPS reprogramming, plasmids pSIN-EF2-OCT4-Pur, pSIN-EF2-SOX2-Pur, pSIN-EF2-Nanog-Pur, and pSIN-EF2-LIN28-Pur (Addgene) were packaged and produced in $20x10^6$ confluent HEK293FT cells using 2nd generation packaging plasmids, psPAX2 and pMD2.G, after incubating plasmids with Lipofectamine LTX for 20minutes at room temperature. Virus was collected 72 hours post transfection and concentrated by ultracentrifugation for 2 hours at 4°C. Virus was titered with HeLa cells according to standard procedures and used for induction of hiPSCs as described previously[2].

For RNAi mediated knockdown of *OCT4 (POU5F1)* and *NANOG*, the lentiviral vector LentiLox3.7 containing the eGFP reporter (Figure 2) under the control of the CMV promoter were constructed as described[118]. Oligonucleotides targeting human *OCT4* and *NANOG* were generated, designed and subcloned into the pLL3.7 vector under the control of the U6 promoter as described by our lab[119]. pLL3.7_eGFP (sh-Control), pLLOct4i_eGFP (sh-OCT4), or pLLNanogi_eGFP (sh-NANOG) plasmids were sequence verified and transfected into HEK293FT cells with 2nd generation packaging plasmids to produce lentivirus as outlined above for iPS generation.

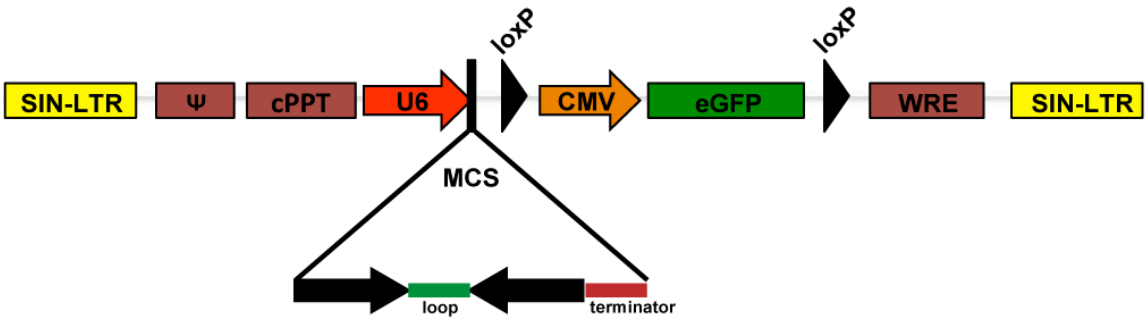


Figure 2. LentiLox3.7 shRNA vector backbone[118].

2.3 Preparation of Primary Cells for Induction of Pluripotency:

Human dermal fibroblasts or human cord blood cells were obtained from healthy consenting donors. $1x10^5$ Human adult fibroblasts were plated onto gelatin coated tissue culture plates in fibroblast medium containing: DMEM, 10% fetal bovine serum, 100 μM nonessential amino acid (Gibco), 1 mM L-glutamine (Gibco) and 1mM sodium pyruvate (Gibco). $CD34^+$ cord blood cells, used for hUCB-iPSCs generation, were isolated by immunomagnetic separation, and then seeded onto Retronectin (Takara BIO, Inc.) coated tissue culture plates prior to lentiviral transduction. Cells were spin-transduced for 90 minutes at 1300rpm at 30°C with concentrated lentiviral vectors in the presence of 8 μg/ml polybrene (Millipore) 24 hours after plating in their respective optimal growth media. The following day, the medium was replaced 1:1 with primary culture medium outlined above and iPSC medium containing 10ng/ml bFGF. Afterwards medium was completely switched to iPSC medium containing 10ng/ml bFGF thereafter. Individual colonies (clones) were mechanically isolated, expanded and frozen. Specific primer sets were used to detect the coding regions of both the endogenous pluripotent genes and the

transgenes used during reprogramming (Total), 3' UTR region (Endogenous), or the region of the viral transgenes (Exogenous).

2.4 Lentiviral spin-transduction of hiPSCs:

LIF/2i-iPSCs were spin-transduced with lentivirus and 8 ng/ml polybrene (Chemicon Int.) 2-3 days after seeding in iPS media supplemented with 3μM CHIR99021 (Stemgent), 1μM PD0329501 (Stemgent), 10ng/ml LIF (Millipore). Plates were wrapped tightly with plastic wrap and centrifuged with lentivirus for 90 minutes at 1300rpm and 30°C. The next day, the media was changed with fresh iPS medium with LIF/2i and daily thereafter until cells were harvested for analysis.

2.5 Assessing signaling dependency:

hiPSCs were plated into 24 or 48 well tissue culture plates coated with Matrigel in their respective growth medium. The next day the medium was changed and replaced with base iPS medium supplemented with either: 100ng/ml human recombinant Activin A (R&D Systems), 10μM SB431542 (TOCRIS), 100ng/ml human recombinant BMP4 (R&D Systems) and 100ng/ml human recombinant NOGGIN (R&D Systems). Controls were either untreated cells in basal iPSC medium or iPSC medium supplemented with either bFGF or LIF/2i. Cells were cultured for 5 days then processed for flow cytometry.

2.6 Clonogenic Assays and FACS sorting:

5,000 of bulk single cell dissociated bFGF-hiPSCs, +/- growth factor (from section 2.5 above) LIF/2i-hiPSCs or EBs cells were plated onto irradiated MEFs in 6 well

tissue culture treated plates in their optimal basal growth medium. Colony initiation was monitored up to 14 days at which time colonies were washed with 1xPBS (with magnesium and calcium) then fixed with 4% paraformaldehyde stained for TRA-1-60 colonies, which were then enumerated. FACS sorting for clonogenic assays: transduced LIF/2i-hiPSCs were dissociated 3 days post-transduction to single cells with cell dissociation buffer and PEF buffer (1xPBS, 1mM EDTA and 0.5% FBS and 1x Penicillin/Streptomycin) then filtered through a 40μm strainer. Cells were counted and resuspended to ~$1x10^6$ cells/ml in PEF buffer. 1:50 dilution of 7-aminoactinomycin D (7-AAD) was added. Cells were sorted using the FACSAria cell sorter (BD Biosciences). LIF/2i-hiPSCs were gated on 7AAD(-)/GFP(+) fraction at a sort purity of 98%. 5,000 and 10,000 cells were sorted into 12 well tissue treated plates containing irradiated feeders in iPS medium with LIF/2i. Colony initiation was also monitored up to two weeks. Colonies were stained for TRA-1-60-Dylight 488 (Stemgent) and Hoechst 33342 then enumerated.

2.7 Microscopy & Image Analysis:

TRA-1-60-Dylight 488 (Stemgent) stained colonies generated from the clonogenic assays were imaged using the Perkin Elmer Operetta high content image analysis system. Images were analyzed with Columbus/Acapella data management and image analysis software package. Image analysis was performed with custom generated scripts where colonies were segmented first using the Hoechst 33342 signal followed by quantifying the fraction of Dylight-488 positive colonies.

2.8 Immunocytochemistry:

Cells were washed once with 1xPBS containing Calcium and Magnesium (Lonza) then fixed with 2% paraformaldehyde using the BD Cytofix fixation buffer. If permeation was needed, cells were treated with 1xCytoperm Wash Buffer containing saponin (BD Biosciences) prior to staining. Appropriate primary and fluorochrome-conjugated secondary antibodies were used. The following antibodies were used for extracellular staining: SSEA3 and SSEA1 (Developmental Hybridoma Bank) and Tra-1-60 DyLight 488 (Stemgent). For intracellular staining: OCT4, SOX2 and NANOG mouse IgG monoclonal antibodies (BD Pharmingen) were incubated in 1xCytoperm wash buffer overnight at 4°C. Cells were stained with either Alexa Fluor 488 or Alexa Fluor 594 secondary goat anti-mouse IgG, goat anti-rat IgM or goat anti-mouse IgM antibodies (Invitrogen) and the nuclei were counterstained with Hoechst 33342 (Sigma).

2.9 hEB formation and directed hematopoietic differentiation:

One clone each of LIF/2i-hiPSC and bFGF-hiPSC were split and seeded onto 1:6 Matrigel-coated plates, and then cultured for 7 days with MEF-CM supplemented with 8 ng/ml bFGF (Invitrogen). hEBs were generated in suspension culture as previously described [24]. Briefly, hiPSCs were treated with Collagenase IV for 2 minutes then cells were gently scraped into large clumps and plated into 6 well ultra-low attachment tissue culture plates in basal EB medium: Knockout-DMEM (Gibco), 20% FBS (Hyclone),1% NEAA, 1 mM L-Glut, 0.1mM β-mercaptoethanol. Medium was changed the next day with EB differentiation medium supplemented with hematopoietic growth factors as

follows: 50 ng/ml granulocyte colony stimulating factor (G-CSF; Amgen, Inc., Thousand Oaks, CA, USA), 300 ng/ml stem cell factor (SCF; Amgen), 10 ng/ml interleukin-3 (IL-3; R&D systems, Minneapolis, MN, USA), 10 ng/ml interleukin-6 (IL-6; R&D systems), 25 ng/ml BMP4 (R&D systems), and 300 ng/ml Flt-3 ligand (Flt-3L: R&D systems). EBs were cultured for 15 days and medium was changed every three days. After which EBs were dissociated to single cells and analyzed by flow cytometry for CD34 and CD45 expression.

2.10 Flow cytometry analysis:

Human iPSCs were dissociated to single cell suspensions using TrypLE Express (Invitrogen). Cells were stained using the following antibodies: CD31-PE (BD Pharmingen), CD34-PE or -FITC (Miltenyi Biotech, Bergisch Gladbach, Germany), CD45-APC (Miltenyi Biotech) for hematopoietic EB differentiation experiments. For pluripotent analysis, hiPSCs were analyzed for the presence of SSEA1-FITC, SSEA3-PE and TRA1-60-Alexa Fluor 647 (BD Pharmingen) for live extracellular pluripotent phenotyping. For detection of intracellular pluripotent transcription factors cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit. Cells were incubated overnight at 4°C with conjugated antibodies OCT4-Alexa Fluor 488, SOX2-Alexa Fluor 647 and NANOG-PE (BD Pharmingen). FACS analysis was performed using the BD LSRII Flow Cytometer with BD FACSDiva software and analyzed with FlowJo software from Tree Star Inc.

2.11 RNA Extraction, cDNA Synthesis & Quantitative RT-PCR:

Total RNA was extracted from cells using RNeasy kit (Qiagen). Transcript was quantified and purity and integrity were measured using the Nanodrop2000. Complementary DNA (cDNA) was made with 500ng of total RNA using the first-strand cDNA synthesis kit (Invitrogen) and subsequent quantitative Real-time PCR (Q-PCR) was carried out in triplicate, using SYBR Green RT-PCR reagents (Applied Biosystems) on an Mx3000P® Q-PCR System according to manufacturer instructions (Stratagene). Amplifications were performed using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. All data were normalized to GAPDH and internal untreated controls ($\Delta\Delta C_T$).

2.12 Western Blotting:

Cells were lysed with RIPA buffer (10mM Tris/HCl, 150mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS) containing 10mg/ml protease inhibitor cocktail and sonicated for 5 minutes at 30sec intervals. Total protein was quantified using the Biorad® DC assay and approximately 30µg of protein cooked with NuPAGE® LDS Sample buffer (4x) was separated using 4-12% SDS-PAGE (NuPAGE® Bis Tris precast gels and NuPAGE® System reagents, Life Technologies). 5 µL of Kaleidoscope protein standard (Biorad) was included. Protein was transferred onto nitrocellulose using a semi-dry transfer with the Biorad® transblot turbo transfer system (Biorad) with NuPAGE® Transfer Buffer. Membranes were blocked with either 5% skim milk or 5% BSA in TBST for minimum of 1 hour then incubated with antibodies overnight at 4°C.

Membranes were incubated with anti-mouse and anti-rabbit secondary antibodies conjugated to HRP and were detected using the Immobilon Western Chemiluminescent HRP substrate. Signal was detected with HyBlot ES Autoradiography film. **Antibodies:** Phospho-p44/42 MAPK(Erk1/2) (Thr202/Tyr204) rabbit mAb IgG (1:2000), p44/42 MAPK(Erk1/2) rabbit mAb IgG (1:2000), Phospho-p79/86 Stat3(Tyr705) mouse mAb IgG (1:500), p79/86 Stat3(Tyr705) mouse mAb IgG (1:2000) (Cell Signaling Technologies). Anti-GAPDH goat anti-mouse mAb IgG (1:500,000) (Abcam) and secondary anti-mouse and anti-rabbit HRP conjugates (1:50,000) (Biorad).

2.13 Data & Statistical analysis:

All results were expressed as the mean ± S.D. and generated from at least three independent experiments on one clone of each of LIF/2i/hiPSCs and bFGF-hiPSCs and managed using Excel and Prism GraphPad software. Statistical significance was determined using either the unpaired Student *t*-test or groups analyzed using the 1-way ANOVA. Results were considered significant or highly significant when $p<0.05$* or $p<0.01$**, or $p<0.001$***, respectively.

Results

3.1 Characterization of mouse-like or "naïve" hiPSCs:

Similar to previous works describing LIF/2i-dependent hPSCs[107,115,117,120], our lab has derived and characterized LIF/2i-hiPSCs from human somatic sources. We generated hiPSC by somatic cell reprogramming to a pluripotent state using the Yamanaka factors (*OCT4, SOX2, c-MYC* and *KLF4*). After isolating hiPSC colonies on mitotically inactivated fibroblasts with 10ng/ml basic fibroblast growth factors (bFGF), hiPSCs (denoted as bFGF-hiPSCs hereafter) cells were adapted to mouse ESC culture conditions. This was achieved through replacement of bFGF with 10ng/ml Leukemia Inhibitory Factor (LIF) and two small molecule inhibitors (2i), 3μM CHIR99021 and 1μM PD0325901[52]; such hiPSCs are denoted as LIF/2i-hiPSCs from here onward.

When cultured in feeder-free conditions (on Matrigel® with F12 media supplemented with LIF/2i) LIF/2i-iPSCs appear as dense, homogeneous colonies that are devoid of niche supportive cells, contrasting bFGF-hiPSCs (Figure 3.1A). Interestingly, LIF/2i-hiPSCs appear enriched for the SSEA3(+) self-renewing fraction[121] compared to bFGF-hiPSCs (Figure 3.1B), suggesting mouse-like hiPSCs may have enhanced self-renewal. The reduced heterogeneity of LIF/2i-hiPSCs, illustrated through heterogeneous SSEA3 expression (Figure 3.1B), has also been reported for variant hESCs in contrast to normal hESCs, indicating that naïve hPSCs may also be impaired for efficient differentiation but superior for pluripotent self-renewal[122]. Furthermore, LIF/2i-hiPSCs

are pluripotent demonstrated through the expression of pluripotent markers SSEA3, SSEA4, SOX2, OCT4 and TRA-1-60 (Figure 3.1C) and the ability to generate teratomas consisting of the three germ layers: ectoderm, endoderm and mesoderm (Figure 3.1D), similar to bFGF-hiPSCs.

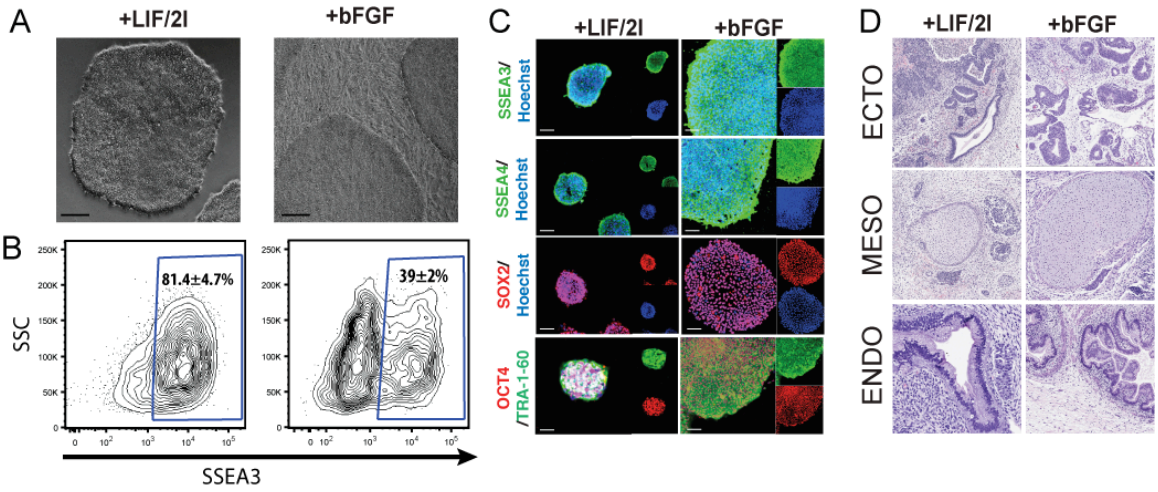


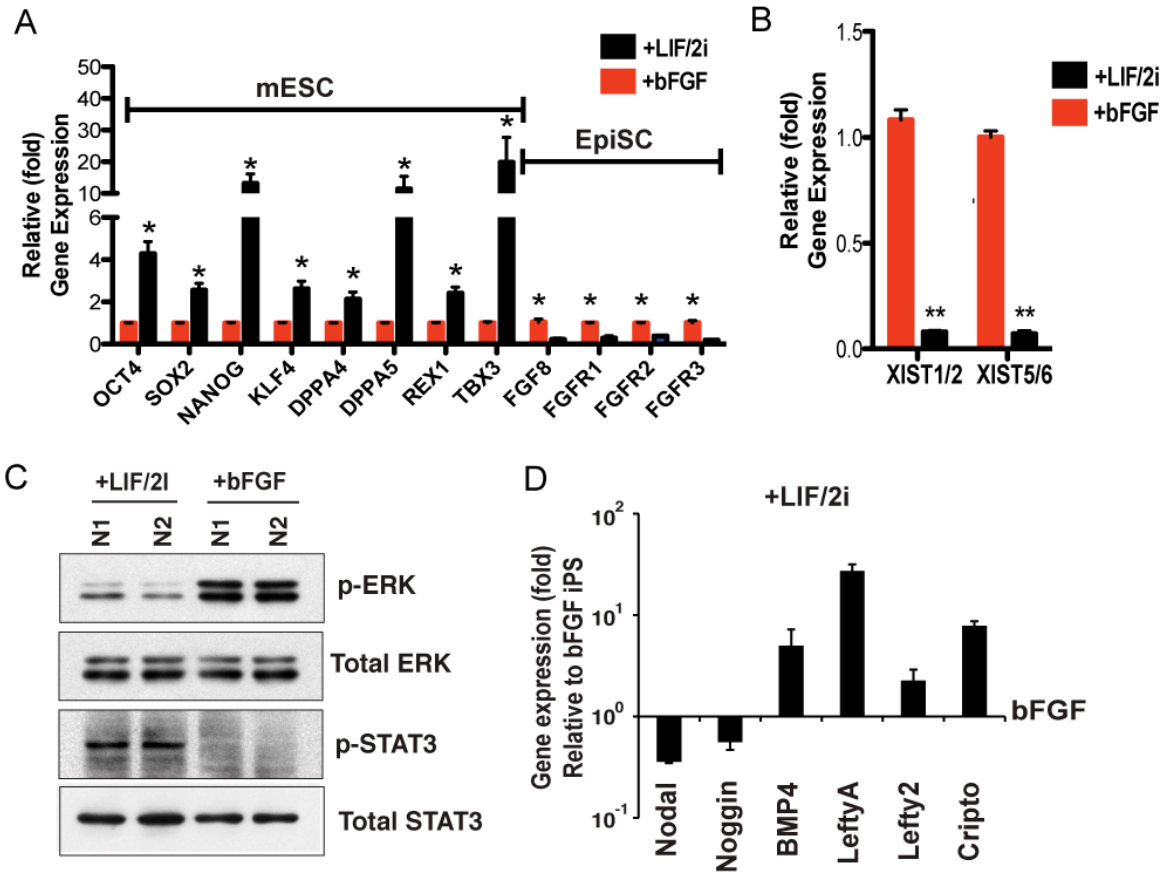
Figure 3.1. Features of Human iPSCs converted to LIF/2i Dependent Conditions:
A) Phase contrast images of hiPSCs dependent on LIF/2i or bFGF growth conditions. **B)** Flow cytometry analysis of the percentage of cells positive for SSEA3, in LIF/2i and bFGF iPS cells (n=3±S.D). **C)** Immunocytochemistry of pluripotent markers SSEA-3, SSEA-4, SOX2, OCT4 and TRA-1-60 counterstained with Hoechst 33342. **D)** Teratoma formation from LIF/2i-hiPSCs and bFGF-hiPSCs injected into the testis capsule of MOD/SCID mice. Mice were scarified 6-8 weeks post-injection and teratomas were harvested and H&E stained to identify tissues representing all three germ layers ectoderm (neural rosettes), mesoderm (cartilage) and endoderm (columnar epithelium). Scale bars = 100µm.

3.2 LIF/2i-hiPSCs have acquired characteristics of a Naïve state:

To determine the likeness of LIF/2i-hiPSC to either naïve or primed-like pluripotent states, several key pluripotency-related genes associated with either the mESC or EpiSC state were analyzed by qRT-PCR. Relative to the expression in bFGF-hiPSCs, LIF/2i-hiPSCs display increased transcript levels of pluripotency markers associated with the naive inner cell mass (*OCT4, SOX2, NANOG, KLF4, DPPA4, DPPA5, REX1, and TBX3*).

Concomitantly, they express reduced levels of transcripts associated with the epiblast (*FGFR1, FGFR2,* and *FGFR3)* (Figure 3.2A).

Differences also manifest between mESCs and EpiSCs through X chromosome inactivation (XCI), an epigenetic phenomenon important for maintaining dosage of X-linked genes in early embryo development[123]. Female mESCs contain two active X chromosomes (XaXa) similar to the early pre-implantation blastocyst[93]. *In vitro* and *in vivo* EpiSCs consistently exhibit X chromosome inactivation (XaXi)[50], measured by the coating of *X-inactive specific transcript (XIST)* mRNA on the inactivating X chromosome, resulting in transcriptional silencing. Although variability in XCI status and the expression of *XIST* transcript has been observed among several hESC and hiPSC lines, only a subset show a pattern of XCI regulation similar to the mouse model[124-126]. Nonetheless, XCI status was assessed here through the expression of *XIST* transcript to keep alignment with the established process in the mouse[123]. Transcript of exons *1/2* and *5/6* of the *XIST* gene revealed a significant underexpression in LIF/2i-iPSCs relative to bFGF-hiPSCs supporting the phenomenon seen in mice, and naïve hPSCs[115] (Figure 3.2B). In addition, LIF/2i-hiPSCs appeared reverted from an EpiSC-like state, which rely on active MEK/ERK1/2 (Figure 3.2C, right), and have acquired activated LIF/STAT3 signaling in LIF/2i dependent conditions. Western blotting clearly illustrates reduced expression of phosphorylated ERK1/2 at residues T202 and Y204 and acquisition of phosphorylated STAT3 at the critical T705 residue in LIF/2i-hiPSCs, similar to mESCs[15,52,53] (Figure 3.2C, left).



3.2 LIF/2i-hiPSCs have acquired characteristics of a Naïve state:
A) RT-PCR analysis for common markers associated with "naive" (mESC) and "primed" (EpiSC) states of pluripotency. **B)** Relative *XIST* gene expression analysis for exons 1&2, 5& 6 in hiPSCs cultured with bFGF or LIF/2i. **C)** Western Blot of MEK/ERK1/2 & LIF/STAT3 signaling in LIF/2i-hiPSC and bFGF-hiPSC cells illustrated as the differential p-ERK (T202 and Y204) and p-STAT3 (T705) expression before and after conditioning in LIF/2i culture conditions. **D)** Gene expression analysis of genes involved in signaling pathways linked to pluripotency. (All gene expression represents the mean expression normalized to *GAPDH* and relative to bFGF-iPS expression ($\Delta\Delta C_T$). Bar graphs represent the mean n=3 ± S.D. Students t-test, *p<0.05; **p<0.01.) (above experiments performed by JH Lee, Bhatia Lab with the exception of A & B)

To gain further insight into the characterization of the signaling requirements of LIF/2i-hiPSCs, several signaling pathway-related genes were assessed for their differential expression in relation to bFGF-hiPSCs (Figure 3.2D). LIF/2i-hiPSCs underexpress *NODAL* (an Activin related TGFβ signaling ligand) and *NOGGIN* (inhibits the binding of BMP4 ligand to its receptor), both of which enhance standard hPSC self-renewal[24,29,54]. At the same time, LIF/2i-hiPSCs show enhanced expression of *LEFTYA*, *LEFTY2* and *CRIPTO* (inhibitors of Activin/Nodal signaling)[127,128] (Figure 3.2D). These gene expression data suggest that LIF/2i-hiPSCs may be less dependent on Activin/TGFβ signaling and inhibitors of BMP4/TGFβ signaling to maintain the undifferentiated state, contradictory to hiPSCs cultured with bFGF[24]. These results appear to corroborate observations that hiPSCs cultured with LIF/2i are independent of Activin/Nodal signaling and may rely on BMP4 to prevent spontaneous differentiation, similar to mESCs[15,56,129]. These data briefly document the reversion of human bFGF-iPSCs to a naïve state of pluripotency, similar to previous generations of human LIF/2i-iPSCs[107,114,115].

3.3 LIF/2i Enhances Pluripotent Self-renewal of Naïve hiPSCs:

Published reports of naïve PSCs, in human or mouse cell lines, suggest pluripotential of these cell lines are enhanced upon acquisition of a naïve state[52,109,115]. Typically, these observations are acquired through analysis of pluripotent stem cell gene expression followed by an observational correlation with enhanced self-renewal. However, a quantitative measurement of enhanced pluripotency at the protein level has not been adequately correlated. In efforts to determine whether protein expression of

pluripotent markers (Figure 3.3A) correlate with the upregulated expression of genes associated with the ground state (Figure 3.2A), flow cytometry analysis measured the mean fluorescent intensity (MFI) to quantify protein expression levels (Figure 3.3B). SSEA3 in LIF/2i-hiPSCs is expressed nearly 2 fold higher than in bFGF-hiPSCs ($p<0.01$). Similarly, OCT4 and NANOG, when gated on the SSEA3(+) fraction, are expressed significantly higher, at 1.5 fold and 1.6 fold respectively, in LIF/2i-hiPSCs vs. bFGF-hiPSCs (Figure 3.3B).

To further explore whether LIF/2i-hiPSCs demonstrate a heightened ability to self-renew relative to bFGF-hiPSCs, a clonogenic assay was performed. The clonogenic assay measures the expansion level of a single pluripotent cell into a colony, referred to as colony-initiation capacity (CIC). Both cell lines were individually dissociated into single cells and 5,000 live cells were plated onto a layer of irradiated fibroblasts (Figure 3.3C). The formation of colonies from single cells was observed for 14 days, at which time colonies were fixed, immunostained and imaged for TRA-1-60 expression. The number of CICs was quantified using microscopy-imaging software. As expected, LIF/2i-hiPSCs are superior ($p<0.01$) in the ability to self-renew compared to bFGF-hiPSCs (Figure 3.3D). This observation agrees with established reports[107,115,120], and appears to correlate with enhanced expression of pluripotent protein and transcript expression. However, enhanced self-renewal within the SSEA3+ fraction requires further exploration by FACS sorting this fraction and comparing pluripotent self-renewal between the naïve and primed states of human pluripotency. This would likely explain if the increased

expression of pluripotent markers directly increases self-renewal in addition to the effects of heterogeneity on self-renewal[121].

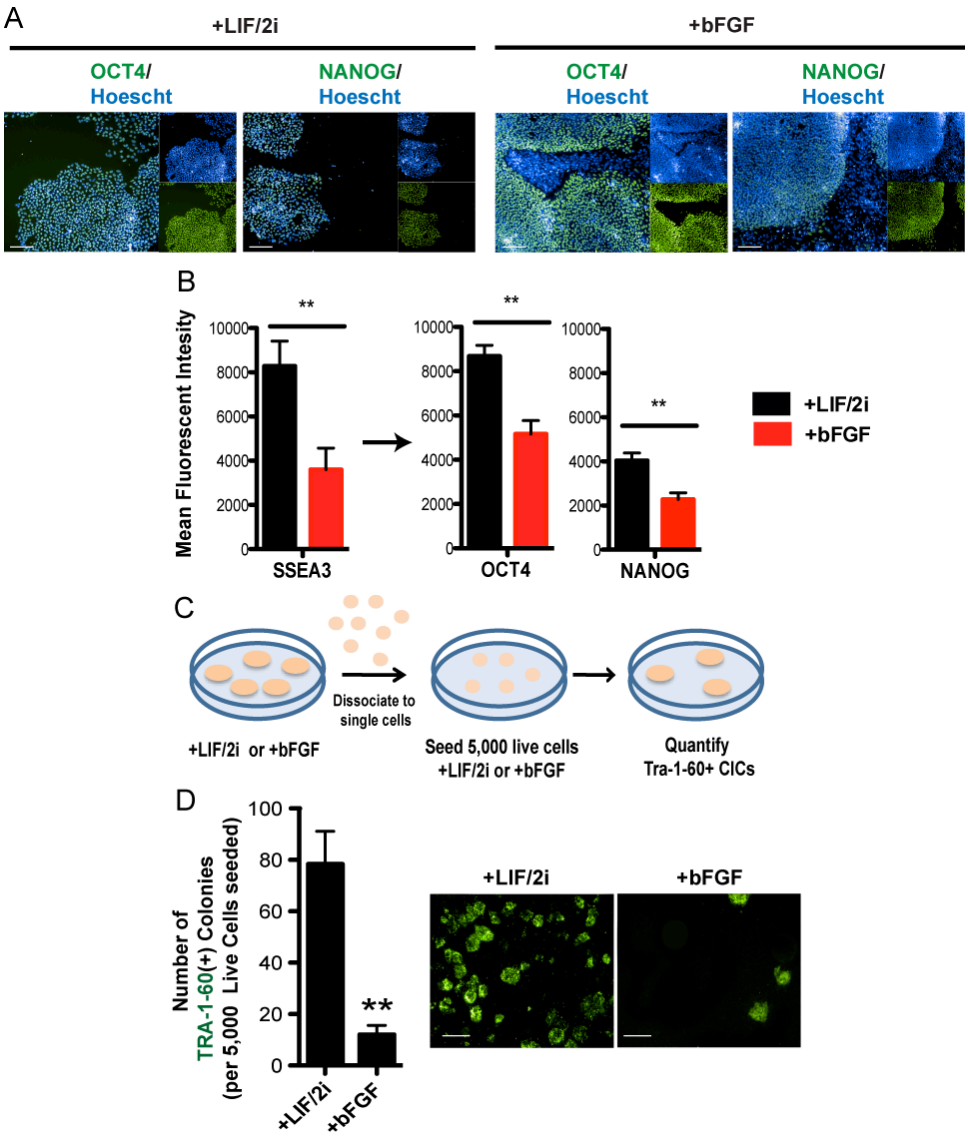


Figure 3.3 Enhanced Pluripotential Predicts Enhanced Self-Renewal in LIF/2i-hiPSCs:
A) Immunocytochemistry analysis of OCT4 and NANOG protein expression in LIF/2i-hiPSCs and bFGF-hiPSCs, counterstained with Hoechst 33342 (4x, scale bar = 100µm). **B)** Mean fluorescent intensity (MFI) of the protein expression of SSEA-3, OCT4 and NANOG acquired via flow cytometry. OCT4 and NANOG MFI was gated on the SSEA-3(+) fraction. **C)** Clonogenic assay schematic for quantifying colony-initiating cells (CIC). Upon single dissociation and filtration through a 40µm strainer, the number of live cells was determined through trypan blue (0.4%) exclusion using an Invitrogen Countess cell counter. **D)** CIC potential of LIF/2i-hiPSCs and bFGF-hiPSCs was enumerated upon immunostaining with Stemgent's Dylight-488 conjugated monoclonal antibody for TRA-1-60. Inset fluorescent images (2x, scale bar = 200µm) were taken with a Perkin Elmer Operetta and analyzed using Columbus/Acapella image analysis software. (Bar graphs; n=3, S.D., **p<0.01 Students T-test).

3.4 Assessing TGFβ Signaling Dependency of LIF/2i-hiPS cells:

To explore whether LIF/2i-hiPSCs are independent of signaling requirements that characterize the putative primed state of hPSCs, but depend on those necessary for naive self-renewal, growth factor requirements pertaining to Activin/Nodal and BMP4 TGFβ signaling pathways were assessed. Removing LIF/2i or bFGF, respectively, from the growth media and providing alternative extrinsic stimulation will delineate whether LIF/2i-iPSCs can sustain a pluripotent morphology and marker expression. Briefly, LIF/2i-hiPS and bFGF-hiPS cells were seeded on Matrigel coated tissue culture plates in their respective optimal growth media. The following day, the media was changed with fresh media supplemented with either 100ng/ml Activin A or 10μM SB431542 (SB) (specific Activin/Nodal antagonist) to evaluate Activin/TGFβ signaling dependency. Similarly, supplementation with either 100ng/ml BMP4 or 100ng/ml NOGGIN (antagonist of BMP4 signaling) assessed BMP4/TGFβ signaling dependency and each condition was compared to the untreated and optimally treated (+LIF/2i or +bFGF) controls. LIF2i-hiPS and bFGF-hiPS cells were cultured in each condition for 5 days[99]. At endpoint, flow cytometry analysis was done to measure OCT4 and NANOG protein expression (Figure 3.4.1). Morphologically, Activin A treated LIF/2i-hiPSCs appeared unaffected and similar to the untreated and LIF/2i controls, however, the addition of SB induced a differentiated morphology (Figure 3.4.1A, top panel). Unexpectedly, BMP4 also induced loss of the pluripotent morphology while NOGGIN did not (Figure 3.4.1A, top panel).

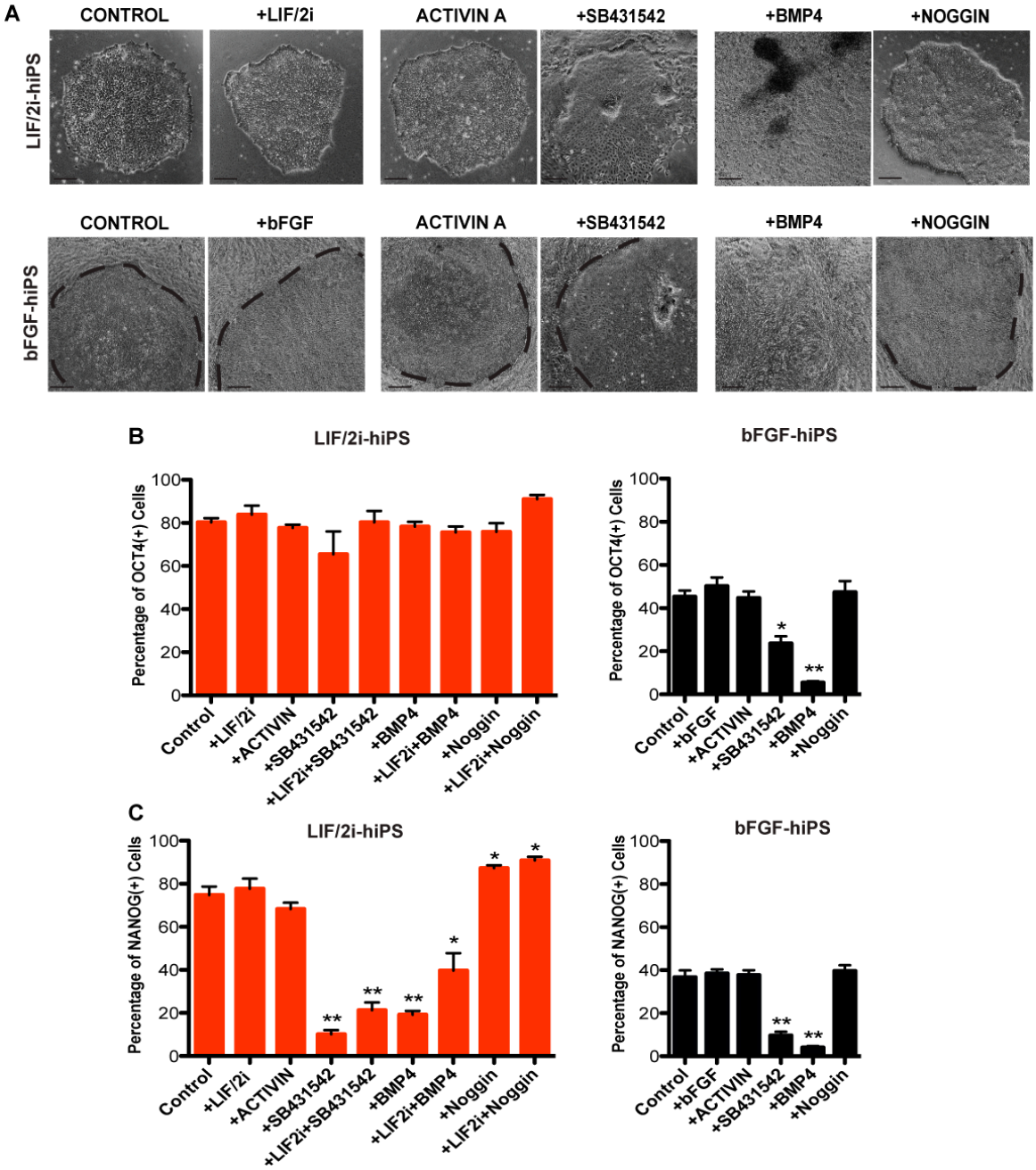


Figure 3.4.1 Assessing the signaling dependency of LIF/2i-hiPSCs:
A) Live phase contrast images (4x, scale bars 100µm) of LIF/2i-hiPSCs (top panel) and bFGF-hiPSCs (bottom panel) after 5 days in culture with either Control (untreated), +LIF/2i, +ACTIVIN A, SB431542, BMP4 and NOGGIN conditions. **B-C)** Flow cytometry analysis of the percent of OCT4 (+) cells **(B)** and NANOG(+) cell **(C)** after fixation and staining for the intracellular OCT4 and NANOG. Percentage positive cells were determined by gating on the live population (Invitrogen fixable LIVE/DEAD Violet discriminator) prior to gating with the FMO (fluorescent minus one) positive controls. (Bar graphs represent n=3 ± S.D. * $p < 0.05$, ** $p < 0.01$, One-way ANOVA relative to the untreated control).

The morphological responses observed for LIF/2i-hiPSCs were likewise observed for bFGF-hiPSCs (Figure 3.4.1A, bottom panel), suggesting that LIF/2i cultured hiPSCs retain signaling dependencies similar to their bFGF-dependent counterpart. The OCT4(+) population versus the morphological observations (Figure 3.4.1A) in LIF/2i-hiPSCs after the various treatments (Figure 3.4.1B, left panel) were not completely overlapping. Unlike in bFGF-hiPSCs, the percentage of OCT4(+) cells did not significantly change across any of the treatments, while SB and BMP4 induced a differentiated morphology in LIF/2i-hiPSCs (Figure 3.4.1B, right panel), results that are similar to published observations examining signaling dependency in LIF/2i cultured hPSCs[115], demonstrating their similarity to mESCs. Moreover, LIF/2i-hiPSCs treated with Activin A would be expected to lose expression of OCT4, but this was not seen, a response also similar to bFGF-hiPSCs (Figure 3.4.1B, right panel). NOGGIN also did not reduce the expression of OCT4, but this may be attributed to an increase in *BMP4* gene expression (Figure 3.2D), possibly negating the inhibitory effects of NOGGIN.

Interestingly, the extended analysis of NANOG in LIF/2i-hiPSCs showed opposite effects to that observed for OCT4. Both SB and BMP4 induced a dramatic drop in the percentage of NANOG(+) LIF/2i-hiPSCs (Figure 3.4.1C, left panel), similar to the response in bFGF-hiPSCs (Figure 3.4.1C, right panel). However, similar to OCT4 expression in both LIF/2i-hiPSCs and bFGF-hiPSCs, NANOG remained unchanged in the presence of Activin A or NOGGIN. These data intimate that LIF/2i-hiPSCs can readout a pluripotent state through OCT4 expression, as previously reported[115], however such analysis could be misinterpreted without the analysis of multiple pluripotent stem

cell factors. This also suggests that LIF/2i-hiPSCs retain signaling similarities to bFGF-dependent hiPSCs and may not be entirely naïve .

The apparent dichotomy between OCT4 and NANOG in LIF/2i-hiPSCs was also confirmed via immunostaining and qRT-PCR (Figure 3.4.2). SB and BMP4 treated LIF/2i-hiPSCs both acquired a differentiated morphology at the colony and cellular levels. However, both conditions clearly retain intensely OCT4(+) colonies, and cells that appear differentiated also remained highly OCT4(+) (Figure 3.4.2A, left panels). NANOG expression does remain in some colony-like cells but the differentiated cell types have lost NANOG expression, supporting the protein analysis (Figure 3.4.1). OCT4 and NANOG protein expression in bFGF-hiPSCs are equally lost upon SB or BMP4 treatment where only a few cells remain positive (Figure 3.4.2A, right panels), an observation in accordance with previous reports for hPSCs[29,61,127]. Relative to the untreated control, the differential response between OCT4 and NANOG protein expression is corroborated through gene expression analysis (Figure 3.4.2B). Interestingly, this suggests OCT4 in LIF/2i-hiPSCs may not be co-dependent on NANOG as previously reported for standard bFGF-dependent hPSCs[96].

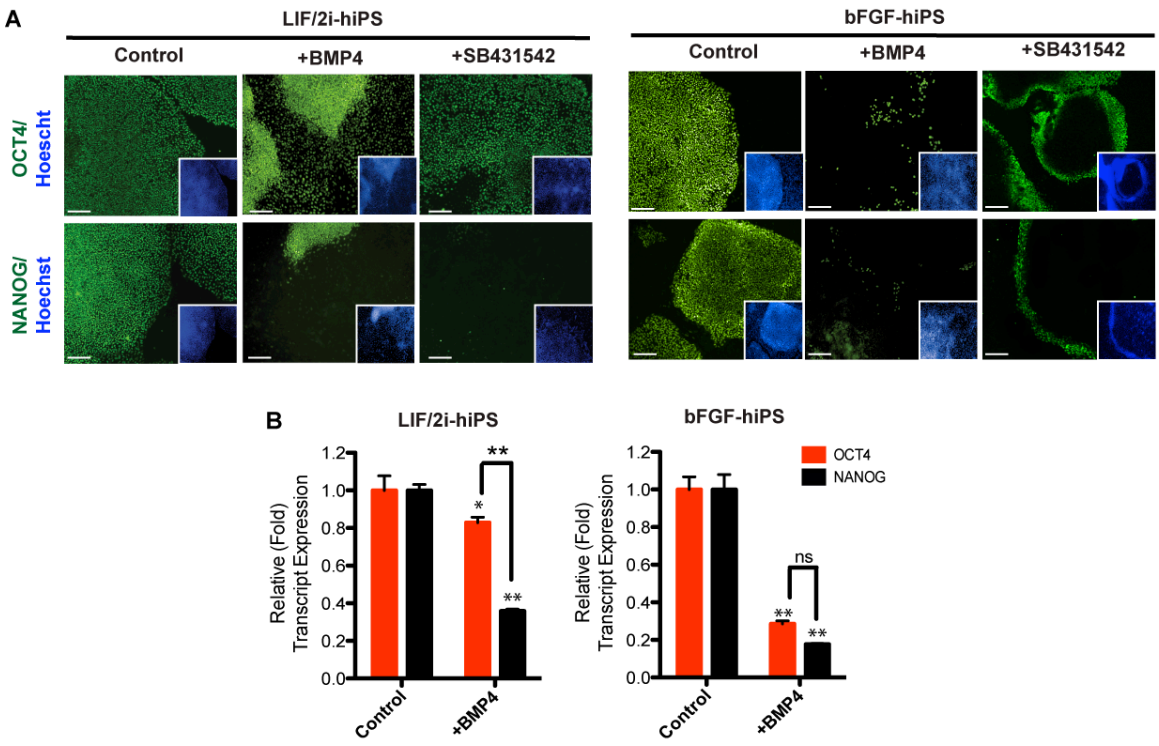


Figure 3.4.2 OCT4 and NANOG display divergent signaling behaviour in LIF/2i-hiPSCs:
A) Immunostaining for OCT4 and NANOG protein (inset image, Hoechst counterstaining) for LIF/2i-hiPSCs and bFGF-hiPSCs (4x, scale bars = 100µm). Cells were treated with 100ng/ml BMP4 or 10µM SB431542 for 5 days without pro-self-renewal growth factors (ie, LIF/2i or bFGF). Cells were fixed, permeabilized and stained for intracellular OCT4 and NANOG. **B)** qRT-PCR gene expression analysis of endogenous OCT4 and NANOG relative to GAPDH and to the untreated control ($\Delta\Delta C_T$); n=3±S.D. *p<0.05, **p<0.01.

3.5 Preserved SSEA3(+)/OCT4(+) LIF/2i-hiPSCs have the potential to self-renew:

To better illustrate and quantify the population differences for the observed divergence between OCT4 and NANOG, flow cytometry analysis of OCT4, NANOG and SSEA3 determined the residual fraction of SSEA3(+) cells that would be predicted to contain clonogenic potential[121] (Figure 3.5). The fraction of SSEA3(+) cells which co-expressed OCT4 (15±5%) or NANOG (12±5%) that remained in BMP4 treated LIF/2i-hiPSCs was significantly higher (p<0.01) than the same populations in BMP4 treated

bFGF-hiPSCs (Figure 3.5A). Relative to the control, nearly all bFGF-hiPSCs lost expression of OCT4, NANOG and SSEA3 equally, similar to Hanna et al.[115]. This corroborates that LIF/2i-hiPSCs can sustain a pluripotent self-renewing state in the presence of BMP4. However, when comparing the same BMP4 treated OCT4(+)/SSEA3(+) or NANOG(+)/SSEA3(+) populations to their untreated LIF/2i-hiPSC controls, a significant reduction in both double positive populations was observed (Figure 3.5B), contrasting Hanna and colleagues description of similar populations. The disparity between reported BMP4 signaling dependency and that presented here is likely the result of using transgenic systems overexpressing pluripotent transcription factors. LIF/2i-dependent hPSCs generated by Hanna and colleagues were completely dependent on the presence of doxycycline to maintain overexpression of *OCT4* and *KLF4* or *KLF2*[115], likely compromising the interrogation of signaling dependency. In addition, Hanna and colleagues utilized SSEA4, instead of SSEA3, which displays a homogeneous expression pattern similar to TRA1-60 that does not illustrate the heterogeneity indicated through the expression of SSEA3. When examining the SSEA3(-) fraction (Figure 3.5 C&D) in BMP4 treated LIF/2i-hiPSCs, most cells (74±7%) become SSEA3(-)/NANOG(-), while the SSEA3(-)/OCT4(-) population (21±4%) remained significantly low (Figure 3.5C). Instead, BMP4 induces SSEA3(+)/OCT4(+)/NANOG(+) cells of the control to become individually OCT4(+) (61±2%) (Figure 3.5D), confirming a divergence between OCT4 and NANOG.

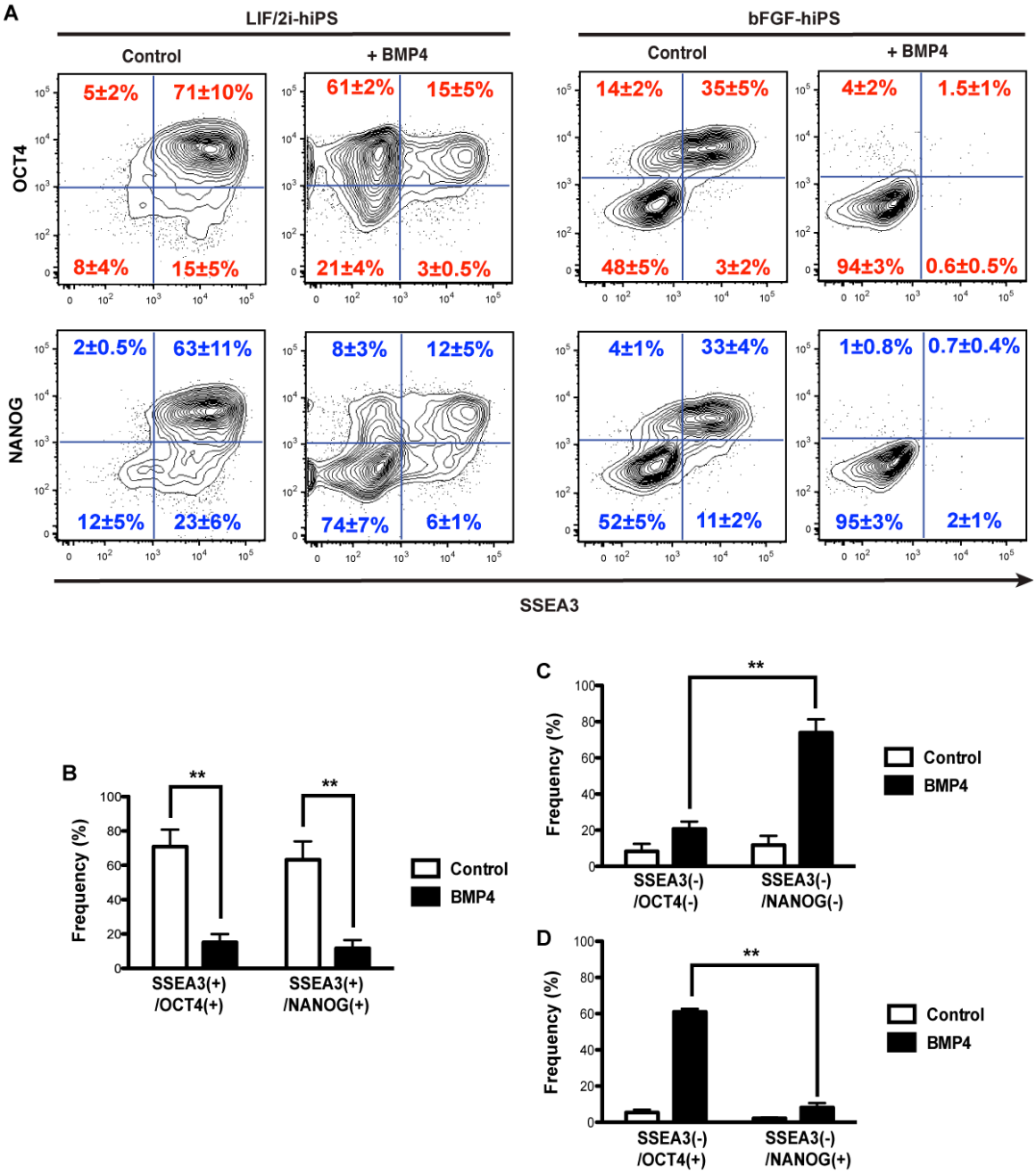


Figure 3.5 LIF/2i-hiPSCs retain an SSEA-3(+)/OCT4(+) population with the potential to self-renew. **A)** Flow cytometry analysis of OCT4, NANOG against SSEA3 for untreated control and BMP4 treated LIF/2i-hiPS and bFGF-hiPS cells. Quadrants report population frequencies (n=3±S.D). **B)** Bar graphs representing flow cytometry analysis of LIF/2i-hiPSCs in (A). **p<0.01, Student's t-test.

3.6 BMP4 treated LIF/2i-hiPSCs are capable of clonogenic pluripotent self-renewal:

Due to the retention of a small fraction of SSEA3(+)/OCT4(+) cells (Figure 3.5A-B), the clonogenic capacity was determined for BMP4 treated LIF/2i-hiPSCs (Figure 3.6). Upon seeding, bulk culture of LIF/2i-hiPSCs were treated for 5 days with or without 100ng/ml BMP4 then dissociated to single cells. 5,000 cells were seeded onto irradiated fibroblasts in growth media containing LIF/2i then observed for colony initiation (Figure 3.6A). Within one week, many colonies formed and stained positive for TRA-1-60, which were quantified and compared. As predicted in Figure 3.5B, BMP4 treated LIF/2i-hiPSCs still retained a fraction of cells with CIC potential similar to murine naïve self-renewal requirements (Figure 3.6B), although clonogenicity was greatly reduced.

Combined, these results suggest NANOG regulation within LIF/2i-iPSCs remains dependent on TGFβ signaling, similar to the observations displayed by hESCs where NANOG is a downstream target in Activin/TGFβ signaling[60,62]. Secondly, NANOG is modulated either through antagonizing the Activin/TGFβ signaling with either the small molecule inhibitor SB or BMP4, while OCT4 appears less dependent on these particular TGFβ pathways. The reduction of NANOG in LIF/2i-iPSCs upon exogenous addition of BMP4 suggests that LIF/2i-iPSCs retain signaling features characteristic of standard hPSCs, which readily differentiate in the presence of BMP4 and Activin/Nodal inhibitors[24,92]. Nonetheless, sustained expression of OCT4 suggests a divergent regulation between OCT4 and NANOG, unlike bFGF-dependent hiPSCs.

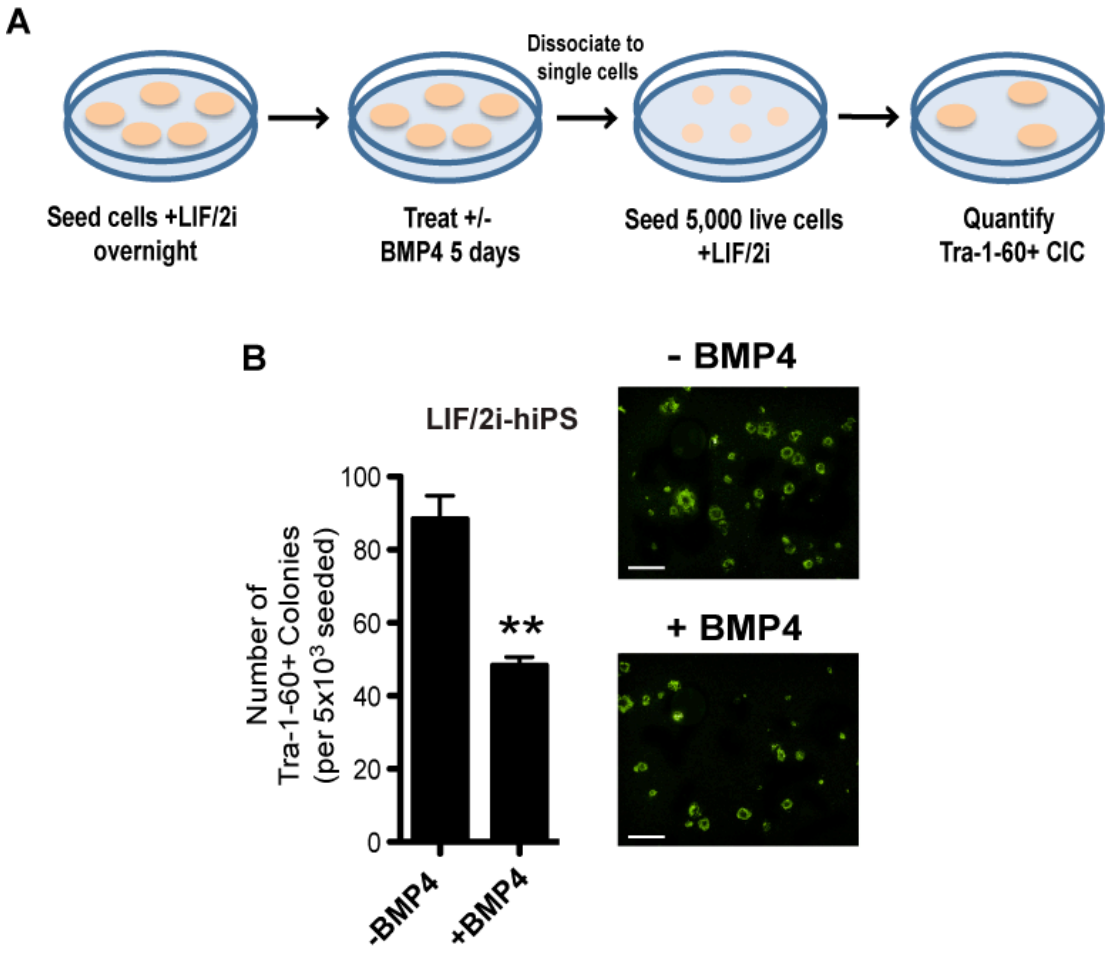


Figure 3.6 BMP4 treated LIF/2i-hiPSCs retain cells capable of clonogenic pluripotent self-renewal: **A)** Schematic of the CIC assay in +/- BMP4 treated LIF/2i-hiPSCs. **B)** Bar graph representing the number of TRA-1-60(+) colonies with and without treatment. Inset fluorescent images were taken with a Perkin Elmer Operetta and analyzed using Columbus/Acapella image analysis software (2x, scale bar = 200μm). (Bar graphs; n=3, S.D., **p<0.01 Students T-test).

These results support published observations in mESCs where the loss of Nanog expression "primes" mESCs for differentiation through an EpiSC-like state while maintaining the expression of OCT4[84]. This suggests that individual roles exist for OCT4 and NANOG in pluripotent self-renewal and lineage commitment, respectively.

3.7 Assessing downregulation of *OCT4* and *NANOG* through RNA interference:

To elucidate whether the divergent behaviour between OCT4 and NANOG can confer a functional explanation, gene-targeting inactivation using (RNAi) was employed to reduce their expression (Figure 3.7). RNAi may suggest that the differential response between OCT4 and NANOG observed in LIF/2i-hiPSCs is not necessarily dependent on TGFβ signaling, but is a unique feature of the pluripotent regulation in hiPSCs cultured in naïve conditions.

Lentiviral constructs containing short-hairpin RNAs (shRNA) targeting either *OCT4* and *NANOG* were transduced into LIF/2i-hiPSCs as described[96,119]. Briefly, upon seeding hiPSCs onto Matrigel® coated plates, LIF/2-iPSCs were transduced with pLL3.7-eGFP (sh-Control), pLL3.7Nanogi-eGFP (sh-NANOG) or pLL3.7Oct4i-2-eGFP (sh-OCT4) containing the LentiLox3.7 backbone[118] (Figure 2, Methods). Transduced cells were maintained in LIF/2i conditions and monitored for signs of differentiation and expression of the GFP reporter (Figure 3.7A). After 72 hours post-transduction, shRNA mediated knockdown of *OCT4* induced differentiation of LIF/2i-hiPSCs, apparent through a distinct change in morphology compared to the empty vector control (Figure 3.7B).

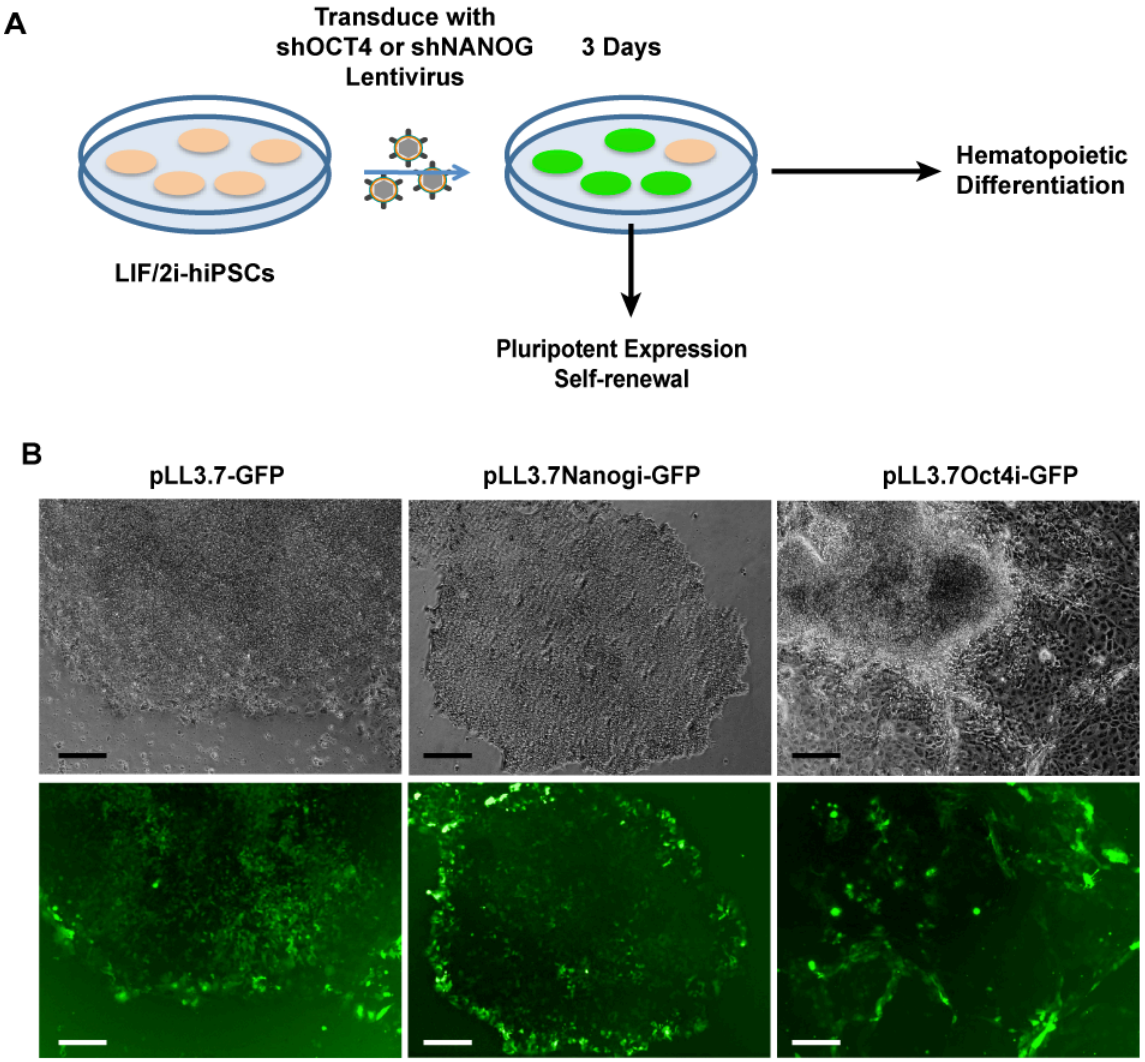


Figure 3.7 RNAi of *OCT4* induces differentiation, while RNAi of *NANOG* does not:
A) Schematic illustrating the transduction of LIF/2i-hiPSCs with lentivirus containing either of pLL3.7-eGFP (sh-Control), pLL3.7-Nanogi-eGFP (sh-NANOG), or pLL3.7-OCT4i-eGFP (sh-OCT4) plasmid vectors. After 3 days post-transduction, OCT4 and NANOG will be interrogated for their roles in pluripotent self-renewal and hematopoietic (HEM) differentiation potential. **B)** Phase contrast and fluorescent images (4x, scale bars = 100µm) showing the biological response as a result of knockdown of OCT4 and NANOG and GFP expression reporting the successful integration of each vector.

In contrast, the knockdown of *NANOG* did not display an obvious biological response and appeared similar to the empty vector control (Figure 3.7B). This finding is unexpected as *Nanog* downregulation in the mESCs results in rapid loss of *in vivo* epiblast and prevents the acquisition of pluripotency during iPSC reprogramming from mouse fibroblasts[18,80,90]. Moreover, this is in complete contrast to previously published work in several hESC lines where knockdown of either *OCT4* or *NANOG* readily induces a reciprocal loss of expression[96].

3.8 RNAi confirms a divergent behaviour between of OCT4 and NANOG in LIF/2i-hiPSC:

Samples from each condition were then analyzed by flow cytometry for the protein expression of OCT4 and NANOG within the GFP(+) fraction to determine the knockdown efficiency. More importantly, this deciphered whether the pluripotent regulation in LIF/2i-iPSCs is similar to that observed in bFGF-dependent hPSCs[96,119]. Knockdown of *OCT4* simultaneously reduced the expression of OCT4 and NANOG protein (Figure 3.8A), in line with previous reports[16,96,119,130]. Similarly, quantification of the percentage of cells positive and the mean fluorescent intensity (MFI) for OCT4 and NANOG protein expression were significantly reduced relative to the empty vector control (Figure 3.8B). Forced knockdown of *NANOG* in LIF/2i-iPSCs also reduced the expression NANOG protein and the percentage of NANOG positive cells in the GFP(+) fraction. Conversely, forced knockdown of *NANOG* did not result in a concomitant downregulation of OCT4 (Figure 3.8B), as previously reported for bFGF-dependent

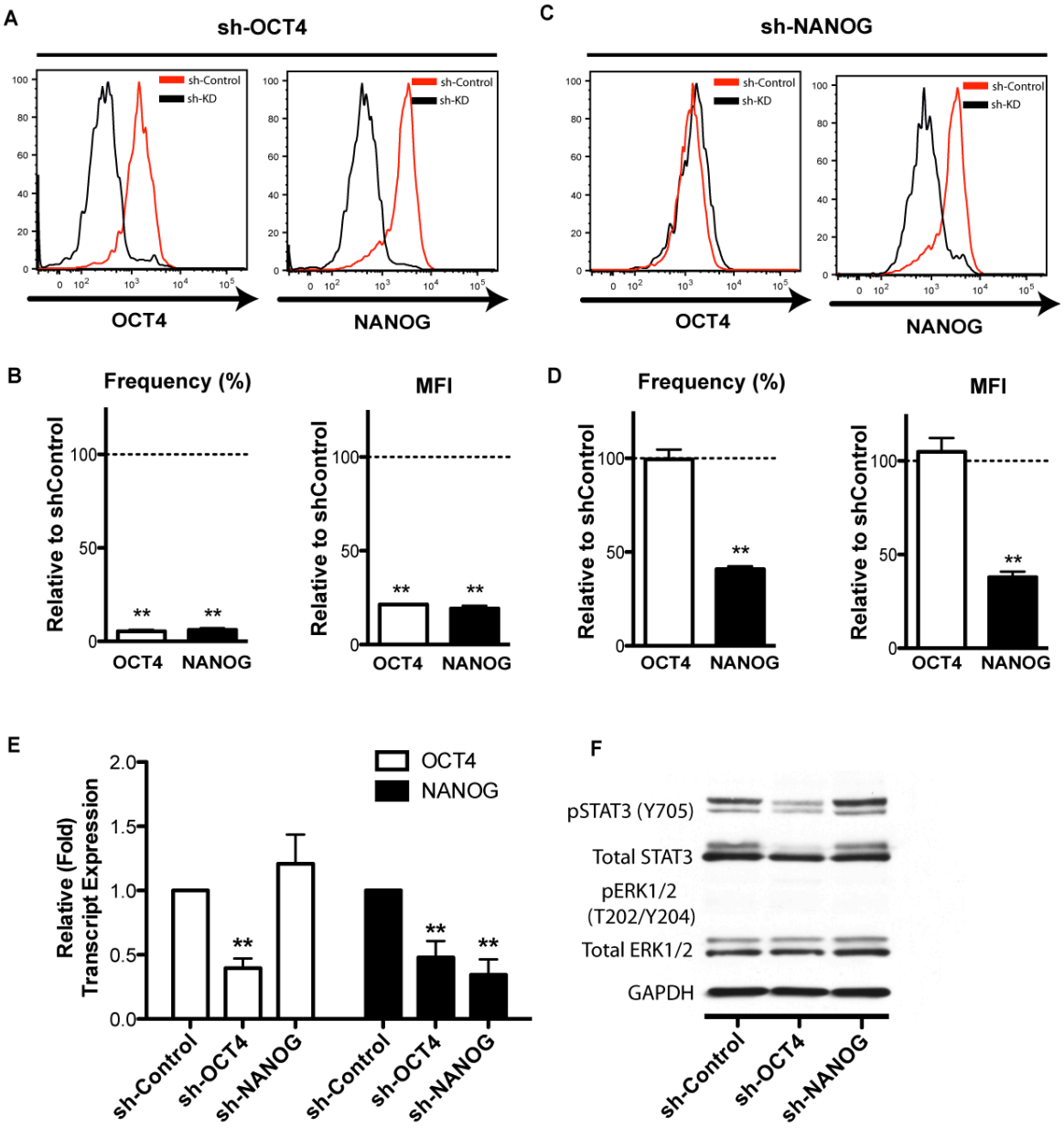


Figure 3.8 RNAi mediated knockdown reveals a divergence in dependency of OCT4 and NANOG: **A-D)** Flow cytometry analysis of the expression of OCT4 and NANOG protein upon RNAi forced knockdown of *OCT4* **(A-B)** and *NANOG* **(C-D)** in LIF/2i-hiPSCs, represented as histogram intensities, frequency (%) of positive cells and the mean fluorescent intensity (MFI) against the empty vector sh-Control. Bar graphs represent n=3±S.D, ***p*<0.01, Student's t-test. **E)** qRT-PCR gene expression analysis of OCT4 and NANOG in transduced LIF/2i-hiPSCs, relative to GAPDH and the sh-Control ($\Delta\Delta C_T$), n=3±S.D, ***p*<0.01, 1-way ANOVA.

hPSCs[96,119]. Transcript expression by qRT-PCR verified the protein expression analysis of OCT4 and NANOG (Figure 3.8E). These data indicate the pluripotent regulation in hiPSCs cultured in LIF/2i induces an alternative mode of regulation, distinct from bFGF conditions. These data also suggest that OCT4 is dominant over NANOG, which appears expendable for maintaining LIF/2i-hiPSCs in an undifferentiated, self-renewing state. This observation appears somewhat in opposition to its role in mESCs, where Nanog is required to maintain and stabilize the "naïve" pluripotent state in mESCs by resisting differentiation, where its downregulation causes progression to a differentiated state[80,84], results not necessarily recapitulated here. However, "a differentiated state" can be interpreted as an EpiSC-like state, that remains pluripotent by retaining the expression of OCT4, but is primed for differentiation[131].

Western blot analysis of activated LIF/STAT3 and FGF/ERK1/2 signaling was assessed to determine whether forced knockdown of either *OCT4* or *NANOG* induces an EpiSC-like state, indicated through phosphorylated ERK1/2 (Figure 3.8F). Active pSTAT3(Y705) appears to be least expressed in sh-OCT4 transduced LIF/2i-hiPSCs compared to the sh-Control and sh-NANOG cells (Figure 3.8F), supporting an induction of differentiation seen in Figure 3.7. The downregulation of STAT3 upon knockdown of *OCT4* also suggests that components of active JAK/STAT signaling are downstream of OCT4, indicating the possibly of a regulatory loop between OCT4 and STAT3. In spite of this, neither *OCT4* nor *NANOG* downregulation induced the concomitant increase in pERK1/2(T202/Y204), suggesting these cells are not progressing through an EpiSC-like state as implied by the mouse[84].

3.9 The functional distinction between OCT4 and NANOG for self-renewal:

To determine the functional differences between OCT4 and NANOG in LIF/2i-iPSCs, further characterization of pluripotent self-renewal and differentiation potential were required. As displayed earlier (Figure 3.5B and D) BMP4 induced OCT4 enrichment with a loss of NANOG, leaving a portion OCT4(+) and SSEA3(+) (15±5%). Forced knockdown of *NANOG* also retained a portion of cells co-expressing OCT4 and SSEA3 (9.6±1.3%), while knockdown of *OCT4* only retained 1.3±0.5% SSEA3(+)OCT4(+) cells (Figure 3.9A). This suggests that targeted downregulation of *OCT4* in LIF/2i-hiPSCs diminishes the fraction of self-renewing cells, thus indicating its hierarchical requirement compared with NANOG. FACS sorting the GFP(+) fraction in the cells harboring the shRNA for either *OCT4* or *NANOG* further resolved a distinction between these two transcription factors for clonogenic self-renewal. After three days post-transduction with the shRNA, 10,000 viable GFP(+) cells were sorted at 98% purity (Figure 3.9B) onto irradiated feeders. Colonies appeared within 7 days, which were fixed, stained for TRA-1-60 and quantified. Relative to the sh-Control (382±83), sh-NANOG transduced LIF/2i-hiPSCs generated significantly less TRA-1-60(+) colonies (60±23, $p<0.01$), while sh-OCT4 transduced LIF/2i-hiPSCs did not produce any TRA-1-60(+) colonies (Figure 3.9C). Although sh-OCT4 cells did yield a few TRA-1-60(+) cells, the colonies that formed were differentiated in morphology, containing cells with large cytoplasm implied through the spacing between nuclei when compared to sh-Control and sh-NANOG colonies (Figure 3.9D).

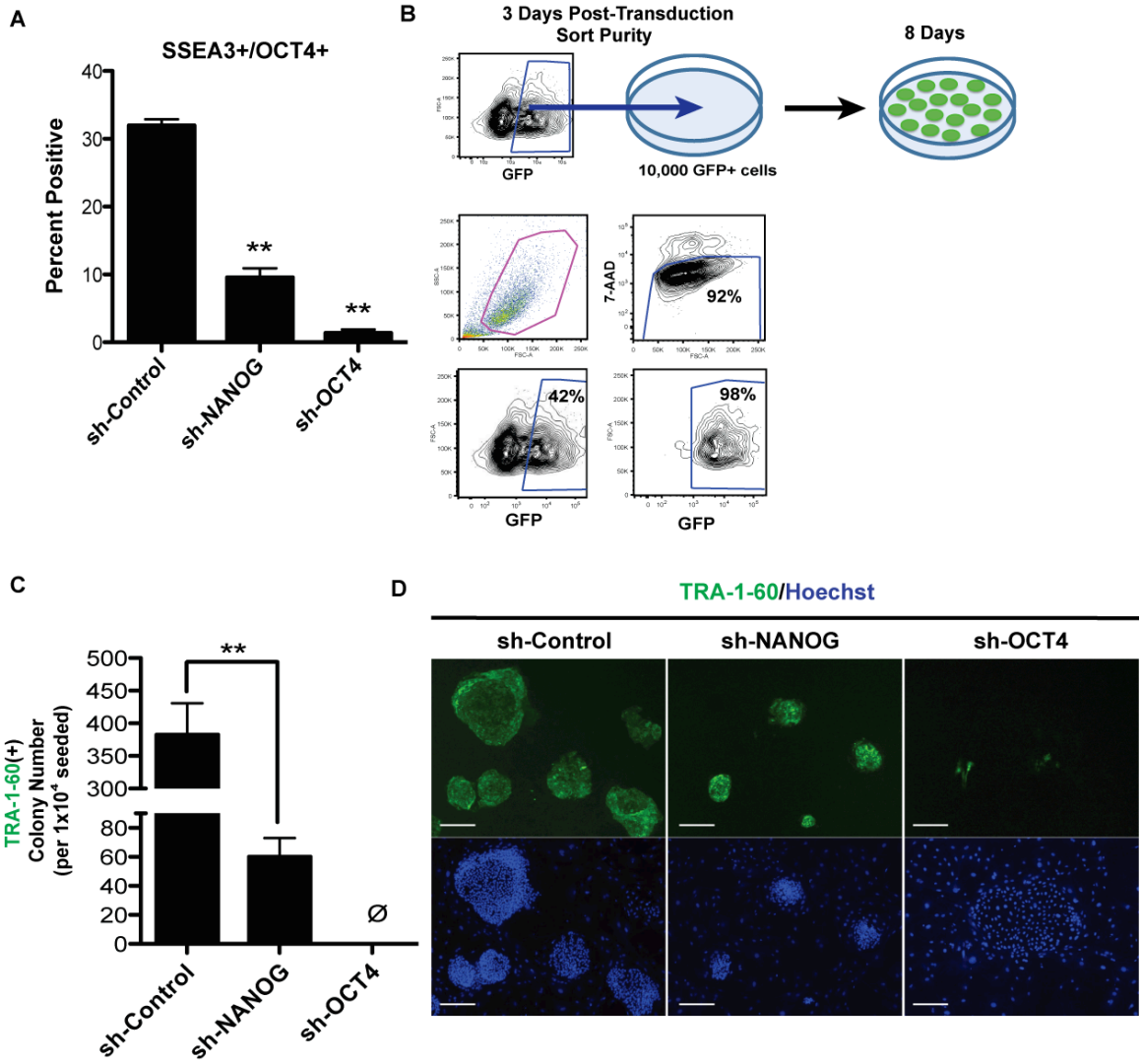


Figure 3.9 OCT4 is essential for pluripotent self-renewal in LIF/2i-hiPSCs:
A) Flow cytometry analysis illustrating the frequency of potentially clonogenic SSEA3(+)/OCT(+) cells in LIF/2i-hiPSCs harboring an shRNA targeting either OCT4 or NANOG. **B)** Schematic illustrating the FACS sorting strategy including the sort purity gates. **C)** Clonogenic assay quantifying the CIC capacity OCT4 and NANOG deficient LIF/2i-hiPSCs. **D)** Fluorescent images (4X, scale bars = 100µm) of fixed TRA-1-60-Dylight 488 immunostaining in LIF/2i-hiPSCs, counterstained with Hoechst 33342.

As predicated from the SSEA3(+)OCT4(+) frequencies indicated in Figure 3.9A, *OCT4* is necessary to maintain the pluripotent state in LIF/2i-hiPSCs. Forced knockdown of *NANOG* produced fewer TRA-1-60(+) colonies that were smaller in size (Figure 3.9D), suggesting NANOG is supportive of self-renewal. Consequently, OCT4 appears to prevent differentiation to sustain the pluripotent state while NANOG provides a supportive role in pluripotent self-renewal, similar to *in vivo* observations[16,18,83].

3.10 Knockdown of *OCT4* recovers Hematopoietic Differentiation of LIF/2i-hiPSCs:

Human PSCs have successfully demonstrated the ability to differentiate toward several cell types, including hematopoietic (HEM) lineages[30]. HEM differentiation from hPSCs is a lineage of interest in our lab, particularly for generating alternative sources of transplantable cells for clinical use in hematopoietic stem cell (HSC) transplantation, and to prevent (or limit) graft versus host disease (GVHD) during cell replacement therapies. In order to achieve successful engraftment into a recipient patient, hPSCs must be able to generate HSCs However, the ability to isolate the HSC has proven difficult to achieve, likely due the retention of embryonic programs and failure to activate adult globins[132], although, our lab has provided a recent explanation suggesting the isolation of a putative adult HSC is possible[133]. HEM potential has been exhibited in hPSCs using the embryoid body (EB) differentiation assay[134] in conjunction with cytokines specific to *in vivo* blood differentiation[133,135]. HEM differentiation from hPSCs[136,137] progresses through a sequence of incremental waves beginning with the generation of a bipotent population capable of endothelial or hematopoietic lineages. This mesodermal phase is marked by the co-expression of surface epitopes, CD31 and CD34. The second wave during HEM

EB differentiation brings about a primitive stem cell and progenitor pool indicated by the expression of CD34(+)CD45(-) (HSC) that remain largely uncommitted until day 10. Beyond day 10 CD45 expression increases and CD34(+)CD45(+) progenitors arise with the potential to generate colony forming units[138]. Lastly, during the final phase of HEM EB differentiation, single CD45(+) definitive HEM cells with erythro-myeloid potential are detectable.

LIF/2i-hiPSCs was further assessed to distinguish the functional roles of OCT4 and NANOG in the propensity to differentiate into hematopoietic cell types. The potential for the primitive cells (CD34+CD45-), progenitors (CD34+CD45+), and mature blood (CD45+CD34-) was compared to bFGF-hiPSCs and LIF/2i-hiPSCs with *OCT4* or *NANOG* knocked down. Briefly, one clone each[139] of LIF/2i-hiPS, bFGF-hiPS, LIF/2i-hiPS+shOCT4, and LIF/2i-hiPS+sh-NANOG were cultured for 5-7 days in their respective growth conditions. Two days prior to EB formation, a 1:6 dilution of Matrigel® was layered upon the cellular layer to facilitate EB formation. hiPSCs were enzymatically dissociated and scraped into large clumps and transferred into EB media containing 10%FBS.

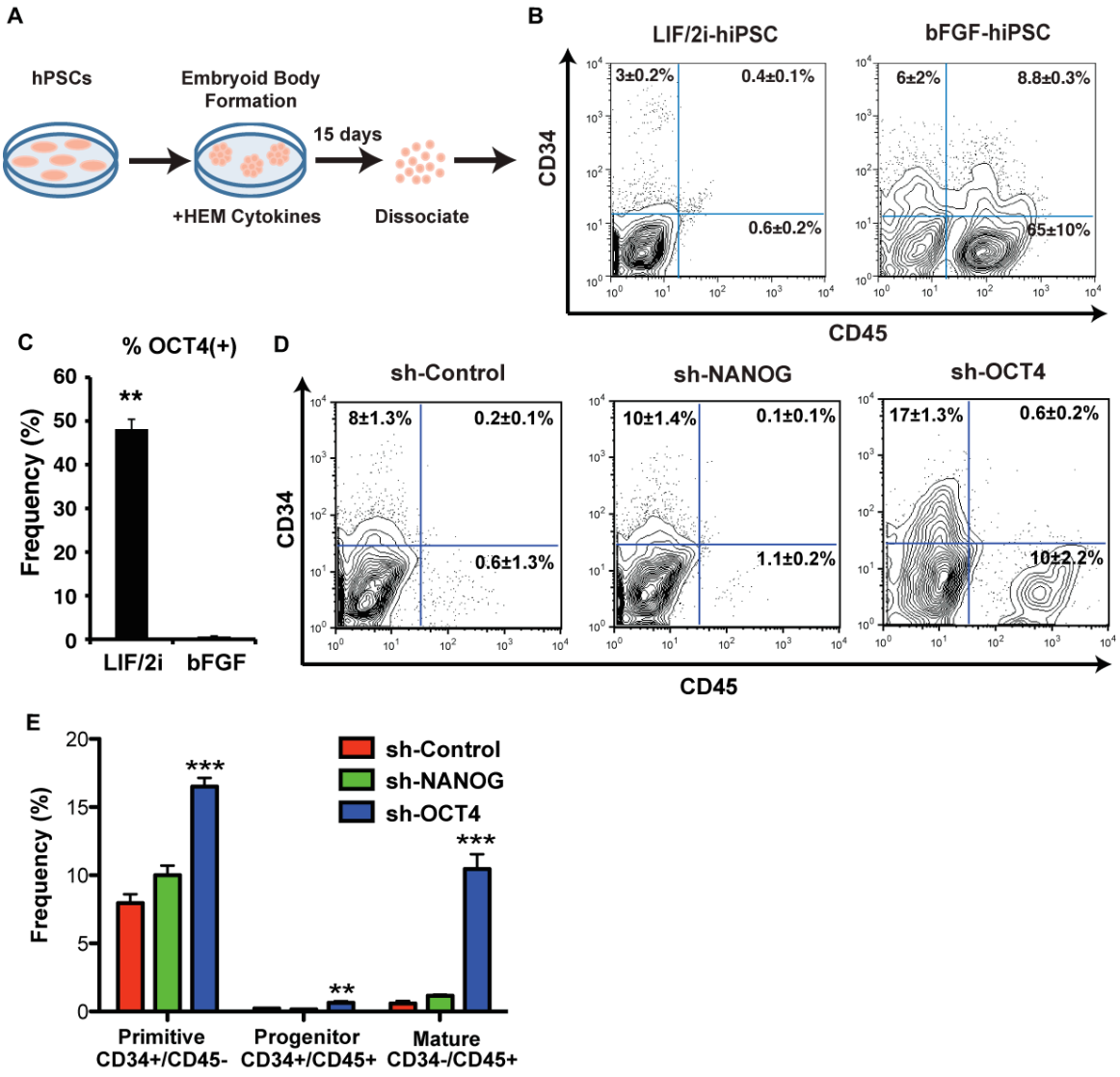


Figure 3.10 Hematopoietic differentiation is compromised inLIF/2i-hiPSCs, but is recoverable by RNAi of *OCT4*: A) HEM EB differentiation assay. **B)** CD34 and CD45 expression in 15 day HEM LIF/2i-hiPSC and bFGF-hiPSC EBs with inset quadrant frequencies. **C)** Flow cytometry of OCT4(+) cells retention in day 15 LIF/2i-hiPSC HEM EBs upon replating in LIF/2i culture conditions, relative to bFGF-hiPSC HEM EBs. **D)** CD34 and CD45 expression in 15 day HEM EBs generated from LIF/2i-hiPSC with shRNAs targeting *OCT4* or *NANOG*. Sh-Control is the empty shRNA vector. **E)** Quantification of HEM efficiency for primitive, progenitor and mature blood cells upon shRNA mediated knockdown of OCT4 and NANOG in LIF/2i-hiPSCs. Bar graphs represent the mean n=3± S.D. *p<0.05, **p<0.01, ***p<0.001, Student's t-test.

The following day the cells were washed to remove debris, which was replaced with fresh EB media containing the HEM cytokines: stem cell factor (SCF), Flt-3L, interleukin-3 and 6 (IL-3 and IL-6), granulocyte colony-stimulating factor (G-CSF) and BMP4[133]. EBs were cultured in suspension for 15 days at which time they were dissociated to single cells and analyzed for CD34 and CD45 expression via flow cytometry (Figure 3.10A).

Compared to bFGF dependent hiPSCs, LIF/2i-hiPSC generated blood at an impressively reduced efficiency as predicted by the homogeneous expression of SSEA3 (Figure 3.1B). bFGF-hiPSCs produced a primitive/progenitor pool (~15%) and mature single CD45(+) cells (65%) that were significantly higher (p<0.01%) than the same populations generated by LIF/2i-hiPSCs (~3.4% and 0.6%, respectively) (Figure 3.10B). Upon replating Day 15 HEM EBs in LIF/2i conditions, LIF/2i-hiPSC EBs regenerated colonies that expressed OCT4 at a frequency close to 50% (Figure 3.10C), supporting the block in HEM differentiation. The inability of LIF/2i-hiPSCs to efficiently produce differentiated HEM cells suggests the naïve LIF/2i extrinsic environment not only induces the loss of supportive niche cells, LIF/2i appears to negatively impact the intrinsic mechanisms involved in differentiation by retaining OCT4. In contrast, the EpiSC-like state effectively downregulates OCT4, and are not devoid of supportive niche thus enabling efficient HEM differentiation potential. This finding does not support similar reports for serum versus LIF/2i cultured mESCs, such that LIF/2i is suggested to be conducive for efficient (neural) differentiation compared to a state that is primed for differentiation[139].

Akin to the HEM potential of untransduced LIF/2i-hiPS, the sh-Control and sh-NANOG transduced cells produced the similar frequencies of HEM primitive/progenitor (CD34+CD45- and CD34+CD45+) and definitive HEM (CD45+) populations (Figure 3.10D) that were not significantly different (Figure 3.10E). Interestingly, LIF/2i-hiPSCs with forced knockdown of *OCT4* appeared to recover HEM potential. However, it is undetermined which population of the bulk culture is contributing to the recovery of CD45+ cells upon delivery of the shRNA, since OCT4 expression in day 15 EBs was heterogeneous (Figure 3.10 B). These data indicate the possibility that bFGF extrinsic conditions prior to EB formation would further improve differentiation, supporting the necessity of differentiation to transition through an EpiSC-like state, as it does *in vivo*[45] and similar to what has been suggested for mESCs[95,131,139]. Most important, it appears the LIF/2i culture system reduces the propensity for differentiation while maintaining a heightened ability for pluripotent self-renewal, questioning the functional utility for culturing human PSCs in conditions defined by the mouse paradigm

Chapter 4

Discussion:

The generation of hiPSC lines from a diverse range of somatic sources[97] and the ongoing evolution towards defined culture conditions[15,24,52,107,115,117,120,140] underscores the need to assess the quality of any given hiPSC line. The excitement surrounding the future of hiPSCs lies in their potential to produce functional cell types for clinical therapeutics. Thus, hiPSC quality must be defined by the ability to efficiently generate targeted cell types of interest, and not by their multilineage "potential". Limiting the use of qualitative multilineage differentiation methods, such as teratoma formation or standard embryoid body (EB) differentiation[141], and replacing such methods with quantitative comparisons of pluripotent self-renewal and lineage specific differentiation between hiPSC lines is critical. Understanding how modifications of the extrinsic environment surrounding hPSCs influence the intrinsic signaling networks, which ultimately govern self-renewal and cell fate specification is essential for defining the utility of new culture systems. For example, a recent study from the Thomson group, Chen *et al.*[140] further refined a chemically defined cell culture system, referred to as E8 (essential 8), that propagates both hESC and hiPSC lines at higher efficiencies than previous generations of defined media formulations[142]. Although hPSCs were maintained in E8 on vitronectin-coated plates, maintenance required the use of chemical inhibitors that promote single cell survivability (i.e., Y27632, Blebbistatin, HA100), perplexing the effects of the defined

media system alone. Most intriguing, differentiation potential was not evaluated. It remains unclear whether new commercially available defined culture systems enable sufficient generation of targeted cell types in comparison to conventional hPSC culture methods. Similarly, hPSCs cultured under conditions defined by mESCs[107,115,117,120], rely on qualitative measurements of differentiation potential suggesting equipotential to bFGF cultured hPSCs. The caveat with qualitative measurements (which rely on immunocytochemistry for verification) is that they provide simplified explanations, resulting in ambiguous conclusions about the quality of new hPSC lines.

Here, we propose that altering the extrinsic environment of hiPSCs with chemically defined conditions (LIF/2i) essential for mESCs, enhances pluripotent self-renewal, while compromising efficient hematopoietic differentiation, through retained expression of the pluripotent transcription factor, OCT4 (Figure 4).

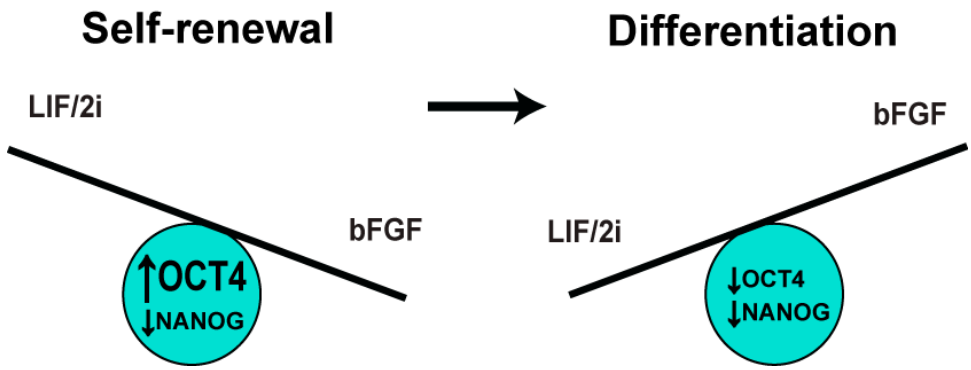


Figure 4. The Balance between Pluripotent self-renewal and differentiation are biased in distinct extrinsic conditions.

The evidence presented here suggests LIF/2i negatively impacts that quality (Figure 3.10B) of hiPSCs by creating a culture that is devoid of supportive niche cells and induces a homogenous state (Figure 3.1 A-B), similar to variant hESCs reported by our laboratory that are also impaired in efficient HEM potential[122]. This indicates that switching the extrinsic conditions to create a state that no longer depends on the interplay between the FGFR+ niche and IFGR+ undifferentiated colonies[44] likely impairs the ability of the putative naïve state to efficiently differentiate, similar to variant hESCs which no longer depend on bFGF for long-term self-renewal and co-express FGFR1 and IGF1R and are devoid of niche-supportive cells[122]. Future studies are necessary to determine the role of the FGFR/IGFR axis in the putative naïve state of hPSCs. For example, exploring whether acquisition of a stem cell niche upon re-establishing the FGFR/IGFR axis by culturing naïve hPSCs with bFGF can recover efficient HEM differentiation would better characterize the role the pluripotent stem cell niche plays in enabling differentiation from a primed state of pluripotency

In addition to the homogenous expression of SSEA3 (Figure 3.1B) and enhanced expression of pluripotent regulators (Figure 3.3B), genes commonly associated with the naïve state (*OCT4, NANOG, SOX2, KLF4, REX1, DPPA4, DPPA5* and *TBX3)* are over-expressed in LIF/2i-hiPSCs, while those genes associated with epiblast (*FGF8, FGFR1, FGFR2, FGFR3, NODAL, NOGGIN*) are downregulated (Figure 3.2A), similar to published reports comparing mESCs to EpiSCs[42,49,50,105,116,143]. Although the direct link between gene and protein expression is generally accepted, the possibility of post-transcriptional mechanisms or protein degradation masking protein expression, and thus a

true biological response, remains. The protein expression analysis of SSEA3, OCT4 and NANOG (Figure 3.3B) appears to correspond with the gene expression (Figure 3.2A), and enhanced pluripotent self-renewal (Figure 3.3D), supporting the notion that LIF/2i-hiPSCs presented here are biologically naïve.

Acquisition of a human naïve state has been reported[107,115,117,120] and employing transgenic overexpression of pluripotent reprogramming factors (OCT4, NANOG, SOX2, KLF4 or KLF2 and c-MYC) to achieve a naïve pluripotent state is prevalent[80,90,108,115,120,144]. As a consequence, such methods rely on their constitutive expression to maintain a human naïve state[115,120], possibly confounding naïve pluripotent gene expression or signaling characterization. Similar to Ding's group[107,117], and those reverting mouse EpiSCs to a naïve state[105,116,143], we were successful at reverting bFGF dependent hiPSCs to a stable naïve state by simply altering the extrinsic signals, thus eliminating the potential for transgene dependency (data not shown here). Initial gene expression data generated from our lab (Figure 3.2D) suggested that LIF/2i-hiPSCs may be supported by BMP4 signaling due to the overexpression of endogenous *BMP4* and the underexpression of the BMP4 inhibitor, *NOGGIN*. At the same, our naïve hiPSCs underexpressed *NODAL* and overexpressed Activin signaling antagonists (*LEFTYA*, *LEFTY2* and *CRIPTO*), supporting their divergence from an EpiSC-like state (Figure 3.2D).

To validate the gene expression data, we characterized the signaling dependency of LIF/2i-hiPSCs with respect to Activin and BMP4 TGFβ pathways by monitoring OCT4, NANOG and SSEA3 protein expression. Naïve hiPSCs maintained the expression of OCT4 gene and protein expression in the presence of SB431542 (selective inhibitor of Activin/Nodal) or BMP4 (Figure 3.4.1B and Figure 3.4.2). These data are similar to reports for mESCs[15,52] and human naïve hPSCs generated by Hanna *et al.*[115],although NANOG expression appeared anomalous to OCT4. Treatment with SB431542 or BMP4 significantly reduced NANOG expression (Figure's 3.4.1C & 3.4.2), pushing LIF/2i-hiPSCs to become predominantly OCT4(+). This implies naïve hiPSCs possibly retain signaling dependencies of conventional hPSCs. When treated with recombinant ACTIVIN A, LIF/2i-hiPSCs are unaffected and maintain an undifferentiated morphology with the simultaneous expression of OCT4 and NANOG (Figure 3.4.1). This is unexpected because Activin/Nodal stimulation does not maintain the naïve pluripotent state and induces endodermal differentiation in mESCs[145]. Collectively, it appears Activin and BMP4 TGFβ signaling pathways induce a response that is similar to conventional hPSCs, implying the reversion to a naïve state was incomplete.

Alternatively, our naïve hiPSCs could be an intermediate of the pre-implantation state, similar to the early-epiblast state in the mouse. Mouse EpiSCs are heterogeneous, like hPSCs, and contain variable degrees of EpiSCs that are reminiscent to the pre-gastrulation stages of the murine blastocyst. Han *et al*[109] demonstrated this by generating transgenic EpiSCs expressing an *Oct4*-GFP reporter (GOF18) under the control of the *Oct4* proximal enhancer (PE). In contrast to naïve mESCs that utilize the distal enhancer

(DE), EpiSCs express the GFP reporter. Sorting positive and negative fractions reveals that GFP(-) EpiSCs primarily utilize the PE of *Oct4*, while the GFP(+) fraction contains residual activation of the DE, suggesting this population better represents the early epiblast when compared to the *in vivo* E5.5 preimplantation epiblast. Analyzing the gene expression profiles, epigenetic status, developmental potential and reversion frequency to an ES-like state, supports their conclusion that EpiSCs are heterogeneous, where GFP(+) EpiSCs are analogous to the pre-implantation epiblast and GFP(-) EpiSCs are epiblastic. Gene expression and epigenetic analysis could support an early-epiblast explanation for our LIF/2i-hiPSCs. However, limitations prevent a complete comparison. First, both LIF/2i- and bFGF-dependent hiPSCs exhibit multilineage teratoma differentiation, similar to murine naïve and primed states. However, we cannot provide a clear distinction between the potential of multiple states of pluripotency without the use of a chimera assay, where primed EpiSCs display poor chimera contribution relative to naïve mESCs. Secondly, it is unclear whether LIF/2i and bFGF-dependent hPSCs rely on differential *OCT4* enhancer utilization seen in the mouse. Nevertheless, Chia *et al.*[146] have demonstrated that hESCs utilize the proximal enhancer of *OCT4,* similar to EpiSCs. After performing a genome-wide RNAi screen in hESCs expressing an *OCT4*-GFP reporter, Chia and colleagues identified the transcription factor, PRDM14, which directly regulates the proximal enhancer of *OCT4* in hESCs and during iPS reprogramming. Lastly, whether heterogeneous bFGF-hiPSCs can give rise to distinct pluripotent states representing truly pre-implantation ICM, and pre- and post-implantation epiblasts states is undetermined, since the epiblast forms upon implantation in the human[45].

Moving forward, LIF/2i-hiPSCs presented here revealed a divergent behaviour between the pluripotent regulators OCT4 and NANOG, which appears unlike typical hPSCs. Wang *et al.*[96] have shown through targeted knockdown of either *OCT4* or *NANOG* in standard hESCs, both transcriptions factors display a reciprocal behaviour, and equally induce the loss of the pluripotent state. This reciprocal behaviour contrasts that of naïve mESCs. *In vivo* and *in vitro*, loss of *Nanog* within pluripotent cells does not induce the loss of *Oct4*, however *Nanog* null blastocysts fail to generate the epiblast[18,83] (marked by the second differentiation event creating the primitive endoderm). Mouse ESCs with forced knockdown of *Nanog* do not lose *Oct4* or *Sox2* expression. In addition, *Nanog* null mESCs are still capable of pluripotent self-renewal and contribute during chimera formation but do not contribute to the primordial germ cells[84]. Similar to this work, our LIF/2i-hiPSCs preserved an SSEA3(+)OCT4(+) population, indicating BMP4 or SB431542 treated cells may retain clonogenic potential (Figure.3.4.2A and Figure 3.5A-B). After 5 days of BMP4 treatment, LIF/2i-hiPSCs were able to produce a number of TRA-1-60(+) colonies at an efficiency ~50% of the untreated control (Figure 3.6), confirming that naïve hPSCs with significantly reduced NANOG, but high OCT4, expression retain the capacity for pluripotent self-renewal. To confirm this, targeted gene silencing of *OCT4* and *NANOG* in LIF/2i-hiPSCs (Figure's 3.7 & 3.8) revealed that *OCT4* knockdown induced differentiation (Figure 3.7 B), similar to mESCs. This was supported by the downregulation of both OCT4 and NANOG protein and gene expression (Figure 3.8B &E) and complete loss of pluripotent clonogenic self-renewal (Figure 3.9A & C). LIF/2i-hiPSCs with *NANOG* silenced, however, did not reciprocally knockdown

OCT4, which remained equivalent to the empty vector control (Figure 3.8 C,D & E) and similar to the observations with BMP4 and SB431542 treatment. *NANOG* deficient LIF/2i-hiPSCs also retained a proportion of SSEA3(+)OCT4(+) cells and retained the ability to regenerate clonogenic pluripotent colonies upon FACS sorting (Figure 3.9 A & C), similar to naïve mESCs.

NANOG deficient LIF/2i-hiPSCs preserved active LIF/STAT3 signaling and did not reactivate an epiblast-like program made evident through inactive p-ERK1/2 (T202/Y204) (Figure 3.8 F). As described for murine naïve and primed states of pluripotency, *Nanog* overexpression enables independent pluripotent self-renewal in mESCs and reverts EpiSCs to a naïve state[80,83,90]. These reports highlight *Nanog* as a critical modulator between these metastable states. Here in the human naïve state, *NANOG* downregulation does not appear to induce an EpiSC state, although *NANOG* deficient LIF/2i-hiPSCs maintain *OCT4* expression and an undifferentiated morphology (Figure 3.7B & 3.9D). A caveat may have circumvented this observation, since the gene-targeting experiments were always in the presence of LIF/2i, possibly confounding the signaling status by preventing the reactivation of p-ERK1/2. It would be necessary to repeat the forced knockdown of *NANOG* and switch the LIF/2i supplemented medium to a basal medium to determine whether FGF/ERK1/2 signaling becomes active. In addition, further analysis of a possible EpiSC-like state would be necessary, including gene expression and epigenetic changes reminiscent of the epiblast. Overexpression of NANOG in bFGF-hiPSCs would enable insight into the interconvertibility between the

two states and determine conclusively whether NANOG regulates hPSCs similar to mPSCs.

To address the ability of LIF/2i-hiPSCs to differentiate, a defining feature for any hPSC line, we simply chose to examine their efficiency at producing blood, representative of a mature lineage commitment. We formed EBs from LIF/2i-hiPSCs and bFGF-hiPSCs and supplemented the medium with hematopoietic-specific cytokines for 15 days (Figure 3.10 A). In comparison to standard hiPSCs, LIF/2i cultured hiPSCs displayed a reduction (0.4±0.1% from 8.8±0.3%) in CD34(+)CD45(+) progenitors and mature CD45(+) blood (0.6±0.2% from 65±10%) (Figure 3.10 B). Upon examining the pluripotent CIC potential in day 15 HEM EBs, we found LIF/2i-hiPSCs retained OCT4(+) cells, bFGF-hiPSCs (Figure 3.10 C). An shRNA vector targeting *OCT4* successfully forced the knockdown of both OCT4 and NANOG in LIF/2i-hiPSCs (Figure3.8 A-B). HEM EBs generated from sh-*OCT4* transduced LIF/2i-hiPSCs recovered primitive CD34(+) and mature CD45(+) blood cells (Figure 3.10 D-E), but CD34(+)CD45(+) progenitors were not detected, suggesting that multilineage differentiation is impaired. Compared to the HEM efficiency displayed by bFGF-hiPSCs this suggests that the putative primed state is a more efficient state to direct differentiation from in human pluripotent stem cells. This finding is in contrast to what has been previously reported by Marks and colleagues[147], who cultured mESCs in 2i versus serum and concluded the LIF/2i naïve state is a better state to induce differentiation from. However, their differentiation analysis only included neural differentiation utilizing one clone of a *SOX1* reporter line to indicate differentiation and they did not include other lineages such as hematopoietic lineages. Similarly, we

analyzed only one clone for each hiPSC line, and our observations would require further analysis on multiple clones from at least three individual hiPSC lines for each of the LIF/2i and bFGF culture conditions in order to be conclusive.

It has been implied that *in vitro* mESCs transition through an epiblast state in order to differentiate, similar to the events *in vivo*. The EB assay appears to be the preferential method for directing differentiation from pluripotent stem cells, mouse and human alike[65,134,136,148]. Similarly, when differentiating mESCs and hPSCs, they are immediately forced into an embryoid structure and directed into a specific lineage with exogenous cues. This step-wise method appears to proceed directly to the primitive streak[65,149], where mESCs bypass an epiblast-like state as described[42,49,50,109]. The evidence presented here suggests (from a developmental point of view) that the epiblast-like state represented by bFGF-dependent hPSCs, is more efficient for inducing lineage specific cell fates. This supports the *in vivo* transition through the epiblast; although murine EpiSCs derived *in vitro* appear to have poor developmental potential in chimera formation.

Conclusions:

The importance of our functional comparison between "naïve" and "epiblast-like" hPSCs lies in the current opinions and practices, which utilize the mouse model as a surrogate for human developmental biology. The direct comparison of mouse and human pluripotent extrinsic conditions and their influence on differentiation, may provide further insight as to whether the mechanisms controlling mouse pluripotency can be accurately

extrapolated to human pluripotency. Importantly, we resolve a divergence in the pluripotent regulation between OCT4 and NANOG that confers an impediment to efficient hematopoietic differentiation in LIF/2i cultured hiPSCs. However, this is likely not limited to HEM lineages and may prove advantageous for other cell fates, including neuronal lineages as indicated by Li *et al*[150], an avenue that is currently being explored in our lab. Although much of the basis of pluripotent regulation is well established in the mouse, this thesis underscores our reluctance to conclude that the mouse is a sufficient model to understand functional human pluripotency. For example, the abilities to derive the ICM of the murine blastocyst in culture conditions that propagate hESCs and EpiSCs[105,108], and revert EpiSCs to a naïve state with LIF/2i[101,108,109,116,143] , suggests the growth factor environment, not developmental origin nor so-called permissiveness of cell lines, dictates multiple potentials of pluripotency. As a result, the distinction of two developmental states, may not be attainable in a Petri dish, and precisely where hPSCs fall within this theory is undefined. Developmental biologists have extensively compared mouse and human development through either *ex vivo* embryology or *in vitro* derived ES cell lines [16,151]. However, such comparisons highlight significant inter-species differences in the signaling cascades that regulate the intrinsic pluripotent networks, as outlined previously, and have indirectly underscored the difficulty of translating mouse biology to human therapeutic applications. To better explain the developmental aspect of hPSCs cultured with LIF/2i, it is necessary to approach the question using a non-human primate model[152]. The successful derivation of LIF/2i-dependent primate ESCs that can generate high-grade chimeras would be a definitive test to resolve multiple states of pluripotency

in a clinically relevant subject. In addition, to make the claim that the LIF/2i system generates "better" cells requires sufficient quantitative measurements of functional differentiation potential as described here. This also includes, but is not limited to, establishing whether differentiated cells types are functionally equivalent to the adult somatic cell type through physiological analysis and xenotransplantation assays.

Currently, it remains unclear whether *in vitro* culture conditions can distinguish two states of pluripotency and whether or not this lack of definitive evidence will influence the future of developmental biology and regenerative medicine. Thus, the practical utility of LIF/2i for maintaining human pluripotent stem cells remains unclear for the time being.

REFERENCES

1. Till, J. E. & McCulloch, E. A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. 1961. *Radiat Res* **175,** 145–149 (2011).
2. BECKER, A. J., McCulloch, E. A. & Till, J. E. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* **197,** 452–454 (1963).
3. Sacchetti, B. *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131,** 324–336 (2007).
4. Mendez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466,** 829–834 (2010).
5. Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L. & Fuchs, E. Self-Renewal, Multipotency, and the Existence of Two Cell Populations within an Epithelial Stem Cell Niche. *Cell Stem Cell* **118,** 635–648 (2004).
6. Morris, R. J. *et al.* Capturing and profiling adult hair follicle stem cells. *Nat Biotechnol* **22,** 411–417 (2004).
7. Vicario-Abejon, C., Johe, K. K., Hazel, T. G., Collazo, D. & McKay, R. D. Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron* **15,** 105–114 (1995).
8. Gritti, A. *et al.* Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J Neurosci* **16,** 1091–1100 (1996).
9. Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449,** 1003–1007 (2007).
10. Bjerknes, M. & Cheng, H. Clonal analysis of mouse intestinal epithelial progenitors. *Gastroenterology* **116,** 7–14 (1999).
11. Martin, G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7634–7638 (1981).
12. Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154–156 (1981).
13. Capecchi, M. R. Altering the genome by homologous recombination. *Science* **244,** 1288–1292 (1989).
14. Koller, B. H. *et al.* Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 8927–8931 (1989).
15. Ying, Q.-L., Nichols, J., Chambers, I. & Smith, A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115,** 281–292 (2003).
16. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95,** 379–391 (1998).
17. Rodda, D. J. *et al.* Transcriptional regulation of nanog by OCT4 and SOX2. *J Biol Chem* **280,** 24731–24737 (2005).

18. Chambers, I. *et al.* Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113,** 643–655 (2003).
19. Thomson, J. A. Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science* **282,** 1145–1147 (1998).
20. Williams, R. L. *et al.* Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336,** 684–687 (1988).
21. Daheron, L. *et al.* LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* **22,** 770–778 (2004).
22. Xu, R.-H. *et al.* BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* **20,** 1261–1264 (2002).
23. Zhang, P. *et al.* Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells. *Blood* **111,** 1933–1941 (2008).
24. Xu, R.-H. *et al.* Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Meth* **2,** 185–190 (2005).
25. Levenstein, M. E. *et al.* Basic Fibroblast Growth Factor Support of Human Embryonic Stem Cell Self-Renewal. *Stem Cells* **24,** 568–574 (2006).
26. Li, J. *et al.* MEK/ERK signaling contributes to the maintenance of human embryonic stem cell self-renewal. *Differentiation* **75,** 299–307 (2007).
27. Beattie, G. M. *et al.* Activin A Maintains Pluripotency of Human Embryonic Stem Cells in the Absence of Feeder Layers. *Stem Cells* **23,** 489–495 (2005).
28. Vallier, L., Alexander, M. & Pedersen, R. A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *jcs.biologists.org*
29. James, D., Levine, A. J., Besser, D. & Hemmati-Brivanlou, A. TGFβ/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *dev.biologists.org*
30. Murry, C. E. & Keller, G. Differentiation of Embryonic Stem Cells to Clinically Relevant Populations: Lessons from Embryonic Development. *Cell* **132,** 661–680 (2008).
31. Boyer, L. A. *et al.* Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122,** 947–956 (2005).
32. Loh, Y.-H. *et al.* The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* **38,** 431–440 (2006).
33. GURDON, J. B. Adult frogs derived from the nuclei of single somatic cells. *Developmental Biology* **4,** 256–273 (1962).
34. Briggs, R. & King, T. J. Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. *Proc. Natl. Acad. Sci. U.S.A.* **38,** 455–463 (1952).
35. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126,** 663–676 (2006).
36. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131,** 861–872 (2007).
37. Yu, J. *et al.* Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. *Science* **318,** 1917–1920 (2007).

38. Pera, M. F. & Tam, P. P. L. Extrinsic regulation of pluripotent stem cells. *Nature* **465,** 713–720 (2010).
39. Kuijk, E. W. *et al.* The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. *Development* **139,** 871–882 (2012).
40. Roode, M. *et al.* ScienceDirect.com - Developmental Biology - Human hypoblast formation is not dependent on FGF signalling. *Developmental Biology* **361,** 358–363 (2012).
41. Schlessinger, J. Cell Signaling by Receptor Tyrosine Kinases. *Cell Stem Cell* **103,** 211–225 (2000).
42. Greber, B. *et al.* Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell* **6,** 215–226 (2010).
43. Greber, B., Lehrach, H. & Adjaye, J. Fibroblast Growth Factor 2 Modulates Transforming Growth Factor β Signaling in Mouse Embryonic Fibroblasts and Human ESCs (hESCs) to Support hESC Self-Renewal. *Stem Cells* **25,** 455–464 (2007).
44. Bendall, S. C. *et al.* IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature* **448,** 1015–1021 (2007).
45. Katie Cockburn, J. R. Making the blastocyst: lessons from the mouse. *The Journal of Clinical Investigation* **120,** 995 (2010).
46. Niswander, L. & Martin, G. R. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse.
47. Yuan, H., Corbi, N., Basilico, C. & Dailey, L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3.
48. Hebert, J. M., Boyle, M. & Martin, G. R. mRNA localization studies suggest that murine FGF-5 plays a role in gastrulation.
49. Brons, I. G. M. *et al.* Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448,** 191–195 (2007).
50. Tesar, P. J. *et al.* New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448,** 196–199 (2007).
51. Kunath, T. *et al.* FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* **134,** 2895–2902 (2007).
52. Ying, Q.-L. *et al.* The ground state of embryonic stem cell self-renewal. *Nature* **453,** 519–523 (2008).
53. Nichols, J., Silva, J., Roode, M. & Smith, A. Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* **136,** 3215–3222 (2009).
54. Shi, Y. & Massagué, J. Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus. *Cell* **113,** 685–700 (2003).
55. Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T. & Miyazono, K. Two major Smad pathways in TGF-beta superfamily signalling. *Genes Cells* **7,** 1191–1204 (2002).

56. Smith, J. R. *et al.* Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. *Developmental Biology* **313,** 107–117 (2008).
57. Vallier, L., Reynolds, D. & Pedersen, R. A. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Developmental Biology* **275,** 403–421 (2004).
58. Shen, M. M. Nodal signaling: developmental roles and regulation.
59. Inman, G. J. *et al.* SB-431542 Is a Potent and Specific Inhibitor of Transforming Growth Factor-β Superfamily Type I Activin Receptor-Like Kinase (ALK) Receptors ALK4, ALK5, and ALK7.
60. Avery, S., Zafarana, G., Gokhale, P. J. & Andrews, P. W. The Role of SMAD4 in Human Embryonic Stem Cell Self-Renewal and Stem Cell Fate. *Stem Cells* **28,** N/A–N/A (2010).
61. Xiao, L., Yuan, X. & Sharkis, S. J. Activin A Maintains Self-Renewal and Regulates Fibroblast Growth Factor, Wnt, and Bone Morphogenic Protein Pathways in Human Embryonic Stem Cells. *Stem Cells* **24,** 1476–1486 (2006).
62. Xu, R.-H. *et al.* NANOG Is a Direct Target of TGFβ/Activin-Mediated SMAD Signaling in Human ESCs. *Cell Stem Cell* **3,** 196–206 (2008).
63. Kubo, A. *et al.* Development of definitive endoderm from embryonic stem cells in culture. *Development* **131,** 1651–1662 (2004).
64. Green, M. D. *et al.* Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. *Nat Biotechnol* **29,** 267–272 (2011).
65. Kattman, S. J. *et al.* Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* **8,** 228–240 (2011).
66. Stewart, C. L. *et al.* Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359,** 76–79 (1992).
67. Smith, A. G. *et al.* Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336,** 688–690 (1988).
68. Yoshida, K. *et al.* Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. *Mech Dev* **45,** 163–171 (1994).
69. Matsuda, T. *et al.* STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J* **18,** 4261–4269 (1999).
70. Onishi, K., Tonge, P. D., Nagy, A. & Zandstra, P. W. Microenvironment-mediated reversion of epiblast stem cells by reactivation of repressed JAK–STAT signaling. *Integr. Biol.* **4,** 1367–1376 (2012).
71. Yang, J. *et al.* Stat3 Activation Is Limiting for Reprogramming to Ground State Pluripotency. *Stem Cell* **7,** 319–328
72. van Oosten, A. L., Costa, Y., Smith, A. & Silva, J. C. R. JAK/STAT3 signalling is sufficient and dominant over antagonistic cues for the establishment of naive pluripotency. *Nat Comms* **3,** 817 (2012).
73. Hall, J. *et al.* Oct4 and LIF/Stat3 additively induce Krüppel factors to sustain

embryonic stem cell self-renewal. *Cell Stem Cell* **5,** 597–609 (2009).
74. Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. & Brivanlou, A. H. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* **10,** 55–63 (2004).
75. Barker, N. in *Methods in Molecular Biology* **468,** 5–15 (Humana Press, 2008).
76. Wray, J. *et al.* Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. *Nat Cell Biol* **13,** 838–845 (2011).
77. Berge, D. T. *et al.* Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. *Nat Cell Biol* **13,** 1070–1075 (2011).
78. Lyashenko, N. *et al.* Differential requirement for the dual functions of [beta]-catenin in embryonic stem cell self-renewal and germ layer formation. *Nat Cell Biol* **13,** 753–761 (2011).
79. Dravid, G. *et al.* Defining the Role of Wnt/β-Catenin Signaling in the Survival, Proliferation, and Self-Renewal of Human Embryonic Stem Cells. *Stem Cells* **23,** 1489–1501 (2005).
80. Silva, J. *et al.* ScienceDirect.com - Cell - Nanog Is the Gateway to the Pluripotent Ground State. *Cell* **138,** 722–737 (2009).
81. Niwa, H., Miyazaki, J. & Smith, A. G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24,** 372–376 (2000).
82. Thomson, M. *et al.* Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. *Cell* **145,** 875–889 (2011).
83. Mitsui, K. *et al.* Cell - The Homeoprotein Nanog Is Required for Maintenance of Pluripotency in Mouse Epiblast and ES Cells. *Cell* **113,** 631–642 (2003).
84. Chambers, I. *et al.* Nanog safeguards pluripotency and mediates germline development. *Nature* **450,** 1230–1234 (2007).
85. Avilion, A. A. *et al.* Multipotent cell lineages in early mouse development depend on SOX2 function. *genesdev.org*
86. Graham, V., Khudyakov, J., Ellis, P. & Pevny, L. SOX2 functions to maintain neural progenitor identity. *Neuron* **39,** 749–765 (2003).
87. Chew, J.-L. *et al.* Reciprocal Transcriptional Regulation of Pou5f1 and Sox2 via the Oct4/Sox2 Complex in Embryonic Stem Cells. *mcb.asm.org*
88. Masui, S. *et al.* Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* **9,** 625–635 (2007).
89. van den Berg, D. L. C. *et al.* An Oct4-centered protein interaction network in embryonic stem cells. *Cell Stem Cell* **6,** 369–381 (2010).
90. Theunissen, T. W. *et al.* Nanog overcomes reprogramming barriers and induces pluripotency in minimal conditions. *Curr. Biol.* **21,** 65–71 (2011).
91. MacArthur, B. D. *et al.* Nanog-dependent feedback loops regulate murine embryonic stem cell heterogeneity. *Nat Cell Biol* – (2012). doi:10.1038/ncb2603
92. Yu, P., Pan, G. & Yu, J. ScienceDirect.com - Cell Stem Cell - FGF2 Sustains NANOG and Switches the Outcome of BMP4-Induced Human Embryonic Stem

Cell Differentiation. *Cell Stem Cell* (2011).
93. Nichols, J. & Smith, A. Naive and Primed Pluripotent States. *Stem Cell* **4,** 487–492 (2009).
94. Buecker, C. & Geijsen, N. Different Flavors of Pluripotency, Molecular Mechanisms, and Practical Implications. *Cell Stem Cell* **7,** 559–564 (2010).
95. Miyanari, Y. & Torres-Padilla, M.-E. Control of ground-state pluripotency by allelic regulation of Nanog. *Nature* **483,** 470–473 (2012).
96. Wang, Z., Oron, E., Nelson, B., Razis, S. & Ivanova, N. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. *Cell Stem Cell* **10,** 440–454 (2012).
97. Wu, S. M. & Hochedlinger, K. Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nat Cell Biol* **13,** 497–505 (2011).
98. Desbordes, S. C. *et al.* High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. *Cell Stem Cell* **2,** 602–612 (2008).
99. Sachlos, E. *et al.* Identification of Drugs Including a Dopamine Receptor Antagonist that Selectively Target Cancer Stem Cells. *Cell* (2012). doi:10.1016/j.cell.2012.03.049
100. Cohen, D. E. & Melton, D. Turning straw into gold: directing cell fate for regenerative medicine. *Nat Rev Genet* **12,** 243–252 (2011).
101. Han, D. W. *et al.* Direct reprogramming of fibroblasts into epiblast stem cells. *Nat Cell Biol* **13,** 66–71 (2011).
102. Selwood, L. & Johnson, M. H. Trophoblast and hypoblast in the monotreme, marsupial and eutherian mammal: evolution and origins. *Bioessays* **28,** 128–145 (2006).
103. Darr, H., Mayshar, Y. & Benvenisty, N. Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features. *Development* **133,** 1193–1201 (2006).
104. Di Stefano, B. *et al.* An ES-like pluripotent state in FGF-dependent murine iPS cells. *PLoS ONE* **5,** e16092 (2010).
105. Najm, F. J. *et al.* ScienceDirect.com - Cell Stem Cell - Isolation of Epiblast Stem Cells from Preimplantation Mouse Embryos. *Cell Stem Cell* **8,** 318–325 (2011).
106. Buehr, M. *et al.* Capture of authentic embryonic stem cells from rat blastocysts. *Cell* **135,** 1287–1298 (2008).
107. Li, W. *et al.* Generation of Rat and Human Induced Pluripotent Stem Cells by Combining Genetic Reprogramming and Chemical Inhibitors. *Stem Cell* **4,** 16–19 (2009).
108. Hanna, J. *et al.* Metastable Pluripotent States in NOD-Mouse-Derived ESCs. *Stem Cell* **4,** 513–524 (2009).
109. Han, D. W. *et al.* Epiblast stem cell subpopulations represent mouse embryos of distinct pregastrulation stages. *Cell* **143,** 617–627 (2010).
110. Lengner, C. J. *et al.* Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. *Cell* **141,** 872–883 (2010).

111. Zwaka, T. P. & Thomson, J. A. Homologous recombination in human embryonic stem cells. *Nat Biotechnol* **21,** 319–321 (2003).
112. Ptaszek, L. M. & Cowan, C. A. New Tools for Genome Modification in Human Embryonic Stem Cells. *Cell Stem Cell* **1,** 600–602 (2007).
113. Li, W. *et al.* Generation of Rat and Human Induced Pluripotent Stem Cells by Combining Genetic Reprogramming and Chemical Inhibitors. *Cell Stem Cell* **4,** 16–19 (2009).
114. Buecker, C. *et al.* A murine ESC-like state facilitates transgenesis and homologous recombination in human pluripotent stem cells. *Cell Stem Cell* **6,** 535–546 (2010).
115. Hanna, J. *et al.* Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 9222–9227 (2010).
116. Bao, S. *et al.* Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells : Article : Nature. *Nature* **461,** 1292–1295 (2009).
117. Xu, Y. *et al.* Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. *Proceedings of the National Academy of Sciences* **107,** 8129–8134 (2010).
118. Rubinson, D. A. *et al.* A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* **33,** 401–406 (2003).
119. Ji, J., Werbowetski-Ogilvie, T. E., Zhong, B., Hong, S.-H. & Bhatia, M. Pluripotent Transcription Factors Possess Distinct Roles in Normal versus Transformed Human Stem Cells. *PLoS ONE* **4,** e8065 (2009).
120. Buecker, C. *et al.* A Murine ESC-like State Facilitates Transgenesis and Homologous Recombination in Human Pluripotent Stem Cells. *Cell Stem Cell* **6,** 535–546 (2010).
121. Stewart, M. H. *et al.* Clonal isolation of hESCs reveals heterogeneity within the pluripotent stem cell compartment. *Nat Meth* **3,** 807–815 (2006).
122. Werbowetski-Ogilvie, T. E. *et al.* Characterization of human embryonic stem cells with features of neoplastic progression. *Nat Biotechnol* **27,** 91–97 (2009).
123. Minkovsky, A., Patel, S. & Plath, K. Concise review: Pluripotency and the transcriptional inactivation of the female Mammalian X chromosome. *Stem Cells* **30,** 48–54 (2012).
124. Shen, Y. *et al.* X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations. *Proceedings of the National Academy of Sciences* **105,** 4709–4714 (2008).
125. Silva, S. S., Rowntree, R. K., Mekhoubad, S. & Lee, J. T. X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells. (2008).
126. Dvash, T., Lavon, N. & Fan, G. Variations of X chromosome inactivation occur in early passages of female human embryonic stem cells. *PLoS ONE* **5,** e11330 (2010).
127. Vallier, L., Alexander, M. & Pedersen, R. A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J Cell Sci*

118, 4495–4509 (2005).
128. Gray, P. C. Cripto forms a complex with activin and type II activin receptors and can block activin signaling. *Proceedings of the National Academy of Sciences* **100,** 5193–5198 (2003).
129. Kelber, J. A. *et al.* Blockade of Cripto binding to cell surface GRP78 inhibits oncogenic Cripto signaling via MAPK/PI3K and Smad2/3 pathways. *Oncogene* **28,** 2324–2336 (2009).
130. Hough, S. R., Clements, I., Welch, P. J. & Wiederholt, K. A. Differentiation of mouse embryonic stem cells after RNA interference-mediated silencing of OCT4 and Nanog. *Stem Cells* **24,** 1467–1475 (2006).
131. Chambers, I. *et al.* Nanog safeguards pluripotency and mediates germline development. *Nature* **450,** 1230–1234 (2007).
132. Chang, K.-H. *et al.* Definitive-like erythroid cells derived from human embryonic stem cells coexpress high levels of embryonic and fetal globins with little or no adult globin.
133. McIntyre, B. A. S. *et al.* Gli3-mediated hedgehog inhibition in human pluripotent stem cells initiates and augments developmental programming of adult hematopoiesis. *Blood* (2013). doi:10.1182/blood-2012-09-457747
134. Wiles, M. V. & Keller, G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *dev.biologists.org*
135. WANG, L. Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. *Journal of Experimental Medicine* **201,** 1603–1614 (2005).
136. Zambidis, E. T., Peault, B., Park, T. S., Bunz, F. & Civin, C. I. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. *bloodjournal.hematologylibrary.org*
137. WANG, L., MENENDEZ, P., CERDAN, C. & BHATIA, M. Hematopoietic development from human embryonic stem cell lines. *Experimental Hematology* **33,** 987–996 (2005).
138. Vodyanik, M. A. Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood* **105,** 617–626 (2005).
139. Marks, H. *et al.* The Transcriptional and Epigenomic Foundations of Ground State Pluripotency. *Cell* **149,** 590–604 (2012).
140. Chen, G. *et al.* Chemically defined conditions for human iPSC derivation and culture. *Nat Meth* **8,** 424–429 (2011).
141. Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. & Kemler, R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *dev.biologists.org*
142. The International Stem Cell Initiative Consortium *et al.* Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. *In Vitro Cell.Dev.Biol.-Animal* **46,** 247–258 (2010).
143. Chou, Y.-F. *et al.* The growth factor environment defines distinct pluripotent

ground states in novel blastocyst-derived stem cells. *Cell* **135,** 449–461 (2008).
144. Guo, G. *et al.* Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* **136,** 1063–1069 (2009).
145. Chen, A. E., Borowiak, M., Sherwood, R. I., Kweudjeu, A. & Melton, D. A. Functional evaluation of ES cell-derived endodermal populations reveals differences between Nodal and Activin A-guided differentiation.
146. Chia, N.-Y. *et al.* A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. *Nature* **468,** 316–320 (2010).
147. Marks, H. *et al.* The Transcriptional and Epigenomic Foundations of Ground State Pluripotency. *Cell* **149,** 590–604 (2012).
148. Kurosawa, H. Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. *Journal of Bioscience and Bioengineering* **103,** 389–398 (2007).
149. Gadue, P., Huber, T. L., Paddison, P. J. & Keller, G. M. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 16806–16811 (2006).
150. Li, W. *et al.* Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors.
151. Shamblott, M. J. *et al.* Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 13726–13731 (1998).
152. Tachibana, M. *et al.* Generation of Chimeric Rhesus Monkeys. *Cell* **148,** 285–295 (2012).