Determining the Signalling Pathways that Govern Human Naïve Pluripotency

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Conventional or "primed" human embryonic stem cells (hESCs) rely on FGF and TGFβ signalling for self-renewal, and occupy a developmentally advanced state of pluripotency comparable to mouse EpiSCs. Recent reports demonstrate that a naïve state of human pluripotency can be consistently derived either through transient histone deacetylase inhibition mediated resetting of conventional hESCs or via isolation of the inner cell mass. Long-term propagation of this state can be achieved using a cocktail of MEK, GSK3 and PKC inhibition in conjunction with leukaemia inhibitory factor (LIF) supplementation (t2iLGö) and a feeder layer of inactivated mouse embryonic fibroblasts. However, the way in which this signalling environment is interpreted in order to maintain naïve pluripotency remains unclear.

I demonstrate a substrate consisting of a high concentration of tissue-derived laminin in combination with t2iLGö is sufficient to replace the feeder layer. Cultures maintained under these conditions are karyotypically normal, maintain a naive pluripotent transcriptional profile and exhibit reduced aberrant expression of mesodermal and endodermal lineage markers.

I utilise the increased stringency of this culture system in combination with small molecule inhibitors to examine the roles of FGF, Activin/Nodal and JAK/STAT signalling in human naïve pluripotency. Naïve hESCs proliferate and maintain pluripotency marker expression in the presence of FGF receptor inhibition. In contrast, TGFβ signalling inhibition leads to rapid downregulation of human specific naïve pluripotency marker, *KLF17*, followed by the eventual collapse of the naïve transcription factor circuitry. Naïve hESCs self-renew in both the absence of LIF and presence of JAK/STAT inhibitors. However, further investigation of JAK/STAT signalling identified the increased potency of Interleukin 6 (IL-6) over LIF to activate the JAK/STAT pathway. Supplemental IL-6 improves colony-forming capacity under self-renewing conditions and attenuates differentiation following inhibitor withdrawal. Furthermore, prolonged activation of IL-6 signalling suppresses expression of *GATA2* and *GATA3* and upregulates *KLF4* transcripts.

Finally, I investigate whether ablation of PKCι is sufficient to replace the activity of the PKC inhibitor, Gö6983. Established naïve cultures that are PKCι null continue to express naïve markers and suppress upregulation of lineage makers following withdrawal of Gö6983. Furthermore, ablation of PKCι in conventional ESCs enables the maintenance of NANOG

expression and the emergence of KLF17 expression in the absence of Gö6983 during histone deactylase mediated resetting.

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Abbreviations

ActA	Activin A
2iL	MEKi GSK3i and LIF
aPKC	Atypical PKC
cR	Prefix indicating chemical resetting
Dox	Doxacycline
E3.5 etc	Embryonic day 4.5 or 4.5 days post coitum
ECM	Extracellular Matrix
Epi	Epiblast
EpiSC	Epiblast stem cell
ES cell	Embryonic stem cell
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FPKM	Fragments per kilobase per million
GFP	Green fluorescent protein
hESC	Human embryonic stem cell
ICM	Inner cell mass
IL-6	Interleukin 6
IVF	*In vitro* fertilisation
JAK	Janus activated kinase
LIF	Leukaemia inhibitory factor
MEFs	Mouse embryonic fibroblasts
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD17	PD173074
PGC	Primordial germ cell
PKC	Protein Kinase C
PrE	Primitive endoderm
qRT-PCR	Quantitative real-time PCR
RNA-seq	RNA sequencing
RPKM	Reads per kilobase per million
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
STAT	Signal transducer and activator of transcription
TE	Trophectoderm
Tet	Tetracycline
VPA	Valproic acid
WT	Wild type

Chapter 1. Introduction

1.1. The developmental origin of pluripotency – lessons from the mouse

Development of a new organism begins with fertilisation of the oocyte, generating the zygote and giving rise to the totipotent state from which both the embryonic and extra embryonic lineage are established. Regulation within the very early stages of development is reliant on maternal factors remaining in the oocyte (Li et al., 2010). These factors initiate the first round of zygote cleavage to generate the two-cell embryo, from which point the embryonic genome is activated and gives rise to the transcripts and factors that guide the subsequent stages of embryonic development (Li et al., 2010).

Following embryonic genome activation (EGA), a further two rounds of cleavage take place to generate the 4-cell and then 8-cell embryo before cell division is properly initiated. The recent use of lineage tracing strategies has provided insight into early lineage bias of the blastomeres within the cleaving embryo. When taken from the 2-cell embryo, blastomeres nearly always contribute to both the trophectoderm and the inner cell mass (ICM) (Fujimori et al., 2003). However blastomeres recovered from the 4-cell stage exhibit a degree of bias, with approximately 30% contributing solely to the trophectoderm, suggesting lineage specification may already be underway at this early stage (Fujimori et al., 2003; Tabansky et al., 2013). Further investigation of lineage bias amongst blastomeres has identified heterogeneity of Prdm14 expression, Oct4 motility and methylation at the arginine 26 residue of histone H3 as potential contributing factors (Burton et al., 2013; Plachta et al., 2011; Torres-Padilla et al., 2007).

At the 8-cell stage the embryo undergoes the processes of compaction and polarisation, whereby the blastomeres flatten against each other and apical domains are established. Cell adhesion protein, E-cadherin and Wnt signalling component, β-catenin are known to be essential for successful compaction (De Vries et al., 2004; Larue et al., 1994; Shirayoshi et al., 1983). During polarisation the apical domain becomes enriched with polarity components Par6, Par3 and atypical PKCs (aPKC) (Yamanaka et al., 2006). The asymmetric division of polarised blastomeres is thought to be one of the earliest lineage specifying events, with the polarised daughters eventually forming the trophectoderm

and the non-polar daughters generating the ICM (Johnson and Ziomek, 1981). Indeed, disruption of apical polarity via expression of a transgenic dominant negative form of aPKC has been reported to direct progeny towards the centre of the embryo, adopting a position associated with an ICM fate (Plusa et al., 2005).

The transcription factor Cdx2 is one of the central factors governing the initial cell-fate decision between trophectoderm and ICM lineages. Cdx2 null embryos show defects in trophectoderm formation and ectopic expression of the ICM factor Oct4 (Strumpf et al., 2005). In support of this, overexpression of Cdx2 in mouse embryonic stem cells induces trophectoderm-like differentiation and suppresses expression of Oct4 (Niwa et al., 2005). It has been suggested that blastomere polarity contributes to trophectoderm specification by recruiting Cdx2 to the apical domain, thereby ensuring asymmetric inheritance in daughter cells following cell division (Jedrusik et al., 2010; Jedrusik et al., 2008). Regulation of Cdx2 expression via the Hippo/Yap pathway is thought to be a key mechanism of lineage specification at this time. In the outer cells of the embryo, a lack of cell-cell contact prevents activation of the Hippo pathway and phosphorylation of Yap. Yap subsequently translocates to the nucleus and acts as a transcriptional activator of Tead4, driving expression of trophectoderm-specifying genes, including *Cdx2* and *Gata3* (Nishioka et al., 2009; Ralston et al., 2010). In the ICM, contact with neighbouring cells leads to activation of Hippo signalling and phosphorylation of Yap by Lats, preventing its translocation to the nucleus (Nishioka et al., 2009). Consistent with this, Tead4$^{-/-}$ embryos exhibit diminished *Cdx2* and *Eomes* expression within the morula, pre-implantation lethality and a failure to generate trophoblast giant cells *in vitro* (Nishioka et al., 2008). Similarly, mutant embryos lacking Eomes also arrest at the blastocyst stage and fail to generate the trophoblast, suggesting both Cdx2 and Eomes are important regulators of trophectoderm development (Russ et al., 2000; Strumpf et al., 2005).

The next lineage decision to be made is the differentiation of the ICM cells into either the pluripotent epiblast that will give rise to the embryo proper, or the primitive endoderm that will form the extra embryonic endoderm, including the yolk sac.

At the 8-cell stage all blastomeres are positive for Nanog and Gata6 expression, however by the 64-cell stage a pronounced salt and pepper distribution can be observed, with

Nanog expression marking epiblast precursors and Gata6 expression marking primitive endoderm precursors (Guo et al., 2010; Kurimoto et al., 2006; Plusa et al., 2008). Furthermore, Gata6 null mutants express Nanog throughout the ICM indicating a shift towards acquisition of epiblast fate (Bessonnard et al., 2014; Schrode et al., 2014).

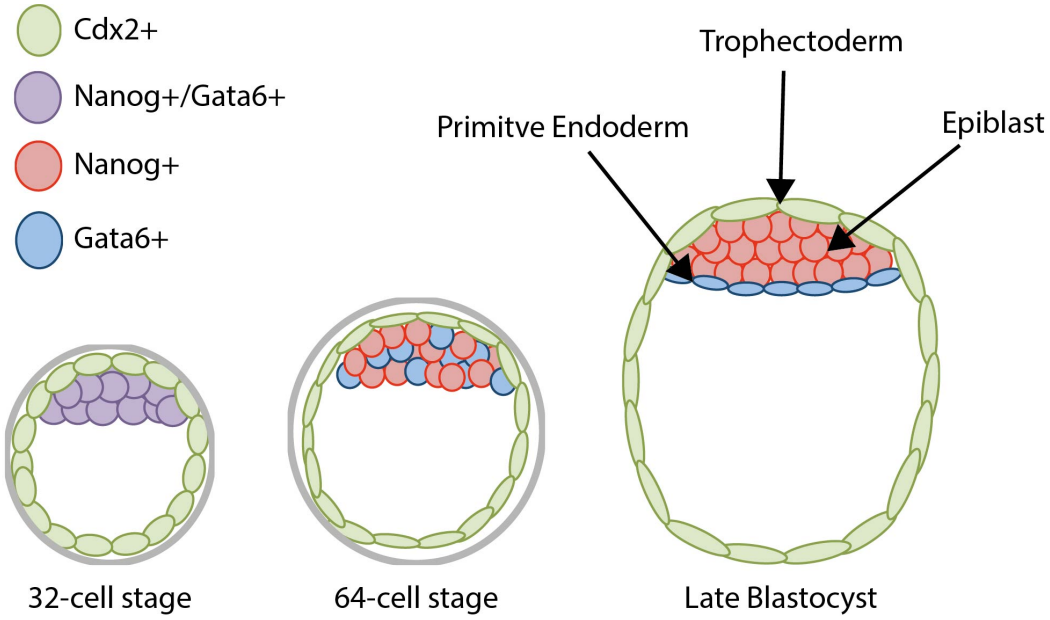

Figure 1.1 Marker expression during specification of the primitive endoderm and epiblast lineages in the pre-implantation mouse embryo

MEK/ERK signalling has been shown to be a key pathway directing specification of the primitive endoderm. Inhibition of MEK/ERK signalling via an FGF receptor inhibitor or MEK inhibitors abrogates the Gata4+ population of the ICM in favour of Nanog+ cells (Nichols et al., 2009; Yamanaka et al., 2010). Similarly, loss of FGF/MEK/ERK signalling via knockout of Grb2 also prevents emergence of a Gata6+ population (Chazaud et al., 2006). Conversely, activation of MEK via FGF supplementation increases the Gata4+ population, while inhibition of this pathway diminishes Gata4+ cells in favour of the Nanog+ population (Nichols et al., 2009; Yamanaka et al., 2010). Recently two groups independently identified activation of pan-ICM FGF receptor, Fgfr1, by Fgf4 as the primary signal triggering primitive endoderm specification and segregation in the developing ICM (Kang et al., 2017; Molotkov et al., 2017). Fgfr2 expression is later restricted to the developing primitive endoderm, possibly as a

secondary driver of MEK/ERK activation to reinforce cell identify (Molotkov et al., 2017). Following this initial fate decision cell identities are reinforced by upregulation of lineage specific transcription factor networks. Sox17, Gata4 and Pdgfra are upregulated in the primitive endoderm, while the epiblast maintains expression of Oct4 and upregulates Nanog and Sox2 to secure pluripotency and self-renewal. At this developmental point the epiblast consists of approximately 10-20 cells with the developmental potential to form all somatic tissues of the embryo proper. Epiblast cells that are isolated and introduced into an alternative blastocyst are integrated into the chimeric organism, contributing to all the somatic lineages including the germ-line (gardener 1998). This stage of development is therefore considered to be the ground state of pluripotency, as there are no function restrictions as to the possible contribution of each epiblast cell (Nichols and smith 2009).

1.2. Capturing pluripotency – murine embryonic stem cells

In 1981 the first embryonic stem cell lines were derived from mouse pre-implantation embryos (Evans and Kaufman, 1981; Martin, 1981). This was achieved by isolating the inner cell mass (ICM) and plating on a feeder layer of mitotically inactivated fibroblasts (MEFs) in the presence of either serum containing medium or serum containing medium conditioned by previous exposure to embryonal carcinoma cells (Evans and Kaufman, 1981; Martin, 1981). Importantly, these cells could be reintroduced into the blastocyst and contribute to the soma and germ-line, demonstrating developmental parity with the *in vivo* epiblast (Bradley 1984).

The vital contribution of the feeder layer that supports self-renewal was later identified as leukaemia inhibitory factor (LIF) and exogenous LIF is sufficient to propagate mouse embryonic stem cells (mESCs) in the absence of feeder (Nichols et al., 1990; Smith et al., 1988; Williams et al., 1988). Furthermore, mESCs cultured on gelatin in the presence of LIF were demonstrated to be fully developmentally competent, differentiating *in vitro* and *in vivo*, and retaining the capacity for germ line-transmission (Nichols et al., 1990).

Since these early derivations the conditions for establishing and maintaining mESCs have been significantly refined. Several other signalling pathways such as Fgfs, Wnts and bone morphogenic proteins (BMPs) in addition to LIF have been demonstrated to play

roles in the regulation of mESC fate (Burdon et al., 1999; Nichols et al., 1990; Sato et al., 2004; Smith et al., 1988; Williams et al., 1988; Ying et al., 2003a) Our understanding of how theses signals are transduced and the downstream factors that promote murine pluripotency has greatly increased.

1.2.1. LIF signalling and the JAK/STAT pathway

Leukaemia inhibitory factor (LIF) has long been known to potently promote the self-renewal of mESCs and, in combination with serum, is sufficient for the derivation and expansion of the naïve epiblast *in vitro* (Nichols et al., 1990; Smith et al., 1988; Williams et al., 1988).

LIF is part of a broader family of cytokines known as the IL-6 family that primarily act through the JAK/STAT signalling pathway. In the case of LIF, binding to the LIF-R/gp130 receptor complex enables recruitment of Janus kinases (JAKs) to the intercellular domain of gp130, enabling binding of STAT1/3/5, which are subsequently phosphorylated at the Tyr705 residue (Boulton et al., 1994). The phosphorylated STATs are then able to dimerize into either homodimers or heterodimers before relocating to the nucleus where they regulate transcription (Ghislain et al., 2001; Rawlings et al., 2004) . In addition to the JAK/STAT pathway, IL-6 family cytokines are also known to activate MAPK signalling and PI3K/AKT signalling though the LIF-R/gp130 complex (Kunisada et al., 1996; Oh et al., 1998).

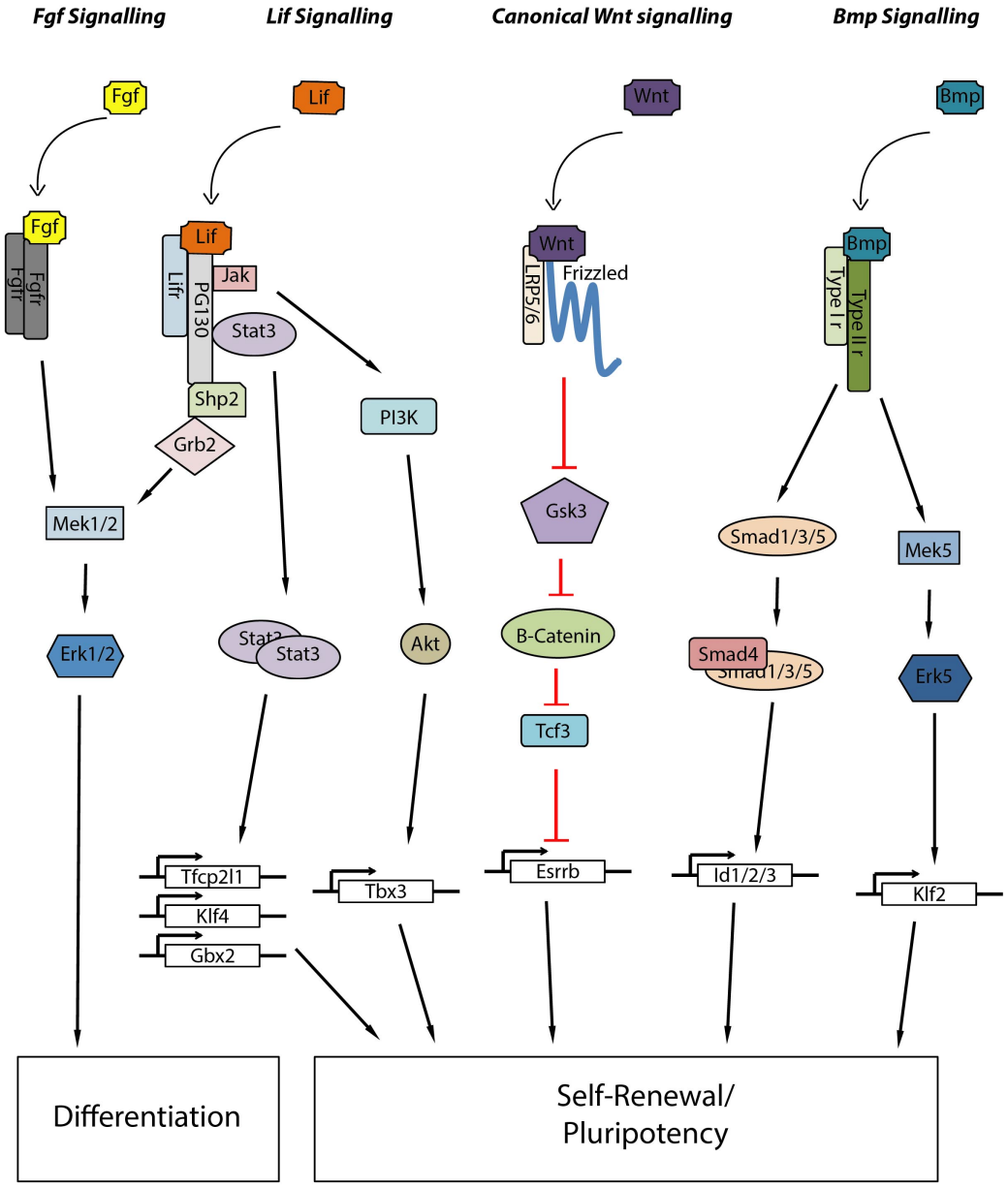

Figure 1.2 The key signalling pathways that govern murine embryonic stem cell self-renewal and commitment.

Numerous extracellular factors converge to either disrupt or promote the pluripotency transcription factor network, thereby regulating mESC self-renewal.

Fgf-mediated activation of Mek/Erk signalling is the major driving force in the differentiation of mESCs. Mek/Erk signalling is also activated in the cell-line variable degree via Lif signalling.

Lif represents the most potent of the self-renewal factors, binding to the Lif receptor/Gp130 complex and activating the Jak/Stat3 signalling axis. Nuclear Stat3 then directly induces expression of pluripotency factors Tfcp2l1, Klf4 and Gbx2. Lif also promotes self-renewal via the PI3K/Akt signalling axis, inducing expression of Tbx3.

The canonical wnt signalling pathways promotes self-renewal by suppressing the activity of differentiation factor Tcf3. Wnt binding to the LRP5/6/frizzled complex sequesters Gsk3, preventing assembly of the b-catenin destruction complex. Accumulating b-catenin then sequesters Tcf3, preventing Tcf3-mediated suppression of pluripotency factor, Esrrb.

Bmp signalling promotes self-renewal via two distinct pathways. Activation of the type I (Alk1/2/3/6)/type II (Bmpr2/Acvr2) receptor complex enables phosphorylation of smad1/3/5 and smad4-mediated nuclear translocation, inducing expression of Id genes. Receptor binding also activates the Mek5/Erk5 signalling cascade, inducing expression of the potent self-renewal factor, Klf2.

Interrogation of JAK/STAT signalling has identified gp130-mediated activation of STAT3 as the critical process in LIF-induced mESC self-renewal (Burdon et al., 1999; Matsuda et al., 1999; Niwa et al., 1998b). Transgenic expression of dominant negative *STAT3* was shown to prohibit LIF-induced self-renewal, while forced activation of STAT3 was sufficient to maintain self-renewal in the absence of LIF (Niwa et al., 1998a). Furthermore, genetic modification of the cytoplasmic domain of gp130 revealed LIF-responsive self-renewal is mediated via STAT activation rather than Shp2-mediated activation of PI3K/AKT or MAPK signalling (Matsuda et al., 1999).

Numerous naïve pluripotency markers are thought to be regulated by LIF signalling. C-*Myc*, *Klf4, Klf5, Tfcp2l1* and *Gbx2* are all reported to be upregulated by activation of STAT3 (Cartwright et al., 2005; Li et al., 2005; Martello et al., 2013; Tai and Ying, 2013). Furthermore, forced expression of many of these factors has been reported to maintain self-renewal in the absence of LIF. One group reported transgenic expression of C-*Myc* sustained SSEA1 immunoreactivity and expression of pluripotency factors Oct4 and Rex1 for at least 14 days after LIF withdrawal (Cartwright et al., 2005). It is also reported that forced *Klf4* expression inhibits proper embryoid body formation (Li 2005). Others report that mESCs cultured in the absence of LIF and maintained on expression of transgenic *Klf4*, which is then excised at the point of microinjection, retain chimera competency (Niwa et al., 2009). Similarly, transgenic *Gbx2* expression enables chimera contribution and can maintain Oct4, Nanog and Klf4 expression in the absence of LIF (Tai and Ying, 2013). Recently, Tfcp2l1 has been implicated as a downstream mediator of LIF-responsive self-renewal. As with many other previously identified STAT3 targets, forced expression of Tfcp2l1 sustains mESC self-renewal when supplemental LIF is withdrawn (Martello et al., 2013; Ye et al., 2013). Importantly though, Knockdown of *Tfcp2l1*, but not *Klf4* or *Gbx2*, was shown to impair LIF-induced self-renewal, indicating not all down-stream targets of LIF are critically required to confer self-renewal (Martello et al., 2013).

Although the critical activity of LIF signalling is known to be transduced via JAK/STAT signalling, the PI3K/AKT pathway has also been implicated in LIF-responsive self-renewal (Niwa et al., 2009). Induction of pluripotency marker *Tbx3* via LIF exposure

was shown to be blocked by addition of the PI3K inhibitor LY29002, indicating a possible requirement for this pathway. As with the factors discussed above, overexpression of *Tbx3* was reported to support self-renewal in the absence of LIF. The authors propose upregulation of *Tbx3* promotes expression of *Nanog* and *Sox2* (Niwa et al., 2009).

1.2.2. BMP signalling

Though LIF has been demonstrated to be a powerful self-renewal signal, it is not sufficient to maintain pluripotency in the absence of serum. When mESCs are cultured in serum-free media differentiation towards a neuronal fate is induced. The presence of LIF attenuates this differentiation but is not sufficient to block the exit from self-renewal. The observation of neuronal differentiation prompted the further supplementation with BMPs, known to inhibit neuronal development in vertebrates and induce acquisition of non-neuronal fates in mESCs (Tropepe et al., 2001; Wilson and Edlund, 2001; Ying et al., 2003b). The addition of BMP4 prevented upregulation of neuronal marker Tuj1 and preserved expression of Nanog and Oct4, as well as chimera competency in the absence of serum (Ying et al., 2003a). In this context BMP4 was shown to induce activation of Smads 1/5/8, which in turn induce upregulation of Id genes. Furthermore, forced expression of Id proteins maintained Oct4 and Nanog expression in the presence of LIF and absence of serum or BMP4, highlighting this pathway as the mediator of BMP4-responsive self-renewal.

In addition to regulation of Id genes, BMP signalling has also been proposed to support mESC self-renewal via suppression of the MAPK pathway (Qi et al., 2004; Zhang et al., 2010). The suppressive activity of BMP signalling on the MAPK pathway was later attributed to upregulation of Dusp9 due to activation of Smads 1 and 5 (Li et al., 2012). Indeed, knockdown of Dusp9 impaired expression of pluripotency markers Nanog and Rex1 within 5 days of culture in BMP4+LIF conditions, while transgenic overexpression of Dusp9 preserved expression of these markers and inhibited ERK activation in the absence of BMP (Li et al., 2012).

Recently it has been suggested that the self-renewal effect of BMP in LIF+BMP4 cultures is actually mediated through activation of ERK5 (Morikawa et al., 2016). Classical BMP

signalling components Smad1/5 were shown to be dispensable for self-renewal in LIF+BMP4, while inhibition of MEK5 or ERK5 lead to differentiation. Furthermore, overexpression of a constitutively active MEK5 mutant induced expression of naïve pluripotency factor *Klf2;* indicating BMP signalling also promotes self-renewal via a BMP/ERK5/Klf2 signalling axis.

1.2.3. FGF and MEK/ERK signalling

The first reports of the involvement of the MEK/ERK pathway in the regulation of mESC fate came about while investigating activation of this pathway downstream of LIF (Burdon et al., 1999). The role of MEK/ERK signalling was investigated via mutation of the cytoplasmic domain of gp130, preventing recruitment of Shp2 and downstream activation of MEK/ERK in response to LIF. However rather than preventing propagation of mESCs as might have been expected, loss of ERK activation rendered the cells far more sensitive to LIF-induced self-renewal. Indeed, after mutation of gp130, self-renewal could be achieved with a 100-fold lower concentration of cytokine (Burdon et al., 1999). Furthermore, over expression of a catalytically inactive version of Shp2 similarly promoted self-renewal, together suggesting LIF-mediated activation of MEK/ERK has a detrimental effect on mESC self-renewal (Burdon et al., 1999).

Consistent with these observations, addition of MEK/ERK signalling inhibitors has since been demonstrated to greatly improve derivation of ESCs from mouse strains that are otherwise not permissive to LIF/serum derivation (Batlle-Morera et al., 2008; Brook and Gardner, 1997). Recently the improved derivation in the presence of MEK/ERK inhibition was linked, at least in part, to differential activation of MAPK signalling by LIF between strains, with recalcitrant strains exhibiting hyper-activation (Ohtsuka and Niwa, 2015).

It has now been demonstrated that activation of MEK/ERK signalling is one of the primary signals triggering exit of self-renewal and proper execution of differentiation (Kunath et al., 2007; Stavridis et al., 2007). mESCs endogenously secrete FGF4, activating MEK/ERK signalling in an autocrine manner (Kunath et al., 2007). Disruption of MEK/ERK signalling, either by pharmacological inhibition or by genetic ablation of FGF4 has been demonstrated to inhibit neural differentiation as well as

BMP-induced non-neural differentiation (Kunath et al., 2007; Stavridis et al., 2007). However enabling FGF/ERK signalling during an initial narrow window of differentiation is sufficient to permit neuronal differentiation (Kunath et al., 2007). Together this suggests FGF signalling is required for mESCs to acquire developmental competency.

Although inhibition of MEK/ERK signalling suppresses differentiation, that alone is not sufficient to maintain self-renewal (Kunath et al., 2007; Ying et al., 2008). However the combination of LIF and MEK/ERK inhibition permits the long-term propagation of mESC in the absence of either serum or BMP4 (Ying et al., 2008).

1.2.4. Canonical Wnt signalling

Canonical Wnt signalling can be artificially simulated via inhibition of GSK3 which prevents GSK3-mediated proteolysis of β-catenin, resulting in accumulation in the cytoplasm (Doble and Woodgett, 2003). Initial reports linking canonical Wnt signalling to self-renewal were based on the observation that an inhibitor of GSK3 could sustain Rex1 expression and teratoma competency over a period of 5 days in the absence of LIF (Sato et al., 2004).

It was subsequently demonstrated that, in combination with MEK/ERK inhibition or LIF supplementation, GSK3 inhibition was sufficient to maintain mESC in a state of self-renewal under defined culture conditions (Wray et al., 2011; Ying et al., 2008). Screening for mutants that confer resistance to differentiation has identified Tcf3 as a promoter of mESC differentiation (Guo et al., 2011; Leeb et al., 2014). GSK3 inhibition is thought to promote self-renewal by stabilising β-catenin, which in turn binds Tcf3 and abrogates its suppressive activity on the pluripotency network (Wray et al., 2011).

Nanog, *Tfcp2l1* and *Esrrb* have all been reported to be upregulated by exposure to the GSK3 inhibition, CHIR99021 (CHIR) (Martello et al., 2012; Pereira et al., 2006; Ye et al., 2013). However $Nanog^{-/-}$ mESCs continue to self-renew in 2i conditions, indicating that upregulation of Nanog is not a critical downstream event in GSK inhibition-mediated self-renewal (Silva et al., 2009). It has subsequently been demonstrated knockout of Esrrb renders mESCs unable to self-renew in 2i, however they remain undifferentiated in the presence of LIF and MEK/ERK inhibition, while forced expression of *Esrrb*

phenocopies GSK3 inhibition or *Tcf3* deletion (Martello et al., 2012). As such, β-catenin-mediated derepression of *Esrrb* appears to be an essential mechanism for canonical Wnt signalling-induced self-renewal.

There is some suggestion that CHIR exposure elicits a small self-renewal response in both β-catenin null and Tcf3 null mESCs, suggesting at least one other mechanism may be involved independently of the β-catenin/Tcf3 axis (Wray et al., 2011).

1.2.5. Capturing the ground state *in vitro* -2iL

While LIF+serum and LIF+BMP conditions are capable of deriving murine pluripotency, the combination of MEK inhibition, GSK3 inhibition and LIF supplementation, termed 2iL, has emerged as the most robust system for derivation of mESCs. 2iL permits robust derivation from multiple strains with homogeneous pluripotency marker expression and little spontaneous differentiation (Brook and Gardner, 1997; Dunn et al., 2014; Wray et al., 2010). Transcriptional profiling of pre-implantation blastocysts show that 2iL mESC cultures are transcriptionally similar to the pre-implantation blastocyst (Boroviak et al., 2014). Similarly, investigation of methylation states demonstrates 2i cultures recapitulate the hypomethylated state of the pre-implantation blastocyst (Leitch 2014).

1.3. Human embryonic stem cells and pluripotent states

1.3.1. Derivation of human embryonic stem cells

Prior to 1995, the main *in vitro* model of human pluripotency and differentiation was embryonal carcinoma cells. These cells are immortal and can be induced to differentiate *in vitro* and form teratomas consisting of all three germ layers when injected into immunecompromised mice (Andrews et al., 1984). However the abnormal karyotype of these cells severely limited their value as a surrogate for human development. Attempts to derive and propagate human embryonic stem cells (hESCs) under mESC LIF+serum conditions were unsuccessful (Bongso et al., 1994). In 1995 Thomson and colleagues derived the first primate ES cells from rhesus monkeys using a combination of a MEF feeder layer with medium containing foetal bovine serum and human LIF (Thomson et

al., 1995). This technique was soon applied to ancillary human IVF blastocysts in order to derive a karyotypically normal immortal hESC line. These cells were highly similar in morphology and expressed pluripotency associated surface antigens, SSEA3, SSEA4 and Tra-1-60 comparably to non-primate ES cell and embryonal carcinoma cells (Thomson et al., 1998; Thomson et al., 1995). Importantly, these cells responded to *in vitro* differentiation cues and formed teratomas consisting of all three germ layers when injected into immunocompromised mice (Thomson et al., 1998).

Since derivation it has become clear that hESCs are distinct from mESCs both in their molecular and morphological characteristics and in their signalling requirements. hESCs require FGF and Activin/Nodal signalling for self-renewal, grow as a epithelial-like mono-player and exhibit poor clonogenicity. Though they continue to express core pluripotency factors *OCT4* and *SOX2* as well as some expression of *NANOG*, they do not express other transcription factors such as *KLF4* or *TFCP2L1* that are present in mESCs.

1.3.2. Mouse EpiSCs and primed pluripotency

Initially the substantial disparities between mESCs and hESCs were attributed to genetic differences between species. However it has since been demonstrated that a distinct murine pluripotent stem cell type can be derived under FGF and activin conditions from the post-implantation epiblast that is highly similar to hESCs (Brons et al., 2007; Tesar et al., 2007). These new pluripotent stem cells, termed EpiSCs, self-renew indefinitely, retaining expression of pluripotency markers *Oct4* and *Sox2* and continue to express *Nanog*, albeit at a reduced level. In addition, they were demonstrated to differentiate into a variety of different tissues *in vitro*, and form teratomas when explanted into immunocompromised mice (Brons et al., 2007; Tesar et al., 2007). However the transcriptional profile of EpiSCs share greater similarities with hESCs and the post-implantation primitive streak stage epiblast than mESCs (Tesar et al., 2007; Tsakiridis et al., 2014). Furthermore, similarly to hESCs, EpiSCs exhibit little to no expression of mESC markers *Klf4*, *Gbx2*, *Tbx3* or *Rex1*, while expression of several lineage markers such as *Eomes* and *Brachyury* are elevated (Tesar et al., 2007). Additionally, EpiSCs also exhibit hypermethylation, preferential use of the proximal

Oct4 enhancer element as well as X chromosome inactivation, all features associated with the murine post-implantation development (Leitch et al., 2013; Tesar et al., 2007; Yeom et al., 1996). Despite retaining the capacity to differentiate *in vitro*, EpiSC contribution to chimeric mice following microinjection into E3.5 embryos is very poor, contrasting mESCs. Similarly, post-implantation epiblast cells also fail to contribute to chimeras (Rossant, 2008). As such chimera contribution has become a key functional determinant between these states of pluripotency. Finally, although mESCs readily convert to an EpiSC state following transfer to FGF and activin containing medium, reversion to the mESC state requires genetic manipulation (Guo et al., 2009). Together these reports have lead to the conclusion that EpiSCs occupy a distinct, developmentally advanced state of pluripotency that has been termed 'primed' pluripotency (Nichols and Smith, 2009). With the exception of chimera contribution, all of the unique attributes of EpiSC primed pluripotency have been demonstrated in hESCs, indicating that these cells represent a more advanced, primed state of pluripotency than their naïve mESC counterparts (Table 1) (Brons et al., 2007; Rossant, 2008; Tesar et al., 2007).

Table 1 Comparison of naïve and primed pluripotent stem cell properties

	Mouse		Human	
	mESCs	**EpiSCs**	**Naïve hESCs (t2iLGö/5iLAF)**	**Conventional hESCs**
Chimera Contribution	High	Low	N/A	N/A
Naïve TF expression (*KLF4/TFCP2L1*)	Yes	No	Yes	No
Preferred *OCT4* enhancer	Distal	Proximal	Distal	Proximal
Metabolic state	Oxidative	Glycolytic	Oxidative/ Undetermined	Glycolytic
Global methylation status	Hypomethylation	Hypermethylation	Hypomethylation	Hypermethylation
X chromosome inactivated	No	Yes	No	Yes
LIF-responsive self-renewal	Yes	No	Undetermined	No
FGF signalling dependence	No	Yes	Undetermined	Yes
Activin/Nodal signalling dependence	No	Yes	Undetermined	Yes
Teratoma formation	Yes	Yes	Yes	Yes
Core TF expression (NANOG/OCT4/SOX2)	Yes	Yes	Yes	Yes

N/A= Not applicable

1.3.3. Extracellular signals governing primed pluripotency

Since the derivation of hESCs considerable effort has gone into delineating the signals that govern the primed state of self-renewal. It was soon noted that culture on laminin with medium previously conditioned by MEF exposure was sufficient to maintain hESC pluripotency, suggesting soluble factors secreted by the MEF are crucial (Xu et al., 2001). FGF and Activin/Nodal signalling have subsequently been identified as key

signalling pathways that promote primed pluripotency (Brons et al., 2007; Chen et al., 2011; Tesar et al., 2007).

1.3.3.1. FGF and Activin/Nodal/TGFβ signalling

Suppression of Activin/Nodal signalling in hESCs has been reported to result in abrupt downregulation of core pluripotency factor *NANOG,* while *NANOG* overexpression sustains self-renewal in the absence of Activin. (Vallier et al., 2009; Xu et al., 2008). These observations have prompted the suggestion that Activin/Nodal signalling promotes self-renewal via *NANOG* induction. However *Nanog*$^{-/-}$ mESCs can contribute to chimeras and readily generate epi-stem cell lines *in vitro* with robust *Oct4* and *Sox2* expression, demonstrating the crucial down-stream mechanism is yet to be resolved (Osorno et al., 2012).

Somewhat surprisingly MEK signalling appears to have disparate effects in the naïve and primed states of pluripotency. While FGF signalling drives differentiation of mESCs, this same pathway promotes self-renewal of hESCs and EpiSCs. However the precise way in which FGF signalling supports self-renewal of primed pluripotency remains largely unresolved. In mouse EpiSCs FGF signalling is reported to suppress neuronal differentiation via co-operation with Activin/Nodal signalling (Greber et al., 2010). While inhibition of neuronal differentiation via FGF signalling appears to be conserved in primed hESCs, this pathway is also purported to prevent induction of trophectoderm and endoderm markers (D'Amour et al., 2005; Li et al., 2007).

1.3.3.2. LIF/JAK/STAT signalling

LIF potently induces self-renewal of mESCs and as a result supplemental human LIF was included as a component in early attempts to derived primate ESCs (Bongso et al., 1994; Smith et al., 1988; Thomson et al., 1998; Thomson et al., 1995; Williams et al., 1988). Although LIF was included for derivation of early primate embryonic stem cells, withdrawal of LIF did not result in growth arrest or differentiation (Thomson et al., 1995). Additionally, exposure to LIF following withdrawal of the feeder layer was not sufficient to prevent differentiation (Thomson et al., 1998). A number of groups have

since attempted to identify a self-renewal response to LIF signalling in hESCs. Several groups have demonstrated that hESCs possess the components necessary for LIF signalling, and that exposure to exogenous LIF induces low level phosphorylation of STAT3 and translocation to the nucleus (Daheron et al., 2004; Humphrey et al., 2004; Reubinoff et al., 2000). However despite activation of this pathway both hESCs and EpiSCs are unresponsive to LIF (Brons et al., 2007; Tesar et al., 2007). The observed lack of self-renewal response is perhaps unsurprising given the lack of expression of genes such as *KLF4*, *TFCP2L1* and *GBX2* that are reported to mediate the self-renewal effect of LIF signalling in mESCs (Brons et al., 2007; Cartwright et al., 2005; Li et al., 2005; Martello et al., 2013; Tai and Ying, 2013; Tesar et al., 2007).

1.4. Capturing human naïve pluripotency *in vitro*

Although they retain the capacity to differentiate into all somatic lineages, conventional primed hESCs possess a number of undesirable characteristics *in vitro*. Of particular note is the apparent variable differentiation propensities of conventional hESC cell lines that is in stark contrast to the efficient unbiased differentiation of naïve mESCs cultured in 2i (Butcher et al., 2016; Martello and Smith, 2014; Ying et al., 2008). Additionally, though conventional primed hESCs have undergone XCI, this state is prone to erosion over time *in vitro*, resulting in the promiscuous expression of X-linked genes (Mekhoubad et al., 2012). Moreover, this leaky X chromosome expression has been reported to persist after prolonged differentiation, presenting potential complications for therapeutic application of these cells (Mekhoubad et al., 2012; Nazor et al., 2012).

Since the derivation of EpiSCs there has been considerable interest as to whether a naïve state of human pluripotency exists in *in vivo* and can be captured *in vitro*. As a result, numerous media formulations based on variations of the 2iL system have be purported to maintain naïve human pluripotency. (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014).

As noted above, the key functional property distinguishing the naïve and primed states of pluripotency in mouse is the ability to efficiently contribute to chimeras. However, such an assay cannot be ethically performed to determine the functional potency of hESCs suggested to be in the naïve state. Interspecies chimerism has been proposed as a

means of functionally assessing human naïve pluripotency. It has been claimed that exposing conventional primed hESCs to a media cocktail consisting of ERK, GSK3, JNK and p38 inhibition, plus LIF, FGF2 and TGFβ1 generates a cell state capable of contributing to mouse and pig chimeras (Gafni et al., 2013; Wu et al., 2017). However more stringent examination of interspecies chimera contribution using a sensitive assay for human mitochondrial DNA has found any contribution to murine embryos to be negligible (Theunissen et al., 2016). Other functional assays such as teratomas formation and *in vitro* differentiation are not capable of distinguishing between the naïve and primed states, thus we must rely on transcriptional, epigenetic and metabolic properties to distinguish these cell states.

Although the *in vivo* naïve human epiblast was not well characterised at the time many of these formulations were published, due to recent advances in single cell sequencing and subsequent Immunostaining we now know much of the murine naïve transcriptional network, such as *KLF4* and *TFCP2L1*, is present in the human pre-implantation epiblast (Blakeley et al., 2015; Petropoulos et al., 2016; Takashima et al., 2014; Yan et al., 2013). Of the multitude of media cocktails reported, two conditions, t2iLGö and 5iLAF, have been demonstrated to maintain cells with a transcriptional profile comparable to murine naïve pluripotency and the human pre-implantation epiblast (Guo et al., 2016; Huang et al., 2014; Takashima et al., 2014; Theunissen et al., 2016; Theunissen et al., 2014).

The t2iLGö medium generated in the Smith lab is based of MEK, GSK3 and PKC inhibition in combination with human LIF and a feeder layer of inactivated mouse embryonic fibroblasts (Takashima et al., 2014). Although initially generated via resetting of convention primed hESCs using transient expression of transgenic *NANOG* and *KLF2*, it has since been demonstrated that these cells can be generated via transient Histone deacetylase inhibition of primed hESCs, or directly form the blastocyst via isolation of the inner cell mass with the addition of ROCK inhibition (Guo et al., 2017; Guo et al., 2016).

The alternative naïve hESC medium, reported by Theunissen *et al,* utilizes MEK, GSK3, ROCK, BRAF and SRC inhibition as well as LIF and Activin with optional FGF, termed 5iLA(F), in combination with a feeder layer of MEFs (Theunissen et al., 2014).

In addition to sharing a similar transcriptome profile, t2iLGö and 5iLA cultures also share key epigenetic features of murine naïve pluripotency. Examining of methylation states in both culture systems indicates genome-wide hypomethylation, in contrast to the hypermethylated states of conventional primed hESCs. Furthermore, expression is detected from both X chromosomes of cells maintained in t2iLGö and 5iLA, indicating that XCI is not present. Metabolic properties can also be used to distinguish between naïve and primed pluripotency. In the mouse, cells in the naïve state employ oxidative phosphorylation, while primed EpiSCs and hESCs are glycolytic. Examination of metabolism to t2iLGö cultures demonstrates these cells utilize that oxidative phosphorylation.

The functional role of naïve pluripotency factors has also been investigated in t2iLGö cells. Knockdown of *TFCP2L1* or *KLF4* was demonstrated to reduce the colony forming capacity of reset naïve hESCs, indicating these factors, which are functional relevant to the murine naïve state, contribute to the stability of human naïve pluripotency (Takashima et al., 2014). Interestingly, both naïve hESCs and the human epiblast do not express murine naïve pluripotency factors *KLF2* and *ESRRB* (Blakeley et al., 2015; Takashima et al., 2014; Yan et al., 2013). Furthermore, naïve hESCs and human naïve epiblast cells express KLF17, a factor not expressed in both mESCs and the murine pre-implantation blastocyst (Blakeley et al., 2015; Guo et al., 2016).

1.4.1. Signalling in the human naïve state

The way in which naïve hESCs interpret the signalling environment to maintain self-renewal is largely unclear. However some preliminary experiments have been performed both in transgene reset hESCs, and those maintained in 5iLAF and t2iLGö that hint at potential differences in the roles of key signalling pathways governing murine naïve and primed pluripotent states.

1.4.1.1. Wnt signalling

Early investigation of Wnt signalling in the human naïve stage suggests possible divergence from its self-renewal activity in the mouse. It is reported that following

transgenic *NANOG* and *KLF2*-mediated reprogramming of conventional hESCs, GSK3 inhibition accelerates the loss of OCT4 distal enhancer activity (Theunissen et al., 2014). Moreover, titration of GSK3 inhibitor, CHIR99021, has been reported to improved propagation under similar conditions (Guo et al., 2017; Takashima et al., 2014). In the mouse, Wnt activity is thought to promote naive self-renewal primarily by stabilizing β-catenin, which in turn leads to de-repression of *Esrrb* expression by Tcf3 (Martello et al., 2012; Wray et al., 2011). The lack of prominent *ESSRB* expression in both the pre-implantation epiblast and naïve hESCs may explain why similar levels of GSK3 inhibition are not beneficial to human naïve self-renewal (Blakeley et al., 2015; Takashima et al., 2014). Suppression of Wnt signalling via tankarase inhibitor, XAV has been demonstrated to suppress lineage marker expression in conventional hESCs transferred to 2iL conditions (Zimmerlin et al., 2016). Recently suppression of Wnt signalling via XAV exposure was demonstrated to significantly reduce expression of *GATA3* and *GATA6* during HDAC-mediated resetting to naïve pluripotency, further indicating a potentially different role for this pathway in naïve human pluripotency (Guo et al., 2017).

1.4.1.2. LIF and JAK/STAT signalling

Human LIF is a ubiquitous component of these formulations, although little is known about its role or efficacy. Previous attempts to derive hESCs under serum+LIF conditions have been unsuccessful, indicating LIF is unlikely to elicit the same potent self-renewal response in the human naïve state as it does in the mouse (Bongso et al., 1994). Nonetheless, upregulation of key mESC JAK/STAT target genes, such as *TFCP2L1* and *KLF4*, in the human naïve pluripotent state raises the question of whether LIF and JAK/STAT signalling influence naïve pluripotency in humans. Indeed, it has been reported that withdrawal of LIF from 5iLAF naïve hESC cultures results in reduced expression of *KLF4* (Theunissen et al., 2014). This indicates that LIF may influence human naïve pluripotency, potentially via the same transcriptional targets as the mESC system.

1.4.1.3. FGF and Activin/Nodal signalling

Similarly to LIF, there is little experimental evidence describing the roles of FGF and Activin/Nodal signalling in the human naïve state. The 5iLAF media formulation includes supplemental FGF2 and activin A, while both 5iLAF and t2iLGö utilise a feeder layer of MEFs, presenting a possible source of factors that may activate FGF and Activin/Nodal signalling. Inhibition of Activin/Nodal signalling via ALK4/5/7 inhibitor, A83-01, did not significantly alter clonogenicity of naïve hESCs maintained in t2iLGö (Takashima et al., 2014).

During 5iLAF-mediated resetting of conventional hESCs the addition of activin A has been shown to result in increased activation of the OCT4 distal enhancer. However subsequent withdrawal of activin A in established 5iLAF naïve cells did not appear to result in a reduction in OCT4 distal enhancer activity (Theunissen et al., 2014). This raises the question of whether Activin is actually promoting the naïve state or stabilizing pluripotency during resetting. Though the authors also report than *NANOG* and *KLF4* expression is reduced in reset cells following withdrawal of activin A, indicating Activin/Nodal signalling may promote naïve pluripotency (Theunissen et al., 2014). *KLF4* expression in 5iLAF cells was also reported to be sensitive to FGF2 supplementation, despite the presence of MEK inhibition.

1.5. Deviating from the paradigm: human pre-implantation development

The inability to derive naïve hESCs under any of the reported mESC media conditions has raised the question of whether pre-implantation development differs significantly between mouse and human. Historically, access to human pre-implantation materials has been severely limited. The advent of IVF treatment has resulted in improved access to fertilized zygotes allowing greater insight into human pre-implantation development. Ethical concerns prevent the use of many genetic and lineage tracing tools that have been used to delineate the regulation of murine pre-implantation development, however a number of recent studies have identified potential mechanistic differences in cell-fate determination in the developing embryo.

The initial stages of human pre-implantation appear to be broadly similar to that of the mouse, however development is somewhat slower and some major morphological events such as compaction are delayed (Figure 1.3) (Iwata et al., 2014; Nikas et al., 1996). Furthermore, embryonic genome activation is thought to take place at the 4 to 8-cell stage in human, in contrast to the 2-cell stage in mouse, although some low level of activation has been reported at the 2-cell stage in human (Blakeley et al., 2015; Taylor et al., 1997; Vassena et al., 2011).

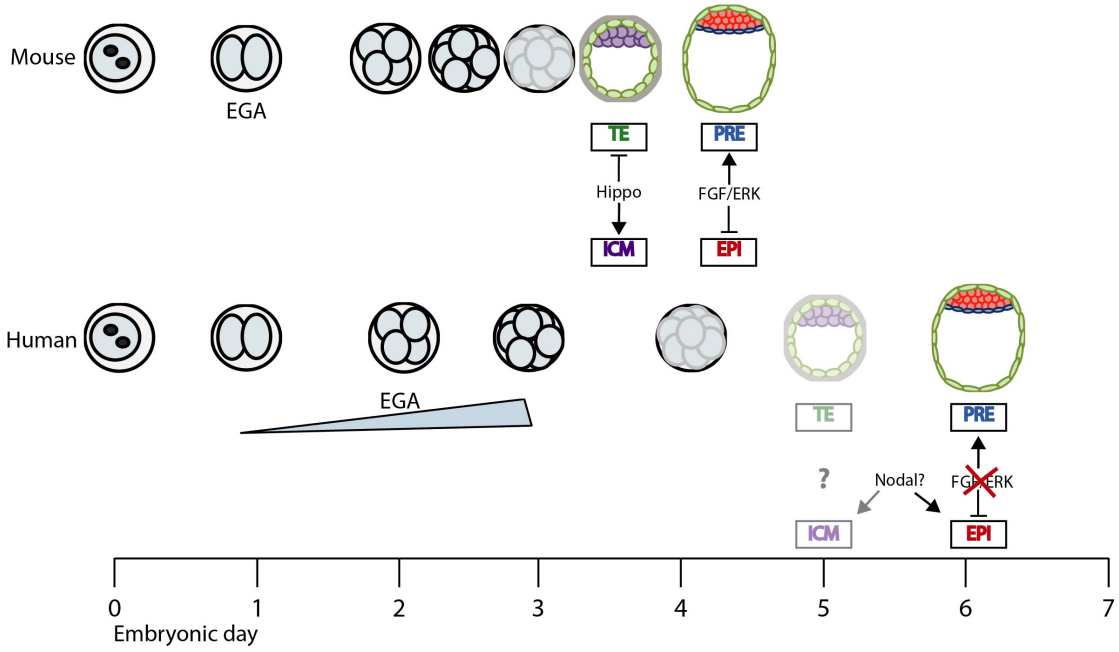

Figure 1.3 A comparison of the timing and signals that govern human and mouse pre-implantation development.

The pre-implantation development of human and mouse embryo appears to be largely conserved; however a number of difference in the timings and signalling pathways have been observed. Mouse embryonic genome activation (EGA) occurs at the 2-cell stage, where as in humans the majority of genome activation takes place during the 4- to 8-cell stage. From the 2-cell stage onwards, human pre-implantation development is delayed in comparison to mouse. At day 3.5 hippo signalling in the mouse segregates the morula into the trophectoderm (TE) and inner cell mass (ICM). The process of TE and ICM segregation in humans is yet to be demonstrated, furthermore recent RNA-seq data suggests segregation of the morula to TE, primitive endoderm (PRE) and epiblast (EPI) fates may occur simultaneously, by-passing the ICM stage entirely (muted greyed). Segregation of the mouse ICM into the EPI and PRE is primarily governed by Erk activation, however inhibition of ERK signalling in human blastocysts does not preclude PRE specification, indicating a deviation in the primacy of this pathway. Recently Nodal signalling has been implicated as a requirement for human epiblast formation, indicating a possible role in the segregation of the ICM or EPI.

One of the earliest lineage decisions made in the murine embryo is the decision to contribute to the ICM or the newly forming trophectoderm. As discussed above, this is

though to be guided by establishment of polarity and the activation of Hippo/Yap signalling (Nishioka et al., 2009). The primitive endoderm and naïve epiblast are then specified from the ICM population at a later stage of development. Recently single cell RNA-seq analysis has been suggested to indicate specification of these three lineages is a simultaneous event in the human pre-implantation blastocyst (Petropoulos et al., 2016).

Another potential difference is in the signalling pathways that direct specification of the primitive endoderm. In the mouse ICM, the choice between contributing to the primitive endoderm or the naïve epiblast is dictated by activation of MEK/ERK via FGF signalling through Fgfr1 (Nichols et al., 2009; Yamanaka et al., 2010). In contrast human embryos exposed to either FGF receptor inhibitor PD173074 or MEK inhibitor PD03259013 from the 8-cell stage to the late blastocyst stage continue to develop as normal, forming a GATA4+ population independent from NANOG+ and OCT4+ cells (Kuijk et al., 2012; Roode et al., 2012).

Table 2 Proposed developmental differences between the human and mouse blastocyst

	Mouse	Human
XIST expression pattern	Monoallelic	Biallelic
MEK-dependent PrE specification	Yes	No
Activin/Nodal/TGFβ-dependent ICM	No	Unclear
CDX2 expression in TE	Yes	Delayed/Mosaic
GATA2/3 expression	TE	*TE/PrE/EPI*
GATA6 expression	PrE	*TE/PrE/EPI*
KLF2/ESRRB expression in EPI	Yes	No
KLF17 expression in EPI	No	Yes

TE=Trophectoderm. PrE=Primitive endoderm. EPI=epiblast. *Italicises* marks differences currently identified by RNA-sequencing only.

Recent single-cell RNA-seq of the human epiblast cells has also indicated significant differences in expression of TGFβ superfamily signalling components (Blakeley et al., 2015; Petropoulos et al., 2016). Expression of Nodal is enriched in the human epiblast when compared to mouse. Exposure to ALK4/5/7 inhibitor SB431542 (40μM) was reported to result in abrogation of ICM lineages, while similar treatment of mouse blastocysts did not impact normal development (Blakeley et al., 2015).

Investigations into the expression and localisation of lineage markers known to be key fate determinants during mouse pre-implantation development suggest a somewhat less restricted, more mosaic expression in the human blastocyst. In the mouse embryo, Cdx2 is proposed to specify the formation of the trophectoderm by mutual exclusion of Oct4 (Niwa et al., 2005). However Immunostaining of human pre-implantation stage embryos at multiple stages of development suggests trophectoderm formation precedes CDX2 expression, which is somewhat mosaic (Niakan and Eggan, 2013). Furthermore,

OCT4 continues to be expressed in the trophectoderm of CDX2+ cells up to the late blastocyst stage, further highlighting potential differences in the specification of the trophectoderm.

Subsequent single-cell RNA-seq of human pre-implantation stage embryos has further supported the notion of inconsistent *CDX2* expression throughout the trophectoderm, and has also hinted at many other transcriptional differences between human and mouse (Blakeley et al., 2015; Petropoulos et al., 2016). *Gata2* and *Gata3* are restricted to the trophectoderm of murine blastocyst, but transcripts of both are detected within cells of the human naïve epiblast and primitive endoderm (Blakeley et al., 2015). Similarly, *Gata6* is a marker of the murine primitive endoderm, however in humans expression is detected in the naïve epiblast and trophectoderm, as well as the primitive endoderm. Elf5 and Eomes are proposed to have central roles is specification and development of the murine trophectoderm, but transcript expression of both is absent throughout the human pre-implantation blastocyst (Blakeley et al., 2015). Similarly expression of pluripotency factors, *KLF2* and *ESRRB* mark the naïve epiblast in mouse but expression is absent or very low in the human epiblast. Finally, the expression pattern of KLF17 during pre-implantation development differs between human and mouse. In the murine embryo *KLF17* peaks in the 4-cell stage, at which point expression beings to tail off and is lost by the morula stage. In contrast, *KLF17* expression peaks at the 8-cell stage in humans, however expression is still detected at a lower level in the pre-implantation blastocyst where it is restricted to the epiblast (Blakeley et al., 2015; Guo et al., 2016).

Another proposed difference between development of the human and mouse epiblast is the regulation of X chromosome inactivation (XCI) in females. XCI is the process by which female embryos repress transcription from one of the two X chromosomes in order to dosage compensate. In the mouse, the X chromosome is reactivated during the cleavage stage and inactivated at implantation, driven in principle by the monoallelic expression of long non-coding RNA, *Xist* (Marahrens et al., 1997; Penny et al., 1996). In contrast *XIST* expression is observed from both X chromosomes within the human pre-implantation stage epiblast, and does not appear to correlate directly with XCI (Okamoto et al., 2011; Petropoulos et al., 2016). It has also been proposed, based on single-cell RNA-seq analysis, that dosage compensation of X-linked genes may take place gradually within the human epiblast (Petropoulos et al., 2016). Recently primate

specific long non-coding RNA, *XACT* was demonstrated to coat eroded X chromosomes in conventional hESC cultures (Vallot et al., 2015). Furthermore, *XACT* and *XIST* have been shown to simultaneous coat the active X chromosomes in the pre-implantation human embryos, while heterologous expression of *XACT* in mESCs biased differentiation-induced XCI towards the X chromosome lacking *XACT* expression (Vallot et al., 2017). The authors propose that in the human epiblast *XACT* works antagonistically at one of the X chromosomes to prevent *XIST* biallaic expression from resulting in inactivation of both X chromosomes. Together this data indicates a primate-specific role for *XACT* in XCI within the human pre-implantation blastocyst.

1.6. Project Aims

Conventional hESCs correspond to the primed state of pluripotency, and with is thought to come a number of undesirable characteristics such as heterogeneity, differentiation bias, and XCI erosion *in vitro*.

It has recently been demonstrated that a naïve state of human pluripotency can be captured *in vitro*, either via transient expression of transgenic NANOG and KLF2, transient exposure to HDAC inhibition, or from the isolated inner cell mass (Guo et al., 2017; Guo et al., 2016; Takashima et al., 2014). Long-term propagation of this state can be achieved via MEK, GSK3 and PKC inhibition in combination with supplemental human LIF and a feeder layer of mitotically inactivate mouse embryonic fibroblasts. However, to date the way in which these signals prevent the exit from naive self-renewal remains to be determined.

The broad aim of this project is to gain a better understanding of the signalling pathways that govern the maintenance and dissolution of human naïve pluripotency. In order to investigate this I have utilized a combination of embryo-derived and reset naïve hESCs in an attempt to address the following questions:

- Is human naïve pluripotency reliant on, or responsive to JAK/STAT signalling?

- Is human naïve pluripotency independent of the conventional primed self-renewal signalling pathways: FGF and Activin/nodal signalling?

- Does disruption of PKCι activity alleviate the requirement for the PKC inhibitor Gö6983?

Chapter 2. Method

2.1. Cell culture

All cell cultures were maintained in humidified incubators at a temperature of 37°C with an atmosphere consisting of 7% CO_2 and 5% O_2.

2.1.1. Media and reagents

2.1.1.1. Inhibitors and cytokines

PD0325901	MEK inhibitor	
CHIR99021	GSK3 inhibitor	
A83-01	AK4/5/7 inhibitor	Tocris. Cat No.2939
SB431542	ALK4/5/7 inhibitor	Cayman Chemical. CAT NO. 13031
GO6983	Pan-PKC inhibitor	Tocris. CAT No.2285
JAK inhibitor 1	JAK inhibitor	EMD Millipore. Cat No.420099
Ruxolitinib	JAK inhibitor	Selleckchem. CAT No. S1378
LIF	IL-6 family cytokine	In house. Dept. Biochemistry. Uni. Cambridge.
IL-6	IL-6 family cytokine	Peprotech. Cat. No.200-06
FGF2	Growth factor	In house. Dept. Biochemistry. Uni. Cambridge
Activin A	Growth factor	In house. Dept. Biochemistry. Uni. Cambridge.
VPA	valproic acid sodium salt – Histone deacetylase inhibitor	Sigma. Cat No. P4543
Y27632	ROCK1/2 inhibitor	Tocris. Cat No.1254
aPKCi	PKCζ Pseudosubstrate peptide inhibitor	Merck Millipore. Cat No. 539624

2.1.1.2. Basal media

N2B27	Equal volumes of DMEM/F12 (No-Glutamine, Gibco, 21331-020) and Neurobasal medium (Gibco, 21103-049) at 1:1 ratio. +1% B27 (Gibco, 17504-044) +0.5% N2 (made in house) +0.1mM β-mercaptoethanol (Life Technologies, 31350-010) +2mM L-Glutamine (Life Technologies, 25030-081)
t2iLGö	N2B27+1μM PD0325901 +1μM CHIR99021 +2μM Gö6983 +10ng/ml human LIF
t2iLGöY	As t2iLGö +10μM Y27632
E8	TSER-E8 (Stem cell technologies. Cat No. 05940)
KSR	DMEM-F12 (No-Glutamine, Gibco, 21331-020) +20% Knockout serum replacement (ThermoFisher Scientific (10828028) +2mM L-Glutamine (Life Technologies, 25030-081) +0.1mM β-mercaptoethanol (Life Technologies, 31350-010) +5ng/ml FGF2
PDL+VPA	N2B27 +1μM PD0325901 +1mM VPA +10ng/ml human LIF
PDLGö	N2B27 +1μM PD0325901 +2μM Gö6983 + 10ng/ml human LIF
Freezing medium	Culture medium + 10% DMSO (Sigma-Aldrich, 472301) + 20% foetal calf serum (Sigma-Aldrich)
Penicillin + Streptomycin	Life Technologies, 15140-122
Accutase	ESGRO Complete Accutase (Millipore, SF006)
PBS	Sigma-Aldrich, D8537

2.1.2. Cell lines

cRShef6 EOS	Female naive hESC line containing a GFP reporter of a mouse OCT4 distal enhancer element (Hotta et al., 2009). Chemically reset using transient HDAC inhibition (Guo et al., 2017).

HNES1	Male naïve hESC line derived directly form isolated inner cell mass under t2iLGöY conditions (Guo et al., 2016).
H9 NK2 EOS	Female naive hESC cell line containing Dox inducible NANOG and KLF2 as well as a GFP reporter of a mouse OCT4 distal enhancer element. Reset to naïve state by transient expression of transgenic NANOG and KLF2 (Takashima et al., 2014).
cRH9 EOS	Chemically reset female naive hESC line containing a GFP reporter of a mouse OCT4 distal enhancer element.
Shef6 PRKCI$^{-/-}$ C1	Clonal Female hESC line containing homozygous deletion of PRKCI. Cells of this line chemically reset via transient VPA exposure are denoted by the prefix: cR
Shef6 PRKCI$^{-/-}$ C3	Clonal Female hESC line containing homozygous deletion of PRKCI. Cells of this line chemically reset via transient VPA exposure are denoted by the prefix: cR
cRShef6 PRKCI$^{-/-}$ C3 +tPRKCI	Clonal Female hESC cells containing homozygous deletion of endogenous PRKCI and a PiggyBac-mediated insertion of transgenic PRKCI under the control of a constitutive CAG promoter.
HNES1 PRKCI$^{-/-}$ C7	Clonal male hESC line containing homozygous deletion of PRKCI.
cRShef6 iKLF17tet	Chemically reset female naive hESC line containing

transgenic KLF17 attached to a TET-on inducible system.

HNES1 iKLF17tet Embryo-derived male naive hESC line containing transgenic KLF17 attached to a TET-on inducible system.

Shef6 Female hESC line. A kind gift from Peter Andrews.

2.1.3. Growth substrates

2.1.3.1. MEFs

Mouse embryonic fibroblasts isolated by dissection from E13.5 mouse embryos were provided by the SCI tissue culture facility. Cells were expanded on gelatin in DMEMF12 (Gibco, 21331-020) 10% FBS (Sigma-Aldrich) and 0.1mM b-macaptoethanol. At passage 4 or 5 cells were collected and inactivated via irradiation for 30 minutes. Inactivated MEFs were plated on gelatin-coated wells at a density of $5.5x10^4$ per cm^2 at least 24 hours before plating hESCs.

2.1.3.2. Geltrex

Gletrex Reduced Growth Factor Basement Membrane Matrix. Soluble basement membrane extracted from murine Engelbreth-Holm-Swarm tumours. Thermofisher scientific (CAT No. A1413202).

Pre-coating: Gletrex aliquots were thawed on ice before diluting in ice-cold DMEM-F12 medium at a 1:100 dilution and coating chilled tissue culture plates at a concentration of $100\mu l/cm^2$. Coated plates were then incubated at 4^oC overnight before returning to room temperature for 1 hour and then aspirating the solution and plating hESCs in culture medium.

Simultaneous coating: Gletrex aliquots were thawed on ice before diluting in chilled culture medium at a dilution of 1:100. Naïve hESCs were resuspended in culture medium +Gletrex before plating at a volume of $100\mu l/cm^2$.

2.1.3.3. Tissue laminin

Laminin isolated from Engelbreth-Holm-Swarm murine sarcoma basement membrane. EMD Millipore (CAT No. CC095-5MG).

Pre-coating: Laminin aliquots were thawed on ice before diluting in PBS and coating tissue culture plates at a concentration of 10μg/cm^2. Coated plates were incubated at 4^{o}C overnight before aspirating the solution and seeding hESCs.

Simultaneous coating: Laminin aliquots were thawed on ice before diluting in culture medium at a concentration that will coat the receiving well with 5μg/cm^2 of laminin. Naïve hESCs were resuspended in culture medium +laminin before seeding and returning to the incubator.

2.1.3.4. Recombinant laminin

Recombinant Laminin 111 (CAT No. LN111-02) and recombinant laminin 521 (CAT No. LN521-02) were purchased from BioLamina. Laminin aliquots were thawed on ice and diluted in PBS containing calcium and magnesium before coating tissue culture plates at the concentration indicated. Coated plates were incubated at 4^{o}C overnight before aspirating the solution and seeding hESCs in culture medium.

2.1.4. Primed hESC maintenance

2.1.4.1. KSR Culture

Primed hESCs were cultured on a feeder layer of mouse embryonic fibroblasts. KSR medium was refreshed daily for a period of 6-8 days before passaging. For passaging, cells were washed once with PBS before adding enough Dispase so as to completely submerge the colonies. Dispase-treated cultures were incubated at 37^{0}c until the edges of the colonies begin to lift and adopt a halo-like appearance (3-6 minutes). The Dispase-solution was then aspirated and the colonies were gently triturated with wash medium so as to detach from the plate and break up large colonies. Cells were pelleted in wash

medium via centrifugation for 3 minutes at 1350rpm before resuspending in KSR medium and plating on fresh MEF-coated wells at a ratio of 1:4-1:6.

2.1.4.2. E8 culture

Primed hESCs were cultured on Geltrex-coated plates for a period of 4-5 days before passaging. For passaging, cultures were washed with PBS before dissociation with 0.5mM EDTA solution at room temperature for 3 minutes. The EDTA solution was then aspirated and the colonies were triturated with wash medium in order to detach and break up colonies. Cells were pelleted in wash medium via centrifugation for 3 minutes at 1350rpm before resuspending in E8 medium and plating on fresh Gletrex-coated wells at a ratio of 1:10. The medium was replenished after 48 hours and then every 24 hours thereafter.

2.1.5. Naïve hESC maintenance

Naïve hESCs were cultured on a substrate of MEFs, simultaneously coated Geltrex, or high concentration tissue laminin in t2iLGö, with or without Y27632 (t2iLGöY), for a period of 4-6 days before passaging. To passage, the medium was aspirated and cells were incubated in Accutase for 4-8 minutes at 37^0c. Accutase was then diluted with wash medium and the colonies were triturated to dissociate into small clusters of approximately 3-10 cells. Dissociated cells were then pelleted by centrifugation for 3 minutes at 1350rpm before resuspending in maintenance medium and plating at a ratio of 1:4-1:8 according to the substrate requirements stated above. The medium was topped-up after 24 hours and replenished every 24 hours thereafter.

2.1.6. Freezing and thawing

2.1.6.1. Freezing

Once confluent, cells were dissociated and pelleted as with standard passaging. Cells were then resuspended in freezing bufer to a concentration of $5x10^6$ cells per ml. 1ml of suspension was aliquoted to each cryovial which was subsequently stored in a CoolCell

LX (Biocision) at -80^{O}C for 48 hours after which the vials were transferred to liquid nitrogen.

2.1.6.2. Thawing

Cryovials were warmed in a water bath at 37^{0}C until 2/3 of the contents became liquid. The cells were then collected with 5ml of warm medium before centrifugation for 3 minutes at 1350rpm and suspension in the appropriate culture medium. Cells were cultured in the presence of 10μM Y27632 for the first 24 hours after thawing.

2.2. Manipulation of cells

2.2.1. Chemical resetting to naïve pluripotency

Primed hESCs were seeded at a density of 11.5x10^{3} cells per cm^{2} on a MEF feeder layer in KSR medium supplemented with 10μM of Y27632. After 48 hours the KSR medium was replaced with resetting medium 1 (PDL+VPA) for the first 3 days of resetting. Following 72 hours of VPA treatment resetting medium 1 was aspirated and the cells were cultured in resetting medium 2 (PDLGö) for a further 8-11 days until tightly packed dome-shaped colonies had established. The cultures were then passaged with accutase and seeded at a ratio of 1:2-1:4 onto MEF-coated wells in PDLGö +Y27632 (10 μM) for a further 6-8days before continuing to passage and culture as normal.

2.2.2. Transfection protocol

2.2.2.1. Primed hESCs

Cells were transfected via electroporation using the NEON Transfection System (MPK5000, ThermoFisher Scientific). E8 cultures were expanded until 80% confluence was achieved, at which point the cells were dissociated to single-cell by incubation in Acctuase for 10-15 minutes. The cells were washed once in PBS before transfection with 2μg of DNA per plasmid according to the Neon Transfection System Protocol. The following system settings were used:

Voltage: 1200

Width: 20

Pulses: 2

Following transfection cells were plated on two 10cm plates in the presence of 10μM of Y27632. The E8 medium was replenished after 48 hours and the appropriate antibiotic was included in the medium after 72 hours. After a further 5 days colonies were picked and expanded for clonal genotyping.

2.2.2.2. Naïve hESCs

Cells were expanded in t2iLGöY medium for 5 days before dissociating with Accutase for 5-10 minutes. The cells were washed once in PBS before transfection with 2μg of DNA per plasmid according to the Neon Transfection System Protocol.

The following system settings were used:

Voltage: 1150

Width: 30

Pulses: 2

Following transfection cells were plated on 6 well plates in t2iLGöY medium for 3 days, at which point the cells were dissociated again and prepared for FACS. After sorting for transfected cells, the recovered GFP+ population was seeded to a 10cm plate in t2iLGöY medium and expanded for 7 days before picking colonies for genotyping.

2.2.3. Colony forming assays

Naïve hESC cultures were dissociated by incubating in Accutase for 5-10 minutes before gently triturating to break up colonies into a majority of single cells. Following centrifugation, the cell pellet was resuspended in N2B27 and 500-1000 cells (as stated) were plated into the medium conditions stated in the assay. Geltrex was used as a substrate unless otherwise stated. The medium was replenished daily for 6 days at which point the colonies were fixed and stained for alkaline phosphatase according to the

manufacturer's instructions (Sigma-Aldrich, AB0300). Colonies were imaged using a colony-scanning microscope and the number determined using ImageJ cell counting software.

2.2.4. Replating assays

Cells were cultured under the conditions specified in the assay before incubating in Accutase for 5-10 minutes before gentle triturating to break up colonies into a majority of single cells. Following centrifugation, the cell pellet was resuspended in N2B27 and 500-1000 cells (as stated) were plated into the t2iLGö media in the presence of ROCK inhibitor. The medium was replenished daily for 6 days at which point the colonies were fixed and stained for alkaline phosphatase according to the manufacturer's instructions (Sigma-Aldrich, AB0300). Colonies were imaged using a colony-scanning microscope and the number determined using ImageJ cell counting software.

2.2.5. Cytokine induction assay

Cells were passaged from tissue laminin cultures and replated with tissue laminin at equal density in the absence of the cytokines of interest. After 24hours the cells were washed with PBS and cultured in the absence of the cytokines of interest for a further 48 hours. 72 hours after plating the cells were then exposed to the cytokines indicated for the time period indicated before collection of cell lysates.

2.3. Immunological detection

2.3.1. Materials and reagents

Table 3 Antibodies and dilutions

Target Name	Description	Source	Use	Dilution
Actin	Goat Polyclonal IgG	Santa Cruz (SC-1615)	WB	1:5000
ERK1/2	Rabbit polyclonal	Cell Signalling Technology (9102)	WB	1:1000
pERK1/2	Rabbit monoclonal IgG	Cell Signalling Technology (4370)	WB	1:2000
(T) BRACHYURY	Goat Polyclonal	R&D Systems (AF2085)	IF	1:500
GKLF(4)	Rabbit Polyclonal IgG	Santa Cruz (SC-20691)	IF	1:400
KLF17	Rabbit Polyclonal IgG	Atlas (HPA024629)	IF (WB)	1:500 (1:500)
NANOG	Goat Polyclonal	R&D Systems (AF1997)	IF (WB)	1:300 (1:500)
OCT4	Mouse Monoclonal IgG_{2b}	Santa Cruz (SC-5279)	IF	1:300
PKCι	Rabbit Polyclonal	Abcam (Ab5282)	WB	1:700
PKCι	Mouse Monoclonal IgG_{2b}	Santa Cruz (SC-17837)	WB	1:200
SMAD2	Mouse Monoclonal IgG1	Cell Signalling Technology (3103)	WB	1:1000
pSMAD2	Rabbit Monoclonal	Cell Signalling Technology (3101)	WB	1:1000
STAT3	Mouse Monoclonal IgG_{2a}	Cell Signalling Technology (9139)	WB	1:1000
pSTAT3	Mouse Monoclonal IgG_{2a}	Cell Signalling Technology (9145)	WB	1:2000

Immunofluorescence secondary antibodies:

All secondary antibodies (Life Technologies) were raised in Donkey, directed against the species of the primary antibody of interest. The antibodies were conjugated with Alexa488 or Alexa647.

2.3.2. Immunofluorescence

Cells were fixed in 4% PFA (Thermo Scientific) for 15 minutes before washing 3 times in PBS and storing at 4^0C until further analysis. To permiabilize the cells they were incubated in PBS +TritonX-100 for 10 minutes. Blocking was performed by incubating in PBS containing 0.1% Tween20 and 5% serum of the second antibody species for 1 hour at room temperature. The primary antibody was diluted in blocking buffer and incubated with the cells at 4^0C overnight. After washing 3 times in PBS +0.1% Tween20, cells were incubated with the secondary antibody blocking buffer solution for 1 hour at room temperature. Following a further 3 washes cells were incubated in PBS +0.1% Tween20 +DAPI for 10 minutes before replacing the solution with PBS and proceeding to imaging.

2.3.3. Western blotting

2.3.3.1. Buffers and reagents

Running Buffer	NuPAGE MOPS SDS Running Buffer (NP0001)
Transfer Buffer	20% MeOH +25mM TRIS +192mM glycine in dH2O
RIPA buffer	EMD Millipore (Cat. No.20-188)
TBS	10mM TRIS +150mM NaCl in dH2O pH7.7
TBS-T	As TBS +0.1% tween20
Blocking Buffer	As TBS-T +5% BSA (Sigma A2153)

2.3.3.2. Electrophoresis, transfer and blotting

Cultures were placed on ice and lysed using RIPA buffer supplemented with a protease inhibitor (Sigma Aldrich, 4693159001) and an inhibitor of phosphorylation (Sigma Aldrich, 4906845001). The lysis solution was then vortexed at 4^0C for degrees before centrifugation at $14x10^3$ RPM for 15 minutes to pellet the DNA fraction. Samples were then fractioned by SDS electrophoresis at 200v for 50 minutes in MOPS buffer and electroblotted at 400mA for 2 hours in transfer buffer to transfer to a nitrocellulose membrane (Hycond ECL, RPN203D). Membranes were incubated in blocking buffer for 2-3 hours at room temperature to block none specific binding. Primary antibodies were diluted in blocking buffer before incubating at 4^0C overnight. The membrane was then washed 3 times in TBS-T for 5 minutes. Secondary HRP conjugated antibody was diluted in blocking buffer and incubated at room temperature for 1 hour. Following a further 3 washes the membrane was incubated in ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, RPN2232) for 5 minutes before detection was HRP activity using an X-ray developer.

2.3.3.3. Stripping and reprobing the membrane

Membranes were washed in mild stripping buffer (glycine 15%w/v, SDS 1%w/v, Tween 10%v/v and H_20, adjusted to pH 2.2) twice for 10 minutes before 4 further washes in TBS for 5 minutes each. The absence of bound antibody was then confirmed using Amersham ECL Prime before washing with TBS and returning to the blocking step.

2.4. Imaging

Imaging of hESC cultures was performed on Olympus 1Z51 for bright-field imaging and a Lacia DMI3000 standard inverted microscope for fluorescence imaging. The filters for the fluorescence channels were A4 (305nm, DAPI), L5 (488nm) and Y5 (647nm).

2.5. Flow cytometry

For analysis of EOS-GFP activity the cells were processed using a CyAn ADP Analyser (Beckman Coulter).

For fractioning of pSpCas9(BB)-2A-GFP (PX458) transfected cells a MoFlow XDP cell sorter (Beckman Coulter) was used. The machine was operated by Andy Riddle.

For sample preparation, the cells were incubated in Accutase for 5-10 minutes to generate a single cell suspension and pelleted in wash buffer. The cells were then resuspended in the appropriate medium before straining with a 50µm filter and proceeding to analysis.

When sorting by MoFlow XDP, the recovered fraction was replated in the presence of Pen/Strep and 10µM of Y27632. Pen/Strep was included in the medium for the following 72 hours.

2.6. Molecular biology

2.6.1. RT-qPCR

2.6.1.1. RNA isolation and Reverse strand synthesis

For RNA extraction from bulk cell populations, the Reliaprep RNA Cell Miniprep System (Promega, Z6012) was used according to manufacturer instructions. RNA extracts were diluted in 25µl of RNase free H_2O before being quantitatively and qualitatively analysed by NanoDrop (Thermofisher Scientific).

cDNA was synthesised from 1µg of extracted RNA using the GoScript Reveserse Transcriptase System (Promega, A5003). Resulting cDNA was stored at -20^{0}C until further use.

2.6.1.2. qPCR reaction

qPCR reactions were performed either on a QuantStudio 12K Flex Real-Time PCR System (Life Technologies) in 384-well format, or on a StepOne Plus system (ThermoFisher Scientific) in 96-well format. qPCR reaction were performed as 10µl reactions using TaqMan and UPL probe systems set up as follows:

TaqMan (1 reaction)

2x standard mix:	5µl
TaqMan probe:	0.5µl
H_20:	2.5µl
cDNA:	2µl

UPL (1 reaction)

2x Standard mix:	5µl
10µM Primers (F+R):	0.4µl
UPL probe:	0.1µl
H_20:	2.5µl
cDNA:	2µl

Table 4 TaqMan Probes

Gene	Probe identifier
KLF4	Hs00358836_m1
TFCP2L1	Hs00232708_m1
NANOG	Hs02387400_g1
POU5F1 (OCT4)	Hs01654807_s1
KLF17	Hs00703004_s1
DPPA5	Hs00988349_g1
ACTB	Hs01060665_g1
SOX17	Hs00751752_s1
GATA3	Hs00231122_m1
DPPA3	Hs01931905_g1

Table 5 UPL Primers and Probes

Gene	Forward	Reverse	UPL Probe
T	gctgtgacaggtacccaacc	catgcaggtgagttgtcagaa	23
GATA4	gcaaaaatacttcccccaca	tctcccgcaccagtcatc	90
GATA6	ggaagcccaagaacctgaat	gttgctggagttgctggaa	17
CDX2	ggtgtacacggaccaccag	tgcggttctgaaaccagatt	34
PDGFRA	ccacctgagtgagattgtgg	tcttcaggaagtccaggtgaa	27
KLF5	gcaggccttaacacacacac	ggcctgttgtggaagaaact	37
SOX2	ttgctgcctctttaagactagga	taagcctggggctcaaact	35
IL6R	ggactgtgcacttgctggt	attgctgagggggctctt	38
IL6ST	accaagttccgtcagtccaa	gccgctcctctgaatctaac	23
SOCS3	gacttcgattcgggaccag	aacttgctgtgggtgaccat	36
PRKCI	gctgcattcttgctttcagac	tgtcgctgcatatgaaacatt	25
MIXL1	ggtaccccgacatccactt	gcctgttctggaaccatacct	32
EOMES	tgagctccgagcggtacta	ctggtacgggaagagtgagc	71

GAPDH reference probe was purchased from applied biosciences (4326317E)

2.6.2. PRKCI genotyping PCR

To test for the presence of wild-type and mutant PRKCI alleles genomic PCR was performed. Genomic DNA was prepared by lysing cell pellets in lysis buffer (10 mM Tris-HCl pH 8.5, 0.5% SDS, Proteinase K) at 55^0C for 60 minutes. Samples were then diluted to 600µl.

The PCR mix, primers and typical cycling program were as follows:

PCR mix

KOD Hotstart master mix (Millipore, 71842):	12.5µl
10µM Primers (F+R):	1.5µl
DNA:	1µl
H_20:	10µl

Primers

Forward - 5'-CTTGAGCTGGCTGGATGGTT-3'

Reverse – 5'-CTCTCAGGAGGCGAGTCTCT-3'

Cycling program

94°C	5 mins
25 cycles	
94°C	20sec
59°C	10sec
70°C	30sec
4°C	Hold

The amplicon size was determined by running 5µl of the reaction on a 1% agarose (Sigma-Aldrich, A9539) gel on a 1X TAE (prepared in house) tank at 100V for 30min. The DNA was visualised by adding ethidium bromide to the gel (Sigma-Aldrich, E1510) and detecting fluorescence under UV illumination.

2.6.3. PKCiota gateway cloning

PKCiota sequence was amplified from Shef6 cDNA using the following primers:

Forward – 5'-caccATGCCGACCCAGAGGGACAGCAGCA-3'

Reverse – 5'-aatctcgagTCAGACACATTCTTCTGCAGACAT-3'

PCR reaction was set up as in 2.6.3 PRKCI genotyping PCR.

The PCR product was recombined with pENTR using the pENTR directional TOPO cloning kit (thermoFisher Scientific, K240020) according to manufacturer instructions. 2µl of the reaction was then transformed in OneShot Top10 chemically competent cells

(Life Technologies, C4040-10) according to manufacture instructions. 5 ampicillin resistant colonies were picked and sequenced.

One clone containing the correct sequence was recombined with the over expression vector containing the Gateway destination sequence (PB PGK Hygro Dest vector provided by Ge Guo). The reaction was step up as follows:

100ng of pENTR clone

150ng of destination vector (PB PGK Hygro Dest)

Xμl H20

2μl of LR Clonase

The mix was made up to a final volume of 8μl and incubated at 25^{0}C for 60 minutes. 1μl of proteinase K was added to the mixture before incubated at 37^{0}C for a further 10 minutes. 2μl of the reaction mixture was OneShot Top10 chemically competent cells (Life Technologies, C4040-10). 5 Kanamyocyin resistant clones were expanded and sequenced.

2.6.4. RNA sequencing

Cell cultures were washed once with PBS before being lysed in 1ml of TRIZOL reagent and stored at -20^{O}C until further processing. RNA extraction was performed using the TRIZOL/chloroform method by Rosalind Drummond. Sequencing libraries were produced by the CSCI genomics facility. RNA sequencing was performed by Cambridge genomic services using a Illumina HiSeq4000. Analysis of raw sequencing data was performed by Sabine Dietmann.

2.7. Chromosomal analysis

The culture medium was supplemented with 0.06ug/ml of KaryoMAX before incubation the cells for 6 hours at 37^{o}C. Cells were dissociated and washed in PBS before incubating 0.075M potassium chloride for 10 min at 37^{o}C. After centrifugation at 1350rpm for 3 minutes the cells were resuspended in PBS and fixed by adding methanol and acetic acid (3:1 ratio) drop-wise as the suspension was agitated.

Fixed samples were sent to Ducan Baker (Centre for Stem Cell Biology, University of Sheffield) for G-banding analysis.

2.8. Statistical analysis

Student's t-test was performed to compare statistical significance of differences between two samples. One-way ANOVA analysis was performed to compare three or more samples. Tukey's range test was applied for post-hoc analysis of one-way ANOVA analysis. All analysis was performed using GraphPad Prism software (GraphPad Software, Inc).

Chapter 3. Feeder-free maintenance of naïve pluripotency

3.1. Introduction

In vivo, stem cells at all stages of development exist within a complex niche supported in part by interactions with the extra-cellular matrix (ECM). Historically, mESC and conventional primed hESC propagation was supported by a feeder-layer of mouse embryonic fibroblasts (Evans and Kaufman, 1981; Martin, 1981).

However, subsequent refinements to the growth medium have relieved the requirement for soluble factors secreted by the MEFs, enabling propagation of pluripotent cells on defined single component substrates such as laminins, fibronectin or vitronection (Chen et al., 2011; Ying et al., 2008). Such robust culture systems have served as a powerful tool for understanding the mechanisms that regulate self-renewal and differentiation in these cell systems.

Recently two different media formulations have been reported to support hESCs with the transcriptional and epigenetic properties of naïve pluripotency (Guo et al., 2016; Takashima et al., 2014; Theunissen et al., 2014). t2iLGö medium in combination with Matrigel or laminin 511-E8 was reported to sustain hESC colonies that continue to express naïve pluripotency markers (Takashima et al., 2014). However, robust expansion of these cells is reliant on a feeder layer of inactivated mouse embryonic fibroblasts (MEFs).

A MEF feeder layer presents a significant obstacle to understanding the signals that direct self-renewal and differentiation of naïve hESCs. MEFs secrete cytokines, respond to signals from the culture medium and the hESCs, and can be highly variable from one batch to the next, confounding attempts to interpret experimental outcomes. Therefore the aim of the work in this chapter was to identify and validate a feeder independent culture system that supports the propagation of naïve hESCs. This culture system will then act as a platform for investigating the signalling requirements of naïve human pluripotency in the following chapters.

3.2. Results

3.2.1. Maintenance of naïve pluripotency marker expression in the absence of feeders

It was recently observed within the Smith lab that HNES1 cells formed homogeneous domed shaped colonies when cultured short-term on a high concentration of tissue-derived laminin. Tissue-derived laminin consists predominantly of laminin 111, nidogen (entactin) and Collagen IV and is purified from murine Engelbreth-Holm-Swarm (EHS) sarcoma according to the method established by Timpl, *et. al.* 1979.

In order to assess the ability of high concentration tissue laminin to replace the feeder layer, the following two differentially established naïve ES cell lines were utilized: HNES1, a male line derived directly from the epiblast under t2iLGöY conditions; and cRShef6 EOS, chemically reset female containing a GFP reporter of a murine OCT4 distal enhancer element that is indicative of naïve pluripotency (Guo et al., 2017; Guo et al., 2016; Hotta et al., 2009; Takashima et al., 2014). HNES1 and cRShef6 EOS cells cultured in the presence of MEF were sorted by forward and side scatter to minimize MEF contamination, before plating on wells coated with 10μg/cm^2 of tissue laminin in t2iLGöY medium. Cells were passaged every 4-5 days via Accutase dissociation to small clumps and re-plated at a ratio of 1:5-1:6. Both HNES1 and cRShef6 EOS cultures maintained a homogeneous domed shaped colony morphology with minimal visual evidence of differentiation (Figure 3.1A). This morphology was consistent over an extended period of passaging. Furthermore, the EOS-GFP reporter remained active in the majority of cRShef6 EOS cells following transition to tissue laminin, indicating preservation of OCT4 distal enhancer activity (Figure 3.1B).

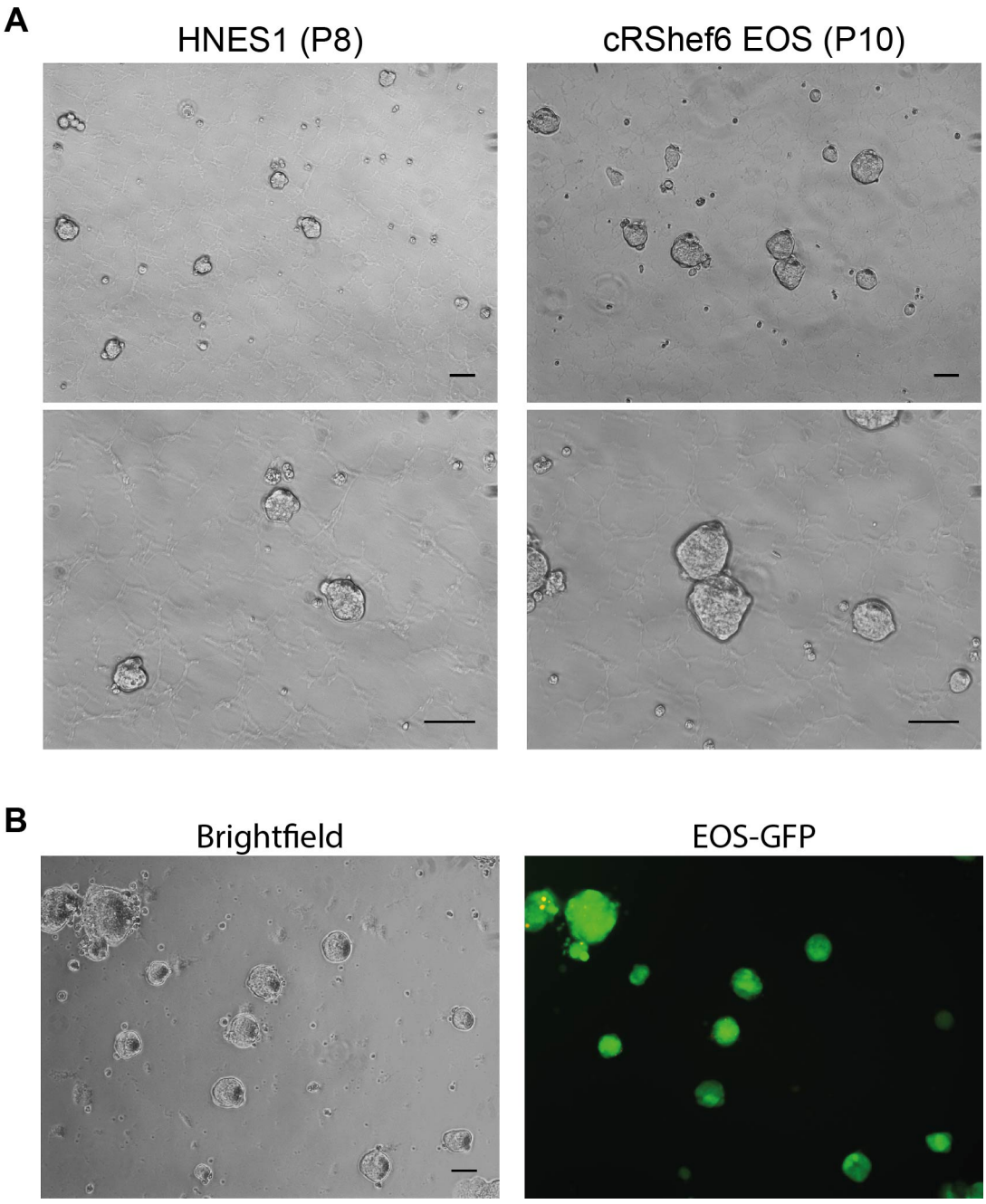

Figure 3.1 Morphology and EOS-GFP reporter activity of tissue laminin cultures

A) Bright-field images of HNES1 and cRShef6 EOS cells cultured in the absence of feeders on tissue laminin to passage 8 and 10, respectively.
B) EOS-GFP reporter activity in cRShef6 EOS cells cultured in the absence of feeders on tissue laminin for 10 passages. Scale bars represent 100µm.

To examine whether laminin cultures maintain expression of pluripotency-associated factors and naïve epiblast markers, cells were stained for OCT4, NANOG, KLF4 and KLF17. Immunoreactivity was detected for all four proteins in the vast majority of cells, both in HNES1 and cRShef6 EOS cultures (Figure 3.2A).

To determine whether transfer to tissue laminin altered the expression levels of pluripotency markers, the cells were cultured in parallel on tissue laminin and MEFs for 25 days before RT-qPCR was performed. Laminin cultures expressed equivalent levels of general pluripotency factors, *NANOG* and *OCT4*, as well as naïve epiblast markers *KLF4*, *KLF17*, *TFCP2L1* and *DPPA3* (Figure 3.2B). An exception was *DPPA5*, which showed consistently elevated expression in tissue laminin cultures.

To investigate whether establishing cultures on tissue laminin may induce chromosomal rearrangement, the karyotype of several established naïve hPSC tissue laminin cultures was examined. G-banding analysis revealed the majority of cells across these cell lines maintained normal karyotypes (Table 6).

Table 6 Karyotypes of established naïve hESC tissue laminin cultures

Cell line	No. passages on tissue laminin	Karyotype
HNES1	12	46 XY [30/30]
cRShef6 EOS	14	46 XX [15/20] add(6)(q2) [5/20]
NCRM2	5	46 XX [30/30]

N.B. Culture and preparation of NCRM2 samples performed by James Clarke.
G-banded metaphase analyses performed by Duncan Baker.

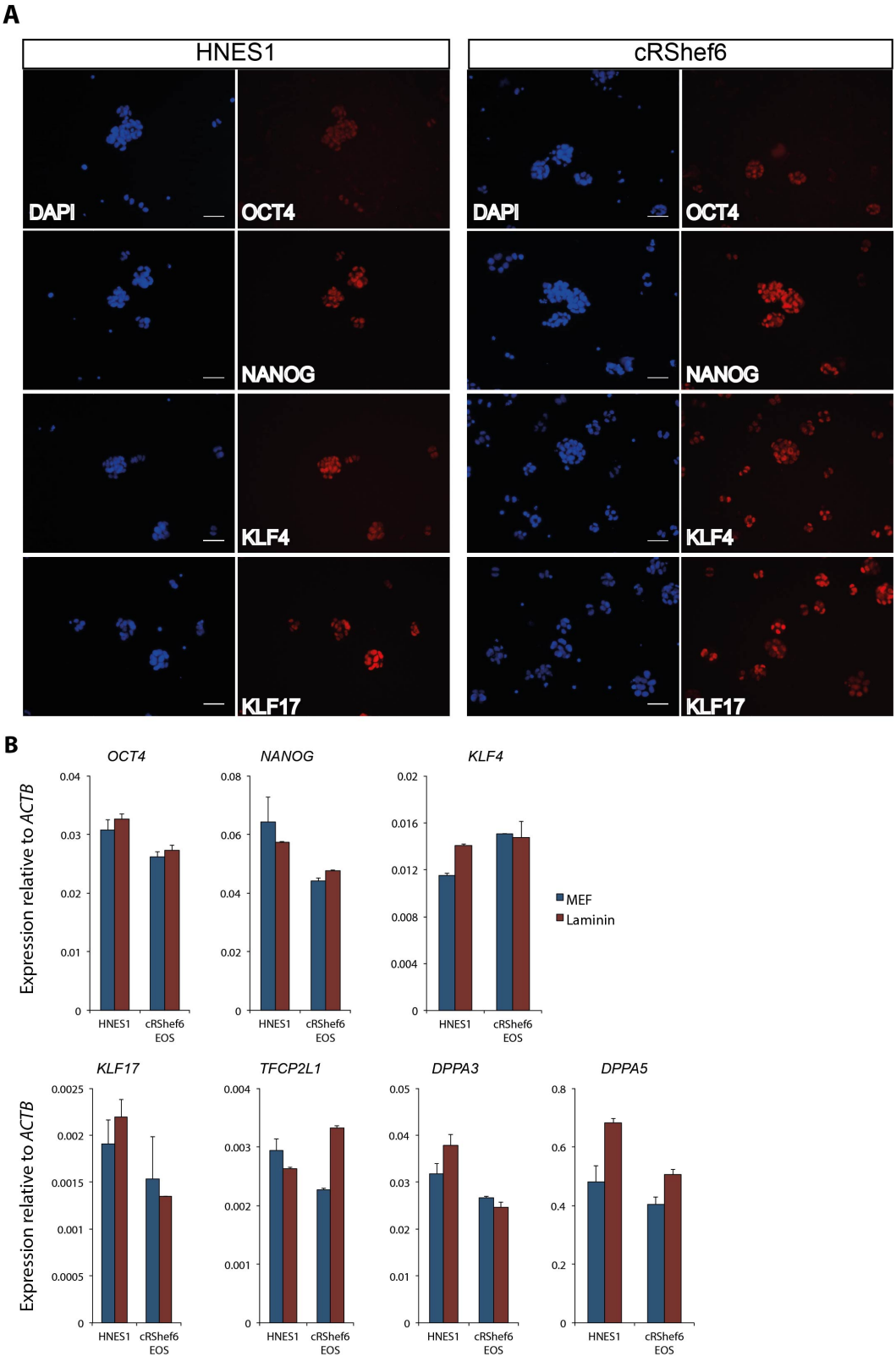

Figure 3.2 Expression of pluripotency markers

A) Immunostaining for core and naïve pluripotency markers in HNES1 and cRShef6 EOS ccells cultured on tissue laminin for 25 days. Scale bars represent 50µm.

B) RT-qPCR showing expression of pluripotency marker transcripts in laminin and MEF cultures. Expression relative to *ACTB*. Error bars represent standard deviation of two independent reactions.

To examine more comprehensively the identity of the tissue laminin cultures, samples were submitted for whole transcriptome analysis. The transcriptomes were sequenced via illumina sequencing and compared to previously published datasets of naïve hESC MEF cultures and conventional primed hESCs using principal component analysis (PCA) (Guo et al., 2016). PCA is a technique that reduces the dimensionality of a data set while maximizing the variance. Analysis was performed by Sabine Dietmann (SCI bioinformatics facility). PCA analysis of the global transcriptomes clearly discriminated naïve hESCs from conventional primed hESCs, regardless of culture on tissue laminin or MEFs. Furthermore, naïve HNES1 and cRShef6 EOS cells cultured on tissue laminin grouped together with naïve cells that had been co-cultured with a MEF feeder layer (Figure 3.3A).

3.2.2. Transfer to tissue laminin-based culture results in downregulation of lineage markers

Although the naive tissue laminin cultures grouped generally with the naïve MEF cultures, they also appeared to form an independent cluster within this grouping (Figure 3.3A). Further investigation of the tissue laminin culture transcriptome data revealed significant downregulation of multiple mesodermal and endodermal marker transcripts that are present at low levels in MEF-based cultures (Figure 3.3B). In order to confirm this differential expression of mesodermal and endodermal lineage marker transcripts, HNES1 and cRShef6 EOS cells were cultured concurrently on tissue laminin or MEF substrates for a period of 8 passages at which point transcript expression was assessed by RT-qPCR. Downregulation of *MIXL1*, *EOMES*, *GATA4* and *T* (*BRACHYURY)* transcripts was observed in laminin cultures (Figure 3.3C). In particular, *T* (*BRACHYURY)* is expressed at relatively high levels in MEF cultures and transcript levels are markedly reduced following transfer to laminin culture.

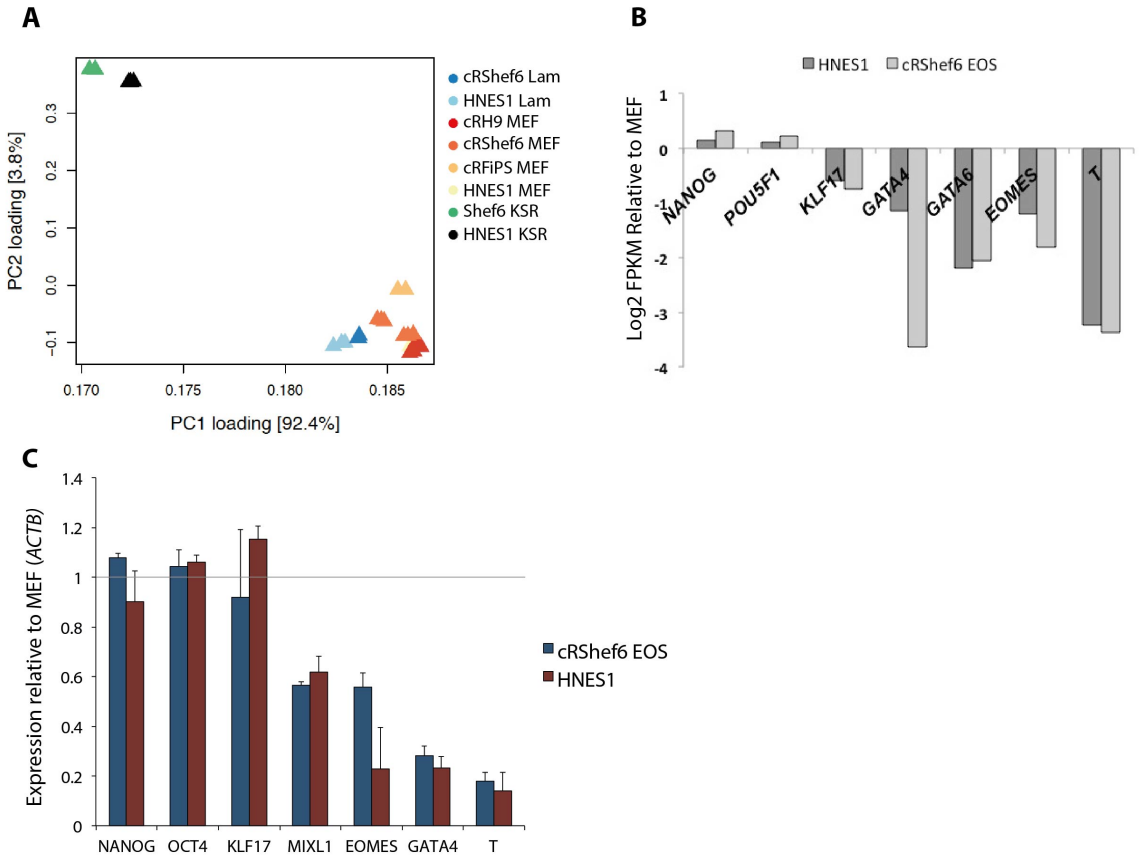

Figure 3.3 Lineage marker expression in MEF and tissue laminin cultures

A) Principle component analysis of whole-transcriptome RNA-Seq data of indicated cultures. Lam=Tissue laminin culture. Analysis and plotting performed by Sabine Dietmann.
B) RNA-Seq data showing expression of pluripotency and lineage marker transcripts in HNES1 and cRShef6 EOS cells cultured on tissue laminin or MEFs. Raw sequencing data analysed by Sabine Dietmann.
C) RT-qPCR data showing expression of lineage marker transcripts in HNES1 and cRShef6 EOS cells following culture on tissue laminin or MEF to passage 8. Expression shown as relative to MEF culture. Expression normalised to *ACTB*. Error bars represent standard deviation of two independent reactions.

Previous experiments in the Smith lab have demonstrated that BRACHYURY protein is detectable in MEF-based naïve hESC cultures by immunocytochemistry (Ge Guo, unpublished). To determine whether suppression of BRACHYURY could also be detected at the protein level, HNES1 and cRShef6 cells were cultured on tissue laminin or MEFs for a period of 4 passages before immunocytochemistry was performed. BRACHURY was detected extensively throughout MEF cultures, although the signal was largely mosaic within the positively stained colonies (Figure 3.4). Importantly, BRACHYURY was detected in tightly packed dome-shaped colonies, and the majority of reactive cells were also positive for the primate-specific naïve epiblast marker KLF17. This indicates that the *T (BRACHYURY)* mRNA expression detected in bulk

preparations of MEF-based cultures is not solely the result of a differentiated sub-population of cells. In contrast to MEF cultures, BRACHYURY immunoreactivity was almost entirely absent from tissue laminin cultures, confirming the downregulation observed at the transcript level.

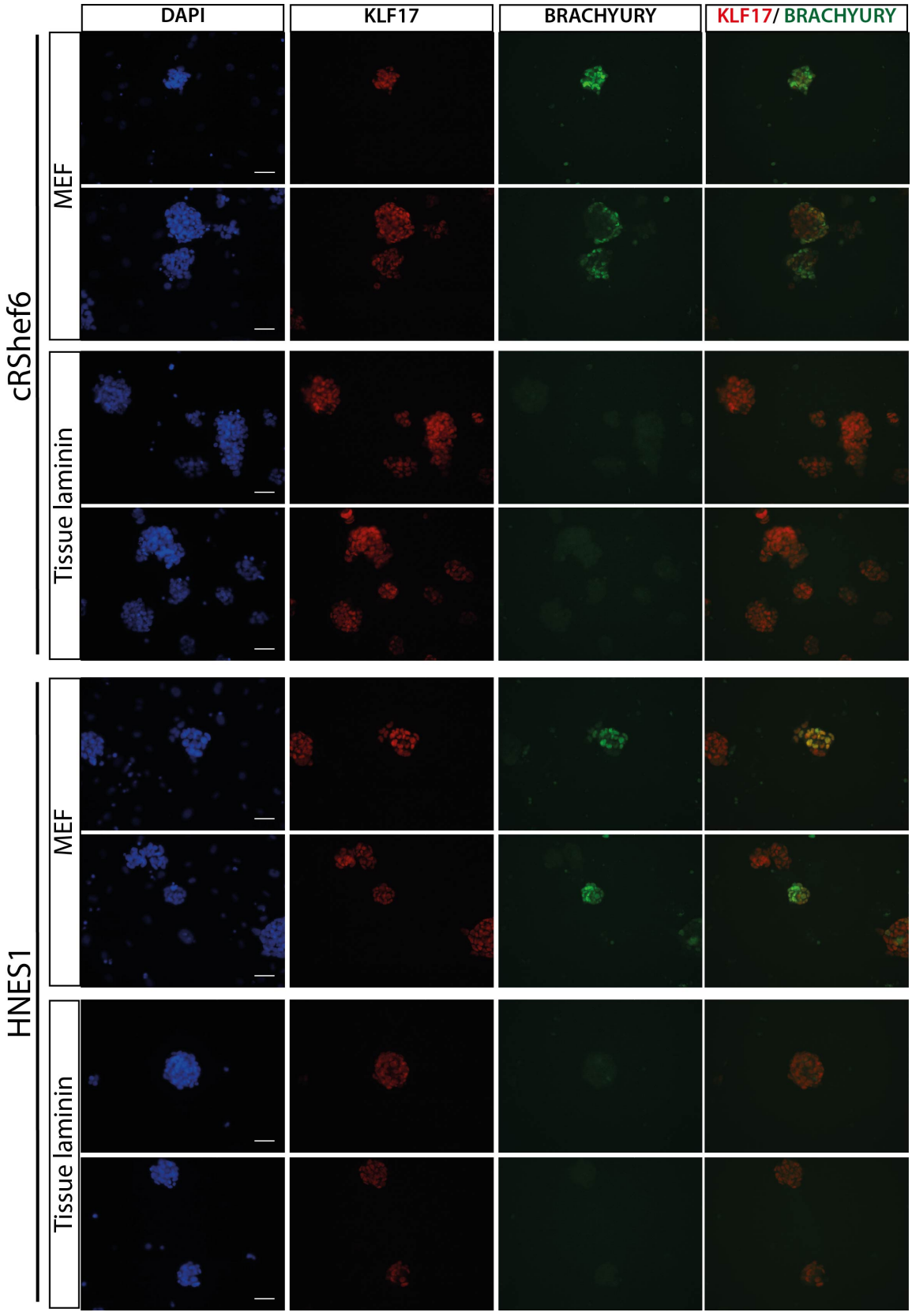

Figure 3.4 BRACHYURY protein expression in MEF and tissue laminin cultures

Representative images of BRACHYURY and KLF17 staining in HNES1 and cRShef6 cells cultured on tissue laminin or MEFs for 4 passages. Scale bars represent 50μm.

3.2.3. Developmental progression of naïve hESCs cultured on tissue laminin

Having observed that naive hESCs transferred from a MEF substrate to high-concentration tissue laminin maintain key features of naïve pluripotency, I next sought to determine whether these cells could exit the naïve pluripotent state and initiate the primed pluripotency transcriptional network.

To assess the ability to progress developmentally from the naïve state to the primed state, HNES1 and cRShef6 EOS tissue laminin cells were plated on Geltrex in t2iLGöY for 24 hours before changing the medium to E8 (Figure 3.5A). Culture in E8 medium for 2 passages resulted in the majority of colonies adopting a monolayer, epithelial-like morphology consistent with conventional primed human pluripotent stem cells cultured under these conditions (Figure 3.5B). Furthermore, cRShef6 EOS cells displayed widespread loss of GFP expression, indicating the *OCT4* distal enhancer element associated with naïve pluripotency was no longer active.

To determine whether HNES1 and cRShef6 EOS tissue laminin cultures transferred to E8 medium had dissolved the naïve transcription factor network and retained core pluripotency markers, RT-qPCR was performed. As expected, mRNA transcripts for naïve markers *KLF4*, *KLF5, KLF17*, *TFCP2L1* and *DPPA5* were almost entirely absent (Figure 3.5C i). In contrast, mRNA levels of *SOX2* and *OCT4* were maintained following priming (Figure 3.5C ii). Taken together with the observed loss of EOS-GFP activity in cRShef6 EOS cells this indicates a switch in the regulation of *OCT4* expression consistent with a transition from naïve to primed pluripotency. Similarly, *NANOG* was still expressed, albeit to a reduced degree, in keeping with previous reports than *NANOG* is downregulated following transition to the primed pluripotency state (Guo et al., 2017; Guo et al., 2016; Takashima et al., 2014).

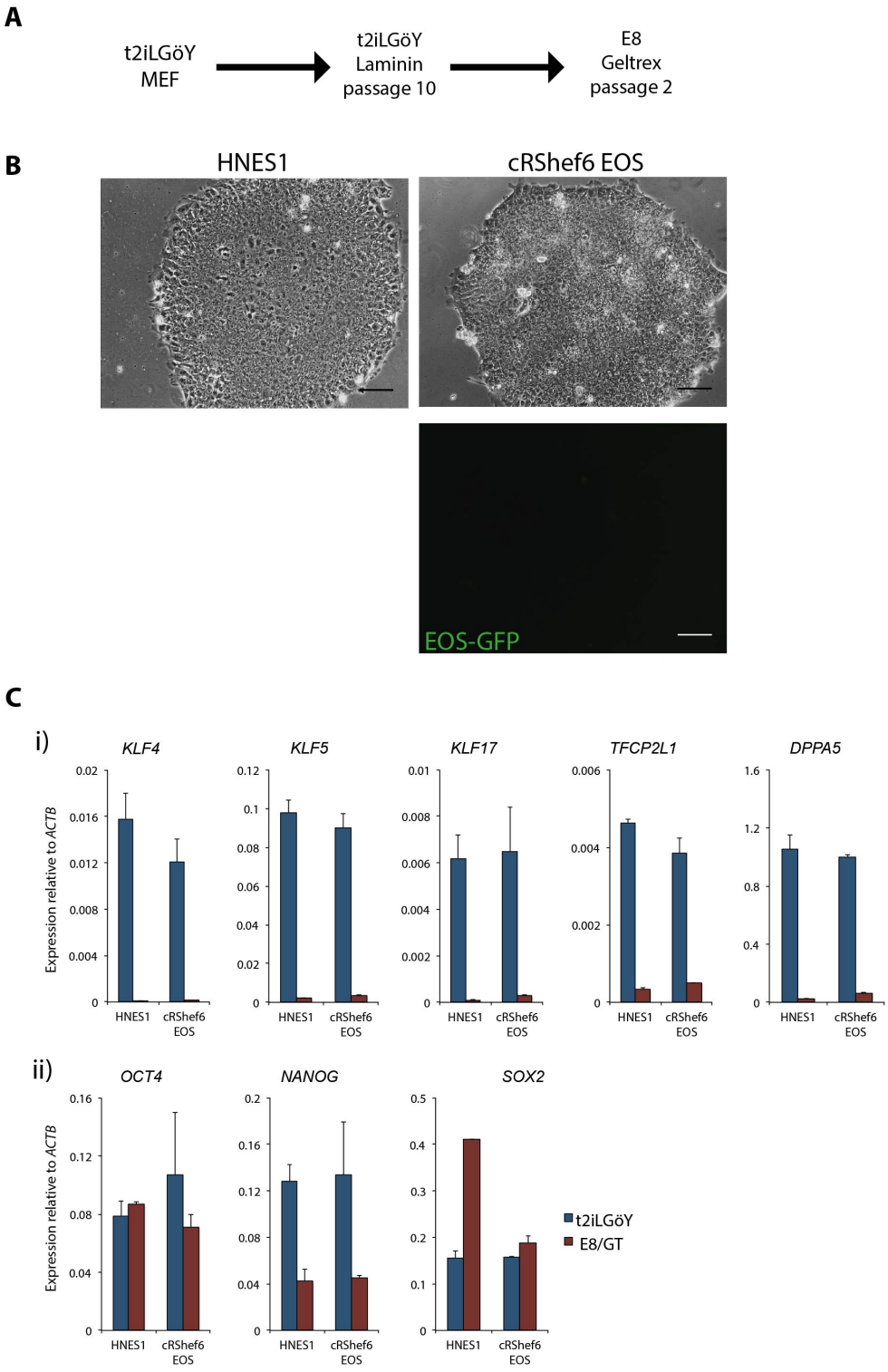

Figure 3.5 Developmental progression to primed pluripotency

A) Diagram showing feeder-free tissue laminin culture and subsequent priming process.
B) Bright-field images showing morphology of HNES1 and cRShef6 EOS cells cultured in E8 on Geltrex for 2 passages (upper). Image showing GFP activity of cRShef6 EOS colony pictured above (lower). Scale bars represent 100µm.
C) RT-qPCR comparing expression of naïve (i) and core (ii) pluripotency marker transcripts in the E8 cultures pictured above and in HNES1 and cRShef6 EOS cells maintained in t2iLGöY on tissue laminin. Expression relative to *ACTB*. Error bars represent standard deviation of 2 independent reactions.

3.2.4. Culture of naïve hESCs on recombinant laminins

The predominant laminin complex present in tissue laminin has previously been identified as Laminin 111 (Timpl et al., 1979). I therefore sought to determine whether a high concentration of recombinant laminin 111 could preserve naïve pluripotency as a defined alternative to tissue laminin. In addition, laminin 521, previously shown to maintain primed hPSCs under defined conditions, was also tested (Lu et al., 2014). To examine this, HNES1 cells previously maintained on tissue laminin were cultured on either 10μg/cm^2 of rLaminin 111, rLaminin 521 or tissue laminin for a period of 3 passages before assessing expression of pluripotency factors. After 3 passages, HNES1 cells culture on rLaminin 521 had adopted a monolayer colony morphology, in contrast to the tightly-packed domed colonies observed in the tissue laminin cultures (Figure 3.6A). When cultured in rLaminin 111 the colonies developed a heterogeneous colony morphology, forming a mixture of flattened monolayer colonies and slightly disorganised dome-shaped colonies more consistent with tissue laminin cultures. RT-qPCR analysis of pluripotency markers revealed general downregulation in rLaminin 521 cultures when compared to expression in tissue laminin cells (Figure 3.6B). With the exception of *TFCP2L1*, expression of these markers was better conserved in cells cultured on rLaminin 111. However a general trend of downregulation was still observed.

Following identification of tissue laminin as a substrate that supports naïve pluripotency, it was observed by James Clarke and Ge Guo that addition of tissue laminin to the medium at the time of plating reduced the required concentration of laminin from 100μg/ml to 50μg/ml (Guo et al., 2017). In verification of this, I observed that HNES1 and cRShef6 cells cultured under these conditions for a period of 6 passages continued to express naïve pluripotency markers (Figure 3.6C).

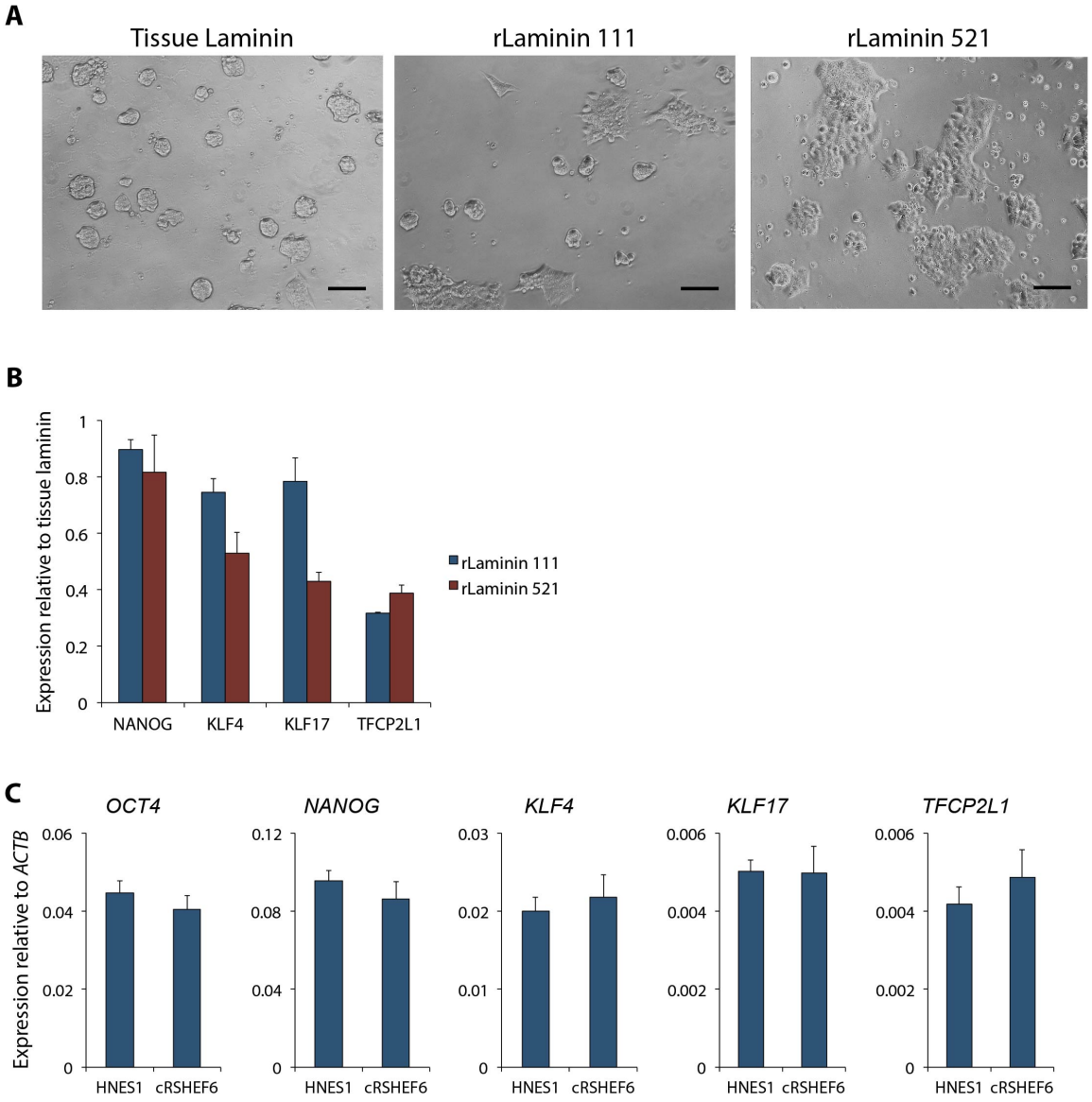

Figure 3.6 Culture on recombinant laminins

A) Bright-field images showing HNES1 cells cultured on either tissue laminin, recombinant laminin 111 or recombinant laminin 521 at a concentration of 10µg/cm^2 for 3 passages. Scale bars represent 100µm.

B) RT-qPCR data showing expression of pluripotency marker transcripts in HNES1 cultures pictured above.

C) RT-qPCR data showing expression of pluripotency markers following culture for 6 passages on tissue laminin (50µg/ml) that was added at the time of plating (simultaneous coating).

Expression relative to *ACTB*. Error bars represent standard deviation of two independent reactions.

3.3. Discussion

Previously, *in vitro* establishment and maintenance of naïve pluripotency has relied on a feeder layer of inactivated mouse embryonic fibroblasts. MEF-based cultures present a variable, reactive and undefined culture system confounding attempts to determine the signalling requirements of naïve human pluripotency. In this chapter I describe a tissue laminin culture system that allows the stable expansion of naïve hESCs. Naïve hESCs cultured under these conditions maintain the transcriptional profile of naïve pluripotency and retain OCT4 distal enhancer activity. Tissue laminin cultures can be maintained as karyotypically normal cultures and retain the ability to progress developmentally to the primed state of pluripotency.

The mechanism by which high-concentrations of tissue laminin contribute to maintenance of naïve pluripotency remains unclear. Replacing tissue laminin with the majority laminin complex, Laminin 111, did not fully preserve morphology or pluripotency marker expression. Although rLaminin 111 did appear to maintain naïve pluripotency more effectively than rLaminin 521, suggesting the type of laminin the cells interact with may be instructive to cell fate.

Previous reports examining the constitution of laminin derived from mouse EHS tumour cultures suggests collagen IV and nidogen (entactin) are likely to also be present in the derivative (Timpl et al., 1979). Furthermore, closer examination of tissue laminin coated wells shows what appears to be a pattern of protein deposits after coating that are not present on recombination laminin coated wells. This may therefore indicate that additional extracellular matrix components present in the extract, other than laminin 111, are important for preservation of human naïve pluripotency. Theses additional undefined components need not directly interact with the cells, but may be important for forming complexes with greater structural similarity to true extracellular basement membranes, allowing the cells to interact with the laminin more appropriately. Consistent with both Timpl et al., 1979 and our own findings, more recent mass spectrometry analysis of tissue laminin indicates the preparation is predominantly comprised of laminin 111, collagen IV and nidogen (personal communication; Paul Bertone). Therefore a future experimental avenue of interest would be to combine

rLaminin 111 with nidogen and/or collagen IV to determine whether these components of tissue laminin are beneficial for naïve cell maintenance.

In addition to co-isolation of other basement membrane components other than laminin, it is also possible that cytokines and growth factors are present after purification. Following transfer to the tissue laminin substrate I observed downregulation of multiple lineage markers that are ectopically expressed in naïve hESCs culture on MEFs. This is consistent with the idea that tissue laminin offers a more stringent signalling environment, and that much of the lineage marker expression previously observed in naïve cultures may result from incongruous signalling from the MEFs. However it is possible that soluble factors present in the tissue laminin solution are important for naïve pluripotency and therefore a larger volume of solution is required. Therefore given the uncertain composition of the tissue laminin used this culture system cannot be termed a fully defined culture system.

Transfer of naïve hESCs cultured in t2iLGöY tissue laminin conditions to E8 Geltrex conditions for 2 passages resulted in the downregulation of naïve markers while maintaining expression of core pluripotency markers. This therefore indicates that tissue laminin cultures retain the capacity to rewire the pluripotency network and transition to the primed pluripotent state. However a thorough examination of the developmental potential of these cells was not performed as part of this thesis and consequently it remains to be demonstrated that cells maintained on tissue laminin still retain the potential to generate somatic cell types.

Chapter 4. Investigation of JAK/STAT signalling

4.1. Introduction

LIF potently promotes self-renewal of naïve mESCs via activation of JAK/STAT signalling but whether this pathway also induces self-renewal in the human naïve state remains undetermined (Smith et al., 1988; Williams et al., 1988).

MEFs are known to secrete numerous cytokines into the growth medium that may influence human naïve pluripotency, including LIF (Rathjen et al., 1990). To date, long-term maintenance of human naïve pluripotency has required a feeder layer of MEFs, confounding attempts to investigate the role of LIF and JAK/STAT signalling in naïve hESC self-renewal. In the previous chapter I described a culture system based on tissue laminin that relinquishes the requirement for a feeder layer. This system provides a more stringent and consistent foundation for interrogation of LIF/JAK/STAT signalling via exogenous cytokine exposure and small molecule inhibitor treatment.

The aim of this chapter was to investigate whether LIF/JAK/STAT signalling plays a role in the maintenance of naïve human pluripotency. I aimed to determine whether self-renewal is independent of JAK/STAT activation and whether supplementation with LIF or other IL-6 family cytokines promotes naive self-renewal. Finally, I also investigated whether key JAK/STAT targets reported in the mESC system are conserved in human naïve pluripotency.

4.2. Results

4.2.1. Prolonged modulation of JAK/STAT Signalling

To determine whether supplemental LIF is required for self-renewal of naïve hESCs, HNES1 and cRShef6 cells were plated on to tissue laminin in the presence or absence of LIF. Cells maintained under these conditions were passaged routinely every 4 days for a period of 20 days. Morphologically both HNES1 and cRShef6 colonies cultured without supplemental LIF were indistinguishable from control cultures, displaying no obvious changes in colony size or shape (Figure 4.1A). Similarly, no overt differences in proliferation were observed between the cultures during routine passaging.

To determine whether withdrawal of LIF altered expression of pluripotency markers RT-qPCR was performed. The absence of LIF did not appear to alter expression of core pluripotency markers, *NANOG* and *OCT4*, or naïve pluripotency makers, *KLF4*, *KLF17*, *TFCP2l1 (*Figure 4.1B*)*. Of particular note, expression of *KLF4* and *TFCP2L1*, downstream targets of LIF and JAK/STAT signalling in the mESC system, were not substantially altered in response to LIF withdrawal.

In order to assess whether omitting LIF resulted in less robust self-renewal the colony forming capacity of the cells was examined.

Initially tissue laminin was used as a substrate for this assay, however I observed a tendency for colonies to detach from the well towards the end of the culture period and during fixation and staining. Therefore, in order to preserve the integrity of the assay Geltrex was utilised as a substrate for plating. Transgene reset H9 NK2tet cells were plated on Geltrex at clonal density in the presence or absence of supplemental LIF. After 7 days alkaline phosphatase staining was used to score the resulting colonies. Consistently, withdrawal of LIF resulted in a slight reduction in colony formation (Figure 4.1C).

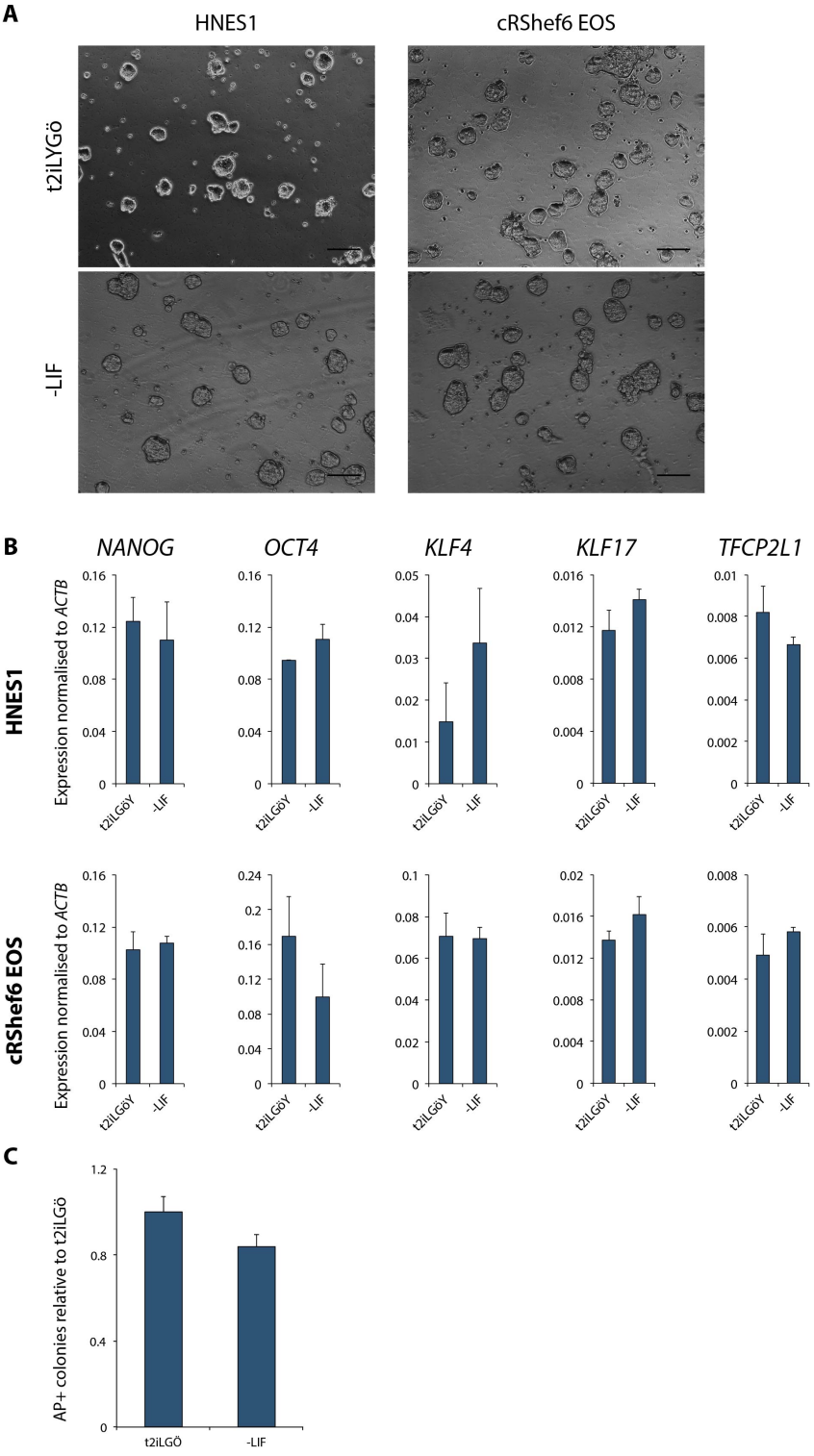

Figure 4.1 LIF supplementation is dispensible for naïve self-renewal

A) Bright-field images showing HNES1 and cRShef6 cells cultured for 20 days on tissue laminin in the presence or absence of human LIF. Scale bars represent 100µm.

B) RT-qPCR comparing expression of pluripotency marker transcripts in HNES and cRShef6 cells cultured under on tissue laminin in the presence or absence of human LIF for 20 days. Error bars represent standard deviation of 2 independent reactions.

C) Colony forming assay showing the number of alkaline phosphatase positive colonies formed from 600 iH9NK2tet cells plated on Geltrex in the presence or absence of human LIF. Cells were cultured for 7 days before fixation and staining. Error bars represent standard deviation of 6 plating replicates.

In order to examine whether endogenous JAK/STAT activation is required for maintenance of naïve identity, LIF was omitted and the cells were exposed to two different inhibitors of JAK activity. HNES1 and cRShef6 cells were treated for 4 passages with either Ruxolitinib (1μM) (Quintas-Cardama et al., 2010), a potent JAK 1/2 inhibitor, or JAK inhibitor 1 (300nM) (Thomson et al., 1998) a broad JAK inhibitor. Cultures of both cell lines could be propagated as normal, regardless of JAK inhibitor treatment. At passage 4 gene expression was assessed via RT-qPCR. Prolonged exposure to either Ruxolitinib or JAK inhibitor 1 did not preclude expression of any of the pluripotency markers examined (Figure 4.2A). However there was a clear trend of reduced pluripotency marker expression. This may indicate endogenous JAK/STAT activation may contribute to stabilisation of the naïve transcription factor network, however off-target effects of the inhibitors cannot be excluded.

To determine whether the reduced pluripotency marker expression observed correlated with changes in lineage marker expression the samples were probed for trophectoderm and primitive endoderm marker transcripts. Interestingly, expression of *GATA2* and *GATA3* increased in the presence of JAK inhibition, particularly in the case of HNES1.

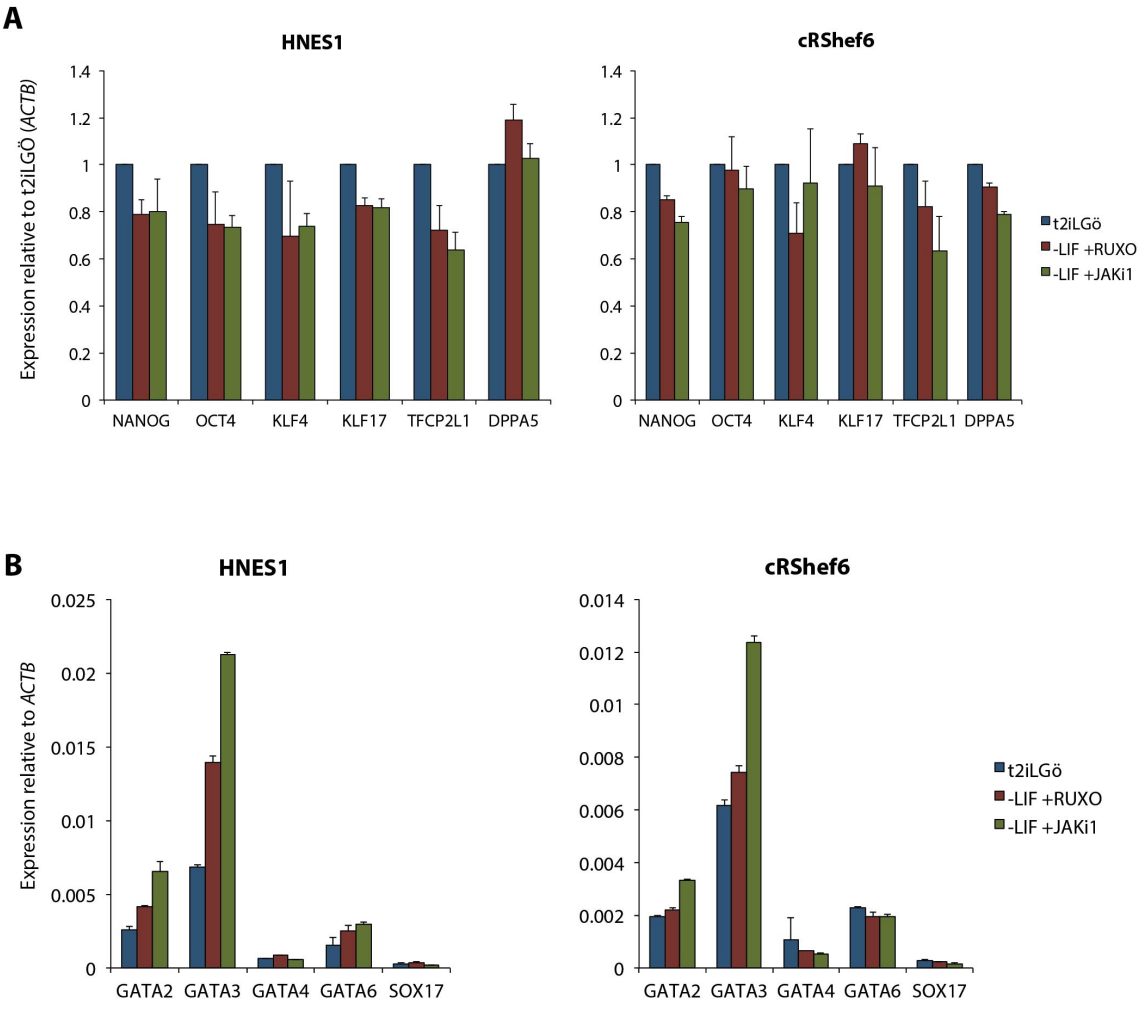

Figure 4.2 Extended inhibition of JAK/STAT signalling

A) RT-qPCR data showing expression of pluripotency marker transcripts in HNES1 and cRShef6 cells cultured on tissue laminin in the presence or absence of human LIF, Ruxolitinib (1μM) and JAK inhibitor 1 (300nM) for 20 days.
B) RT-qPCR showing lineage marker transcript expression in samples shown above.
Error bars represent standard deviation of 2 independent replicates.

4.2.2. Identification of an alternative activator of JAK/STAT signalling

JAK/STAT mediated self-renewal of mESCs has been demonstrated to be directed not only through LIF signalling, but also through other members of the Interleukin 6 (IL-6) family, such as IL-6 when its soluble receptor is also applied (Nichols et al., 1994). To determine whether other members of the IL-6 family may direct JAK/STAT activation in the human naïve state published RNA-seq data was interrogated. Transcripts of the IL-6 receptor (*IL-6R*) were significantly more abundant in naïve hESCs in comparison to hESCs maintained in conventional primed culture conditions (Figure 4.3A). Interestingly, *IL-6R* was generally expressed to a much higher level than the LIF receptor (*LIF-R*) in the naïve state, suggesting IL-6 may activate the JAK/STAT pathway more potently than LIF. To determine whether this observation could be a genuine feature of naïve hESC identity or a product of the incongruous signalling environment inherent to MEF culture systems, *LIF-R* and *IL-6R* expression were determined under more stringent signalling conditions. In addition to these cytokine specific components, I also examined expression of *IL-6ST* (gp130), an essential component of IL-6 family cytokine receptor complexes. RT-qPCR analysis of HNES1 and cRShef6 EOS cells cultured under naive conditions on tissue laminin and primed conditions on Geltrex independently verified the increased *IL6-R* expression in the naïve state (Figure 4.3B). In addition to increased expression of *IL-6R* I also observed greater expression of essential signalling component *IL-6ST* (gp130) in naïve cultures. Furthermore, this data shows that both *IL6-R* and *IL6-ST* (gp130) expression is heavily suppressed upon entering the primed pluripotent state, suggesting any potential role of this signalling pathway in pluripotency is restricted to the naïve state.

In order to determine whether a greater abundance of *IL-6R* transcripts translates into more potent activation of the JAK/STAT signalling pathway, STAT3 phosphorylation in response to LIF and IL-6 was examined. HNES1 cells were cultured on tissue laminin in the absence of LIF for 4 days. The cultures were then treated with either LIF or IL-6 at a concentration of 20ng/ml for a period of 20, 40 and 60 minutes before cell lysis and western blotting. Additionally, LIF and IL-6 treatment was also performed in the presence of JAK inhibitor 1 for 60 minutes to confirm any resulting STAT3 phosphorylation signal is dependent on JAK/STAT activation.

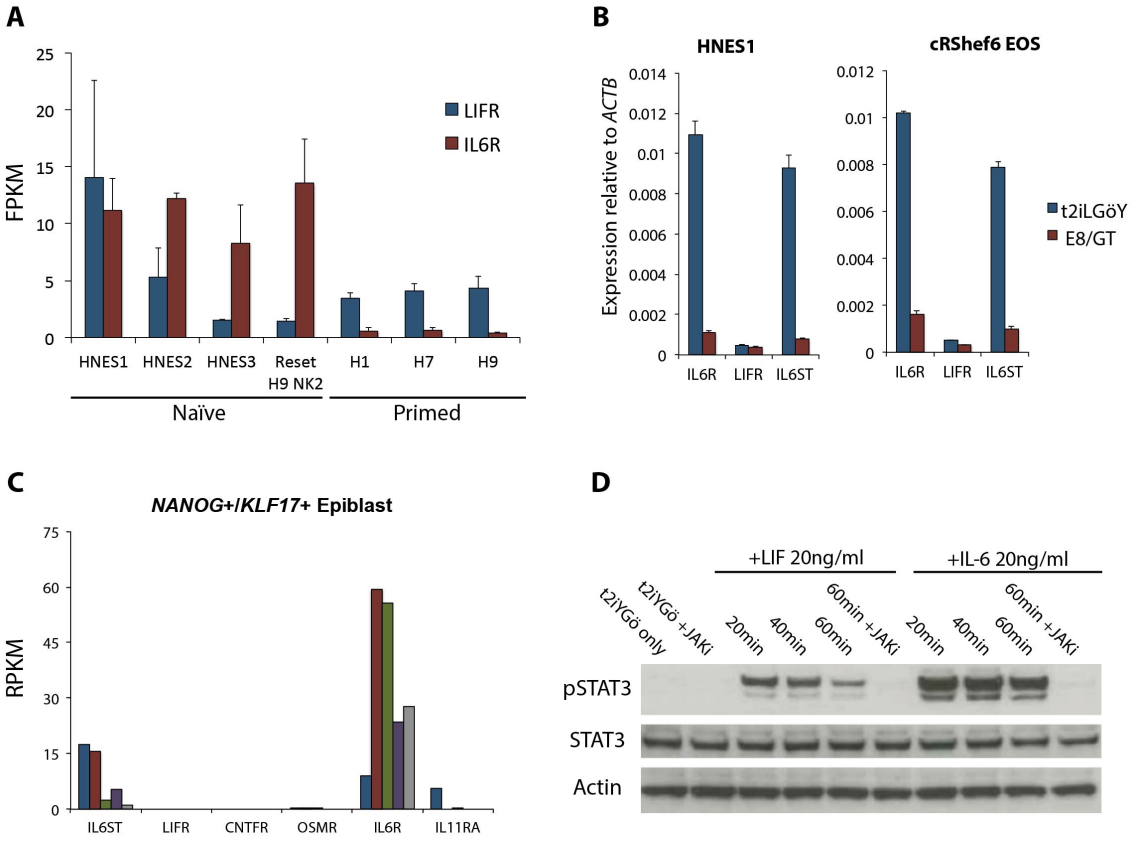

Figure 4.3 Analysis of alternative activators of JAK/STAT signalling

A) RNA-seq data showing expression of LIF and IL6 receptor transcripts in cell lines cultured under naïve (t2iLGöY/MEF) and primed (KSR/FGF2) conditions. Chart produced from publicly available data set (Guo *et al.,* 2016). Error bars represent standard deviation of 3 independent sequencing reactions.
B) RT-qPCR data showing expression of LIF and IL-6 receptor component transcripts in HNES1 and cRShef6 EOS cells cultured under naïve (t2iLGöY/Lam) and primed (E8/Geltrex) ESC culture conditions. Error bars represent standard deviation of 2 independent reactions.
C) Single-cell RNA-seq data showing expression of JAK/STAT receptor transcripts in 5 individual *NANOG* and *KLF17* double positive (>5RPKM) cells isolated from pre-implantation stage human blastocysts. Chart produced from publically available data set (Yan *et al.,* 2013).
D) HNES1 cells were cultured on laminin in the absence of LIF for 4 days at which point LIF or IL6 was introduced in either the presence of absence of JAK inhibitor 1 (1μM) for 20, 40 or 60 minutes. Western blotting of resulting protein lysates shows detection of phosphorylated STAT3 (705Y) and total STAT3. Actin served as a loading control.

Reintroduction of LIF to the cultures resulted in moderate phosphorylation of STAT3, peaking before the 40-minute mark, followed by rapid reduction in signal by the 60-minute mark. In contrast, introduction of IL-6 resulted in significantly stronger STAT3 phosphorylation that persisted beyond the 60-minute time point. In the case of both LIF and IL-6, phosphorylation of STAT3 was blocked by addition of JAK inhibitor 1, demonstrating dependence on the JAK/STAT signalling pathway. This immunoblotting data therefore indicates that IL-6 is a more potent activator of JAK/STAT than LIF in naïve HNES1 cells.

To gain insight into whether the dominant activity of IL-6 is an artefact of the culture system or a *bona fide* feature of the naïve human epiblast, a published human embryo single-cell RNA-seq dataset was examined to determine expression of IL-6 family receptor components (Yan et al., 2013). Cells of the naïve epiblast were identified as those expressing *NANOG* and *KLF17* at a level greater than 5 RPKM. Of the 5 putative epiblast cells identified, none showed expression of *LIF-R* transcripts (Figure 4.3C). In contrast, *IL-6R* transcripts were detected in all 5 cells. In addition, Interleukin 6 Signal Transducer (IL-*6ST*/gp130), an essential component of IL-6 family receptor complexes was also detected in all 5 cells. Recent combined reanalysis of Petropoulos et al., 2016 and Blakeley et al, 2014 single-cell RNA-seq datasets also indicates prevalent expression of *IL6R* in the human naive epiblast, with little to no expression of *LIFR* (personal communication, Giuliano Stirpraro, unpublished).

4.2.3. IL-6 signalling promotes naïve self-renewal

Given that IL-6 supplementation results in significantly stronger activation of STAT3, I next sought to determine whether IL-6 supplementation positively influences self-renewal of naïve hESCs.

In order to determine whether IL-6 induces a more robust state of self-renewal, HNES1 cells were plated at clonal density on Geltrex and cultured for 7 days in the presence or absence of LIF and IL-6. IL-6 supplementation resulted in improved capacity to generate alkaline phosphatase positive colonies, suggesting self-renewal may be more robust (Figure 4.4A).

I next sought to determine whether IL-6 supplementation is able to safeguard naïve pluripotency under differentiating conditions. cRH9 EOS cells were cultured either in t2iLGö, N2B27, N2B27+LIF or N2B27+IL-6 for a period of 7 days before using FACs to quantify the number of cells still expressing GFP driven by the murine OCT4 distal enhancer element in the reporter (Figure 4.4B). Under t2iLGö control conditions 90% of the cell population was GFP positive (Figure 4.4C). In contrast, culture for 7 days in N2B27 alone resulted in a substantial drop to around 43%. Supplementation of N2B27 with LIF resulted in an increase to approximately 51%, while supplementation with IL-6 further increased the GFP positive population to 62%.

Increased GFP activity in the presence of IL-6 indicates reduced dissolution of naïve transcriptional regulation, however the correlation between the activity of this reporter and functional maintenance of pluripotency is not well established. Therefore, to functionally assess the self-renewal capacity HNES1 and cRShef6 cells were challenged under the same conditions for 7 days, at which point they were re-plated at equal density into standard self-renewing conditions (Figure 4.4B). After 7 days of culture pluripotent colony formation was assessed via alkaline phosphatase staining. Exposure to N2B27 alone for 7 days reduced the colony forming capacity of both HNES1 and cRShef6 cells to around 10% that of t2iLGö cultures (Figure 4.4D&E). Exposure to LIF did not result in a significant increase in colony formation compared to N2B27 alone. However the addition of IL-6 lead to a 2.5-fold increase in colony formation, returning approximately 25% of the colonies formed form t2iLGö cultures. Taken together these data indicate IL-6 signalling acts to promote naïve hESC self-renewal.

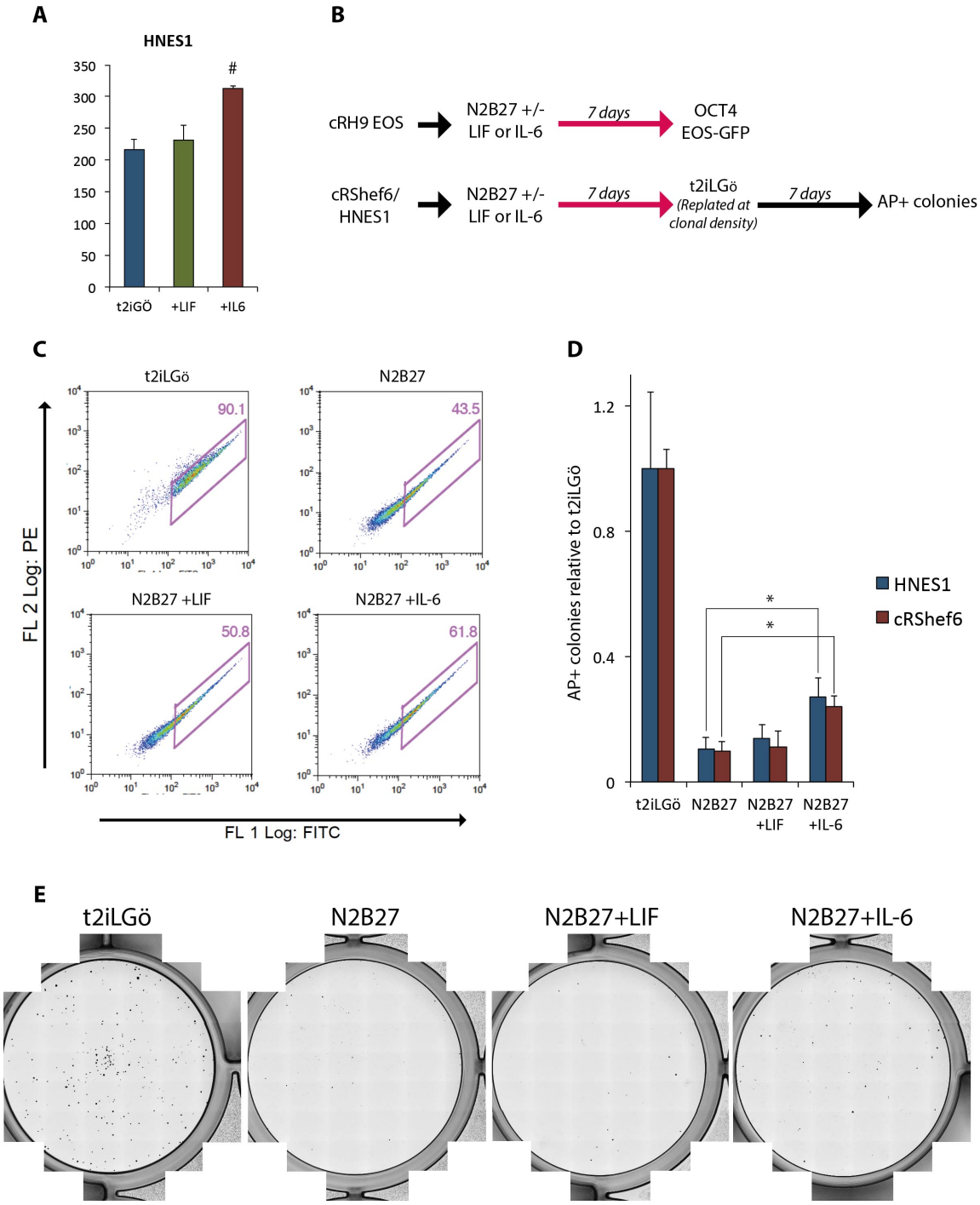

Figure 4.4 IL-6 promotes naïve self-renewal

A) Colony forming assay showing the number of alkaline phosphatase positive colonies formed following plating of 600 cells in the presence or absence of LIF or IL-6. Error bars represent standard deviation of 3 plating replicates.
B) Outline of assays shown in C (upper) and D (lower).
C) FACS plot showing OCT4 distal enhancer reporter activity (EOS-GFP) in cRH9 EOS cells following 7 days culture in t2iLGö, N2B27, N2B27+LIF or N2B27+IL-6.
D) Re-plating assay showing the relative number of AP+ colonies formed from 1000 HNES1 or cRShef6 cells plated in t2iLGö on Geltrex following 7 days culture under conditions noted in C. Error bars represent standard deviation of 2 independent cultures with 3 re-plating replicates each.
E) Representative images of alkaline phosphatase-stained re-plated cRShef6 cultures mentioned in D.
*Anova Tukey's post hoc $p<0.001$. # Anova post hoc $p<0.05$.

4.2.4. Changes in gene expression resulting from IL-6 signalling

Having observed improved self-renewal in response to IL-6 exposure, I next aimed to determine whether prolonged exposure also altered expression of naïve pluripotency or lineage markers.

HNES1 and cRShef6 cells were cultured on tissue laminin in the presence or absence of LIF or IL-6 for 4 passages, at which point gene expression was determined via RT-qPCR. In general, pluripotency marker expression was slightly higher when cells were cultured in the presence of IL-6 (Figure 4.5A). Interestingly, the addition of IL-6 resulted in up-regulation of *KLF4*, a well-established down-stream target of JAK/STAT signalling in mouse ES cells (Li et al., 2005). However, *TFCP2L1*, another JAK/STAT target, was not consistently upregulated across cell lines. Activation of JAK/STAT signalling, via either LIF or IL-6, in mouse ES cell 2i cultures has recently been suggested to lead to induction of primitive endoderm marker expression and suppression of the trophectoderm marker, *cdx2* (Morgani and Brickman, 2015). Long-term culture of naïve hESCs with IL-6 appeared to result in slight upregulation of *GATA4,* however expression levels still remained very low. Alternative primitive endoderm markers, *GATA6* and *SOX17* failed to be consistently upregulated by either IL-6 or LIF and continued to be expressed at very low levels. Conversely, both cell lines exhibited downregulation of murine trophectoderm markers *GATA2* and *GATA3* in response to IL-6 treatment. Of particular note, *GATA3* was relatively highly expressed in t2iGö cultures and was reduced by approximately 50% when IL-6 was present in the medium.

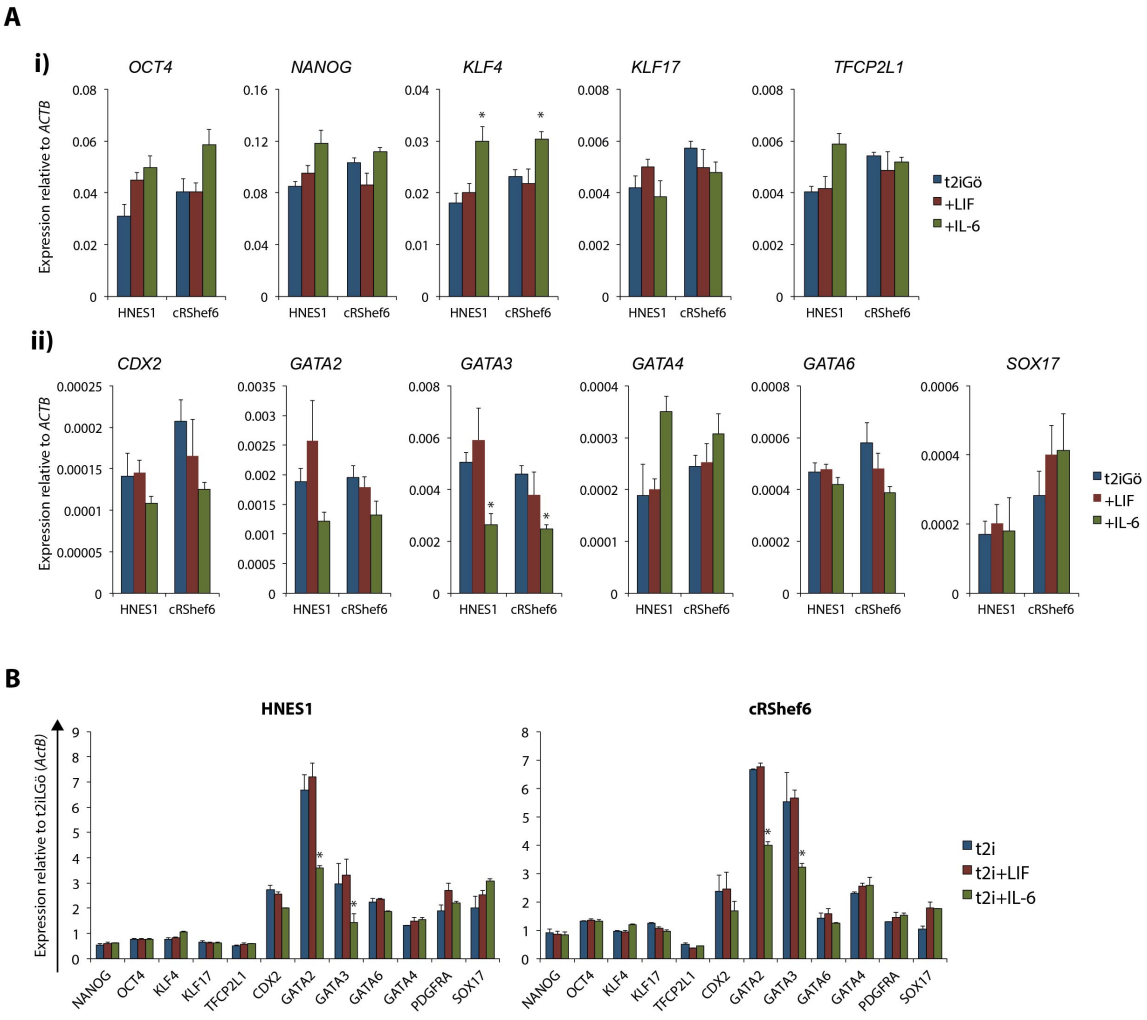

Figure 4.5 IL-6 signalling upregulates *KLF4* and suppresses expression of *GATA2*/3

A) RT-qPCR showing expression of pluripotency (i) and lineage (ii) marker transcripts in HNES1 and cRShef6 cells cultured on tissue laminin in the presence or absence of LIF or IL-6 for 3 passages.
B) RT-qPCR showing expression of pluripotency and lineage marker transcripts in HNES1 and cRShef6 cells cultured on tissue laminin in the absence of Gö6983 (t2i) and the presence of absence of LIF or IL-6 for 3 passages. Expression shown relative to t2iLGö tissue laminin cultures. Error bars represent standard deviation of 2 independent reactions.
*Anova Tukey's post hoc $p<0.01$.

I had previously observed that withdrawal of Gö6983 from tissue laminin based cultures for successive passages results in the downregulation of pluripotency factors and upregulation of GATA factors. I therefore attempted to use this system to determine whether IL-6 treatment promotes pluripotency factor expression and suppresses trophectoderm marker expression in these differentiating conditions. Following withdrawal of Gö6983, widespread colony flattening and increased cell death were observed, regardless of LIF or IL-6 supplementation. After a period of 3 passages gene expression was assessed via RT-qPCR. Consistent with the previous expression data, *KLF4* transcripts were more abundant when cells were exposed to IL-6 (Figure 4.5B). However increased *KLF4* was not accompanied by upregulation of other pluripotency transcripts. Withdrawal of Gö6983 in the absence of LIF or IL-6 resulted in strong upregulation of *GATA2* and *GATA3* (5-7 fold) relative to t2iLGö cultures (Figure 4.5B). Exposure to IL-6 markedly reduced upregulation of *GATA2* and *GATA3*, as well as *CDX2*. Expression of primitive endoderm markers was examined to determine whether prolonged Gö6983 withdrawal permitted induction via IL-6 signalling. Expression of *GATA4*, *GATA6* and *PDGFRA* was not altered by addition of LIF or IL-6 (Figure 4.5B).

4.2.5. Induction of IL-6 target genes

As observed above, long-term modulation of IL-6 and JAK/STAT signalling consistently resulted in altered expression of naive marker *KLF4* and trophectoderm markers *GATA2* and *GATA3*. However, it is unclear whether these changes in expression result from direct activation by IL-6 signalling or as a subsequent result of other transcriptional changes. To attempt to identify direct target genes of IL-6 signalling, cRShef6 cells cultured on tissue laminin were exposed to LIF or IL-6 for 1 hour or 24 hours before assaying transcript expression. To determine whether changes in gene expression could be attributed to JAK/STAT activation, cultures were also exposed to these cytokines in the presence of JAK inhibitor 1. Negative regulator of JAK/STAT signalling, *Socs3* is known to be particularly sensitive to JAK/STAT activation in mouse cells, and is heavily upregulated in mESCs upon LIF exposure (Auernhammer et al., 1999; Niwa et al., 2009). In naïve hESCs exposure to LIF resulted in a 5-fold increase in

SOCS3 expression by the hour mark, however by 24 hours this increased expression was almost entirely lost (Figure 4.6). Consistent with my previous observations of STAT3 phosphorylation, exposure to IL-6 resulted in much stronger upregulation of *SOCS3*, reaching approximately 12-fold the level of expression observed in cells cultured in the absence of LIF or IL-6. Furthermore, this elevated expression was still apparent 24 hours after IL-6 has been added to the culture. Down-stream mediator of JAK/STAT signalling, *STAT3*, is itself positively regulated by activation of this pathway. Addition of LIF did not result in significant upregulation of *STAT3* at either the 1-hour or 24-hour mark. In contrast, IL-6 resulted in a greater than 2-fold increase in *STAT3* transcripts by the 24-hour mark. Upregulation of *SOCS3* and *STAT3* in response to either cytokine was blocked by addition of JAK inhibitor 1. These results further demonstrate IL-6 activates the JAK/STAT signalling pathway in naïve human ES cells and that this mode of activation is sufficient to regulate expression of downstream target genes.

Consistent with reports of JAK/STAT activation in mouse ES cells, supplementation with either LIF or IL-6 did not result in rapid changes in *OCT4* or *NANOG* expression (Figure 4.6). Somewhat surprisingly though, neither LIF nor IL-6 induced upregulation of *KLF4* or *TFCP2L1* by 1 or 24 hours of exposure. However both naïve markers were downregulated within 24 hours when JAK/STAT signalling was inhibited.

In addition to phosphorylation of STAT3, LIF and IL-6 signalling is also known to activate STAT5. Unlike STAT3, which is reported to function solely as a transcriptional activator, STAT5 has been demonstrated to act as both an activator and suppressor of transcription (Mandal et al., 2011). I therefore sought to examine whether the previously observed downregulation of *GATA3* might be a result of direct transcriptional regulation by JAK/STAT activation. Exposure to IL-6 did not result in significant changes in *GATA3* expression relative to t2iGö control cells (Figure 4.6). Once again, expression of primitive endoderm markers *GATA4* and *GATA6* were also examined and consistent with previous observations, expression remained unchanged regardless of LIF or IL-6 exposure.

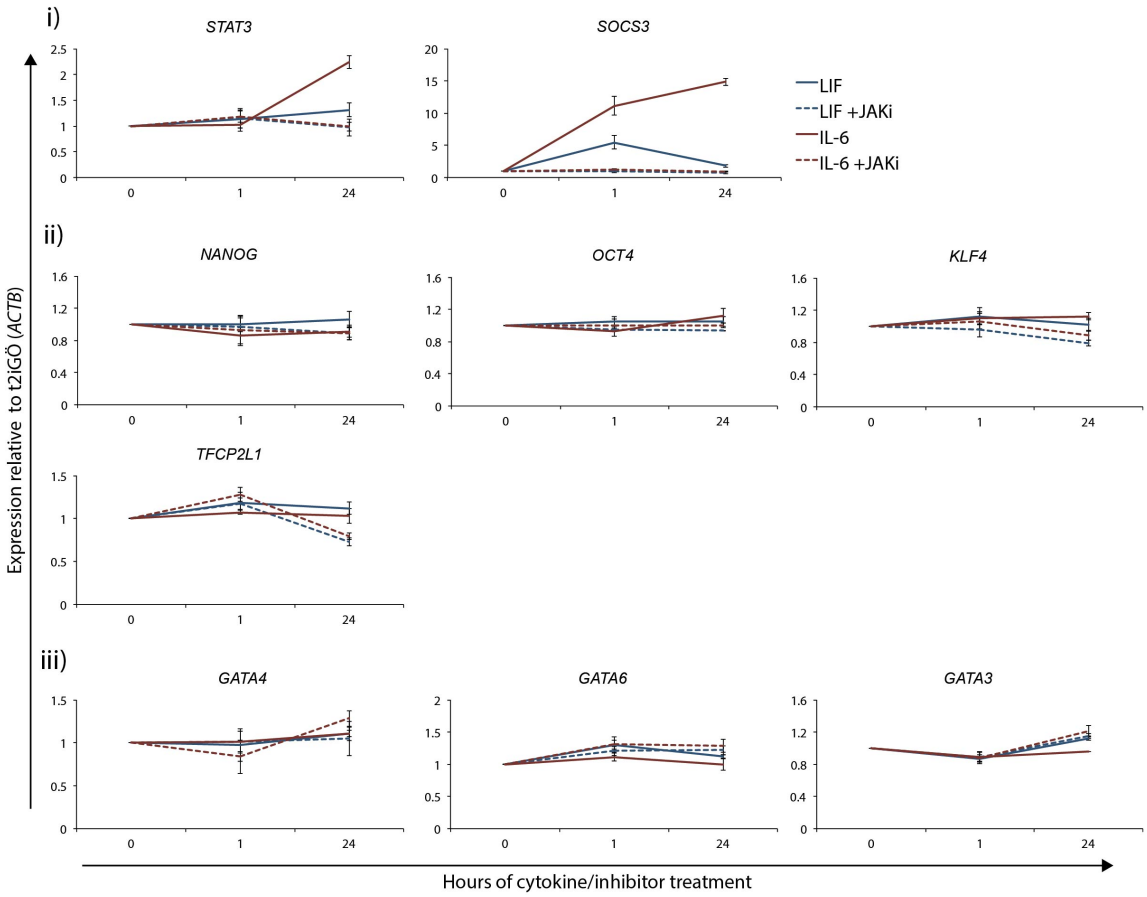

Figure 4.6 Identification of JAK/STAT target genes

RT-qPCR data showing expression of JAK/STAT signalling component **(i),** pluripotency marker **(ii)** and lineage marker (**iii**) transcripts in tissue laminin cultures following exposure to LIF or IL6 for 1 or 24 hours. Cells cultured in the presence of JAK inhibitor 1 (JAKi) served as negative controls of JAK/STAT signalling. LIF was withdrawn from the medium 72 hours before commence the assay. Expression relative to 0 hour cultures. Error bars represent standard deviation of 2 independent reactions.

4.3. Discussion

Derivation of a naïve state of human pluripotency has raised the question of whether the potent self-renewal effects of LIF and JAK/STAT signalling are conserved between mouse and human. In this chapter I utilised a feeder-free, tissue laminin substrate in combination with inhibitors of JAK activity to determine maintenance of human naïve pluripotency is unlikely to require JAK/STAT activation. Higher activation of JAK/STAT signalling could be achieved with addition of IL-6, rather than LIF. Furthermore, IL-6 supplementation appeared to increase colony forming capacity and promoted self-renewal in differentiating conditions. Long-term exposure to IL-6 consistently promoted expression of naïve marker *KLF4* and suppressed expression of trophectoderm-associated markers. However, short-term exposure to IL-6 failed to induce upregulation of canonical JAK/STAT target genes, *KLF4* and *TFCP2L1*.

In contrast to reports of LIF withdrawal in the 5iLAF culture system, long-term withdrawal of supplemental LIF did not appear to alter expression of naïve factors, including *KLF4* (Theunissen et al., 2014). The cause or causes of this disparity could potentially range from disparate signalling environments resulting from different inhibitor cocktails, to slightly different cell identities. However the muted induction of *SOCS3* and lack of *STAT3* upregulation observed following LIF supplementation indicates that LIF does not potently activate the JAK/STAT pathway in naïve hESCs.

Withdrawal of human LIF and exposure to 2 separate JAK inhibitors did not prohibit cell propagation or expression of naïve pluripotency markers, indicating that the JAK/STAT signalling pathway is unlikely to be required for human naïve pluripotency. This is consistent with our understanding of mESC naïve pluripotency, in which the JAK/STAT pathway is dispensable when inhibitors of MEK and GSK3 (2i) are present (Ying et al., 2008). Nevertheless, long-term exposure to JAK inhibition did result in transcriptional changes, including a general reduction in pluripotency marker expression and upregulation of *GATA* factors. These results imply endogenous JAK/STAT activation may contribute to stabilisation of the naïve transcription factor network. However, the potential for off-target effects is intrinsic to small molecule inhibitors and in the absence of complementary genetic interrogation it is impossible to definitively attribute these changes wholly to loss of JAK/STAT activity.

Both RNA-seq and RT-qPCR transcript expression data revealed that *IL-6R*, rather than the *LIF-R*, is predominantly expressed in human naïve cells. Furthermore, treatment with IL-6 resulted in greater phosphorylation of STAT3, as well as greater induction of *SOCS3* and *STAT3* expression. Consistent with previous reports of enriched expression in the human epiblast; IL-6R transcripts were detected in putative human naive epiblast cells, whereas *LIF-R* expression was absent. This would therefore indicate a species difference in the mode of JAK/STAT activation between mouse and human in the pre-implantation blastocyst.

Treatment with IL-6 increased the clonogenicity of t2iGö cultures, suggesting activation of IL-6 signalling results in a more robust state of self-renewal. Importantly, exposure to IL-6 attenuated the loss of murine OCT4-distal enhancer reporter activity and more than doubled the re-plating efficiency of cells subjected to differentiation via removal of MEK, GSK3 and PKC inhibition. This indicates that IL-6 family cytokines may have a conserved role in promotion of naïve pluripotency. However, while no direct comparison was performed in this thesis, JAK/STAT signalling did not appear to promote self-renewal to the same degree that is reported in mESCs. Surprisingly, expression of naïve marker *TFCP2L1* did not increase with IL-6 treatment, regardless of the length of exposure. *TFCP2L1* is reported to be a key mediator of LIF-responsive self-renewal in mESCs (Martello et al., 2013). Therefore lack of *TFCP2L1* induction in human naïve cells may contribute to the reduced self-renewal effect of JAK/STAT activation. Additionally, differences in transcriptional regulation between LIF and IL-6 signalling may contribute to disparity in self-renewal response. Although combined supplementation of IL-6 and a soluble IL-6 receptor is sufficient to maintain mESCs in serum, reports suggest the resulting cultures exhibit a greater degree of spontaneous differentiation (Nichols et al., 1994; Yoshida et al., 1994). This therefore suggests LIF and IL-6 signalling may not be absolutely equivalent in the context of naïve self-renewal. Differences in IL-6 and LIF signalling have also been reported in other cell systems. It is reported that LIF and IL-6 have counter-acting effects in the specification of CD4+ T cells (Metcalfe, 2011). Exposure to LIF promotes expression of *TREG,* and therefore a regulatory T cell fate, while IL-6 signalling promotes a TH17 effector T cell fate. The mechanism for this is currently unknown, however it has been proposed that the differential recruitment of IL-6ST (gp130) may be involved. The LIF receptor complex is

comprised of one LIFR subunit and one IL-6ST (gp130) subunit, while the IL-6 receptor complex consists of one IL-6R subunit and two IL6-ST (gp130) subunits. How this would influence signal transduction is yet to demonstrated.

As yet, the down-stream mediators of IL-6 induced self-renewal of naïve hESCs remains unclear. Long-term exposure to IL-6, in both self-renewing and differentiating conditions, consistently resulted in increased expression of canonical JAK/STAT target *KLF4*. Somewhat surprisingly though, *KLF4* transcript expression failed to be induced by acute exposure to IL-6, either for 1 or 24 hours. These results suggest IL-6-induced upregulation of *KLF4* could be a subsequent result of other transcriptional changes rather than a direct response of this signalling. However the lack of observed induction may also result from low expression of JAK/STAT signalling components at the start of the assay. *KLF4* expression was shown to be robust, even in the absence of LIF or IL-6 supplementation. It is possible that an initial period of upregulation of signalling components is required before the strength of the signal is such that it can further increase *KLF4* expression. Therefore further fine-tuning of this assay may be required to reliably identify down-stream mediators of IL-6-induced self-renewal. Alternatively, numerous other pluripotency factors have been reported to be directly regulated by JAK/STAT signalling, including *GBX2*, *KLF5* and *TBX3*, that may act to confer the observed self-renewal effect of IL-6 (Niwa et al., 2009; Tai and Ying, 2013).

Activation of JAK/STAT signalling is reported to promote expression of primitive endoderm markers, both in the pre-implantation blastocyst and in mESC cultures (Morgani and Brickman, 2015). When exposed to IL-6, naïve hESCs failed to consistently upregulate primitive endoderm markers; both in the presence or absence of Gö6983, suggesting this mechanism of primitive endoderm expansion may not be conserved. Nonetheless, single-cell RNA sequencing of pre-implantation stage human embryos indicates expression of IL-6R and the potential down-stream target *KLF4* in both primitive endoderm and epiblast cell populations (Blakeley et al., 2015). Therefore, JAK/STAT signalling might still have some role in specification of the primitive endoderm.

While expression of primitive endoderm markers was not affected, expression of murine trophectoderm markers, *GATA2* and *GATA3*, was consistently reduced in the

presence of IL-6. This was apparent in t2iLGö cultures, though it was more pronounced following prolonged culture in the absence of Gö6983. However, given the increased cell death observed when Gö6983 was omitted it is unclear whether IL-6 signalling actively suppressed *GATA2* and *GATA3* upregulation; or whether IL-6 signalling prevented propagation of cells that had upregulated *GATA2* and *GATA3.* Additionally, in tissue laminin t2iLGö cultures expression of *GATA2* and, in particular, *GATA3* is in general higher than the other lineage markers assayed. Therefore increased suppression of these factors could potentially result from generally more robust naïve transcription factor expression acting to suppress lineage marker expression, rather than a specific IL-6-induced repression of these genes. Nonetheless, IL-6 downstream signalling component STAT5 is known to act both as a repressor and activator of transcription, thought to depend on formation of either a homodimer or tetramer at the promoter region, as well as recruitment of 3 Lys27 methylase, EZH2 (Farrar and Harris, 2011). STAT5 is also thought to bind directly to the promoter regions of GATA factors, presenting a possible mechanism for direct regulation by IL-6 (Li et al., 2015). If IL-6 signalling is acting to suppress expression of trophectoderm-associated genes it may signal a somewhat conserved role of IL-6 family cytokines in the pre-implantation blastocyst. Where as LIF may specify the mouse ICM by promotion of epiblast and primitive endoderm factors; IL-6 may contribute towards specification of the ICM by suppressing trophectoderm markers.

Chapter 5. Determining independence from conventional primed pluripotent signals

5.1. Introduction

The way in which FGF and Activin/Nodal signals are interpreted is thought of as a key distinguishing feature separating naïve and primed pluripotency. In the mESC system, MEK/ERK activation via FGFs induces differentiation, while blockade of MEK/ERK activation in combination with GSK3 inhibition or LIF supplementation is sufficient to support self-renewal (Ying et al., 2008).

Interrogation of Activin/Nodal signalling in mESCs suggests this pathway is largely inconsequential to naïve self-renewal. Supplementation of Activin, Nodal or TGFβ is not required for self-renewal under defined conditions (Ying et al., 2008). Furthermore, prolonged inhibition of endogenous nodal signalling does not appear to alter clonal capacity in 2i conditions (Mulas et al., 2017). In contrast to naive mESCs, primed pluripotent EpiSCs require exposure to exogenous FGF and Activin/Nodal for stable expansion (Brons et al., 2007; Tesar et al., 2007).

Conventional human pluripotent stem cells share many of the functional, transcriptional and epigenetic characteristics of mouse EpiSCs, indicating they too occupy a primed state of pluripotency. Like mouse EpiSCs, conventional human pluripotent stem cells require exogenous FGF and Activin/Nodal/TGFβ for derivation and expansion (Chen et al., 2011; Thomson et al., 1998).

Activators of FGF and Activin/Nodal signalling have appeared in numerous media formulations purported to support human naïve pluripotency (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014). Two culture systems, t2iLGö and 5iLAF, have been demonstrated to derive hESCs with the transcriptional and epigenetic characteristics related to the human naïve epiblast (Guo et al., 2016; Takashima et al., 2014; Theunissen et al., 2014). 5iLAF utilizes FGF2 and Activin A supplementation, in combination with LIF and inhibitors of MEK, GSK3, BRAF, SRC and ROCK to derive and sustain naïve pluripotency (Theunissen et al., 2014). Furthermore, withdrawal of either FGF2 or Activin A was reported to result

in down-regulation of naïve pluripotency factor *KLF4*. While the t2iLGö formulation does not include activators of FGF or Activin/Nodal signalling, previously this system relied on a feeder layer of MEFs providing a potential source of exogenous growth factors (Takashima et al., 2014). This has therefore raised the question of whether naïve human pluripotency is independent of these two pathways.

Recent data has also suggested FGF and Activin/Nodal signalling may behave differently in the human pre-implantation epiblast. In contrast to mouse, inhibition of FGF/MEK signalling does not prohibit specification of the primitive endoderm, suggesting a divergence in the primacy of this pathway (Kuijk et al., 2012; Roode et al., 2012). Recent single-cell RNA-seq of the human epiblast cells has also indicated significant differences in expression of TGFβ superfamily signalling components. Expression of Nodal appears to be enriched in the human epiblast, when compared to the mouse. Furthermore, exposure to ALK4/5/7 inhibitor SB431542 (40μM) resulted in abrogation of the ICM, while similar treatment of mouse blastocysts did not impact normal development (Blakeley et al., 2015).

In chapter 4 I described a culture system based on tissue laminin that relinquishes the requirement for a feeder layer. This system provides a stringent and consistent foundation for interrogation of FGF and Activin/Nodal signalling via exogenous growth factor exposure and small molecule inhibitor treatment.

The aim of the work in this chapter was to investigate whether FGF or Activin/Nodal signalling is required to maintain naïve human pluripotency. Following this, I aimed to determine whether exposure to FGF2 or Activin A positively influences naïve pluripotency and begin to identify any potential down-stream mediators of self-renewal.

5.2. Results

5.2.1. Modulation of FGF Signalling

To investigate whether FGF signalling is required for maintenance of naïve pluripotency, HNES1 and cRShef6 cells were continuously cultured on tissue laminin while exposed to 0.1μM of FGF receptor inhibitor, PD173074 (PD17) (Mohammadi et al., 1998). In addition, separate cultures were supplemented with 8ng/ml FGF2 to determine whether robust FGF signalling improves naïve hESC pluripotency marker expression as previously reported (Theunissen et al., 2014). After 5 passages in the presence of either PD17 or supplemental FGF2 no overt differences were observed in colony morphology or cell proliferation (Figure 5.1A). Furthermore, RT-qPCR analysis of these cultures failed to detect changes in core or naïve pluripotency marker expression (Figure 5.1B).

FGF signalling promotes primed pluripotency through activation of MEK/ERK (Li et al., 2007). Conversely, activation of MEK/ERK signalling in mESCs triggers the exit from naïve self-renewal (Kunath et al., 2007; Stavridis et al., 2007). It was previously reported that ERK1/2 is not phosphorylated in naïve reset iH9 NK2 cells cultured in t2iLGö on MEFs (Takashima et al., 2014). Western blotting of HNES1 and cRShef6 cells cultured in t2iLGö on tissue laminin confirmed ERK1/2 is not phosphorylated in naïve culture conditions, as expected due to the presence of MEK/ERK inhibitor PD0325901 (PD03) (Figure 5.1C). Furthermore, supplementing the medium with FGF2 for 48 hours failed to induce phosphorylation of ERK1/2. Withdrawal of inhibitors (denoted by N2B27), including MEK inhibitor PD03, at the point of FGF2 addition enabled phosphorylation of ERK1/2. Further addition of FGF receptor PD17 to N2B27+FGF2 cultures suppressed phosphorylation of ERK1/2. Thus indicating PD17 was proficiently inhibiting FGF receptor activity.

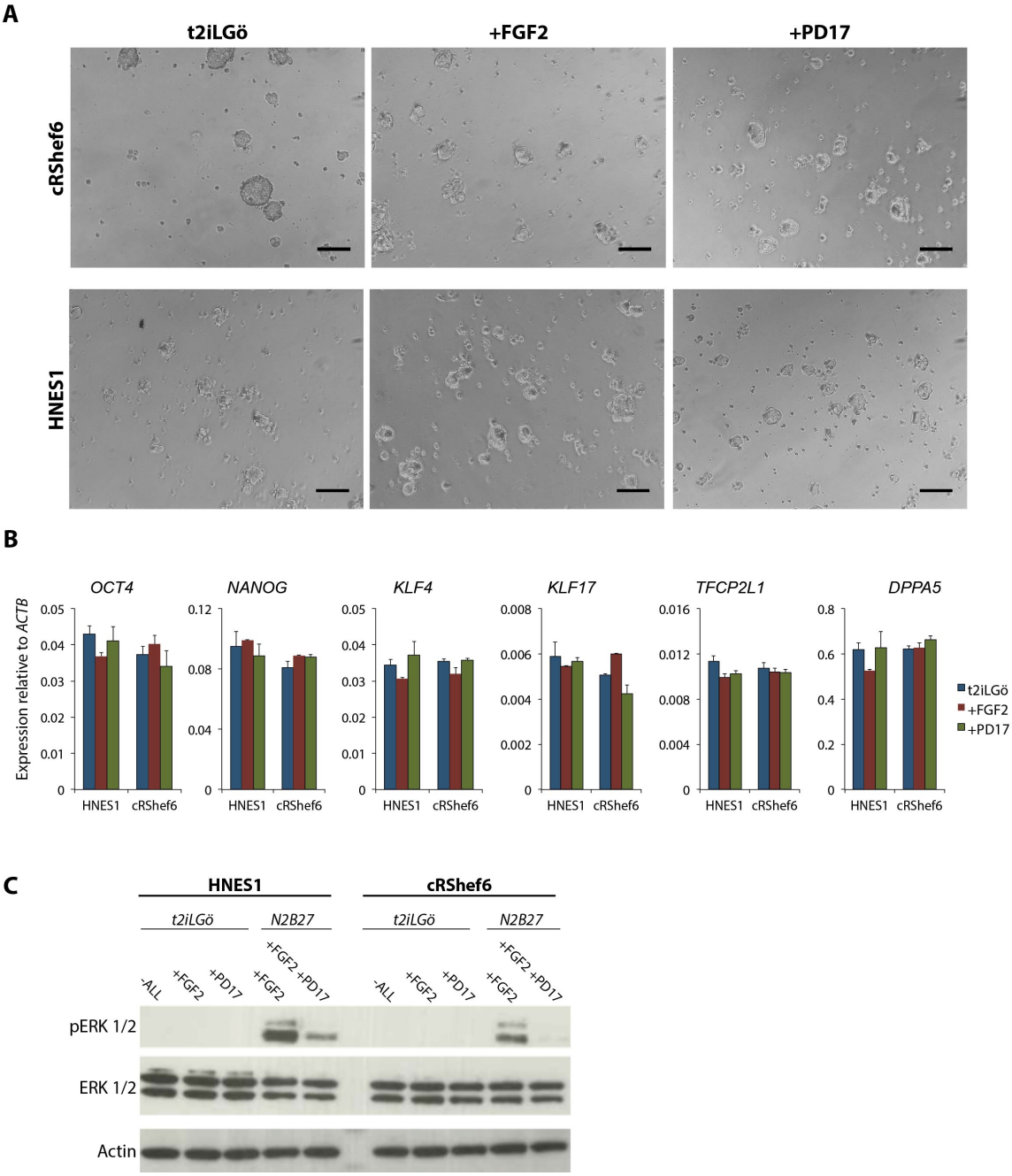

Figure 5.1 Modulation of FGF signalling

A) Bright-field images showing HNES1 and cRShef6 cells cultured on tissue laminin for 5 passages in the presence or absence of FGF2 (8ng/ml) and PD17 (0.1μM). Scale bars represent 100μm

B) RT-qPCR data showing pluripotency transcript expression in HNES1 and cRShef6 cultures shown above. Expression normalized to *ACTB*. Error bars represent standard deviation of 2 independent reactions.

C) Western blot showing detection of phosphorylated ERK1/2 and total ERK1/2 protein in HNES1 and cRSHEF6 cells. Cells were cultured on tissue laminin in t2iLGö in the presence or absence of FGF2 (8ng/ml) and PD17 (0.1μM) for 48 hours. Concurrent cultures in the absence of MEK, GSK3 and PKC inhibition (N2B27) acted as positive controls of FGF2 and PD17 activity. Actin served as a loading control.

5.2.2. Modulation of Activin/Nodal signalling

Next, I sought to determine whether Activin/Nodal signalling is required for self-renewal of naïve hESCs. I employed Alk4/Alk5/Alk7 inhibitor A83-01 to inhibit the transduction of Activin, Nodal, and TGFβ signals (Tojo et al., 2005). Exogenous stimulation of this signalling pathway was achieved via supplementation of 20ng/ml of Activin A, a concentration previously reported to increase expression of *KLF4* in 5iLAF naïve hESC cultures (Theunissen et al., 2014). After 5 passages addition of Activin A did not appear to alter colony morphology of either HNES1 of cRShef6 EOS cultures (Figure 5.2A). Conversely, after several passages in the presence of A83-01 the cultures were noticeably more heterogeneous, with many morphologically irregular colonies emerging and fewer cells persisting after subsequent passages. Furthermore, the few remaining cRShef6 EOS colonies did not exhibit robust activation of EOS-GFP.

To examine the expression of core and naïve pluripotency markers in these cultures, RT-qPCR was performed. After 5 passages in the presence of Activin A expression of pluripotency transcripts was more varied between cell lines (Figure 5.2B). *OCT4* expression appeared to be consistently reduced; however in general all factors assayed continued to be expressed at levels comparable to control cultures. In contrast, prolonged exposure to A83-01 resulted in widespread downregulation of pluripotency markers. Expression of core pluripotency factors, *OCT4* and *NANOG*, as well as naïve markers *KLF4* and *KLF17* was dramatically reduced. Naïve marker *TFCP2L1* appeared more resistant to A83-01.

To attempt to quantify the effect of Activin/Nodal signalling modulation on naïve hESC self-renewal, HNES1 and cRShef6 cells previously maintained in t2iLGö medium were plated at clonal density in the presence or absence of Activin A or A83-01. After 7 days of culture self-renewing colonies were quantified by alkaline phosphatase staining. Neither Activin A or A83-01 treatment appear to consistently alter the colony forming capacity of naïve hESCs (Figure 5.2C).

I had previously observed that prolonged culture in the presence of A83-01 was required before morphological abnormal colonies were apparent. I therefore hypothesised that prolonged inhibition of Activin/Nodal signalling may be required to destabilize naïve self-renewal. In order to investigate this, HNES1 and cRShef6 cells were subjected to

A83-01 treatment for a period of either 5 days or for 1 passage (10 days) before replating at low density back into standard self-renewing conditions. 5 days of A83-01 exposure resulted in a 30% reduction in HNES1 AP+ colonies formed when compared to control cultures (Figure 5.2D). After 1 passage in the presence of the inhibitor both HNES1 and cRShef6 cells clearly exhibited a reduced capacity to form self-renewing colonies in t2iLGö.

Taken together, these data indicate that Activin/Nodal signalling may be required for maintenance of human naïve pluripotency.

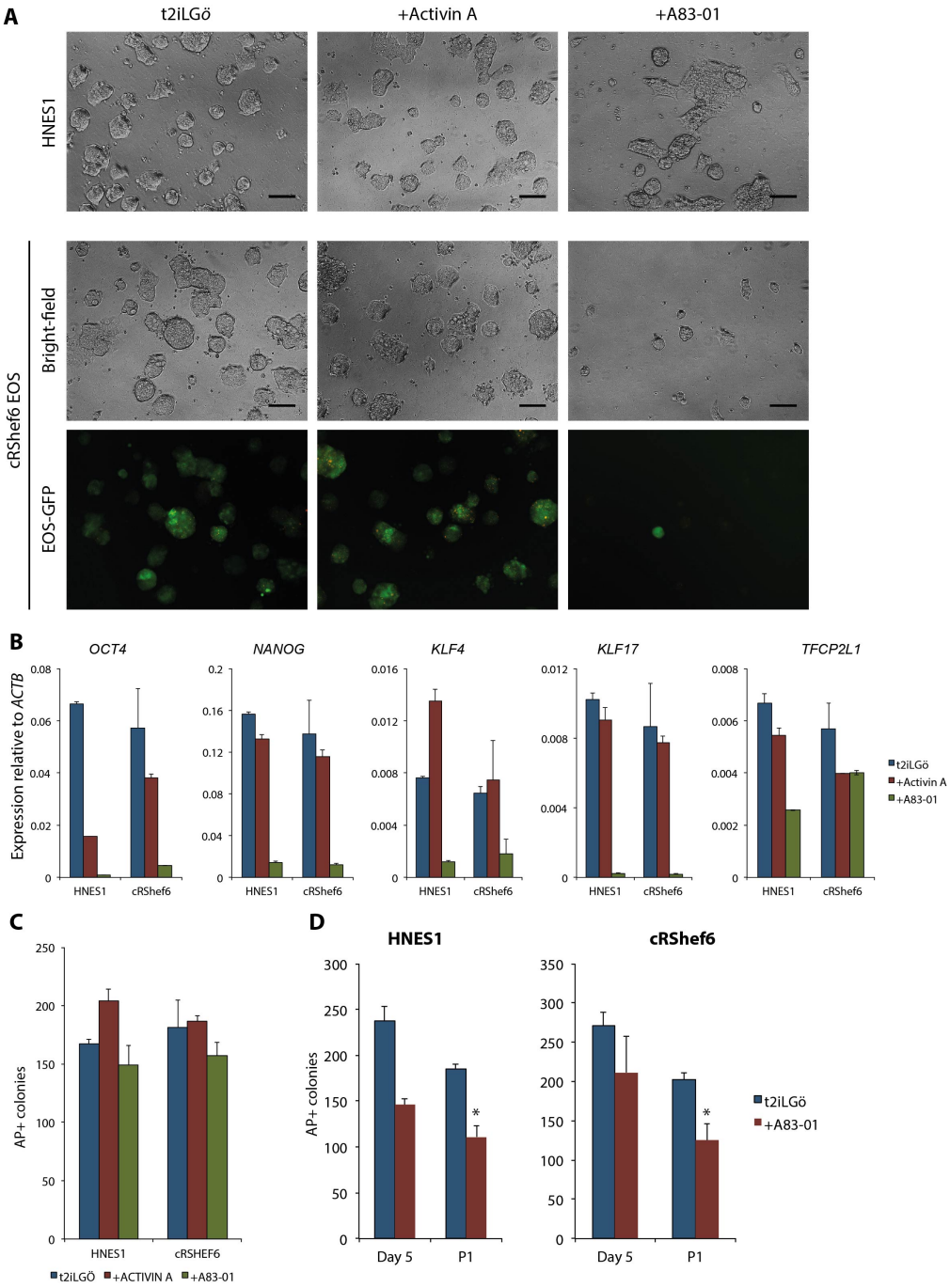

Figure 5.2 Modulation of Activin/Nodal signalling

A) Bright-field images (upper) showing HNES1 and cRShef6 EOS colonies cultured on tissue laminin in the presence or absence of Activin A (20ng/ml) and A83-01 (1μM) for 4 passages. OCT4 EOS-GFP activity (lower) in cRSHEF6 EOS colonies pictured above. Scale bars represent 100μm.

B) RT-qPCR data showing expression of pluripotency marker transcripts in HNES1 and cRShef6 cultures shown above. Expression normalized to *ACTB*. Error bars represent standard deviation of 2 independent replicates.

C) Colony forming assay showing the number of alkaline phosphatase positive colonies formed after plating 1000 cells in the presence or absence of Activin A and A83-01. Error bars represent standard deviation of 3 plating replicates.

D) Re-plating assay showing the number of AP+ colonies formed in t2iLGÖ following either 5 days or 1 passage in the presence or absence of A83-01. Error bars represent standard deviation of 2 independent cultures with 2 plating replicates.

*Anova Tukey's post hoc $p<0.01$.

5.2.3. Endogenous activation of Activin/Nodal signalling

Mouse ESCs cultured in 2i exhibit phosphorylation of smad2/3, likely through endogenous Nodal and/or Gdf3 signalling (Mulas et al., 2017). To examine whether naïve hESCs may be capable of endogenous activation of Activin/Nodal signalling RNA-seq data from naïve cultures was examined. Consistent with reports of the pre-implantation epiblast, appreciable levels of *NODAL* and *GDF3* transcripts were detected (Figure 5.3A). For efficient signal transduction, Nodal signalling requires post-translational cleavage and formation of a receptor complex consisting of multiple components (Beck et al., 2002). Transcripts of proprotein convertase, *Furin*, were present, as well as transcripts for the type 1 receptor component, *ACVR1B* (ALK4), type 2 receptor components, *ACVR2A* and *ACVR2B* (Figure 5.3A). Receptor co-factor, *TDGF1* (Cripto) was also expressed (Beck et al., 2002). Lastly, expression of down-stream mediators *Smad2* and *Smad3* was detected, as well as negative regulator *Smad7*.

To establish whether Activin/Nodal signalling is active in the naïve pluripotent state the phosphorylation status of Smad2 was examined. HNES1 and cRShef6 cells were cultured on tissue laminin in the presence or absence of Activin A and A83-01 for a period of 48 hours. The resulting cultures were lysed and subjected to western blotting. When cultured in t2iLGö on tissue laminin both HNES1 and cRShef6 cells exhibited phosphorylation of Smad2, indicating endogenous activation of the Activin/Nodal signalling pathway (Figure 5.3B & Figure 5.4B). Supplementation with Activin A further increased phosphorylation of Smad2. Treatment with A83-01 efficiently blocked endogenous phosphorylation, confirming the blocking effect of the inhibitor.

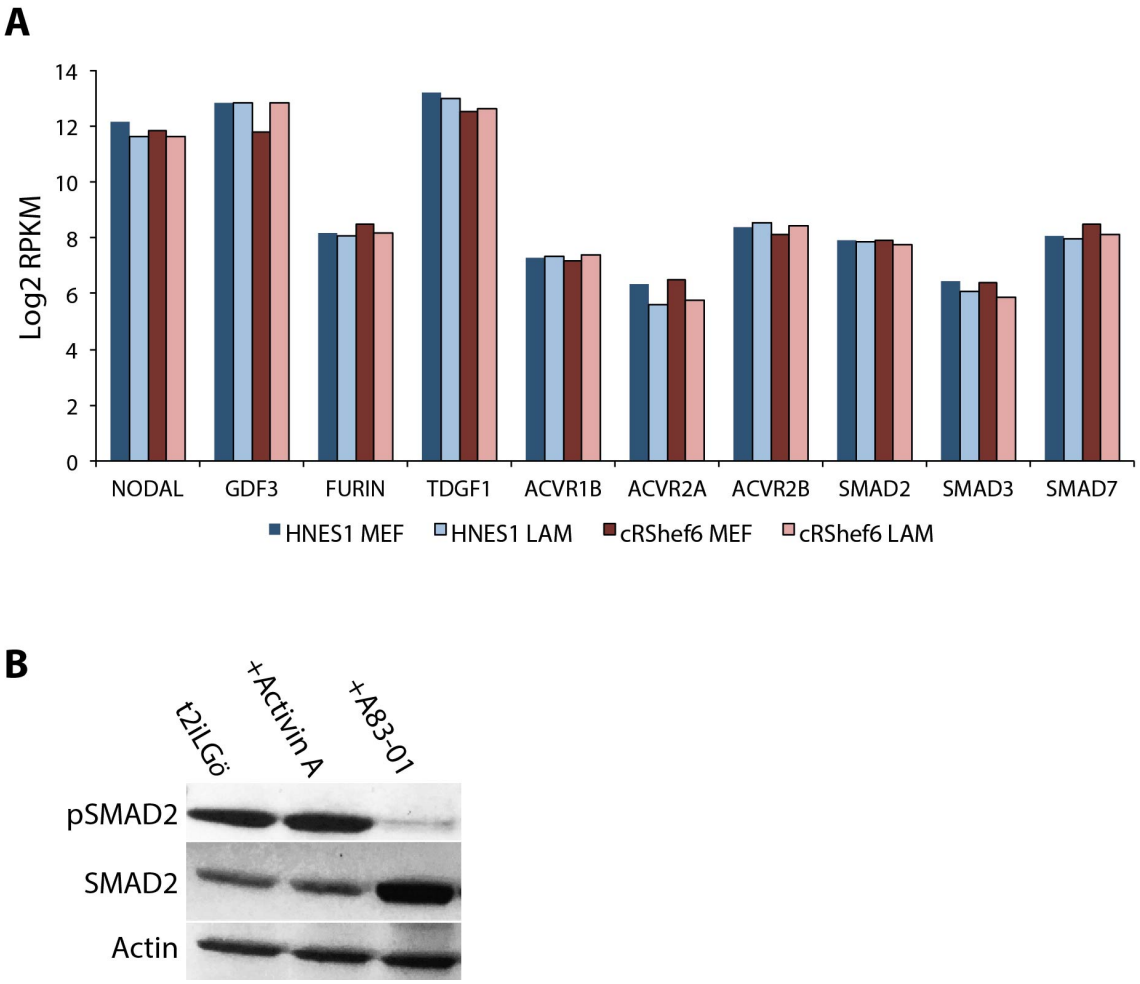

Figure 5.3 Endogenous activation of Activin/Nodal signalling

A) RNA-seq data showing expression of Nodal signalling components in HNES1 and cRShef6 cells cultured either on MEFs or tissue laminin. Sequencing analysis performed by Sabine Dietmann.
B) Western blot showing detection of phosphorylated smad2 and total smad2 in HNES1 cells cultured on tissue laminin in the presence or absence of Activin A and A83-01. Actin served as a loading control.

5.2.4. Identification of pluripotency markers responsive to inhibition of Activin/Nodal signalling.

Given that continued exposure to A83-01 resulted in downregulation of pluripotency marker expression, I next investigated whether Activin/Nodal signalling might directly regulate pluripotency markers in the naïve state. To determine this, transcript expression was examined after 6, 24, 48, and 96 hours of A83-01 treatment. Additionally, to examine the responsiveness of this pathway, after 96 hours of A83-01 exposure the inhibitor was removed and the medium was supplemented with 25ng/ml of Activin A. In the primed state of pluripotency Activin/Nodal signalling is proposed to

support self-renewal via upregulation of *NANOG* expression (Vallier et al., 2009; Xu et al., 2008). While exposure to A83-01 caused a small reduction in *OCT4* transcript expression over the first 6 hours, neither *OCT4* nor *NANOG* expression changed significantly over the course of inhibitor treatment (Figure 5.4A). Albeit, *NANOG* expression did increase slightly with the withdrawal of A83-01 and addition of Activin A. Similarly to *OCT4* and *NANOG*, naïve markers *KLF4*, *TFCP2L1* and *DPPA5* failed to consistently respond to modulation of Activin/Nodal signalling. Interestingly though, primate-specific naïve marker *KLF17* was downregulated within 6 hours of treatment in both HNES1 and cRShef6 cells, and continued to be steadily downregulated across the 96 hours of exposure (Figure 5.4A). Furthermore, withdrawal of the inhibitor and stimulation of the pathway with Activin A saw transcript expression recover to near normal levels within 6 hours.

To confirm independence of *NANOG* expression and the downregulation of *KLF17* expression in response to A83-01, western blotting of cRShef6 cells was performed after 48 hours of treatment. Consistent with previous data, A83-01 treatment proficiently blocked endogenous phosphorylation of SMAD2 (Figure 5.4B). Consistent with the RT-qPCR data, 48 hours of treatment with A83-01 was sufficient to reduce expression of KLF17. Furthermore, levels of NANOG protein were unaffected by short-term exposure to this inhibitor.

Next, I investigated whether the reduced clonogenicity and *KLF17* downregulation observed upon short-term A83-01 exposure also correlated with changes in lineage marker expression. Utilizing the same A83-01 time course samples probed in Figure 5.4A, I examined expression of a number of lineage markers. With the exception of *T (BRACHYURY)*, which was further downregulated upon exposure to the inhibitor, all lineage markers assayed exhibited upregulation within 96 hours of A83-01 treatment (Figure 5.4C).

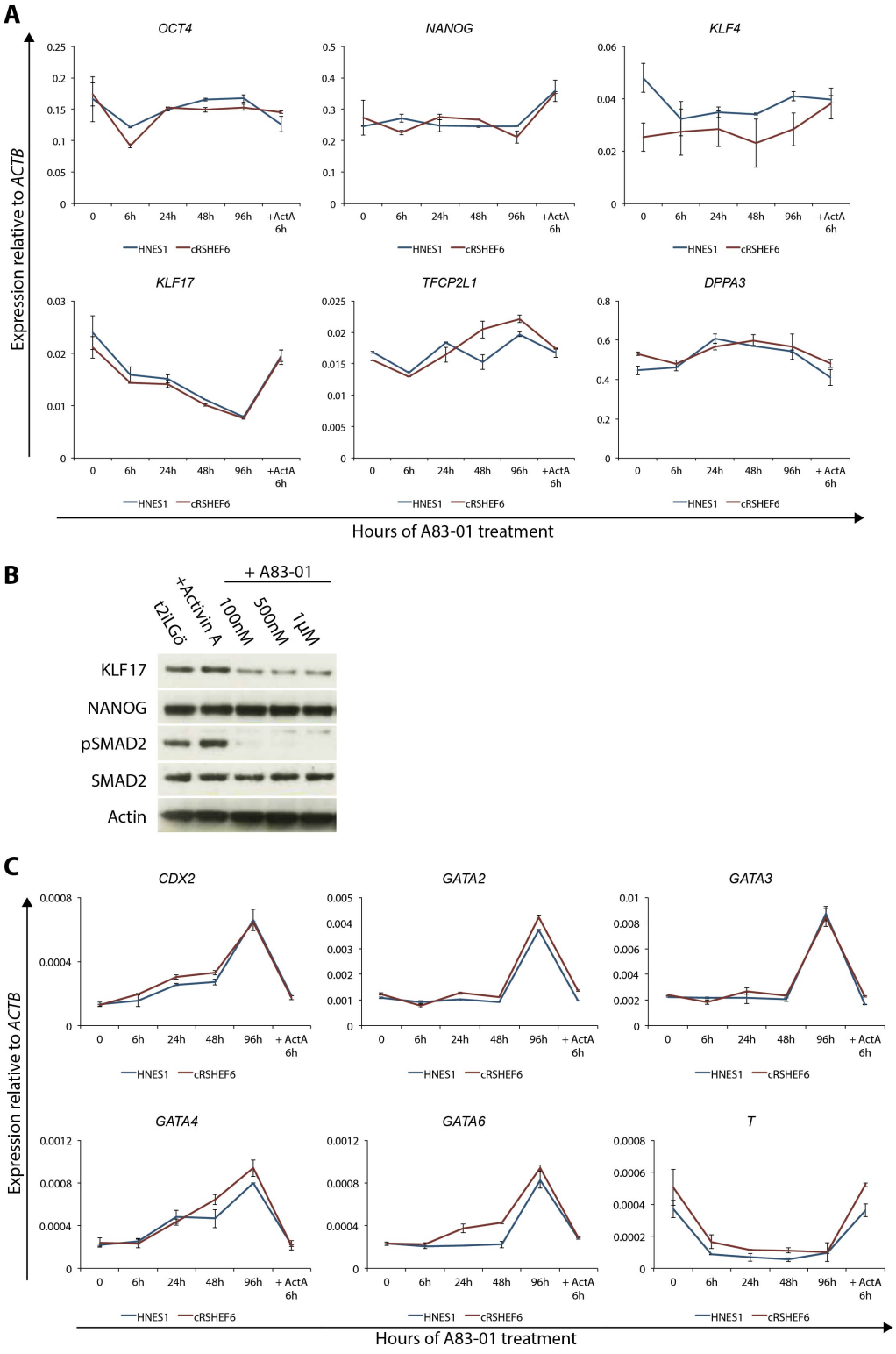

Figure 5.4 Identification of markers responsive to A83-01 treatment

A) RT-qPCR showing expression of pluripotency markers in HNES1 and cRShef6 following exposure to A83-01 for 6, 24, 48 and 96 hours. Final time point represents 96 hours of A83-01 treatment followed by inhibitor withdrawal and activin A (20ng/ml) treatment for 6 hours (+ActA 6h). Expression normalized to *ACTB*. Error bars represent standard deviation of 2 independent reactions.
B) Western blot showing detection of KLF17, NANOG, phosphorylated smad2 and total smad2 protein in cRSHEF6 cells following exposure to Activin A or A83-01 for 48 hours. Actin served as a loading control.
C) RT-qPCR showing expression of lineage markers in cultures detailed in Figure 6.4A.

5.2.5. Transgenic overexpression of KLF17 during A83-01 treatment

Relatively little is known about the function of KLF17 during development, and to date there are no reports regarding a functional role in naïve human pluripotency. Having observed acute downregulation of KLF17 in response to inhibition of Activin/Nodal signalling, I next inquired whether transgenic expression of KLF17 was sufficient to prevent the destabilisation of naïve pluripotency resulting from A83-01 exposure.

HNES1 and cRShef6 cells were transfected with an inducible human KLF17 TET-on construct (KLF17tet) provided by Ge Guo (Figure 5.5A). To determine the efficacy of the inducible system in these cells RT-qPCR was performed. In the absence of doxycycline (Dox), expression of *KLF17* was approximately 4 to 5-fold higher in transfected HNES1 (HNES1 KLf17tet) and cRShef6 (cRShef6 KLF17tet) cells than the wild-type (WT) parental cells (Figure 5.5B). Supplementing the medium with 5ng/ml of Dox further increased *KLF17* expression by approximately 50-fold (HNES1 KLF17tet) and 80-fold (cRShef6 KLF17tet), relative to WT cells. Interestingly, transfected cells generally adopted a more disorganised, heterogeneous colony morphology than typical WT cultures. Furthermore, within 48 hours of Dox treatment wide spread cell dissociation could be observed; in many cases to the point of total colony dissolution (Figure 5.5C). RT-qPCR analysis of pluripotency marker expression in these cultures revealed substantial downregulation of *KLF4*, with some variable reduction in *NANOG* and *OCT4* expression (Figure 5.5D).

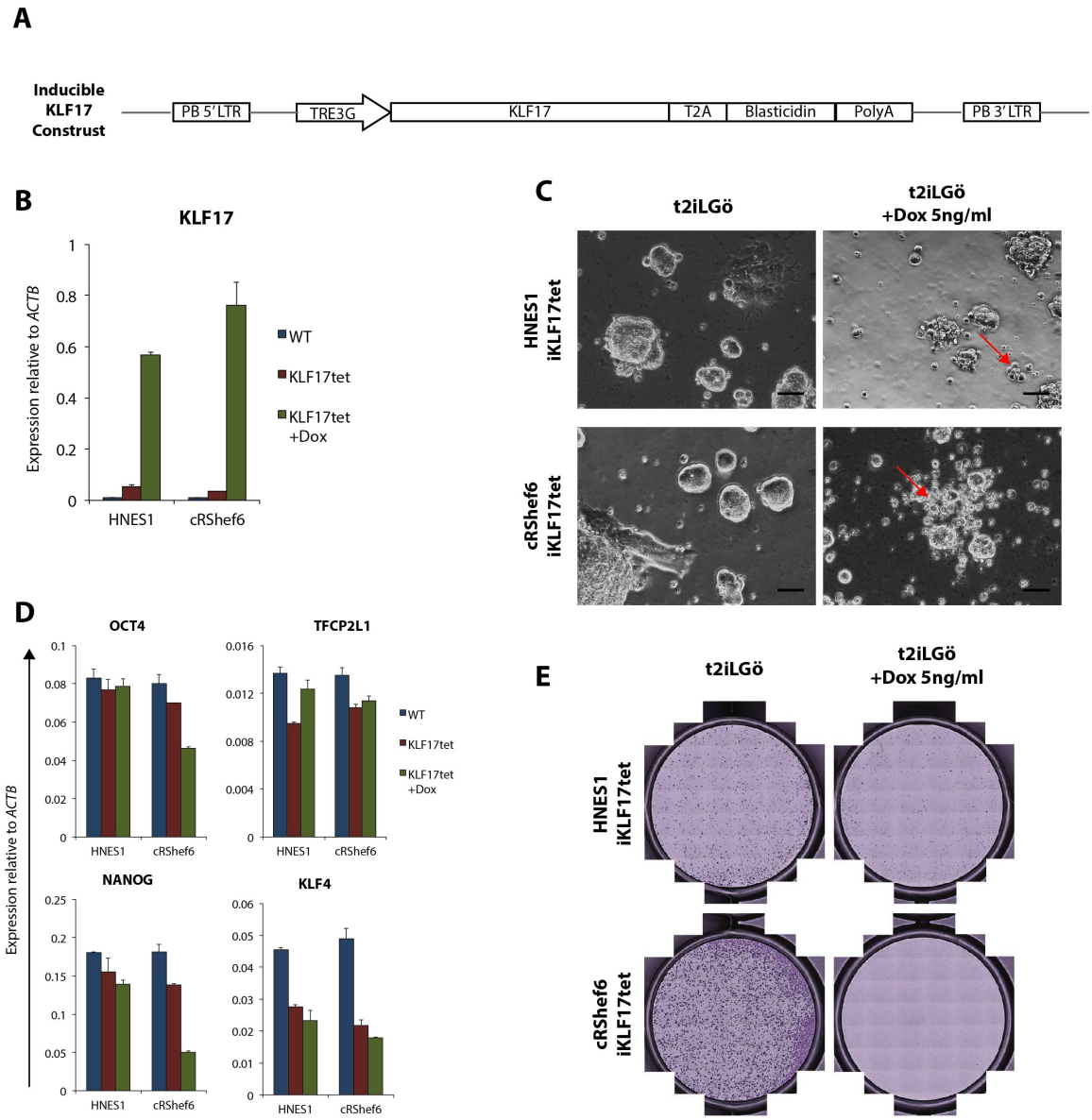

Figure 5.5 Overexpression of transgenic *KLF17*

A) A schematic of the TRE3G KLF17 inducible construct.
B) RT-qPCR showing expression of *KLF17* in wild-type and KLF17tet cells cultured in the presence or absence of Doxycycline (5ng/ml). Error bars represent standard deviation of 2 independent replicates.
C) Bright-field images showing HNES1 KLF17tet and cRSHEF6 KLF17tet colonies following exposure to doxycycline for 48 hours. Red arrows indicate disassociating cells. Scale bar represents 100µm.
D) RT-qPCR showing expression of pluripotency marker transcripts in WT and iKLF17tet HNES1 and cRSHEF6 cells following exposure to doxycycline for 48 hours.
E) Alkaline phosphatase positive colonies remaining following 7 days of culture in the presence and absence Doxycycline 5ng/ml.

To investigate the effect of *KLF17* overexpression on colony forming capacity, HNES1 KLF17tet and cRShef6 KLF17tet cells were passaged at an equal ratio and cultured in the presence or absence of 5ng/ml Dox. After 7 days pluripotent colonies were determined via alkaline phosphatase staining. In the presence of Dox, HNES1 KLF17tet colonies

appeared to reduce in both number and size, while Dox treatment of cRShef6 KLF17tet cells resulted in virtually no alkaline phosphatase positive colonies persisting by day 7 (Figure 5.5E). Taken together this preliminary data would suggest that, when over expressed, *KLF17* is detrimental to the maintenance of naïve pluripotency.

Despite the apparent limitations of this inducible system, I attempted to utilise the promiscuous expression of transgenic *KLF17* observed in the absence of Dox to rescue the effect of continuous A83-01 exposure. After 14 days of exposure to A83-01, *KLF17* expression was suppressed in WT cells but remained elevated in both KLF17tet cell lines (Figure 5.6A). However expression of pluripotency markers *NANOG*, *OCT4* and *DPPA5* was reduced to levels either comparable or lower than that of wild-type cells. Similarly, both HNES1 KLF17tet and cRShef6 KLF17tet cells continued to exhibit upregulation of lineage markers *GATA2* and *GATA3* (Figure 5.6). A notable exception to this trend was *SOX17*, which showed reduced upregulation relative to wild-type cultures.

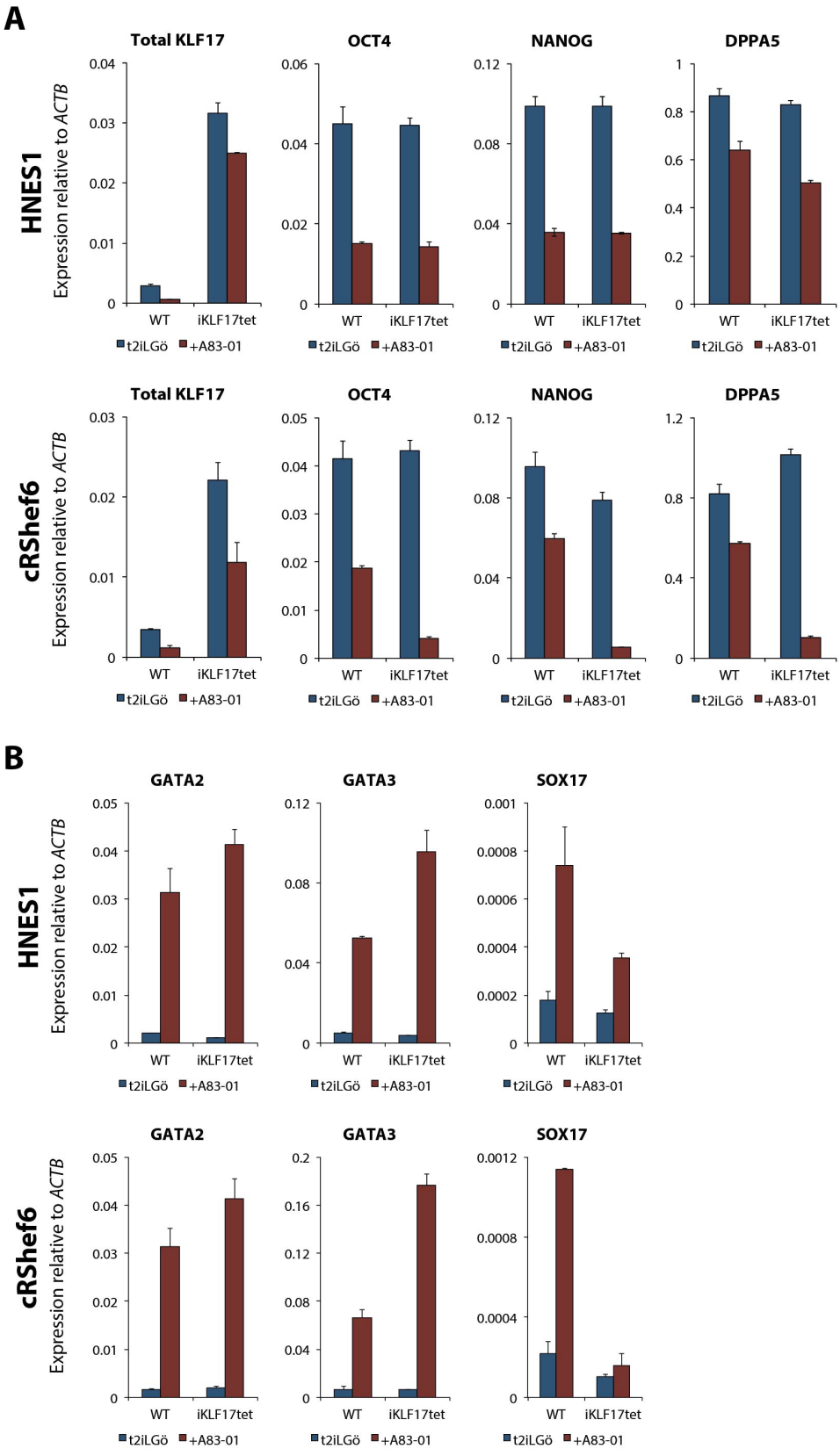

Figure 5.6 Overexpression of *KLF17* during A83-01 treatment

RT-qPCR showing expression of pluripotency (A) and lineage (B) markers in wild-type and KLF17tet HNES1 and cRShef6 cells cultured in the presence or absence of A83-01 for 3 passages.

5.3. Discussion

Independence from FGF and Activin/Nodal signalling are hallmarks of mouse naïve pluripotency. Whether this is a true paradigm of naïve pluripotency is a topic of much discussion within the field. FGF and Activin/TGFβ have regularly been included in media cocktails purported to support human naïve pluripotency (Chen et al., 2011; Gafni et al., 2013; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014). Recently it was reported that addition of both FGF2 and Activin A may be beneficial for the derivation and maintenance of hESCs that exhibit transcriptional and epigenetic characteristics related to murine naïve pluripotency and the human epiblast (Theunissen et al., 2016; Theunissen et al., 2014). To address whether FGF and Activin/Nodal signalling are required for naïve self-renewal I employed the feeder-independent tissue laminin culture system described in chapter 3 as a platform for small molecule interrogation of these pathways.

FGF receptor inhibitor PD17 was employed to disrupt endogenous activation of FGF signalling. Under these conditions cells could be readily expanded and maintained expression of naïve transcription factors comparable to control cultures, indicating naïve human pluripotency is likely not reliant on FGF signalling for self-renewal. This is consistent with reports that inhibition of both MEK and FGF receptor activity does not prohibit emergence of NANOG and OCT4 positive cells in human blastocysts (Roode et al., 2012).

Withdrawal of FGF from 5iLAF cultures has been reported to result in reduced expression of *KLF4* (Theunissen et al., 2014). FGF2-mediated activation of MEK was comprehensively blocked in t2iLGö tissue Laminin cultures. Nonetheless, FGF2 signalling has been shown to activate alternative pathways that may influence naïve pluripotency, including STATs, AKT and PLCs (Ornitz and Itoh, 2015). In my system supplemental FGF2 failed to increase *KLF4* expression. Moreover, none of the pluripotency markers assayed were upregulated in response to exogenous FGF2 treatment. Given the substantial differences in signalling environment between t2iLGö tissue laminin cultures and 5iLAF MEF cultures the potential reason for this discrepancy is difficult to ascertain. Possible explanations could range from Gö6983-

mediate block of PKC activation by PLCγ, to induction of changes in feeder layer viability or cytokine secretion, or simply different cell states.

While the addition of FGF2 did not appear to alter expression of naïve factors, further investigation is required to fully discount the relevance of FGF signalling to human naïve pluripotency. In particular, FGFs confer a growth advantage in many cell systems and the possibility remains that FGF supplementation may aid in the robust proliferation and expansion of naive cells.

Naïve HESCs expressed the components necessary for autocrine Nodal signalling and exhibited phosphorylation of SMAD2, indicating activation of the Activin/Nodal pathway. Long-term exposure to the ALK4/5/7 inhibitor, A83-01, resulted in the eventual loss of naïve transcription factor expression and reduced colony forming capacity. In contrast, although SMAD2 is phosphorylated in the mouse ESC 2i system, similar clonogenic assays following A83-01 exposure do not result in differential colony forming capacity (Mulas et al., 2017). Consistent with this observation, treatment of human blastocysts with an alternative ALK4/5/7 inhibitor, SB431542, has been reported to result in a reduction in OCT4 and NANOG positive cells, while the epiblast cells of comparative mouse blastocysts were unaffected (Blakeley et al., 2015).

Somewhat surprisingly, modulation of Activin/Nodal signalling did not initially influence the efficiency of colony formation, instead requiring prolonged A83-01 exposure before colony formation was effected. Examining lineage marker expression across the initial 4 days of inhibitor treatment revealed upregulation becomes most apparent between days 2 and 4. At present it is unclear whether the upregulation observed is biologically significant, however it is possible that the observed upregulation is important for driving destabilisation of naïve self-renewal, and that it takes place to late in order to influence the vulnerable earlier stages of colony formation.

The important role of Activin/Nodal signalling in early differentiation processes may also contribute to the delayed exit from self-renewal upon A83-01 exposure. Activation of this pathway is known to induce expression of mesoderm and endoderm lineage markers such as *T (BRACHYURY)* and *EOMES* during gastrulation (Tam and Loebel, 2007). Consistent with this, the low level of *T (BRACHYURY)* expression present in t2iLGö cultures was further suppressed by A83-01 treatment. This raises the possibility

that A83-01 treatment may be suppressing upregulation of genes that drive the differentiation processes while simultaneously destabilising the naïve pluripotent state.

To date, all studies examining the role of Activin/Nodal signalling in naïve human pluripotency, including this investigation, have relied upon the use of small molecule inhibitors to suppress activation of this pathway. Unintended inhibition of unrelated targets is an inherent limitation of this approach. The findings of this chapter support the notion that, contrary to mouse ESCs, Activin/Nodal signalling may have a role in establishing and maintaining human naïve pluripotency. However, the absence of genetic ablation of key signalling components such as Nodal, SMAD2 and SMAD3 precludes a strong conclusion at this time. Given that naïve hESCs express high levels of GDF3, which may potentially activate SMAD2/3 in the absence of endogenous Nodal signalling, the cleanest approach for ablation of this signalling activity may be inducible double knockout of SMAD2 and SMAD3.

In the primed pluripotent state Activin/Nodal signalling is proposed to drive expression of *NANOG* via SMAD2/3 binding to the proximal promoter region (Vallier et al., 2009; Xu et al., 2008). Inhibition of Activin/Nodal signalling with SB431542 for a 48-hour period has been reported to result in significant downregulation of *NANOG*. Similar inhibition of Activin/Nodal signalling in naïve cells resulted in loss of SMAD2 phosphorylation but did not alter expression of *NANOG* transcripts, even after 96-hours of exposure. This therefore demonstrates that, in contrast to reports of primed pluripotency, naïve pluripotency does not rely on Activin/Nodal signalling to directly sustain expression of *NANOG*. However, *NANOG* expression increased when A83-01 was withdrawn and the cells were exposed to Activin A for 6 hours. This raises the question of whether the transcriptional machinery for activin/Nodal signalling to induce *NANOG* expression is present but functionally redundant in the naïve state. However, given the relatively long period of Activin/Nodal signalling induction (6 hours) and the array of lineage markers subsequently downregulated, it is possible the observed *NANOG* upregulation is a consequence of earlier transcriptional changes. A simple approach to delineate this would be to expose the cells to Activin A for 1, 2, 6 and 24-hour periods and then assay expression of NANOG mRNA. Increased expression of NANOG mRNA in response to 1 or 2 hours of Activin A treatment would indicate direct transcriptional control.

Short-term exposure to A83-01 resulted in a steady decline of *KLF17* mRNA and protein expression. Withdrawal of the inhibitor and stimulation of the Activin/Nodal pathway with Activin A quickly returned transcript expression to normal levels. These data indicate that expression of the primate-specific naïve marker *KLF17* may be regulated by Activin/Nodal signalling in the human naïve state. To date, the regulation and function of KLF17 in pre-implantation development is unknown. However, KLF17 has been studied in the context of epithelial-mesenchymal transition, where loss of TGFβ signalling correlates with downregulation of *KLF17* and increased invasiveness and metastasis (Gumireddy et al., 2009). Investigation of *KLF17* regulation in the context of the HepG2 liver cancer cell line demonstrated TGFβ-induced upregulation of *KLF17*, as well as SMAD3 binding at the *KLF17* promoter (Ali et al., 2015). Therefore a precedent exists for this mode of *KLF17* regulation outside of pre-implantation development.

Overexpression of *KLF17* appeared to have a disruptive effect on both pluripotency marker expression and colony integrity. Single-cell RNA-seq data of human pre-implantation stage blastocysts indicates that *KLF17* expression peaks at the 8-cell stage and is gradually reduced through the morula stage, into the epiblast where low level expression persists (Blakeley et al., 2015; Petropoulos et al., 2016). While in the mouse blastocyst, *KLF17* expression appears to peak at the 4-cell stage and remains at low levels through to the morula stage (Blakeley et al., 2015). This expression pattern raises the question of whether KLF17 is a mediator of naïve pluripotency or whether expression in the epiblast is a vestige of an earlier developmental network in the process of dissolution. This expression pattern also raises the possibility that transgenic overexpression may activate earlier developmental transcription factors, instigating reprogramming of naïve cells to a cleavage-like state. Although beyond the scope of this thesis, it may be informative to examine expression of 8-cell stage markers following induction of *KLF17* expression.

Constitutive overexpression of *KLF17* failed to rescue downregulation of pluripotency markers and upregulation of lineage markers in response to prolonged A83-01 exposure. However it is difficult to draw a proper conclusion from these results due to the exceptionally high expression of *KLF17* in these cells. To confidently determine

whether downregulation of *KLF17* contributes to A83-01-mediated loss of self-renewal, expression of transgenic *KLF17* will need to be direct at near normal levels.

Of potential interest was the observed suppression of *SOX17* expression in cells over-expressing *KLF17*, both in the presence and absence of A83-01. SOX17 was recently shown to be a critical factor directing primordial germ cell-like cell specification in human but not mouse (Irie et al., 2015). This raises the question of whether *KLF17* expression is required in the human naïve epiblast in order to regulate SOX17-mediated primordial germ cell specification. However a more robust assay and *KLF17* inducible cell system is required in order to draw firm conclusions on this relationship.

Chapter 6. Characterising the role of PKCι in the acquisition and maintenance of human naïve pluripotency

6.1. Introduction

The *in vitro* propagation of the mouse naïve epiblast is efficiently achieved using a combination of MEK and GSK3 inhibition together with the cytokine LIF (2iL) (Boroviak et al., 2014; Ying et al., 2008). The 2iL formulation does not permit propagation of the human naïve epiblast *in vitro*, however it has formed the basis for numerous media cocktails proposed to sustain human naïve pluripotency (Chan et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014). In particular, addition of the PKC inhibitor Gö6983 (t2iLGö) enables derivation of human pluripotent stem cells with the transcriptional and epigenetic features of naïve pluripotency (Guo et al., 2016; Takashima et al., 2014). The requirement for one additional component makes t2iLGö a potentially attractive system for delineating the distinct mechanisms that govern naïve human pluripotency.

At present, the way in which Gö6983 inhibits the exit from naïve self-renewal remains unclear. A large scale *in vitro* screen for inhibition of recombinant kinase activity suggests Gö6983 is a broad-spectrum PKC inhibitor with varying degrees of inhibitory activity over many other kinase families (Gao et al., 2013). Previously it was demonstrated that chimera competent mESCs could be maintained using Gö6983 in combination with serum (Dutta et al., 2011). In this system, the self-renewal response was proposed to be directed via inhibition of atypical PKC, PKCζ. More recently however, transposon-based knockout screening of haploid mESCs identified atypical PKC, PKCι as a driving factor of mESC differentiation (Leeb et al., 2014). Moreover, knockdown of PKCι was demonstrated to maintain *OCT4* expression and naïve colony morphology of reset hESCs cultured in t2iL conditions following withdrawal of transgenic *NANOG* and *KLF2* expression (Takashima et al., 2014).

Atypical PKCs (aPKC) form a key component of the PAR3/PAR6/aPKC epithelial polarity complex and they have been studied extensively within the context of early

embryonic development (St Johnston and Ahringer, 2010; Suzuki and Ohno, 2006). In the pre-implantation blastocyst loss of aPKC activity has been associated with disruption of tight junction formation and proper blastocyst cavitation, suggesting a vital role in trophectoderm function (Eckert et al., 2004a, b). More recently, disruption of aPKC function via Gö6983 or siRNA treatment has been reported to disrupt ICM segregation and prevent maturation of primitive endoderm precursors (Saiz et al., 2013). Together these reports identify PKCι as regulator of pre-implantation development and a leading candidate for Gö6983-induced self-renewal.

In this chapter I aimed to assess the ability of disruption of PKCι activity to sustaining naïve pluripotency. Further to this, I also sought to determine whether loss of PKCι activity is sufficient to enable activation of the naïve transcriptional circuitry during chemical resetting in the absence of Gö6983 (Guo et al., 2017).

6.2. Results

6.2.1. Inhibition of atypical PKC activity

To examine which PKCs might be functionally relevant to the maintenance of human naïve pluripotency, previously published RNA-seq data from embryo-derived and transgene reset naïve cell lines were examined (Guo et al., 2016). Although some low-level expression of novel PKCs, delta (PRKCD) and theta (PRKCQ), and atypical PKCzeta (PRKCZ) was detected, transcripts for PKCiota (PRKCI) were the most abundant (Figure 6.1A).

In order to further investigate the importance of PKCι activity to the differentiation of naïve human ES cells in the absence of Gö6983, an atypical PKC pseudo-substrate inhibitor (aPKCi) was employed. This short peptide inhibitor mimics the pseudo-substrate region of atypical PKCs that blocks the kinase activity when the protein is in the off conformation. This region is highly conserved between PKCι and PKCζ suggesting dual inhibition is highly likely (Rosse et al., 2010). In order to determine whether aPKCi is able to replace the self-renewal activity of Gö6983, reset H9 EOS NK2tet cells containing a murine *OCT4* distal enhancer GFP reporter were cultured in the presence or absence of Gö6983 and aPKCi. After a period of 2 passages the cells were re-plating at low density for 7 days before counting GFP+ colonies. Withdrawal of Gö6983 resulted in a significant reduction in colony formation, returning approximately 30% of the colonies formed in t2iLGö cultures (Figure 6.1B). Replacement of Gö6983 with aPKCi at 5 or 10μM resulted in a significant improvement to the GFP+ colony forming capacity in comparison to cells cultured in t2iL alone. However GFP+ colony formation was still significantly reduced in comparison to t2iLGö cultures.

Next I sought to determine whether addition of aPKCi to the culture media could attenuate the loss in pluripotency factor expression observed when Gö6983 is omitted. Reset H9NK2tet cells were again cultured in the presence of absence of Gö6983 and aPKCi for a period of 12 days before RT-qPCR was performed. Withdrawal of Gö6983 resulted in reduced expression of *OCT4, NANOG, KLF4* and *TFCP2L1* expression relative to t2iLGö control cultures. Supplementing t2iL cultures with aPKCi attenuated

the down regulation of pluripotency markers but failed to maintain the levels observed when Gö6983 is present in the culture.

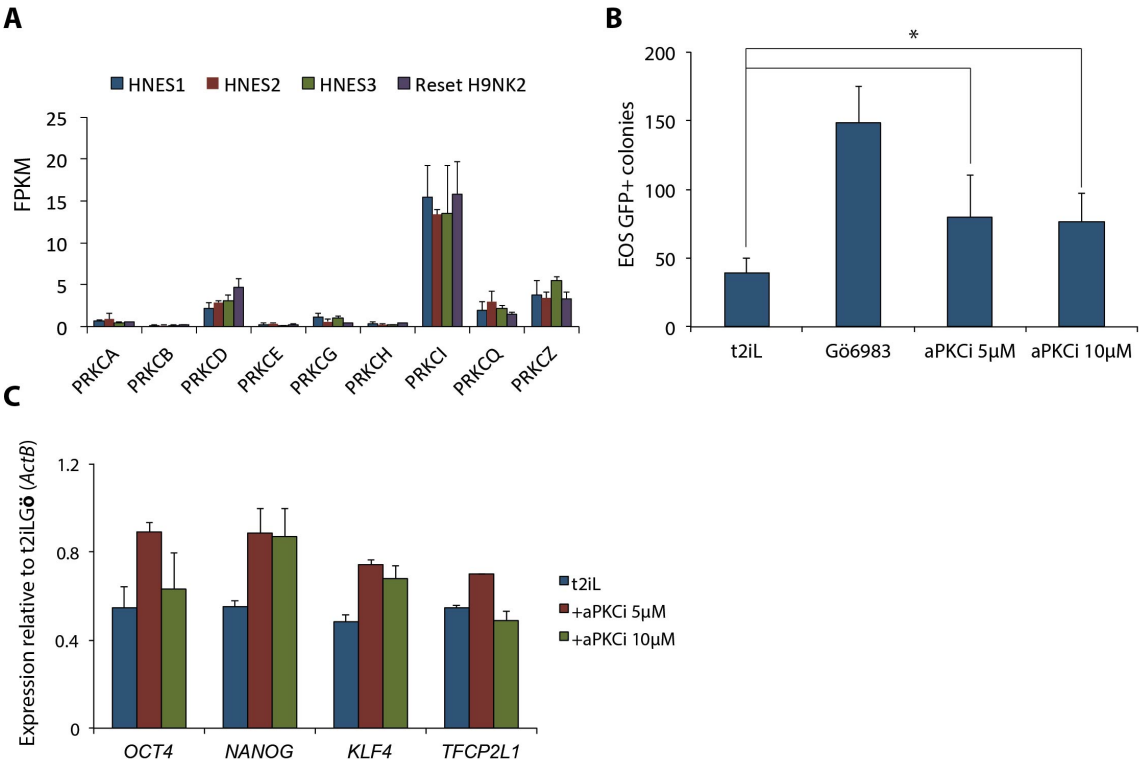

Figure 6.1 Inhibition of Atypical PKC Activity

A) RNA-seq data showing expression of PKC family transcripts in embryo-derived and reset naïve hESC cell lines. Chart produced from publicly available data set (Guo *et al.,* 2016). Error bars represent standard deviation of 3 independent sequencing reactions.
B) Replating assay showing EOS-GFP+ colonies formed from iH9NK2 EOS cells culture in the presence or absence of Gö6983 and atypical PKC inhibitor (aPKCi) for 2 passages. Error bars represent standard deviation of 3 plating replicates.
C) RT-qPCR showing expression of pluripotency markers following culture for 2 passages in the presence or absence of GÖ6983 and aPKCi. Expression relative to *ACTB*. Error bars represent standard deviation of 3 independent reactions.
*Anova Tukey's post hoc $p<0.05$.

These results are somewhat consistent with previous reports that disruption of PKCι activity reduces differentiation in the absence of Gö6983 (Takashima et al., 2014). However exposure to aPKCi did not appear to fully rescue naïve factor expression or the colony forming capacity of the cells when Gö6983 was omitted. In the absence of an appropriate assay to determine the activity of PKCι in the presence of either Gö6983 or aPKCi it is difficult to determine whether these results arise from incomplete blockade

of PKCι function or failure to inhibit the full range of Gö6983 targets. In order to generate a cleaner system for determining the contribution PKCι makes towards naïve hESC fate decisions, the CRISPR/Cas9 system was used to generate PKCι null cell lines.

6.2.2. PKCι ablation preserves key pluripotency marker expression in the absence of Gö6983.

To allow for differences that may results from line variation or methods of deriving naïve pluripotency, PKCι was ablated in both embryo derived naïve HNES1 cells and conventional primed Shef6 HESCs that were subsequently chemically reset to the naïve state.

6.2.2.1. Strategy and Validation of PKCι Ablation.

Shef6

To generate conventional Shef6 hESCs that lack expression of PKCι, dual guide RNAs (gRNAs) generated from the pSpCas9(BB)-2A-GFP (PX458) plasmid were used in combination with a targeting vector containing antibiotic resistance selection cassette (Figure 6.2A). A pair of gRNAs were designed to target the region immediately downstream of the translational start within the first coding exon of *PRKCI*. In addition the two gRNAs were spaced exactly 70 base pairs from one cleavage site to the other, creating a frame shift when the intervening sequence is excised. In order to allow for selection of targeted cells a targeting vector was generated containing a G418/Neomycin antibiotic resistance gene flanked at the 5' end by the 800 bp sequence upstream of gRNA1, while the 3' end was flanked by the 800 bp sequence downstream of gRNA2. These flanking sequences therefore allowed for homologous recombination of the selection cassette into the region that was excised following CRISPR/Cas9 cleavage.

The drawbacks of this strategy are twofold: firstly, the use of multiple gRNAs increases the likelihood of off target cleavage; and secondly, requiring two cleavage events raises the barrier for successful homologous recombination. Nonetheless this approach was adopted because once the initial recombination event is achieved at the first allele it raises the likelihood of disruption to *PRKCI* at the second allele. This could either be in the form of homologous recombination of the selection cassette, a frame shift caused by

excision of the intervening coding DNA sequence between the gRNA pair, or small DNA insertions or deletions at either cleavage site.

Following generation of gRNA and targeting vector plasmids Shef6 cells were expanded in E8 culture conditions and transfected via electroporation before plating at low density (Figure 6.2B). 48 hours after transfection the medium was supplemented with G418 for a period of 10 days at which point clones were picked for genotyping and expansion. Two independent clones, Shef6 *PRKCI*$^{-/-}$ clone 1 (Shef6 C1) and Shef6 *PRKCI*$^{-/-}$ clone 3 (Shef6 C3), showed disruption of the translational sequence at both alleles, either by integration of the selection cassette or via deletion of the 70bp sequence between the gRNAs resulting in a frame shift (Figure 6.2C, D & E). Westernblotting showed that PKCι protein was absent in these two clones, confirming the knockout of *PRKCI* (Figure 6.2F). Each clonal line was reset to the naïve state of pluripotency using transient exposure to MEK inhibition, human LIF and valproic acid, as described in (Guo et al., 2017).

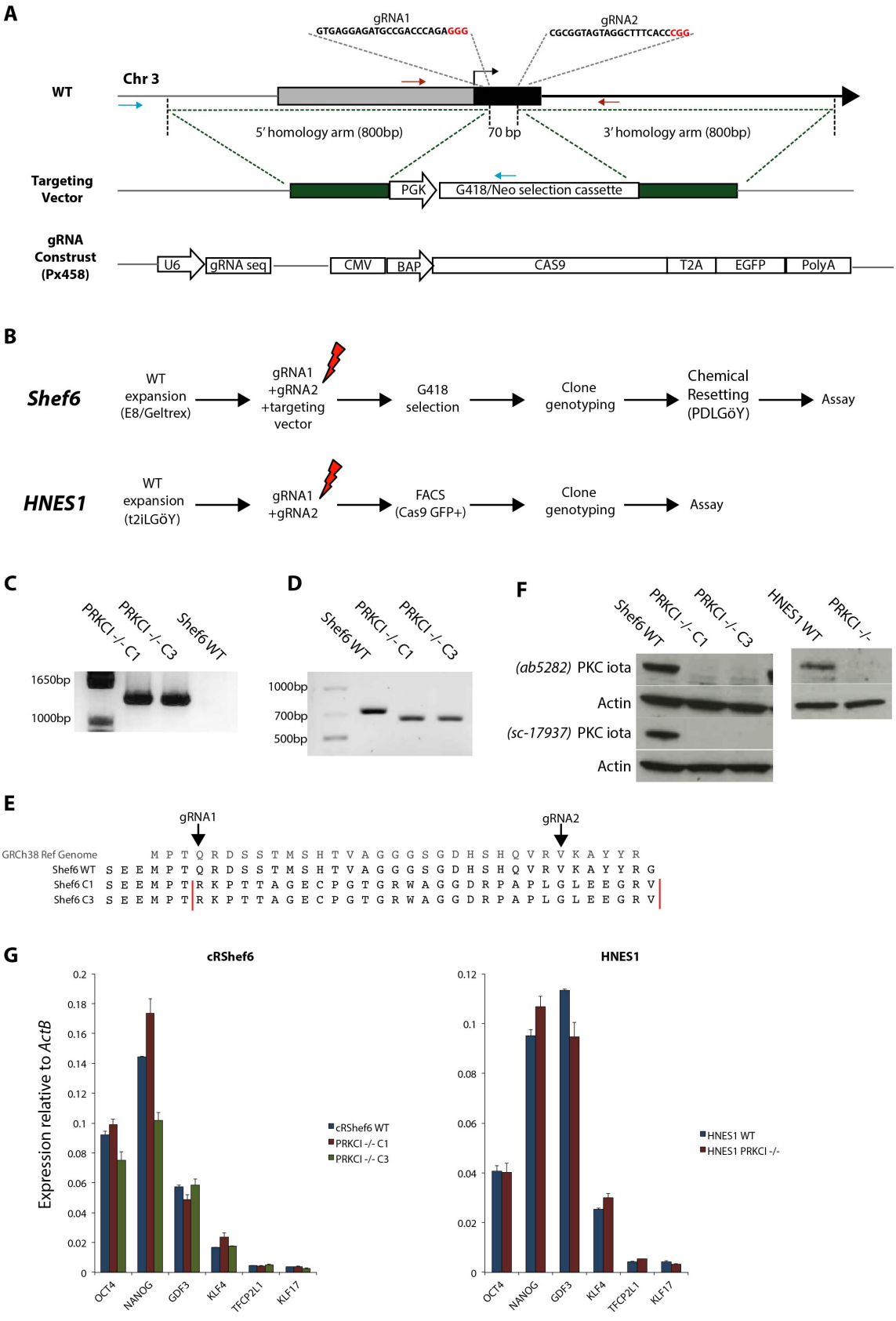
A
gRNA1
GTGAGGAGATGCCGACCCAGAGGG
gRNA2
CGCGGTAGTAGGCTTTCACCCGG
WT
Chr 3
5′ homology arm (800bp)
70 bp
3′ homology arm (800bp)
Targeting Vector
PGK
G418/Neo selection cassette
gRNA Construst (Px458)
U6
gRNA seq
CMV
BAP
CAS9
T2A
EGFP
PolyA
B
Shef6
WT expansion (E8/Geltrex)
gRNA1 +gRNA2 +targeting vector
G418 selection
Clone genotyping
Chemical Resetting (PDLGöY)
Assay
HNES1
WT expansion (t2iLGöY)
gRNA1 +gRNA2
FACS (Cas9 GFP+)
Clone genotyping
Assay
C
PRKCI -/- C1
PRKCI -/- C3
Shef6 WT
1650bp
1000bp
D
Shef6 WT
PRKCI -/- C1
PRKCI -/- C3
1000bp
700bp
500bp
F
Shef6 WT
PRKCI -/- C1
PRKCI -/- C3
HNES1 WT
PRKCI -/-
(ab5282) PKC iota
Actin
(sc-17937) PKC iota
Actin
E
gRNA1
gRNA2
GRCh38 Ref Genome M P T Q R D S S T M S H T V A G G G S G D H S H Q V R V K A Y Y R
Shef6 WT S E E M P T Q R D S S T M S H T V A G G G S G D H S H Q V R V K A Y Y R G
Shef6 C1 S E E M P T R K P T T A G E C P G T G R W A G G D R P A P L G L E E G R V
Shef6 C3 S E E M P T R K P T T A G E C P G T G R W A G G D R P A P L G L E E G R V
G
cRShef6
HNES1
Expression relative to ActB
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
cRShef6 WT
PRKCI -/- C1
PRKCI -/- C3
HNES1 WT
HNES1 PRKCI -/-
OCT4
NANOG
GDF3
KLF4
TFCP2L1
KLF17

Figure 6.2 Generation of PKCι null naïve hESC lines

A) Schematic showing the translational start site of *PRKCI*, the region targeted by CRISPR/Cas9 and the gRNA and targeting constructs.
B) Outline of differential targeting strategies for Shef6 and HNES1 cells.
C) Genotyping PCR showing correct insertion of targeting construct. Primer locations indicated by blue arrows (A).
D) Genotyping PCR showing deletion of sequence between gRNA target sites. Primer locations indicated by red arrows in (A).
E) Amino acid sequence of PCR product (D) indicating disruption of protein coding sequence.
F) Western blots showing expression of PKCι protein in Shef6 and HNES1 targeted clonal lines.
G) RT-qPCR showing expression of pluripotency markers in chemically reset cRShef6 *PRKCI*$^{-/-}$ clonal lines and a HNES1 *PRKCI*$^{-/-}$ clonal line. Expression relative to *ACTB*. Error bars represent standard deviation of 2 independent reactions.

HNES1

At present, robust protocols for human naïve hESC transfection are yet to be determined. Previous early efforts to genetically manipulate naive hESCs in the Smith lab have indicated that electroporation is significantly less efficient in this cell system than conventional primed E8 culture conditions. In order to increase the likelihood of achieving homozygous disruption of *PRKCI* the strategy was adjusted to exclude the targeting vector and reduce the quantity of plasmid DNA required for transfection (Figure 6.2B).

HNES1 cells cultured in t2iLGöY were transfected with gRNA1 and gRNA2 via electroporation. 48 hours after plating, transfected cells were sorted from the bulk population by using GFP constitutively expressed from the CAS9 encoding plasmid. GFP positive cells were then plated at clonal density for 6 days before picking and expanding individual colonies. Putative *PRKCI*$^{-/-}$ clones were identified by PCR amplification of the 500 bp genomic region containing the two cleavage sites. Ablation of PKCι was then confirmed in one of the clonal lines via western blotting (Figure 6.2F).

To investigate whether loss of PKCι function permitted the acquisition and maintenance of naïve pluripotency, naïve transcript expression was determined. After 5 passages in t2iLGöY medium following chemical resetting both WT and *PRKCI*$^{-/-}$ clonal lines maintained expression of core pluripotency factors, *NANOG* and *OCT4 (*Figure 6.2G*)*. Furthermore, expression of naïve pluripotency markers *KLF4, KLF17* and

TFCP2L1 was similar between all three lines. Similarly, targeting of naïve HNES1 cells did not significantly alter expression of any of the pluripotency markers assayed.

6.2.2.2. Withdrawal of Gö6983

Having established *PRKCI*$^{-/-}$ human naïve ESC clones from two separate parental lines, I now sought to test whether loss of PKCι is sufficient to maintain pluripotency following withdrawal of Gö6983. cRShef6 WT and *PRKCI*$^{-/-}$ lines were cultured on MEF either in t2iLGöY or with Gö6983 withdrawn from the medium. By passage 3 the dome-shaped morphology associated with naïve HESCs was almost entirely lost from cRShef6 WT cultures when Gö6983 was omitted (Figure 6.3A). Instead, colonies adopted a flattened epithelial cobblestone-like morphology. I had previously observed that HNES1 cells show greater sensitivity to Gö6983 withdrawal. Thus, under the same conditions similar morphological changes could be observed in HNES1 WT cells within 5 days of inhibitor withdrawal (Figure 6.3A). In contrast both cRShef6 and HNES1 *PRKCI*$^{-/-}$ lines maintained the typical naïve hESC dome-shaped colony morphology throughout this period of culture. However *PRKCI*$^{-/-}$ cultures lacking Gö6983 appeared to contain a greater number of flattened colonies than those that continued to be exposed to the inhibitor.

Given that colony morphology was mostly preserved, I next looked to see whether naive pluripotency marker expression was also conserved by PKCι ablation. RT-qPCR was performed on the cultures shown in Figure 6.3A and the transcript expression of a panel pluripotency markers was compared between cells cultured in the presence and absence of Gö6983. To allow for changes in expression arising from the cloning or chemical resetting processes, comparison of marker expression was made within each cell line only. By passage 3, cRShef6 WT cells had downregulated expression of all 8 markers assayed (Figure 6.3C). In contrast, expression of these markers was mostly preserved in cells that lacked PKCι activity. Following 5 days of Gö6983 withdrawal, HNES1 WT cells had also downregulated transcript expression all 8 pluripotency markers (Figure 6.3C). However this downregulation was less advanced than cRShef6 cultures, likely due to the reduced duration of Gö6983 withdrawal. This would suggest changes in morphology, in particular flattening of the colonies, precede the extinguishing of

pluripotency marker expression. Consistent with my observations in cRShef6 cells, downregulation of these markers was attenuated in HNES1 cells lacking PKCι.

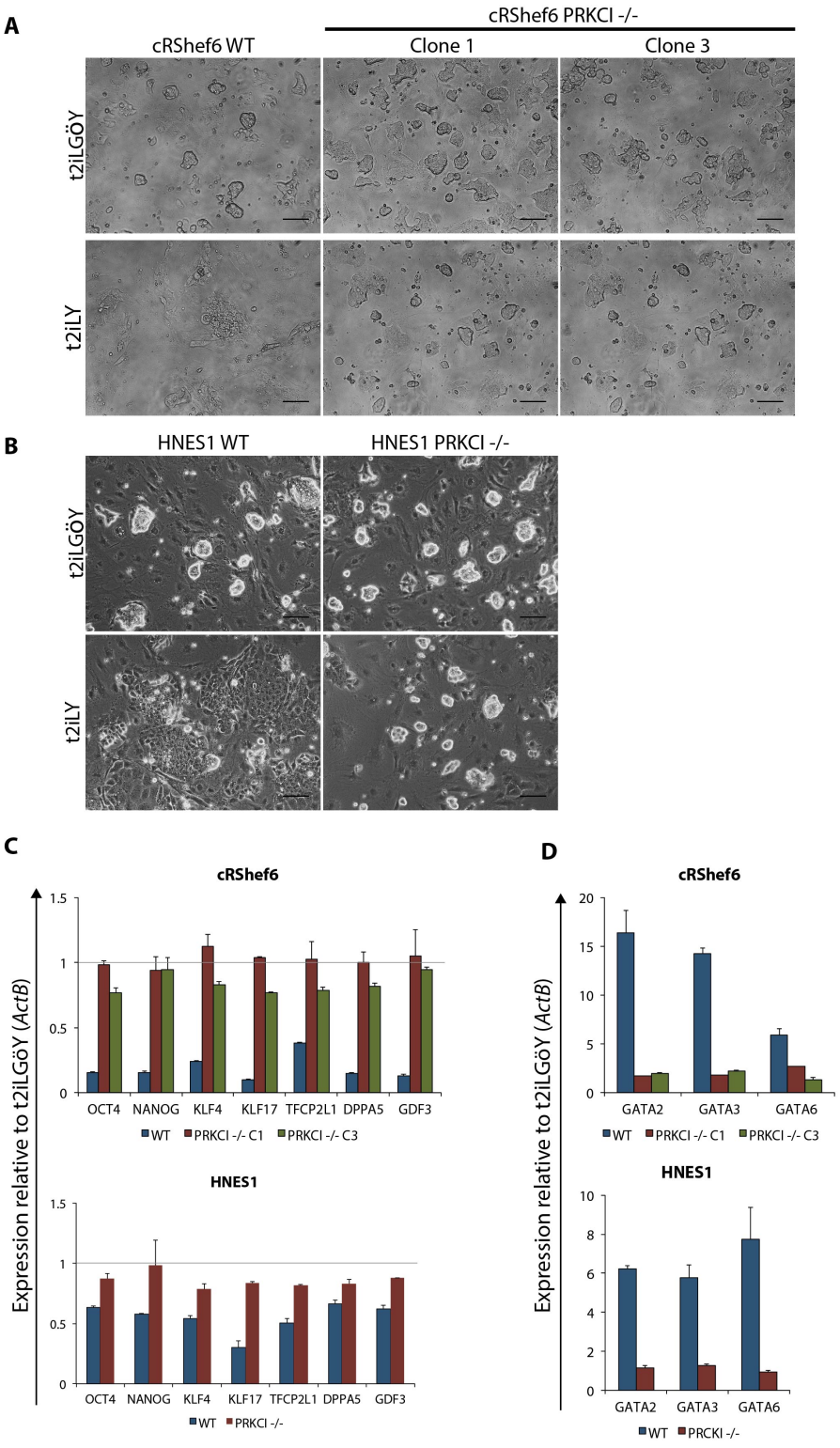

Figure 6.3 PKCι ablation preserves naïve features in the absence of Gö6983

Bright-field images of cRShef6 **(A)** and HNES1 **(B)** wild-type and *PRKCI*$^{-/-}$ colonies cultured in the presence and absence of Gö6983 for a 3 passages. Scale bars represent 100µm.
RT-qPCR showing expression of pluripotency markers **(C)** and lineage markers **(D)** in cultures pictured above. Represented as expression of each cell line cultured in t2iLY medium relative to that cell line cultured in t2iLGöY. Expression normalized to *ACTB*. Error bars represent standard deviation of 2 independent reactions.

Having observed that ablation of PKCι preserved expression of naïve factors in the absence of Gö6983; I next sought to determine whether loss of PKCι activity also influenced upregulation of lineage markers. In cRShef6 WT cells withdrawal of Gö6983 resulted in a strong upregulation of *GATA2, GATA3* and *GATA6* (Figure 6.3D). In contrast, upregulation of all three GATA factors was strongly diminished in both *PRKCI*$^{-/-}$ clonal lines. Similarly, the increase in GATA factor expression observed in HNES1 WT cells cultured without Gö6983 was barely evident in HNES1 cells lacking PKCι.

6.2.2.3. Rescuing PRKCI expression

To confirm the observed phenotype resulted from disruption of *PKRCI*, rather than as a consequence of clonal line adaptation from the targeting or resetting processes, constitutively expressed transgenic *PRKCI* was introduced. Shef6 PRKCI$^{-/-}$ C3 cells were chemically reset as before until the culture had stabilized, at which point the culture was transfected with transgenic human *PRKCI* driven by a constitutive CAG promoter and integrated into the genome via the PiggyBac system (Figure 6.4A). 100ug/ml of Hygromycin was applied to the culture for a period of 10 days, after which the cells were passaged and maintained as a pool. RT-qPCR of transfected cells revealed approximately an 8-fold increase in *PRKCI* expression above endogenous levels following transfection (Figure 6.4B). Expression of pluripotency markers *NANOG, OCT4, KLF4* and *TFCP2L1* was unaltered by overexpression of *PRKCI*, however levels of *KLF17* transcripts appeared to be reduced (Figure 6.4C). Interestingly, a number of lineage markers were expressed at elevated levels in cells over expressing transgenic *PRKCI* (Figure 6.4D).

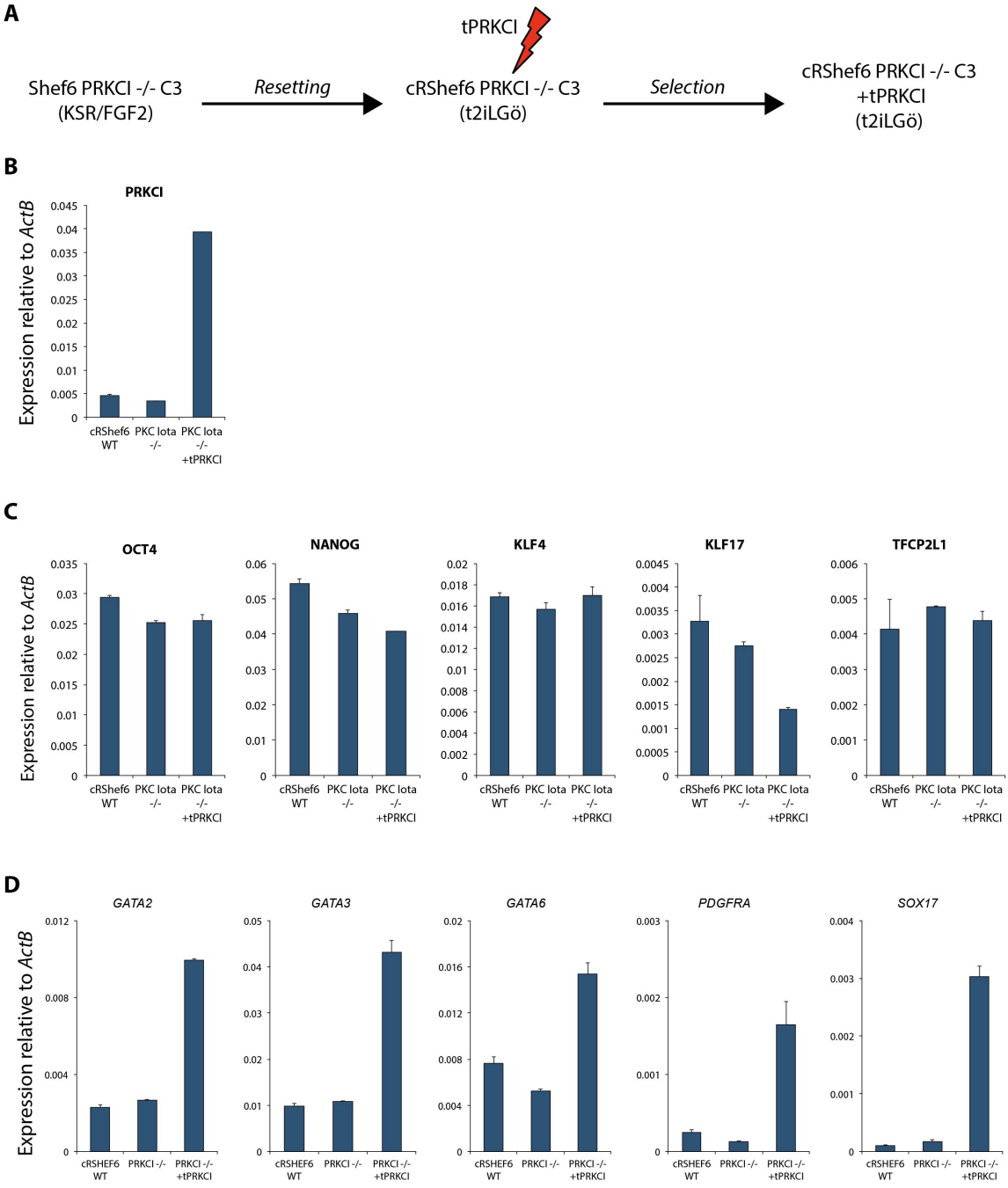

Figure 6.4 Rescue of PKCι expression

A) Outline of protocol for introduction of transgenic PRCKI.
B) RT-qPCR showing expression of *PRKCI* in transfected cRShef6 PRKCI$^{-/-}$ cells.
RT-qPCR showing expression of pluripotency markers **(C)** and lineage markers **(D)** in cRShef6 wild-type, PRKCI$^{-/-}$ and transgenic *PRKCI* expressing cultures.
Expression normalised to *ACTB*. Error bars represent standard deviation of 2 independent reactions.

I next sought to determine whether rescuing PKCι activity via transgenic expression restores differentiation following Gö6983 withdrawal. cRShef6 WT, cRShef6 C3 and cRShef6 C3 +t*PRKCI* cells were cultured either in t2iL in the presence or absence of Gö6983 for 3 passages. By passage 3 the flattened cobblestone-like morphology associated with Gö6983 omission was apparent in wild type colonies (Figure 6.5A). Consistent with previous experiments colonies of *PRKCI*$^{-/-}$ cells mostly maintained a dome-shaped morphology, however those cells now overexpressing *PRKCI* exhibited wide spread flattening.

RT-qPCR analysis of these cultures showed WT cell heavily downregulated pluripotency markers following Gö6983 withdrawal, while expression of these markers persisted in PKCι ablated cells (Figure 6.5B). Expression of transgenic *PRKCI* in mutant cells resulted in a greater more pronounced downregulation of pluripotency marker expression following Gö6983 withdrawal, however this downregulation did not reach the levels of WT cells. To investigate whether these differences in gene expression altered the self-renewal capacity of theses cells, Gö6983 deficient cultures from each line were re-plated back into self-renewing conditions. After 3 passages in the absence of Gö6983, cRShef6 WT, cRShef6 PRKCI$^{-/-}$ C3 and cRShef6 PRKCI$^{-/-}$ C3 +tPRKCI cells were plated at clonal density in t2iLGö /Geltrex culture conditions. After 7 days the number of alkaline phosphatase positive colonies was determined. Ablation of PKCι drastically improved the colony forming capacity of naïve hESCs cultured for 3 passage without Gö6983, returning 16-fold more alkaline phosphatase positive colonies than wild-type cells (Figure 6.5C). In contrast, over expression of tPRKCI in knockout cells greatly reduced the colony forming capacity.

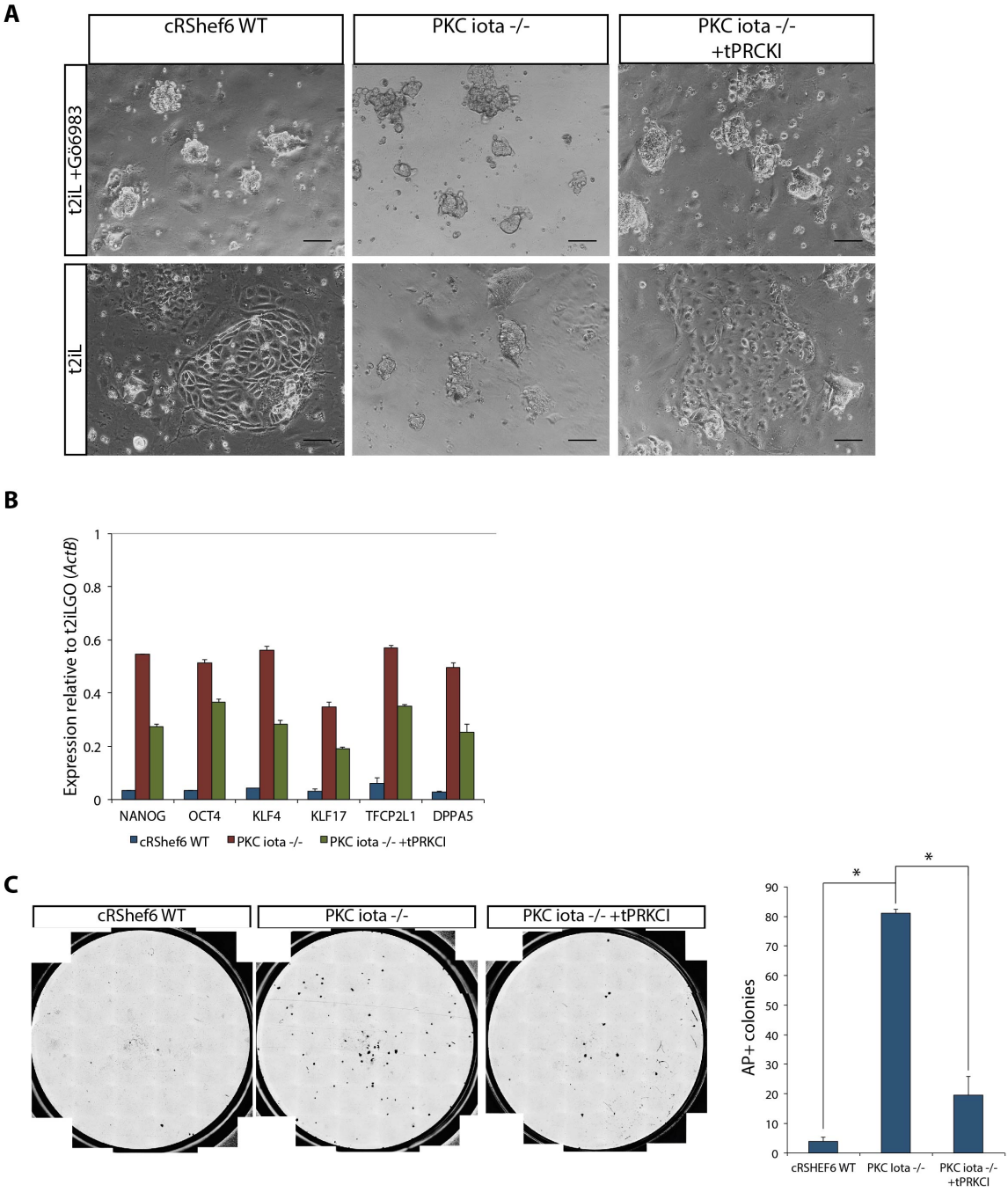

Figure 6.5 Rescuing PKCι activity diminishes preservation of naïve features

A) Bright-field images of Shef6 WT, PRKCI$^{-/-}$ or transgenic *PRKCI* expressing cells cultured in the presence or absence of Gö6983 for 3 passages. Scale bars represent 100µm.

B) RT-qPCR expression of pluripotency markers in the cultures pictured above. Represented as expression of each cell line cultured in t2iLY medium relative to that cell line cultured in t2iLGö. Expression normalized to *ACTB*. Error bars represent standard deviation of 2 independent reactions.

C) Images and chart showing alkaline phosphatase positive colonies formed from cultures picture above when re-plated in t2iLGö. Error bars represent 3 plating replicates.

*Anova Tukey's post hoc $p<0.05$.

6.2.3. Ablation of PKCι influences activation of the naïve transcription factor circuitry during chemical resetting

Given that PKCι ablation is sufficient to preserve expression of pluripotency markers following Gö6983 withdrawal, I next examined whether it is also sufficient to enable activation of the naïve transcription factor circuitry when Gö6983 is omitted during chemical resetting.

To address this question the two Shef6 PRKCI knockout clonal lines previously generated were exposed to the chemical resetting protocol detailed by Guo *et al. (Guo et al., 2017)(*Figure 6.6*A*). Briefly, cells were expanded in KSR/FGF2 medium before single cell dissociation and plating on MEF at a density of $11.5x10^3$ cells per cm^2. After 2 days the medium was changed to medium containing PD03, LIF and the HDAC inhibitor VPA (PDL+VPA) for a further 3 days. VPA was then withdrawn from the medium and the cells were cultured either in the presence (PDLGö) or absence (PDL) of Gö6983 for 9 more days until naïve-like colonies had emerged.

6.2.3.1. Ablation of PKCι enable emergence of colonies with naïve morphology

6 days after commencing HDAC inhibitor treatment WT colonies had largely adopted a heterogeneous cobblestone-like morphology typically associated with ES cell differentiation (Figure 6.6B). While some cobblestone-like colonies were present in the knockout cultures, the vast majority of colonies maintained the more compact homogeneous colony morphology they possessed before commencing the resetting process.

By day 14 of resetting, dome-shaped colonies associated with naïve pluripotency had emerged in the PDLGö cultures of all cell lines (Figure 6.7). Domed colonies were more abundant in PKCι ablated cultures. When Gö6983 was omitted from the medium resetting of Shef6 WT cells failed to yield any colonies with naïve morphology. In contrast, colonies with the characteristic naïve appearance emerged from both PKCι null lines cultured under the same conditions.

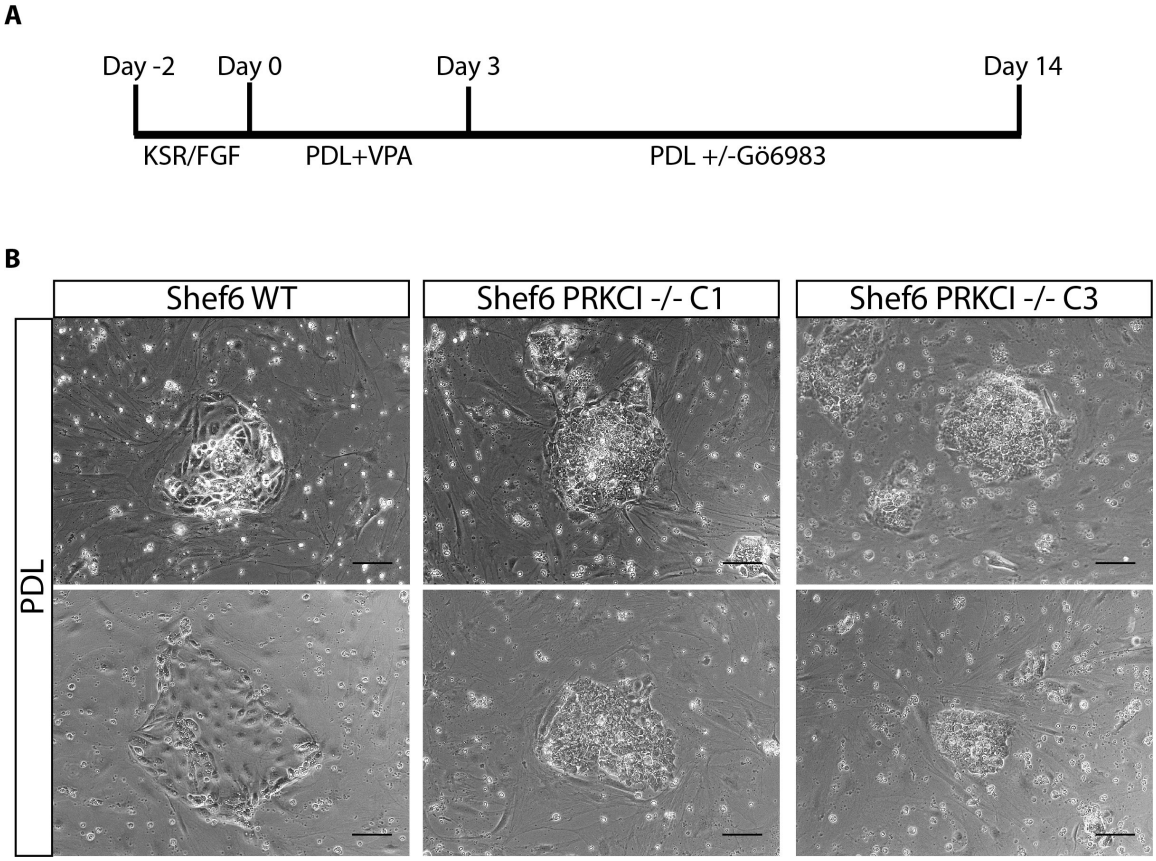

Figure 6.6 PKCι ablation preserves undifferentiated morphology during resetting

A) Outline of the chemical resetting protocol.
B) Bright-field images of WT and PRKCI$^{-/-}$ cells after 6 days of resetting in the absence of Gö6983. Scale bars represent 100μm.

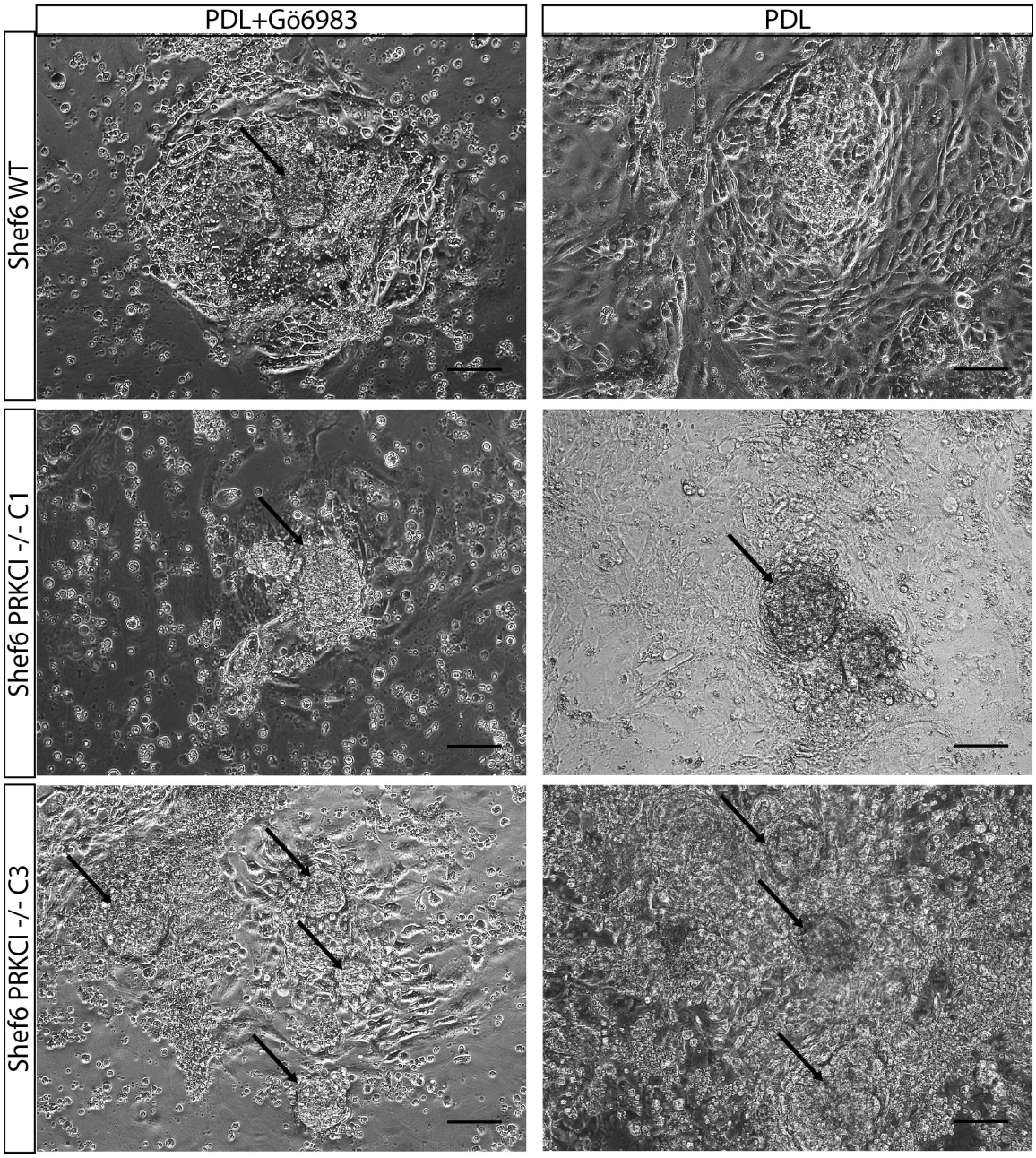

Figure 6.7 PKCι ablation enables formation of colonies with naïve appearance when Gö6983 is omitted

Bright-field images of wild-type and PRKCI$^{-/-}$ cultures after 14 days of resetting in the presence or absence of Gö6983. Black arrows indicate colonies with naïve morphology. Scale bars represent 100μm.

6.2.3.2. Tracking activation of naïve pluripotency markers during resetting in the presence and absence of Gö6983

I next sought to determine whether emergence of naïve morphology in PKCι ablated cells corresponded with Gö6983-independent activation of naïve pluripotency marker expression.

To monitor the acquisition of naïve pluripotency, bulk culture expression of *NANOG*, *OCT4*, *KLF4* and *KLF17* was monitored during the resetting process at 3, 6 and 14 days after commencing VPA treatment. In the presence of Gö6983, all cell lines had significantly upregulated naïve marker *KLF4* prior to day 6 and expression had further increased by day 14 (Figure 6.8A). Similarly, Gö6983 cultures activated expression of primate naïve marker *KLF17* between days 6 and 14. Interestingly, with the exceptions of *KLF4* and *TFCP2L1*, all other pluripotency markers assayed were expressed to a higher degree in PKCι ablated cultures by day 14.

In the absence of Gö6983 treatment, Shef6 WT cells downregulated core pluripotency factors *NANOG* and *OCT4* over the course of the assay (Figure 6.8A). In contrast, PKCι null cultures steadily upregulated *NANOG* as resetting progressed. Furthermore, *OCT4* downregulation was significantly attenuated during the early stages of resetting and was restored to pre-resetting levels by day 14.

Upregulation of naïve marker *DPPA5* was severely stunted when Gö6983 was omitted from WT cultures, while primate-specific naïve marker *KLF17* failed to be significantly upregulated at all (Figure 6.8A&B). In contrast, PKCι ablated cultures activated expression of *KLF17* between days 6 and 14. Similarly, upregulation of *DPPA5* was far greater in PRKCI$^{-/-}$ cells.

Surprisingly, the absence of Gö6983 did not prevent WT cells from upregulating naïve markers *KLF4*, *KLF5* and *TFCP2L1*, which were all expressed at levels comparable to Gö6983 treated cultures by day 14 (Figure 6.8A&B). Furthermore, ablation of PKCι appeared to result in reduced upregulation of *KLF4* and *TFCP2L1* following resetting when compared WT cells, both in the presence and absence of Gö6983.

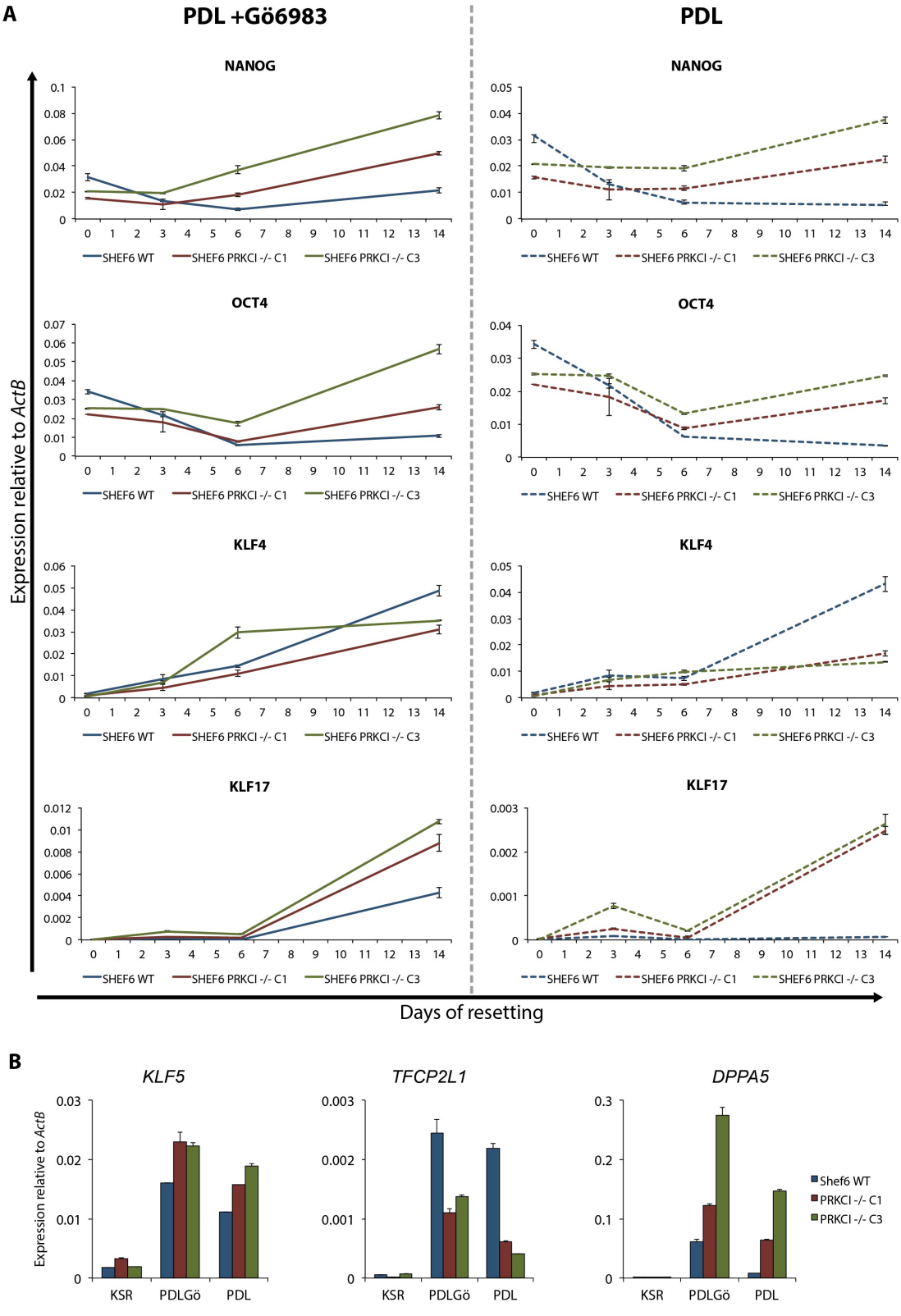

Figure 6.8 PKCι ablation enables expression of *KLF17* following resetting in the absence of Gö6983

A) RT-qPCR data showing expression of pluripotency markers in wild-type and PRKCI$^{-/-}$ bulk cultures following 3, 6 and 14 days of chemical resetting in the presence (PDLGö) or absence (PDL) of Gö6983. **B)** RT-qPCR data showing expression of naïve pluripotency markers in WT and PRKCI$^{-/-}$ bulk cultures in KSR/FGF or after 14 days of chemical resetting in PDL or PDLGö conditions. Expression normalized to *ACTB*. Error bars represent standard deviation of 2 independent reactions.

6.2.3.3. Localisation of NANOG and KLF17 expression

To investigate the localisation of naïve factors in these resetting bulk cultures Immunostaining was performed. 14 days after commencing VPA treatment the cultures were fixed and co-immunostained for NANOG and KLF17. As expected, in the presence of Gö6983, NANOG and KLF17 expression was restricted to densely packed regions of the domed colonies, while the more epithelial-like edges of these colonies were negative for both proteins (Figure 6.9). In all three cell lines the vast majority NANOG+ cells were also KLF17+ suggestive of acquisition of naïve pluripotency.

When Gö6983 was omitted NANOG+ cells could still be identified throughout the Shef6 WT cultures, however the signal appeared weaker and NANOG+ cells were less tightly packed and more mosaic within the colonies (Figure 6.9). In contrast, PKCι ablated cells exhibited extensive NANOG staining that was restricted to the cell-dense regions of the colonies. As expected based on RT-qPCR data, KLF17 was entirely absent from WT cultures when Gö6983 was omitted, including cells that were NANOG+. Importantly, in PKCι null cultures KLF17 expression could be seen prevalently throughout NANOG+ regions of colonies. However NANOG+/KLF17+ cells were less prevalent when Gö6983 is omitted.

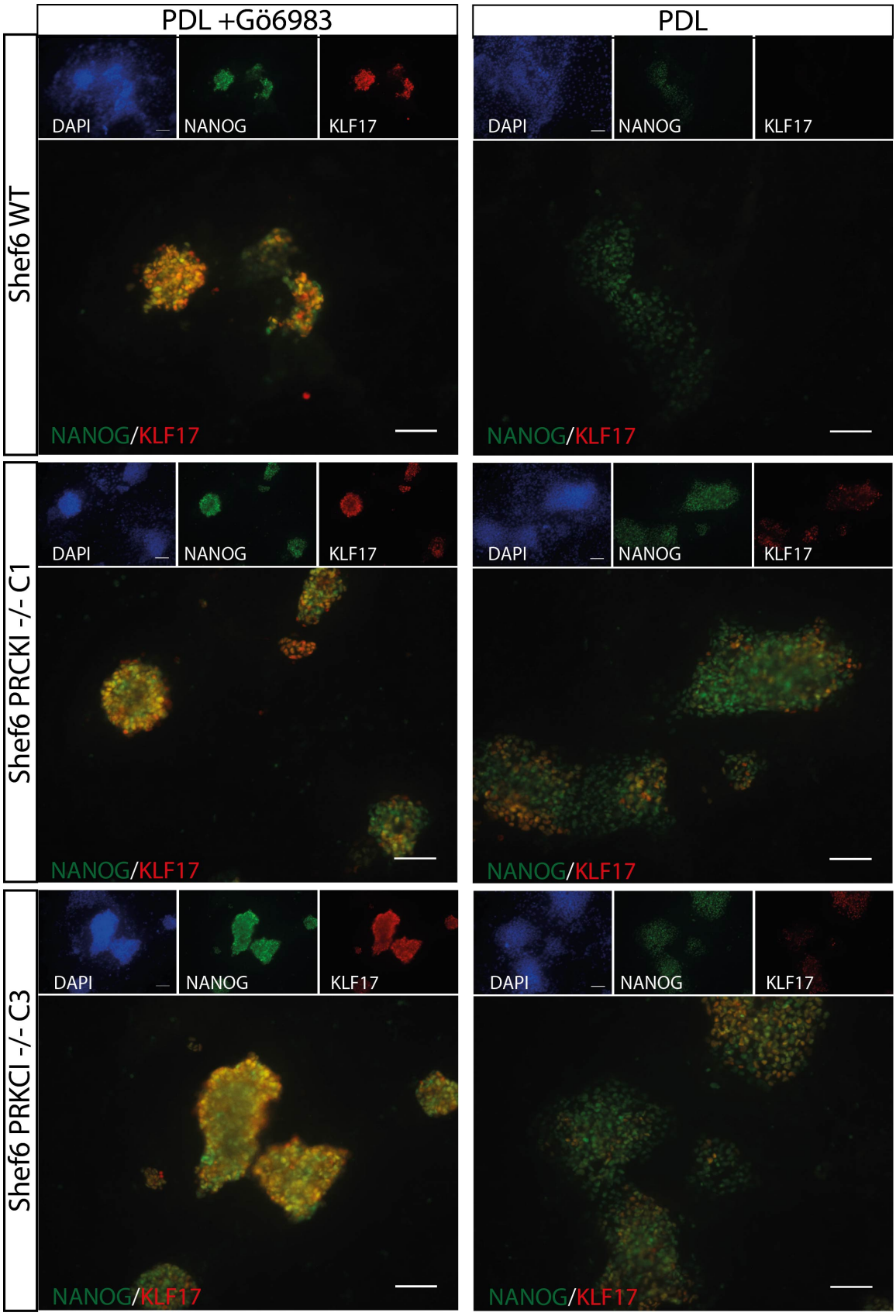

Figure 6.9 Immunostaining of NANOG and KLF17 in day 14 chemical resetting cultures

Immunostaining of WT and PRKCI$^{-/-}$ colonies chemically reset for 14 days in the presence (left) or absence (right) of Gö6983. Green= NANOG, Red= KLF17. Scale bar represents 50µm.

6.2.3.4. Preservation of naïve pluripotency features after passaging

Having demonstrated that PKCι ablation enables acquisition of morphological and transcriptional features of naïve pluripotency in the absence of Gö6983, I next sought to determine whether acquisition of these features is stable over successive passages. To investigate this, wild-type and PKCι knockout cells were chemically reset as before, in the presence of absence or Gö6983, before being maintained for 2 passages in the final resetting medium, PDL, with or without Gö6983. As anticipated, by passage 2 an absence of Gö6983 resulted in widespread differentiation of wild-type cells, adopting the cobblestone morphology also observed with Gö6983 withdrawal (Figure 6.10). In contrast, both PKCι null clonal lines contained a heterogeneous mix of either flat epithelial-like colonies or tightly packed domed colonies with a perimeter of cells that had a differentiated appearance. In the presence of Gö6983 all three cell lines adopted a far more homogeneous appearance of naïve-like colonies.

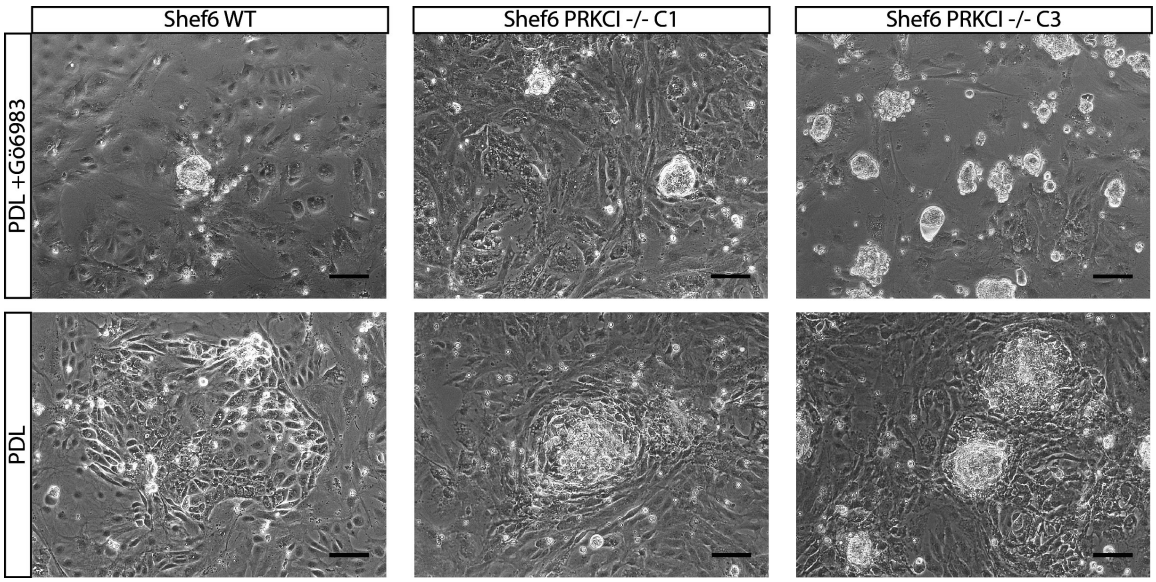

Figure 6.10 PRKCI$^{-/-}$ colonies with naïve-like morphology persist after passaging in PDL

Bright-field images showing WT and PRKCI$^{-/-}$ cultures after 14 days of resetting and 2 subsequent passages in PDL or PDLGö. Scale bars represent 100μm.

To determine whether the naïve-like colonies that persisted in PDL PRKCI$^{-/-}$ cultures continue to express pluripotency factors the cells were fixed and stained for OCT4 and KLF17.

Somewhat surprisingly, OCT4 immunoreactivity was detected in the few remaining cell-dense regions of WT cells (Figure 6.11). However, by passage 2, KLF17 expression could still not be identified, even in OCT4+ cells. Conversely, KLF17 expression continued to be prevalent in PKCι ablated cultures after multiple passages. Furthermore, the majority of cells that were OCT4+ cells were also KLF17+.

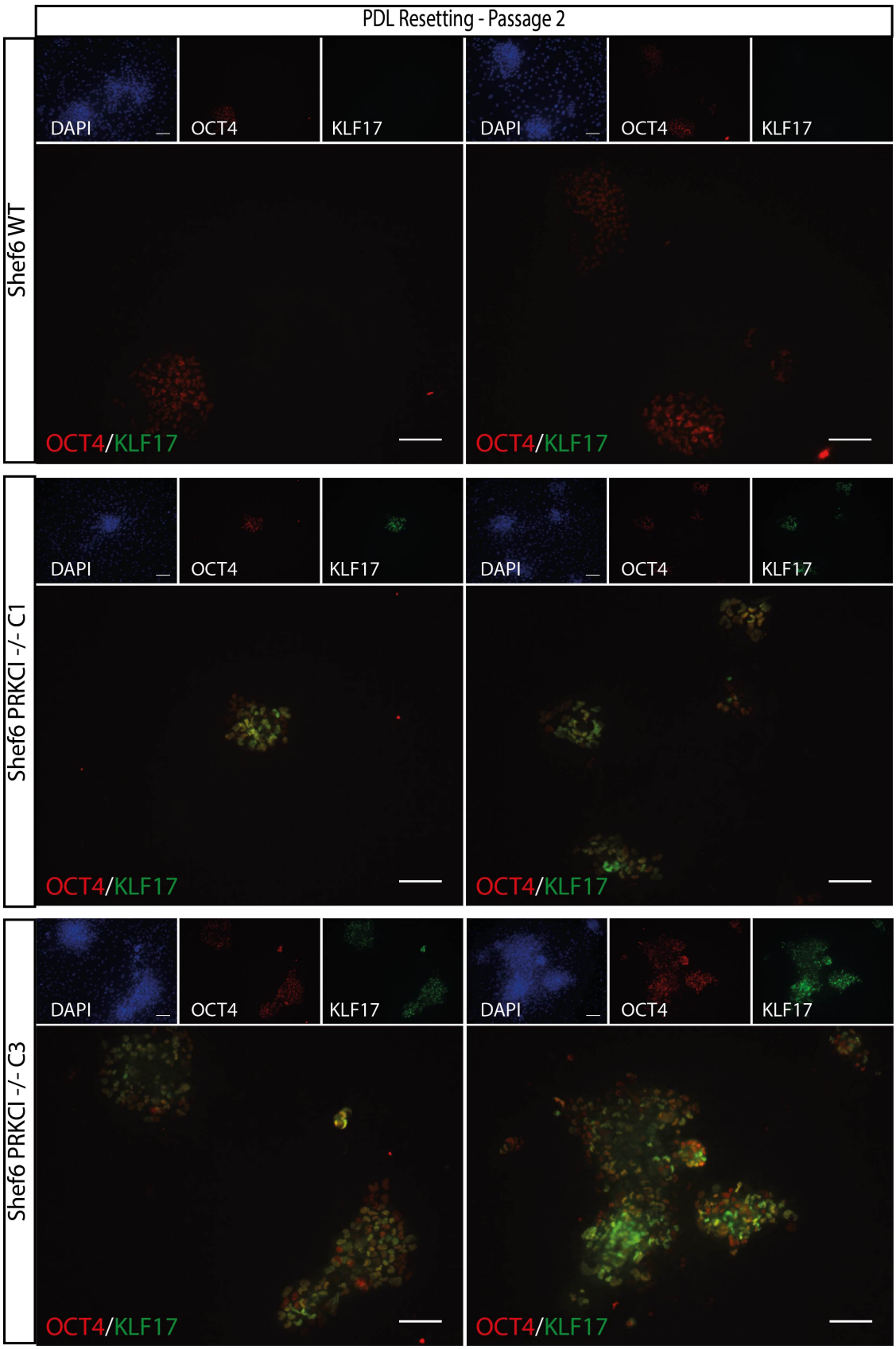

Figure 6.11 Expression of KLF17 in PRKCI$^{-/-}$ cultures persists for at least 2 passages in the absence of GÖ6983

Immunostaining of WT and PRKCI -/- cultures after 14 days of resetting and 2 subsequent passages in PDL or PDLGö. Red= OCT4, Green= KLF17. Scale bar represents 50μm.

6.3. Discussion

Though 2iL permits the efficient derivation and propagation of naïve mESCs it is not sufficient to derive naïve hESCs. However with the further addition of the PKC inhibitor Gö6983, a naïve state of human pluripotency can be derived and propagated *in vitro*. It has been proposed that the critical target of Gö6983 that enables the maintenance of naïve hESCs might be the atypical PKC, PKCι.

In this chapter I demonstrate that inhibition or ablation of PKCι increased colony forming capacity following prolonged Gö6983 withdrawal. *PRKCI*$^{-/-}$ cells continued to express naïve markers that were otherwise lost in wild-type cells when Gö6983 was withdrawn for multiple passages. Furthermore, ablation of PKCι suppressed the resulting upregulation of GATA factors observed in wild-type cells. These observations were consistent between mutant naïve cells generated from both primed Shef6 hESCs and embryo-derived HNES1 parental lines. Together these data indicate that PKCι activity promotes the exit from naïve pluripotency in the absence of Gö6983. This is consistent with previous reports that PKCι disruption inhibits differentiation of both naïve hESCs and haploid mESCs (Leeb et al., 2014; Takashima et al., 2014). However, to date the mechanism by which PKCι facilitates the exit from naïve pluripotency remains unknown. Atypical PKCs are a well-established and prominent component of epithelial polarity complexes (Suzuki and Ohno, 2006). mESCs acquire epithelial polarity during transition from the naïve state to the primed state of pluripotency, while conventional primed hESCs are known to possess features of epithelial polarity (Krtolica et al., 2007). This therefore presents the possibility that acquisition of polarity may be required in order to exit naïve pluripotency, and that ablation of PKCι may interfere with this process.

Expression of transgenic PKCι failed to fully rescue differentiation of PRKCI$^{-/-}$ cells when Gö6983 was withdrawn from the medium. At present the reason for this discrepancy is unclear. This observation may be explained by the persistence of a small population of untransfected cells following antibiotic selection or some degree of transgene silencing over the course of the assay. Prolonged culture in the absence of Gö6983 is required for differentiation and the passaging process provides opportunity

for cells that lack transgenic expression of *PRKCI* to be preferentially selected. Alternatively, the use of a single clonal line, in this case cRShef6 PRKCI$^{-/-}$ C3, presents the opportunity for clonal adaptions to influence the out come of the assay. Adaptions acquired during the cloning or resetting process may have made the cells more resistant to differentiation in the absence of Gö6983 regardless of PKCι activity. Nonetheless, rescue of PKCι expression in ablated cells still resulted in reduced pluripotency marker expression and reduced clonal capacity following Gö6983 withdrawal, supporting the notion that PKCι facilitates differentiation of naïve hESCs.

Interestingly, overexpression of transgenic PKCι appeared to have little effect on expression of naïve markers except for *KLF17*, which was reduced by approximately 50%. However, PKCι overexpression did appear to result in upregulation of a number of lineage markers, far above the levels observed in WT cells or the PKCι null parental line. This may be indicative of a role in the regulation of lineage marker expression rather than suppression of pluripotency factors. However the considerable overexpression of transgenic PKCι raises the possibility that these transcriptional changes result from an overexpression artefact. Therefore to draw a firm conclusion PKCι overexpression would need to be directed at near normal levels in multiple cells lines.

In this chapter I also utilised PKCι null primed hESCs in combination with a chemical resetting protocol to investigate whether ablation of PKCι permits activation of the naïve transcription factor circuitry in the absence of Gö6983.

Knockout of PKCι enabled upregulation of all the naïve markers examined when Gö6983 was omitted from the resetting medium. In particular, ablation of PKCι permitted expression of *KLF17* and *DPPA5*, which was otherwise absent in WT cultures when Gö6983 was not present. Co-localisation of NANOG and KLF17 further indicated the emergence of naïve colonies within these bulk cultures. This data indicates that PKCι activity is a barrier to proper activation of the naïve transcription factor circuitry during the resetting process.

Following consecutive passages a naïve-like morphology and OCT4/KLF17 expression could still be detected in PKCι null cells reset and maintained without Gö6983 treatment. This suggests that the naïve-like phenotype acquired during resetting is

stable to a degree. However, consistent with the Gö6983 withdrawal data previously discussed, the mutant cultures were more heterogeneous in appearance in the absence of Gö6983 than in the presence of the inhibitor, indicating a less robust state of naïve self-renewal.

Somewhat surprisingly a number of the naïve factors examined were upregulated during resetting of wild-type cells regardless of Gö6983 exposure, while others such as *KLF17* and *DPPA5* failed to be induced. These results may be indicative of differences in the regulatory mechanisms that control expression of these naïve markers, possibly indicating expression of *KLF17* and *DPPA5* may be more closely linked to the activity of Gö6983 than other markers such as *KLF4*, *KLF5* or *TFCP2L1*. Differential expression of these markers could potentially be indicative of resetting towards different cell fates. RNA-seq data of pre-implantation stage human blastocysts suggests all three of the naïve markers upregulated in WT cells reset without Gö6983 are also expressed in the primitive endoderm (Blakeley et al., 2015; Petropoulos et al., 2016; Yan et al., 2013). Furthermore, in contrast to mouse, primitive endoderm formation in the human blastocyst is reported to occur even when MEK is inhibited, raising the possibility that PDL medium may present a permissive signalling environment (Roode et al., 2012). Therefore, upregulation of these markers could result from a subpopulation of resetting cells acquiring a primitive endoderm-like fate when Gö6983 is omitted. Understanding the identities of cells generated in the absence of Gö6983 may be important for understanding the roles of Gö6983 and PKCι in fate acquisition during chemical resetting. It may therefore be informative to examine induction of markers of the primitive endoderm, such as *GATA4*, *SOX17* and *PDGFRA*, in these resetting cultures.

Ablation of PKCι appeared to prevent acquisition of a differentiated cobblestone-like morphology during the early stages of chemical resetting. Consistent with this observation, expression of core pluripotency factors *NANOG* and *OCT4* was heavily downregulated in WT cells at the same stage, but was mostly preserved in PKCι null cells. This could possibly indicate that PKCι has a more general role in exiting the pluripotent state, however at present it is unclear if knockout of PKCι renders conventional primed cells refractory to differentiation or if this observation is unique to the conditions presented by this resetting protocol. Additionally, it is possible that resistance to differentiation during the resetting process renders the cells more

permissive to reprogramming, contributing to the activation of naïve factors in the absence of Gö6983.

Previous studies have suggested PKCι promotes the differentiation of both mESC and naïve hESCs, making it a leading candidate for Gö6983-mediated inhibition. Although evidence of differentiation was significantly reduced, ablation of PKCι did not appear to fully recapitulate the self-renewal response of Gö6983 when the inhibitor was withdrawn. This would indicate that, if PKCι is indeed a target of Gö6983, it is not the sole target of this inhibitor. Furthermore, PKCι ablation appeared to enhance induction of pluripotency markers *NANOG*, *KLF17* and *DPPA5* during chemical resetting in the presence of Gö6983. This may suggest loss of PKCι activity has an additive effect in addition to the activity of Gö6983. This indicates that any inhibitory activity of Gö6983 might have on PKCι may not result in a complete blockade of function. Taken together the results of this chapter suggest the Gö6983-induced self-renewal either unrelated or only partially related to inhibition of PKCι activity.

A number of other potential inhibitory targets of Gö6983 may account for this discrepancy. PKCζ, an atypical PKC highly similar in structure to PKCι, may be capable of functionally substituting for PKCι. Potential functional redundancy is particularly relevant when considering the differences between pharmacological inhibition and genetic ablation. In the presence of an inhibitor PKCι may still be able to sequester other proteins but the enzymatic activity would be blocked. When PRKCI is disrupted the total absence of PKCι would prevent those proteins from being sequestered, therefore presenting greater opportunity for functionally similar but lowly expressed kinases such as PKCζ to act in its place.

In vitro analysis of Gö6983 targets suggests this inhibitor may strongly suppress GSK3 activity (Gao et al., 2013). In mESCs GSK3 suppression via CHIR stabilizes β-catenin signalling and prevents TCF3 mediated repression of murine naïve factor ESRRβ thereby supporting self-renewal (Martello et al., 2012; Wray et al., 2011). However GSK3 is also a regulator of numerous other cellular processes that may positively or negatively influence naïve self-renewal (Doble and Woodgett, 2003). Maintenance of human naïve pluripotency is reported to benefit from titration of CHIR, possibly due to the absence of ESRRβ expression in the human epiblast. However, titration of CHIR

may also be beneficial due to otherwise excessive inhibition of GSK3 when Gö6983 is present. It is therefore possible that PRKCI$^{-/-}$ cells might require an increase in CHIR concentration in order to properly maintain self-renewal following Gö6983 withdrawal. Such an experiment could be performed by exposing PRKCI$^{-/-}$ cells to a range of CHIR concentrations (0-3μM) at the time of Gö6983 withdrawal and then assaying the colony forming capacity and pluripotency transcript expression of the cells after 3 passages. Finally, it has been reported that inhibitors of vascular endothelial growth factor receptors (VEGF-R) preserve *OCT4* distal enhancer activity in reset naïve hESCs following withdrawal of transgenic *NANOG* and *KLF2* expression (Theunissen et al., 2014). In vitro assays of Gö6983 inhibitor activity suggest inhibition of VEGF-R activity, presenting a potentially relevant alternative target of this inhibitor. It may therefore be informative to assay the self-renewal response of wild-type and PKCiota null cells in response to VEGF-R inhibitor exposure.

Chapter 7. General discussion & future directions

7.1. Towards a defined culture system

A key step towards therapeutic application of hESCs and towards delineation of the signals that regulate self-renewal is to fully define the culture system. In thesis I identify a tissue laminin culture system that replaces feeders and maintains the key transcriptional features of naïve pluripotency with reduced lineage marker expression (Figure 7.1). Culture on this substrate eliminates the potential for signalling response from the feeder layer and reduces undefined soluble factors in the culture. These properties made this culture system a more appropriate tool for interrogation of signalling pathways during this project. However, due to the origin and purification of this substrate it cannot be termed a fully defined system. Initial attempts to maintain naïve hESCs on recombinant laminin 111, the major laminin component of tissue laminin, or recombinant laminin 521 resulted in reduced pluripotency marker expression suggesting this may not be sufficient to support self-renewal. However, downregulation was more pronounced on laminin 521 than on laminin 111. This observation may be explained by differentiation interaction with integrins on the cell membrane. Laminin-integrin binding through the RGD site is capable of activating a number of intracellular signalling pathways, such as MEK, PI3K, SRC and RhoA signalling (Harburger and Calderwood, 2009). Interestingly, the RGD binding site of laminin 111 is thought to be masked by the globular domain 1Iva, suggesting there may be differential activation of integrin signalling between these two laminin isoforms (Schulze et al., 1996).

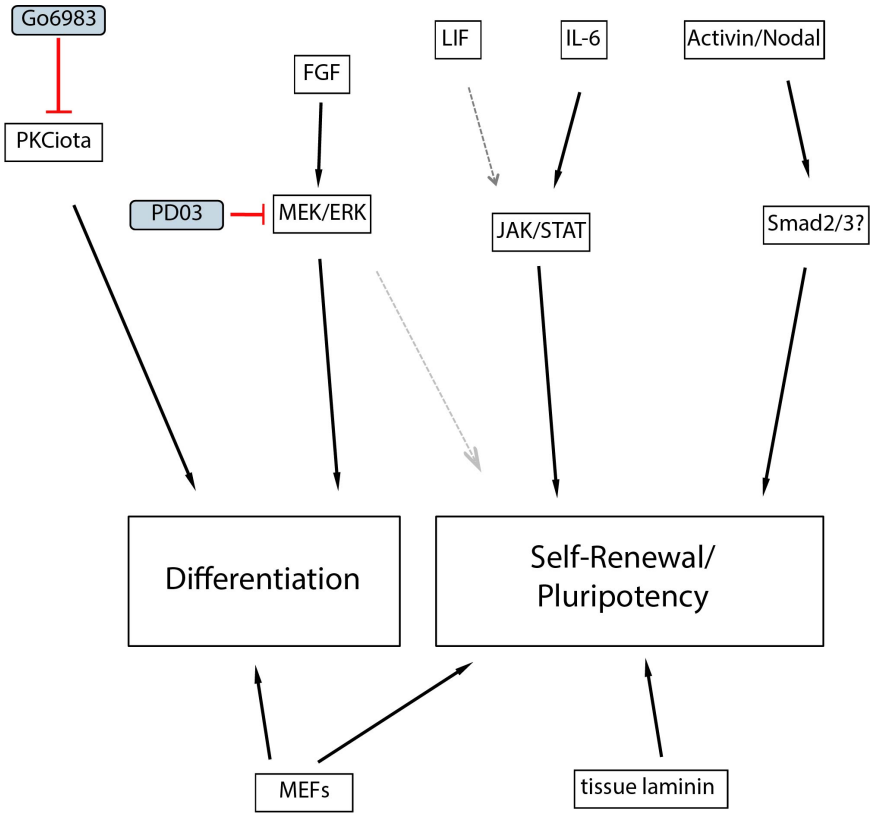

Figure 7.1 A diagram placing the findings of this thesis in the context of the wider literature.

PKCiota promotes differentiation of naïve hESCs through a yet undefined mechanism. Disruption of PKCiota activity via Gö6983, aPKC inhibitor, or genetic ablation inhibits differentiation. Exposure to JAK/STAT activator, IL-6, promotes self-renewal. Exposure an inhibitor of Activin/Nodal signalling indicated endogenous Nodal signalling might be required for self-renewal. Expression of primate-specific naïve marker, KLF17 was demonstrated to be responsive to modulation of Activin/Nodal signalling, indicating possible direct regulation.
The presence of MEF culture substrate induces expression of mesodermal and endodermal lineage markers, indicating the presence of signals promoting differentiation.
Grey arrows indicate signalling processes reported in the literature that were not substantiated by the data in this thesis.

Previous analysis of tissue-derived laminin indicates co-purification of basement membrane proteins, nidogen (entactin) and collagen IV (Timpl et al., 1979). Although immunostaining of human pre-implantation stage blastocysts suggest laminin is more prominent in the trophectoderm, more recent RNA-seq data indicates expression of laminin 111 components, *LAMA1*, *LAMB1* and *LAMC1* throughout the epiblast and primitive endoderm (Blakeley et al., 2015; Niakan and Eggan, 2013). NID1 (nidogen) shares a similar pattern of expression, whereas *COL4A1* (collagen IV) is restricted to the primitive endoderm (Blakeley et al., 2015). Therefore the major components of tissue laminin may be biologically relevant *in vivo*. Furthermore, this raises the possibility that nidogen and/or collagen IV is required in addition to laminin 111, either to directly

interact with the cells or possibly to form a substrate structure that ensures laminin111 is functionally available to the cell.

Recently the Guo *et al.,* demonstrated that naïve hESCs can be propagated for a prolonged period using Geltrex instead of feeders when added to the medium at the time of plating (Guo et al., 2017). In contrast, pre-coating the plate supported short-term propagation but ultimately significant cell death and differentiation is observed. It has also been observed that adding tissue laminin to the medium at the time of plating reduces the concentration required for maintenance of self-renewal. These observations present the question: Why is mixing soluble ECM components in with the cell suspension beneficial for self-renewal? One explanation might be that low-concentration cytokines in these preparations are important for maintaining the naïve state; therefore addition of the solution to the medium increases the concentration over the initial 48 hours after plating. It is also possible that this plating method enables the ECM proteins to coat the cells, therefore interacting with cell membrane receptors on all sides of the cell. This may potential be beneficial for preventing the cell from establishing the baso-lateral domain, thereby preventing acquisition of polarity and any of the potential down-stream transcriptional changes discussed below. The most pressing question regarding tissue laminin and Geltrex substrates is to divorce the effect of substrate interaction from the possible influence of growth factors in the solution. A simple preliminary experiment would be to determine whether MEF-conditioned medium enables the propagation of naïve hESCs on rLaminin 111. If conditioned medium+rLaminin 111 is sufficient this may indicate that further modification of the culture medium is required.

7.2. JAK/STAT signalling

Historically, JAK/STAT signalling was thought to be essential for self-renewal of mESCs however more recently it has been demonstrated that this pathway is dispensable when inhibitors of MEK and GSK3 are present (Ying et al., 2008). Human LIF is a component of both t2iLGö and 5iLAF human naïve pluripotent media formulations. To date, both systems have also required a feeder layer of MEFs, know to produce LIF (Rathjen et al., 1990). It has therefore unknown whether JAK/STAT signalling is dispensable for the

maintenance of naïve human pluripotency. In this thesis I utilised a feeder-independent culture system combined with inhibitors or JAK activity to demonstrate that JAK/STAT signalling is unlikely to be required for maintenance of naïve hESC self-renewal. Additionally I present data indicating that IL-6 potently activates JAK/STAT signalling in naïve hESCs, and that IL-6 supplementation results in resistant to differentiation and leads to upregulation of naïve pluripotency marker *KLF4*. This would indicate some degree of conserved activity between mouse and human embryonic stem cells, whereby activation of this pathway promotes naive self-renewal but is not absolutely required to maintain this state. Whether IL-6 signalling in the human naïve state operates by the same JAK/STAT3 signalling axis as mESCs remains to be determined. Prolonged exposure to IL-6 upregulated *KLF4* but failed to upregulate *TFCP2L1*, a critical downstream target of JAK/STAT signalling in mESCs (Martello et al., 2013). Furthermore, short-term exposure to IL-6 did not elicit upregulation of any of the pluripotency markers assayed, although as previously discussed this could be the result of limitations to the experimental approach.

The next question that needs addressing is whether the self-renewal effect of IL-6 is directed though the classical mESC JAK/STAT3 axis. In order to robustly address this question genetic disruption of STAT3 is required. This would definitively discern whether the JAK/STAT3 signalling axis is dispensable for naïve hESC self-renewal. This would also allow investigation of whether IL-6 is still able to promote self-renewal in the absence of STAT3, providing valuable mechanistic insight.

7.2.1. How might JAK/STAT signalling behave *in vivo*?

IL-6 signalling appears to promote naïve hESC self-renewal, however the role of IL-6 signalling in human pre-implantation development remains unclear. In the mouse ablation of essential JAK/STAT signalling component gp130 has been demonstrated to be compatible with normal development of mouse blastocysts (Nichols et al., 2001). However induction of diapause, a process by which murine blastocysts enter a prolonged stasis, results in loss of the naïve epiblast population (Nichols et al., 2001), leading some to hypothesise that potent LIF-induced self-renewal is intrinsically linked to diapause. More recently, mouse embryos lacking both maternal and zygotic STAT3

were shown to be defective at specifying the ICM, but not the trophectoderm lineage (Do et al., 2013). This data would suggest that, in the mouse, JAK/STAT signalling does in fact have a role in regulating the ICM. Consistent with this, JAK/STAT signalling receptor, IL-6R, is expressed in both the primitive endoderm and naïve epiblast of human pre-implantation stage embryos (Blakeley et al., 2015).

While the experiments in this thesis failed to detect IL-6-induced upregulation of primitive endoderm markers in the presence of MEK and GSK3 inhibition as previously suggested in mESCs, upregulation of pan-ICM marker, *KLF4* was observed (Morgani and Brickman, 2015). Prolonged IL-6 exposure resulted in downregulation of murine trophectoderm markers, *GATA2* and *GATA3*. Furthermore, when Gö6983 was withdrawn from naïve cell cultures IL-6 supplementation attenuated the upregulation of *CDX2*, *GATA2* and *GATA3*. These results could be indicative of a role whereby IL-6 signalling in the human pre-implantation embryo supports specification of the ICM by promoting expression of pan-ICM marker *KLF4* and suppressing expression of trophectoderm-associated markers.

7.2.2. FGF and Activin/Nodal signalling

Independence from FGF and Activin signalling are hallmarks of murine naïve pluripotency, however whether this is a true in other species is unclear. The 5iLAF naïve hESC media formulation contains FGF2 and Activin A, both of which were reported to be beneficial for maintenance and naive marker expression (Theunissen et al., 2014). Though t2iLGö does not contain activators of FGF or Activin/Nodal signalling, the presence of MEF in the culture system presents the possibility that these signals may be active. Additionally, recent single-cell RNA-seq data identifying enrichment of Activin/Nodal signalling components in the human pre-implantation stage epiblast has raised further questions about the role of this pathway in naïve human pluripotency (Blakeley et al., 2015).

In this thesis I utilised the tissue laminin culture system in combination with an inhibitor of FGF receptor activity to further demonstrate that human naïve pluripotency is likely independent of FGF signalling. However exposure to an inhibitor of Activin/Nodal signalling, A83-01, appeared to be detrimental to the maintenance of

naïve pluripotency. Short-term exposure to the inhibitor resulted in downregulation of *KLF17*, followed by upregulation of a number of lineage markers. Prolonged exposure resulted in reduced colony forming capacity and general downregulation of pluripotency markers. However, the conclusions that can be reached from these experiments are limited by the unknown off-target activity of the inhibitor. In order to definitively determine whether Activin/Nodal signalling is required to maintain human pluripotency ablation of signalling components is required. Nodal presents the mostly appealing target for ablation, seeing as it is likely the primary endogenous cytokine activating this pathway and would allow rescue of any phenotype by Activin A supplementation. However similar recent experiments in the mESC system identify SMAD2 phosphorylation following Nodal knockout, likely resulting from GDF3 stimulation (Mulas et al., 2017). RNA-seq of naïve hESC cultures also detected an abundance of GDF3 transcripts in these cells, suggesting this approach may not result in elimination of SMAD2/3 activation. Another possible candidate for genetic disruption is SMAD4, which is required for nuclear translocation of SMAD2/3 downstream of Activin/Nodal signalling. However SMAD4 is also a key component of BMP signalling, potentially complicating the interpretation of any resulting phenotype. Experimentally, the cleanest approach would be to generate double knockout of SMAD2 and SMAD3. It is reported that SMAD3 deletion is tolerated by conventional primed hESCs (Sakaki-Yumoto et al., 2013). A potential approach to this issue may be to generate a SMAD3 null conventional primed hESC line and then introduce a conditional knockout of SMAD2 to be induced after resetting to naïve pluripotency.

7.2.2.1. Downstream of Activin/Nodal signalling

Assuming these observations are not the result of off-target inhibition it would suggest there is a currently unknown mechanism by which Activin/Nodal signalling regulates human naïve pluripotency.

Downregulation of *KLF17* was preliminarily investigated as a potential downstream mechanism for A83-01-induced loss of self-renewal, however a firm conclusion could not be reached based on the experimental system used. Very little is known about the activity of KLF17, however it is heavily linked to inhibition of epithelial-mesenchymal

transition (Gumireddy et al., 2009). Within the context of metastasis, TGFβ signalling is proposed to be anti-metastatic via induction of *KLF17* expression, which in turn suppresses activation of *Id* genes (Ali et al., 2015). However in mESCs BMP signalling prevents neural differentiation by promoting expression of *Id* genes, suggesting this is unlikely to be the mechanism at play in the human naïve state (Ying et al., 2003a).

7.3. aPKCs, polarity and pluripotency

A unique feature distinguishing the human and rodent naïve pluripotent states is the inability to maintain naïve hESCs in 2iL conditions. The t2iLGö naïve hESC media formulation presents an attractive system for determining the additional requirements of human naïve pluripotency due to the addition of a single inhibitor, Gö6983. Previously, it was proposed that the self-renewal activity of Gö6983 might be mediated via inhibition of PKCiota (Takashima et al., 2014).

In this study I present data indicating that PKCι activity facilitates the differentiation of naïve hESCs. Genetic ablation of PKCι resulted in continued pluripotency marker expression and greater colony-forming capacity following Gö6983 withdrawal. Furthermore, ablation of PKCι enabled upregulation of primate-specific naïve marker *KLF17* and naïve pluripotency marker *DPPA5* when cells were subjected to a chemical resetting protocol in the absence of Gö6983. A more longitudinal study of PRKCI$^{-/-}$ cells maintained or reset in the absence of Gö6983 is required to truly determine the stability of this phenotype, however the data presented here indicates that loss of PKCiota activity in combination with LIF and inhibitors of MEK and GSK3 is sufficient to support a naïve-like state.

The mechanism by which PKCι promotes the acquisition of alternative cell fates remains to be determined. The activity and regulation of aPKCs is generally less well understood than classical PKCs or novel PKCs. However, a function of aPKCs that has been well described is their role in the establishment and maintenance of cell polarity. Briefly, aPKC forms part of the PAR complex, along with PAR6 and PAR3, which localizes to the apical domain. The basal polarity complex, consisting of LGL, DLG and SCRIB, localizes to the basal-lateral domain, driven by cell-cell contacts. The kinase activity of aPKC then acts to phosphorylate LGL, enforcing restriction of the

LGL/DLG/SCRIB complex to the basal-lateral domain, thereby establishing polarity (Assemat et al., 2008). A lack of apico-basal polarity is a somewhat unique feature of the pre-implantation epiblast. In the mouse blastocyst, both trophectoderm and primitive endoderm cells exhibit polarity, while the naïve epiblast will only acquire polarity as it develops into the post-implantation epiblast (Bedzhov and Zernicka-Goetz, 2014). Disrupting the acquisition of polarity therefore is a potential mechanism by which PKCι ablation and Gö6983 exposure may impede the exit from naïve pluripotency.

So how might the acquisition of polarity dictate the fate of naïve hESCs? There are a number of reports indicating polarity may directly contribute to cell-fate decisions in the early embryo. It is suggested that a potential mechanism determining the acquisition of a trophectoderm or ICM fate is the asymmetric distribution of mRNA transcripts arising from apico-basal polarity. In the polarised blastomeres of compaction stage embryos transcripts of a key trophectoderm specifying factor, *CDX2*, are reported to localise apically in an aPKC dependent fashion (Skamagki et al., 2013). Interestingly, expression of transcripts associated with specific blastocyst lineages in the mouse, such as *GATA6* for primitive endoderm and GATA2/3 for the trophectoderm, exhibit mosaic expression throughout the human pre-implantation blastocyst (Blakeley et al., 2015; Petropoulos et al., 2016). Low-level expression of *GATA2* and *GATA3* in particular, is also observed throughout naïve hESC cultures. Asymmetric partitioning of these or other lineage determining transcripts upon acquisition of polarity may tip the balance of cell fate, initiating differentiation of daughter cells following cell division. Apical localisation of signalling molecules is also suggested to promote a trophectoderm fate in blastomeres. As discussed in chapter 1, the Hippo/YAP signalling pathway has emerged as an important regulator of trophectoderm and ICM fate. Knockdown of polarity component PAR6 is reported to result in cytoplasmic accumulation of YAP, preventing upregulation of trophectoderm specifying genes (Hirate et al., 2013). Establishing polarity is thought to prevent Hippo/YAP signalling by sequestering AMOT to the apical domain, preventing its interaction with LATS and the resulting phosphorylation of YAP (Hirate et al., 2013).

The role of cell polarity in PKCι null- and Gö6983-mediated self-renewal requires investigation. A simple means to do this would be to look at the localisation of polarity and tight junction components in wild type and PRCKI$^{-/-}$ cells in the presence and

absence of Gö6983. If such a scenario exits, the expectation would be that in wild type and PRKCI$^{-/-}$cells treated with Gö6983 there would be little to no localisation of tight junction marker ZO-1 at the membrane and, in the absence of aPKC kinase activity, baso-lateral complex component SCRIB would extend up into the apical domain (Hirate et al., 2013). Upon withdrawal of Gö6983, WT cells would be expected to exhibit localisation of ZO-1 at the membrane while SCRIB would become restricted to the baso-lateral domain. The expectation would be that PRKCI$^{-/-}$ would still lack ZO-1 and SCRIB localisation.

In order to determine whether polarity is mechanistically important it is also necessary to directly disrupt other components of these complexes. PAR6 is required for recruitment of aPKC to the apical domain and therefore presents a possible target for genetic investigation. Targeting of other polarity components may also be beneficial for circumventing the possible problem that the reduced self-renewal response observed in PRKCI$^{-/-}$ cells upon Gö6983 withdrawal results from the functional redundancy with PKCζ.

7.4. Genetic dissection of human naïve pluripotency

Genetic ablation is a powerful experimental approach that is extensively utilized throughout developmental biology. Recent development of the CRISPR/Cas9 system has provided a simple and efficient tool for targeted homozygous knockout in a variety of cell systems (Sander and Joung, 2014). However, to date there have been no demonstrations of successful gene targeting in either of the current naïve hESC culture systems. The impetus to apply such genetic tools to human naïve pluripotency is further strengthened by the current lack of defined culture systems, limiting the conclusions that can be drawn from modulation of signalling pathways.

In this thesis I demonstrate two strategies for generation of human naïve hESC homozygous mutants using CRISPR/Cas9 technology. For the initial strategy, targeting was achieved using standard protocols for transfection of conventional primed hESCs cultured in E8 medium, following which verified homozygous mutants were reset to the naïve state via transient HDAC inhibition (Guo et al., 2017). In the second strategy naïve HNES1 cells were targeted using two gRNAs spaced 72 base pairs apart, so as to

introduce a frame shift when the intervening sequence is excised. 48 hours later, transfected cells were sorted using GFP expression from the CAS9 vector and the resulting clonal lines were screened for homozygous deletion.

These strategies were applied to generate PKCι null clonal lines, thereby demonstrating disruption of PRKCI is sufficient to sustain pluripotency marker expression for multiple passages following Gö6983 withdrawal, and initiate *KLF17* expression during chemical resetting in the absence of Gö6983.However, there are many other questions surrounding the regulation of human naïve pluripotency that these strategies could be utilized to address.

7.4.1. Primate-specific naive epiblast marker KLF17

The identification of KLF17 as a primate-specific marker of the naïve epiblast has sparked considerable interest as to its role in the human pre-implantation epiblast (Blakeley et al., 2015; Guo et al., 2016). Given the lack of expression of potent self-renewal and reprogramming factors, *KLF2* and *ESSRB*, in the human epiblast, it is tempting to predict that KLF17 may be required to stabilize human naïve self-renewal. To date, there has been no functional analysis of KLF17 within the context of naïve pluripotency. In this thesis I generated naïve hESCs overexpressing transgenic *KLF17* in an attempt to rescue loss of pluripotency factor expression upon A83-01 treatment. Overexpression of *KLF17* under standard self-renewing conditions clearly had a detrimental effect on both colony integrity and naïve marker expression. However given that transgenic *KLF17* expression in these cells far exceeded endogenous levels it is difficult to draw a proper conclusion as to whether KLF17 promotes naïve pluripotency. Currently a key question concerning human naïve pluripotency is whether KLF17 is functionally required for self-renewal. Generation of KLF17 null cells both in the primed state and the naïve state would allow us to assess the requirement of this factor for resetting and maintenance in t2iLGö. Similarly, disruption of endogenous KLF17 and introduction of inducible transgenic *KLF17* would allow the more stringent assessment of any potential function in differentiation or self-renewal.

7.4.2. Conserved naïve circuitry

Genetic interrogation of mESCs demonstrates they are surprisingly resistant to loss of individual pluripotency factors. In the absence of NANOG mESCs continue to self-renew *in vitro* and readily integrate into the blastocyst, generating chimeric mice (Chambers et al., 2007; Osorno et al., 2012; Silva et al., 2009). Similarly ablation of *KLF2*, *KLF4* or *ESRRB* does not prohibit propagation of mESCs *in vitro* (Hall et al., 2009; Jiang et al., 2008; Martello et al., 2012; Ye et al., 2013). This would indicate some degree of functional redundancy is present in the mouse naïve pluripotency network. Naive hESCs also express many components of the murine naïve pluripotency network, such as *KLF4* and *TFCP2L1*. Takashima et al report that siRNA-mediated suppression of *KLF4* or *TFCP2L1* expression in naïve hESCs results in a marked reduction in alkaline phosphatase positive colony formation. However whether naïve pluripotency can be maintained in the absence of *NANOG*, *KLF4* or *TFCP2L1* remains to be determined. The gene targeting strategies laid out in this thesis allow us to approach this issue from two angles. Generation of null mutants in the primed state allows the assessment of whether the factor is necessary for acquisition of naïve pluripotency, and targeting in the naïve state allows the determination of requirement for self-renewal. Of course it is important to consider that the current culture conditions may be limiting for properly determining the requirement of any specific pluripotency factor. For instance, although mESC tolerate the loss of *ESRRB* in 2iL or in PD03+LIF, they will differentiate in the presence of 2i or GSK3 inhibition plus LIF (Martello et al., 2012). Therefore any deletions resulting in a block to self-renewal may simply be due to the absence of other stabilising signals that would otherwise permit self-renewal of the mutant cells.

7.5. Assaying naïve pluripotency: experimental limitations

A key consideration both for interpretation of the data presented in this thesis and within the field of human naïve pluripotency in general is the lack of robust assays for determining naïve pluripotency. True identification of naïve hESCs will always be more problematic due to the inability to apply the gold standard rodent pluripotency assay: chimera contribution. Indeed the current best practice for determining naïve human pluripotency involves multiple assays examining XCI, global methylation,

transcriptional profile and metabolic state. However the application of other routine mESC self-renewal assays may also require additional considerations when applied to the current naïve hESCs culture systems. For example, the ability to generate alkaline phosphatase positive colonies in 2i at clonal density is used extensively in the field as an initial measure of mESC self-renewal. Other cell types, including hESCs and EpiSCs, will also stain positive for alkaline phosphatase, however the 2i culture system is highly selective, limiting the possibility of propagation of other cell types. Low-density colony formation in t2iLGö was applied to assay naïve hESC self-renewal throughout this thesis, however the same assumption of selectivity cannot necessarily be applied. This is particularly true in the case of assays performed in the presence of MEFs, which may produce many soluble factors that permit propagation of other alkaline phosphatase positive cell states that do not reflect naïve pluripotency.

Expression of naïve pluripotency markers was also used to determine naïve pluripotency throughout this thesis. General downregulation of pluripotency markers is likely to be indicative of a loss of naïve self-renewal, however which factors most faithful track this process is currently unclear. In the mESC system, *Rex1* expression has been demonstrated to faithfully represent the self-renewal capacity of naive mESCs. Generation of a *Rex1* reporter line has enabled the tracking of the maintenance and loss of naïve pluripotency within a population in an easily determined way (Kalkan et al., 2017). Currently such a reporter system is not available for the assessment of naïve hESC self-renewal. The OCT4-EOS reporter system was utilised as a surrogate measure of naïve pluripotency in this thesis, however how faithfully activation of this reporter tracks naive pluripotency is yet to be determined.

As our knowledge of the human naïve pluripotent state increases more robust tools and assays will likely become available, however in the meantime it is important be particularly cautious when interpreting experimental outcomes.

7.6. Concluding remarks

The ability to harness developmental processes in order to generate therapeutic tissues and disease models is a key aim of modern biomedical research. The capacity of pluripotent stem cells to generate all cell types of the soma makes them a particularly attractive avenue of research. However conventional hESCs occupy a developmentally advanced, primed state of pluripotency, which is thought to carry with it a number of undesirable characteristics, such lineage bias and X-chromosome erosion *in vitro*, making them a sub-optimal starting point from which to execute differentiation (Butcher et al., 2016; Mekhoubad et al., 2012). This has therefore driven the pursuit to capture the human naïve epiblast *in vitro*, before the transition to the heterogeneous primed pluripotent state. Recently a modified version of the mESC culture system, 2iL, containing an additional PKC inhibitor was demonstrated to be sufficient to maintain hESCs in a naive-like state, however the way in which this signalling environment acts to capture naïve pluripotency remains unclear. The broad aim of this thesis was to gain a better understanding of the signals that govern naïve hESC self-renewal and differentiation as an eventual means to efficiently direct generation, propagation and differentiation of these cells.

In summary, I have demonstrate that naïve hESCs can be propagated on tissue laminin in the absence of feeders and continue to maintain the transcriptional profile of naïve pluripotency. I provide data that indicates maintenance of human naïve pluripotency is not reliant of JAK/STAT signalling. However exposure to the JAK/STAT activator IL-6 elicits strong phosphorylation of STAT3 and appears to result in more robust self-renewal and upregulation of naïve marker *KLF4*. I further demonstrate that human naïve pluripotency is likely independent of FGF signalling and highlight a possible role for Activin/Nodal signalling in the maintenance of naïve self-renewal and the transcriptional regulation of primate-specific naïve pluripotency marker *KLF17*. Finally, I expand on previous preliminary reports that loss of PKCι activity promotes naïve self-renewal and demonstrate ablation of PKCι is sufficient to enable upregulation of naïve markers *KLF17* and *DPPA5* during chemical resetting in the absence of Gö6983.

This work poses a number of questions regarding the maintenance of naïve pluripotency: To what degree is JAK/STAT signalling-induced self-renewal is conserved

in humans, Is Activin/Nodal signalling required for naïve pluripotency, and by what mechanism does PKCι facilitate differentiation of naïve hESCs in t2iL conditions. The work laid out in this thesis provides a foundation from which to investigate these and other key question regarding the signalling pathways that govern human naïve pluripotency.

Chapter 8. Bibliography

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