Mitochondrial and Transcription Rate

Heterogeneity of Mouse Embryonic Stem Cells

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Abstract

Cell-to-cell variation in expression of pluripotency- and lineage-determining factors has been proposed to be integral to the process of cell fate commitment in pluripotent cells both in vitro and in vivo. Understanding the sources of this heterogeneity in pluripotent stem cells promises greater insight into the mechanisms underlying cell fate choice. I identify mitochondrial membrane potential as an axis of heterogeneity in mouse embryonic stem cell populations, and show that high mitochondrial membrane potential marks cells that are in a stable self-renewing state. Partial overlap with previously described metastable subpopulations is demonstrated through gene expression analysis. I present evidence that similarly to previous findings in HeLa, heterogeneity in mitochondrial membrane potential is associated with variation in global transcription rate in mESCs. The direct impact of global transcription rate on differentiation propensity is demonstrated through manipulation of RNA Pol II transcription elongation rate. Mitochondrial variability is therefore likely a functionally relevant source of extrinsic gene expression variability in mouse embryonic stem cells.
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**Glossary and abbreviations**

2-DG: 2-Deoxy-D-glucose 2-DG

2i: defined culture medium for mouse embryonic stem cells with inhibitors of two kinases (MEK and GSK3) that is postulated to induce and maintain the naïve ground state of pluripotency.

BMP: bone morphogenetic protein

BrdU: Bromodeoxyuridine

BrU: 5-Bromouridine

Differentiation potential: the set of cell types that a stem or progenitor cell may give rise to. OR All the possible cell fates that a stem or progenitor cell has available to it.

DRB: 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole

EC: embryonal carcinoma

EPI: epiblast

EpiSC: epiblast stem cell

ERK: extracellular receptor kinase

ESC: embryonic stem cell

ETC: electron transport chain

FGF: fibroblast growth factor

FUCCI: fluorescence ubiquitination Cell cycle indicator

GRN: gene regulatory network

GSK3: glycogen synthase kinase 3

hESC: human embryonic stem cell

HSC: hematopoietic stem cells
ICM: inner cell mass

iPSC: induced pluripotent cell

LIF: leukemia inhibitory factor

Lineage bias: the higher likelihood of a stem or progenitor cells to adopt one cell fate over others that are also available to it.

MAPK: mitogen-activated protein kinase

MEF: mouse embryonic fibroblast

MEK: mitogen-activated protein kinase kinase

MMP: mitochondrial membrane potential

NAD: nicotinamide adenine dinucleotide

OCR: oxygen consumption rate

OXPHOS: oxidative phosphorylation

PE: primitive endoderm

PI3K: phosphoinositide 3-kinase

PSC: pluripotent stem cell

RNAP: RNA Polymerase

rRNA: ribosomal RNA

STAT: signal inducer and activator of transcription

TE: trophectoderm
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Chapter 1: General Introduction

1.1 Cell types and cellular decision switches

Multicellular organisms consist of a large variety of cell types with dramatically different functions and morphologies, yet almost all cells of an individual share the same genome. Instead of genetic diversification, cell types owe their differences to the expression of different sets of genes, the synthesis of different sets of RNA and protein molecules, which carry out the cells’ specialized functions. The \(4 \times 10^{13}\) cells (Bianconi et al., 2013) of the human body all descend from the zygote through rounds of cell division, during which their gene expression profiles diverge in the process of cellular differentiation. Gene expression therefore must be regulated process, namely genes must turn on and off. Jacob and Monod (1961) told us how: there is a class of molecules that specialise in regulating the synthesis of ‘structural messengers’ (mRNAs) by binding to operator sites on DNA.

In another paper Monod and Jacob (1961) pointed out that two regulators with mutual negative “cross-feedback” would create a system of two alternative stable states. The suggestion was that the two stable states of the circuit are analogous to the different cell types of multicellular organisms, and that if the two regulators were connected to other parts of the genome by regulatory interactions, they could define alternative stable states of genome expression. Kauffman showed that even randomly constructed gene regulatory networks
(GRNs) with few, but specific regulatory inputs per gene also exhibit multiple stable expression states (Kauffman, 1969, 1974). The existence of multiple cell types is then a natural consequence of the large-scale structure of gene regulatory networks, and cellular differentiation represents the cells’ settling into the different stable states of the genome-wide gene regulatory network. We now know that transcription factors indeed regulate the expression of not only effector genes, but also that of other transcription factors, often including that of their own, and form extensive GRNs. Large-scale gene expression profiling has allowed us to classify cell types based on their genome-wide gene expression profiles. Conceiving cell types as distinct stable states of a genome-wide gene regulatory network has provided the framework for understanding the process of cellular differentiation and its molecular underpinnings.

1.1.1 Multipotency, pluripotency and stem cells

The identities of differentiated cells are stable; they do not change into other cell types under normal conditions. In contrast, the speciality of pluripotent cells is their ability to give rise to multiple different cell types. All cell types that make up the body descend from the pluripotent cells of the early embryo, and differentiation is apparently hierarchical and directional, and characterised by binary branching points. In vivo pluripotent cells proliferate transiently, and eventually differentiate into multipotent stem and progenitor cells that are restricted in their developmental potential to a subset of cell types.
Not all cells reach terminal differentiation during embryonic development or even during the lifetime of the individual. Somatic stem cells maintain tissues such as the blood and the gut epithelium. Stem cells are characterised by their dual ability to give rise to daughter cells that differentiate along multiple lineages and to self-renew indefinitely, propagating their full lineage potential to daughter cells (Gardner and Beddington, 1988). Stem cells differentiate into progenitor cells, which lack long-term self-renewal capacity and usually have the potential to give rise to a few types of terminally differentiated cell types.

1.1.2 Multistability in gene regulatory circuits: the paradigm case

The small regulatory circuit consisting of two transcription factors (X1 and X2), each of which mutually represses expression of the other, functions as bistable toggle switch, and serves to illustrate the possibility of multiple alternative expression states in gene regulatory circuits. Expression of X1 and X2 is mutually exclusive, but cells expressing one or the other can coexist. This is because the system has two stable steady states corresponding to high expression of one gene and low expression of the other. The third steady state corresponds to co-expression of the two factors, but it is unstable. A cell expressing X1 can be induced to flip states by alleviating its inhibitory impact on the expression of X2, which consequently accumulates and eventually results in repression of X1. The basin of attraction of a steady state is the set of expression values of X1 and X2 from which the system returns to the particular steady state. A cell, whose gene expression state falls within its basin of attraction in state space, will return
to the steady state. Suppose that the two transcription factors each activate and repress different sets of genes, each imposing a distinct gene expression profile when expressed, acting as ideal master regulators (Chan and Kyba, 2013). Then the two stable states of the X1-X2 switch determine distinct cell types, and switching between the states is analogous to the differentiation of one cell type from another.

1.1.3 Binary cell fate choice

Multipotent cells make binary decisions between two alternative lineages. The general theoretical framework for understanding multipotency and binary cellular decision making is based on the observation that many lineage-specifying transcription factors, in addition to forming a mutual repression circuit with a specifier of an alternative fate, also bind to the genomic regulatory regions of their own respective genes and promote expression (Graf and Enver, 2009; Zhou and Huang, 2011). Adding auto-regulatory loops to the X1-X2 circuit modifies the attractor landscape by stabilising the previously unstable steady state corresponding to co-expression of transcription factors both X1 and X2 so that the landscape now consists of three basins of attraction (Huang et al., 2007). Expression states that fall within the middle basin correspond to the multipotent cell type, while the two basins, dictating mutually exclusive expression of the two transcription factors, correspond to the two differentiated lineages. Cell fate commitment corresponds to attractor-transition from the multipotent state to one of the differentiated attractor basins. The
correspondence between the third stable state in this model and the multipotent state is also consistent with the characteristic low-level co-expression of markers of multiple lineages in multipotent cell types, termed multilineage priming (Hu et al., 1997; Månsson et al., 2007; Miyamoto et al., 2002).

1.1.4 Intrinsic and extrinsic cell fate determination and the significance of heterogeneity multipotent populations

Diversification of cell types requires that cells within multipotent populations in fact choose different fates. Variability must be introduced in some form to break the uniformity. External cues, for example molecular signals from surrounding tissues that asymmetrically promote expression of one of the transcription factors, can drive differentiation. However, commitment of two cells to different lineages then requires exposure to different signals and environmental heterogeneity.

Alternatively, pre-existing cell-to-cell variation between individual multipotent cells at the time of commitment can cause cells to differentiate along alternative lineages (Klein and Simons, 2011). Because of the stochastic nature of biophysical processes, the actual copy numbers of each of the transcription factors fluctuate around the steady state values. Such noise manifests as gene expression heterogeneity, which has indeed been observed in both adult and embryonic pluri- and multipotent cell populations (reviewed in Graf & Stadtfeld 2008). Symmetric change in parameters of the cell fate switch, for instance decrease in the strength of auto-induction of both X1 and X2, alter the attractor
landscape, and the multipotent state becomes destabilised (Huang et al., 2007). The attractor basin of the multipotent steady state shrinks with weakening auto-induction, and at the limit the system is reduced to the bistable toggle switch. With the central steady state destabilised, multipotent cells are forced to differentiate. The fate of each cell is determined by its position in state space at the time of forced commitment, and cell fates diverge because of the pre-existing cell-to-cell gene expression variability.

Withdrawal of a signalling molecule that stabilises stem cell self-renewal in culture or in an in vivo niche could result in such a symmetric, destabilising change, enforcing differentiation, but leaving the choice of fate up to the pre-existing lineage bias of each cell. Note that even partial destabilisation of the multipotent attractor can cause differentiation of cells whose expression state falls outside the shrunken basin. Deviation of gene expression from the steady state manifest as increased differentiation propensity under certain conditions and can result in spontaneous differentiation in self-renewing conditions. In contrast, cells whose gene expression state falls close to the steady state remain undifferentiated and continue to stably self-renew even if the multipotent attractor shrinks temporarily. Dynamic variation in the expression of specific lineage-affiliated factors such as signalling pathway components can even result in heterogeneity in responsiveness to instructive differentiation signals. Cell-intrinsic and extrinsic components of cell fate choice are not mutually exclusive, and both are at work to different degrees in specific types of multipotent cells (Palani and Sarkar 2009).
1.2 Gene expression noise

Gene expression variability, also known as gene expression noise, underlies the cell-intrinsic component of lineage choice (Enver et al. 1998; McAdams and Arkin 1999; Huang 2009). Complete understanding of cell fate commitment will include knowledge of the origins of gene expression variability in pluri- and multipotent cells.

Randomness is the rule rather than the exception in cellular processes operating with low copy numbers of participating molecules and in a space crowded with other molecular species at the whims of Brownian motion. Gene expression for instance is a multistep process that involves a sequence of discrete physical stochastic events, starting with binding of transcription factors, recruitment and binding of RNA polymerase, promoter escape and transcription elongation. Translation involves ribosome binding to the mRNA, which is impacted by the strength of the ribosomal binding sites as well as codon usage. The consequence is that there is a lower limit to homogeneity of isogenic populations in homogeneous environments (Maheshri and O’Shea 2007; Ross et al. 1994). However, living systems have evolved to function in presence of stochastic fluctuations, and in some cases molecular noise is even exploited for generation of phenotypic heterogeneity (McAdams and Arkin, 1999). Noise is an evolvable trait tuned by natural selection (Newman et al., 2006; Raser and O’Shea, 2004). The noisiness of the expression of individual genes depends for instance on the strength of promoters and ribosomal binding sites and the efficiency of
transcription and translation. Negative auto-regulation is another method of noise reduction (Becskei and Serrano, 2000).

Bursts of translation of mRNA molecules present at low copy numbers is a major source of gene expression noise in bacteria (Kierzek et al., 2001; McAdams and Arkin, 1997; Thattai and van Oudenaarden, 2001a). In eukaryotic cells, mRNA molecules are produced not with constant probability, but in large bursts of transcription punctuated by silent periods due to the slow process of chromatin remodelling-dependent transitioning of promoters between transcriptionally active and inactive states (Blake et al., 2003; Kaern et al., 2005; Raj et al., 2006; Ross et al., 1994). Variation due to the inherent stochasticity in physical interactions involved in the process of gene expression is referred to as intrinsic noise. It is intrinsic to the expression of the specific gene and results from randomness that is implicit in the biophysical process of gene expression. Transcriptional and translational bursting are manifestations of intrinsic stochasticity.

Variation in the level of transcriptional regulators propagates, and manifests as noise in expression of their targets (Blake et al., 2003; Hooshangi et al., 2005; Pedraza and van Oudenaarden, 2005). Variability in a regulator is one source of extrinsic noise, the fraction of expression noise of a gene that is due to pre-existing variation in factors that impact its expression. If the variation is not pathway-specific but potentially impacts the entire genome, then the noise source is not only extrinsic but also global. By definition, extrinsic and global
sources of expression noise cause coordinated fluctuations in expression of two copies of a gene in a cell, but manifest as cell-to-cell heterogeneity in the population (Elowitz et al., 2002; Swain et al., 2002). Sources of global noise include variation in the number of available RNA polymerase II complexes, ribosomes, the level of cellular energy, cell-to-cell variation in upstream factors originating from intrinsic gene expression noise or partitioning error at cell division (Becskei et al., 2005; Huh and Paulsson, 2011; Volfson et al., 2006).

1.3 Early embryonic development

1.3.1 The first cell fate decision

The first cell fate decision in embryonic development initiates after three rounds of cell division, at the 8-cell stage, and sets apart the trophectoderm (TE) and the inner cell mass (ICM) (Fig. 1.1). The TE becomes the outer layer of the pre-implantation embryo and is destined to give rise to the embryonic portion of the placenta that supports the embryo through development. Its characteristic markers include Cdx2 and Eomes (Strumpf et al., 2005). The ICM forms the inner portion of the early embryo and goes on to give rise to the yolk sac, allantois and amnion, the membrane surrounding the fetus, and the embryo proper (Rossant and Tam, 2004). It is marked by continued expression of the transcription factors Oct4 (Nichols et al., 1998; Palmieri et al., 1994) and Nanog (Chambers et al., 2003; Chazaud et al., 2006; Mitsui et al., 2003). Starting at the 8-cell stage, cells that are born positioned on the outer layer of the morula tend
to adopt the TE fate, while cells on the inside tend to give rise to the ICM without much spatial reorganisation, suggesting that lineage choice is not random. Indeed, segregation of the two lineages is driven by asymmetric inheritance of lineage specifying factors due to polarisation of blastomeres prior to cell division (reviewed in Zernicka-Goetz et al. 2009)

**Figure 1.1. Pre-implantation development.** The first cell fate decision initiates at the 8-cell stage. Outer cells differentiate into trophectoderm (TE), while the cells on the inside form the inner cell mass (ICM). Subsequently ICM cells give rise to the pluripotent epiblast (EPI) and the primitive endoderm (PE), which forms at the surface of the ICM. (Figure reproduced from Zernicka-Goetz et al. 2009)

### 1.3.2 The second cell fate decision

Once established, the ICM cells face the choice between the pluripotent epiblast (EPI), which goes on to give rise to the embryo proper, and the primitive endoderm (PE), which forms extra-embryonic tissues. The transcription factors Nanog and Gata6 are associated with establishment of these two respective lineages (Fujikura et al., 2002; Mitsui et al., 2003; Schrode et al., 2014). Primitive endoderm forms on the outer layer of the ICM, at the interface with the blastocoel, a fluid-filled cavity (Fig. 1.1). Exposure to different environmental cues was thought to drive lineage segregation (Dziadek, 1979; Rossant, 1975).
Contrary to the hypothesis that PE and EPI fate induction is purely position-dependent, E3.5 ICM cells express Gata6 and Nanog in a random, mutually exclusive, ‘salt-and-pepper’ pattern. Gene expression profiles of each cell at this stage are largely predictive of ultimate fate, but the cells remain uncommitted until at least E3.75 (Chazaud et al. 2006; Yamanaka et al. 2010). PE- and EPI-biased progenitors shift to their respective location at the interface with the blastocoel and in the inside of the blastocyst by the time that differentiation is complete. While the model according to which EPI and PE precursors emerge in a random pattern across the ICM has gained much experimental support, it has been noted that positional information may also play a role in ultimate lineage specification (Meilhac et al., 2009; Ohnishi et al., 2014; Plusa et al., 2008).
Figure 1.2. Segregation of the epiblast and primitive endoderm lineages. Nanog and Gata6 form a mutually antagonistic gene regulatory circuit. Gata6 expression is directly induced by Fgf4 signalling through the receptor Fgfr2 and the receptor tyrosine kinase (RTK) – extracellular signal-mediated kinase (ERK) pathway. The first clear cell-to-cell differences are represented by inverse correlation between the expression of Fgf4 and Fgfr2. This results in paracrine rather than autocrine Fgf4 signalling. Fgf4 signalling reinforces the cell-to-cell differences by further induction of Gata6 expression and direct or indirect repression of Nanog. In the early blastocyst cells biased towards epiblast fate are characterised by Fgf4 expression and high Nanog levels, while cells biased towards the primitive endoderm fate express Fgfr2, have high levels of Gata6, but low Nanog. Eventually cells become committed and primitive endoderm-specific gene expression is further reinforced by Fgf4 signalling. (Figure reproduced from Frankenberg et al. (2011))

Nanog and Gata6 are co-expressed at the 8-cell stage, and their expression becomes mutually exclusive in the ICM by E3.75 (64-cell stage) (Chazaud et al. 2006; Guo et al. 2010; Plusa et al. 2008; Kurimoto et al. 2006) (Fig. 1.2). The two transcription factors form a mutually inhibitory circuit that resembles the canonical cell fate switch (Singh et al., 2007). Each factor positively regulates its own expression either directly or indirectly (Artus and Chazaud, 2014; Boyer et al., 2005; Loh et al., 2006; Schrode et al., 2014). Initiation of Gata6 expression and primitive endoderm differentiation crucially depend on Fgf4 signalling through extracellular receptor kinase (ERK). Embryos that lack Fgf4, and those
in which its action is circumvented by inhibitors, fail to form primitive endoderm (Chazaud et al., 2006; Nichols et al., 2009; Yamanaka et al., 2010).

As mentioned above, the ultimate fates of biased ICM cells can be modulated through blastocyst maturation. Daughters of some biased ICM cells contribute to both lineages (Meilha et al., 2009; Plusa et al., 2008), and transplanted PE- and EPI-biased cells can give rise to cells of both lineages, and only loose this capacity at late blastocyst stage (Grabarek et al., 2012). Further, modulation of Fgf4 signalling with pharmacological inhibitors or supplementation with exogenous Fgf4 shifts the fates of ICM cells between EPI and PE at least until E3.75 (Yamanaka et al., 2010).

Fgf signalling through ERK initiates Gata6 expression at the 8-cell stage, after which the mutually exclusive heterogeneous expression pattern of Gata6 and Nanog gradually emerges in the ICM (Frankenberg et al., 2011) (Fig. 1.2). Nanogexpressing EPI-biased cells secrete Fgf4, but lack the receptor Fgfr2 (Guo et al. 2010; Ohnishi et al. 2014). Conversely, Gata6-expressing PE-primed cells express Fgf2r, which transmits the Fgf4 signal and represses Nanog expression in these cells, reinforcing their lineage bias (Frankenberg et al., 2011). At the later stage, primitive endoderm maturation and upregulation of its characteristic markers directly depend on Fgf4 secreted by Nanog-expressing epiblast precursors. The negative correlation between Nanog and Gata6 is lost in the ICM of embryos lacking Fgf4, and ultimately the primitive endoderm and epiblast fail to segregate (Kang et al., 2013). Global gene expression analysis on
the single cell level revealed that Fgf4-null embryos not only fail to form primitive endoderm, but the Nanog-high epiblast-like tissue is also abnormal (Ohnishi et al., 2014).

Lineage segregation in the ICM depends on establishment of distinct identifiable cell subtypes prior to cell fate commitment. Initial heterogeneities are reinforced by the gene regulatory circuit and through cell-cell interactions until the distinct salt-and-pepper patterning emerges. How the cell-to-cell variation initiates is not yet clear.

One study using live-cell imaging to track cells of intact mouse embryos found that ICM cells that originate from the first wave of asymmetric cell divisions of blastomeres are biased towards epiblast fate, while those originating from the later waves of division tend to differentiate into primitive endoderm (Morris et al. 2010). Temporal separation between the births of ICM cells could allow the first wave to start maturing toward a default epiblast fate accompanied by upregulation of Fgf4. The later waves of ICM cells would then be met by a signalling environment that promotes primitive endoderm fate (Guo et al. 2010). However, another study detected no impact of developmental history on the eventual fate of ICM cells (Yamanaka et al., 2010).

Some reports support the hypothesis that initially incoherent cell-to-cell differences due to stochastic gene expression initiate the emergence of the distinct EPI- and PE-biased precursor populations (Ohnishi et al., 2014; Plusa et
Early blastocyst stage ICM cells have been found to exhibit stochastic variation in the expression of individual lineage-associated genes, and the cell-to-cell expression variability is too high for distinguishing subpopulations (Ohnishi et al., 2014; Plusa et al., 2008). The earliest detected pattern is the inverse correlation between the expression of Fgf4 and its receptor Fgf2r (Guo et al. 2010; Ohnishi et al. 2014). Early emergence of this inverse correlation is thought to further reinforce the divergence of EPI- and PE-biased states through cell-cell signalling interactions that feed into the gene regulatory circuit.

Animal development is a self-organising process that crucially depends on the continuously changing interactions between emerging cell types. Analogously, in early embryonic development lineage divergence depends on interactions between different cells within heterogeneous multipotent populations. A population of initially equivalent multipotent cells can exploit the stochasticity inherent in cellular processes to break its symmetry. Feedback regulation in GRNs and cell-to-cell interactions amplify and reinforce initially stochastic cell-to-cell differences, and ensure diversification of the two lineages.

1.3.3 The fate of the epiblast

The epiblast forms the inner epithelial layer of the embryo around the time of implantation, and goes on to give rise to the three germ layers of the embryo proper. Cell fate choice in the epiblast occurs concurrently with gastrulation, the process of the epithelium folding in on itself, establishing the core of the body
plan in the process. Mediation of cell movement and exposure to different signals during gastrulation modulate cell fate, while cell-intrinsic state in turn is likely to play a role in the mediation of gastrulation (reviewed in Heisenberg and Solnica-Krezel 2008). The major signalling pathways involved in gastrulation, Nodal, Fgf, BMP and Wnt affect both cell movement and fate choice.

1.4 Embryonic stem cells

1.4.1 Derivation of embryonic stem cells from the inner cell mass

The first in vitro cultured pluripotent cells, embryonal-carcinoma (EC) cells were derived from tumours (teratocarcinomas) formed by embryos engrafted into mice (Solter et al., 1970; Stevens, 1968, 1970). Evans and Kaufman (1981) and Martin (1981) established the first pluripotent cell lines with normal karyotype directly from the blastocyst stage mouse embryo. These are the cells that are now known as embryonic stem cells (ESCs).

Mouse ES cells are the in vitro equivalents of the ICM (Brook and Gardner, 1997). In contrast with somatic stem cells such as haematopoietic stem cells (HSCs), which continue to self-renew and to replenish the blood of the animal throughout its lifetime, pluripotent cells do not have long-term self-renewal capacity in vivo. The ICM exists only transiently, quickly undergoing differentiation into primitive endoderm and epiblast, followed by commitment to the definitive germ layer fates. The self-renewal capacity of pluripotent cells is
stabilised *in vitro* by the culture conditions, enabling indefinite propagation of ES cells. ES cells have the capacity to contribute to all embryonic lineages, but not to extraembryonic tissues in chimeras (Gardner, 1985; Rossant et al., 2003). In contrast, ES cells can be induced to differentiate into trophectoderm *in vitro*, albeit this requires forced expression of *Cdx2* or forced down-regulation of *Oct4* (Niwa et al., 2000, 2005). ES cells can spontaneously differentiate into primitive endoderm, an event that can also be induced by down-regulating Nanog or forcing Gata-4 or -6 expression (Fujikura et al., 2002; Mitsui et al., 2003). These discrepancies between the *in vivo* and *in vitro* differentiation potentials of ES cells highlight the dependence of the cell’s actual potential on its environment.

Demonstrating pluripotency of mouse ES cells requires either *in vivo* or *in vitro* derivation of tissues of all three definitive germ layers. ES cells expanded from a single clone and allowed to congregate differentiate in the process of embryoid body formation (Martin and Evans, 1975). They differentiate and form teratocarcinomas when injected subcutaneously (Martin, 1981). Pluripotent ES cells are capable of contributing to all three germ layers and the germ line of a developing individual when injected into the blastocyst (Bradley et al., 1984).

**1.4.2 Molecular and signalling underpinnings of pluripotent self-renewal of ESCs**

The intrinsic tendency of ES cells to differentiate is demonstrated by the transient nature of their *in vitro* equivalents. ES cells were first derived and propagated on fibroblast feeders and in serum-supplemented medium (Evans and
Kaufman, 1981; Martin, 1981). Feeders were subsequently replaced by conditioned medium, whose essential component was found to be leukemia inhibitory factor (LIF) (Smith and Hooper, 1983; Smith et al., 1988; Williams et al., 1988). LIF- and serum-supplementation support long-term self-renewal and pluripotency of ES cells. Culture conditions became fully defined in 2003, when Ying et al. (2003) found that in presence of LIF, bone morphogenetic protein 4 (BMP4) can substitute for serum. However, identification of intrinsic determinants of pluripotency was a prerequisite of understanding how extracellular signals are able to support self-renewal.

1.4.2.1 Transcriptional regulators

Oct4 is a POU-domain transcription factor encoded by the Pou5f1 gene whose expression is restricted to toti- and pluripotent cells during development (reviewed in Pesce et al. 1998). It is expressed in the unfertilised egg, in the blastomeres, and it continues to be expressed in the pluripotent cells of the inner cell mass and then the epiblast, while its expression is lost in the trophectoderm and in the primitive ectoderm (Pesce et al., 1998; Rosner et al., 1990; Schöler et al., 1990). Oct4 is indispensable for the formation of the pluripotent ICM and epiblast, and for the maintenance of pluripotent ES cells in vitro, but its expression is not sufficient to maintain pluripotent self-renewal in absence of LIF (Nichols et al., 1998; Niwa et al., 2000). ES cells are sensitive to the dosage of Oct4: its overexpression induces endodermal/mesodermal differentiation, and its severe reduction results in trophectoderm-specification (Niwa et al., 2000).
Sox2 is a regulatory partner of Oct4. The two transcription factors interact at and co-bind genomic regulatory regions of many pluripotency-associated genes, including those of Fgf4 and Nanog (Ambrosetti et al., 1997; Chambers and Tomlinson, 2009; Rodda et al., 2005; Yuan et al., 1995). The Oct4-Sox2 complex induces expression of their own genes, forming a positive feedback loop (Chew et al., 2005; Tomioka et al., 2002). Complete absence of Sox2 results in differentiation, similarly to lack of Oct4. Loss of Sox2 however can be compensated for by expression of wild-type levels of Oct4 from a transgene, suggesting that the main role of Sox2 is in maintenance of Oct4 expression (Masui et al. 2007).

Forced expression of Nanog supports LIF- and BMP-independent constitutive self-renewal of ES cells, but it does not compensate for loss of Oct4 (Chambers et al., 2003). LIF further enhances self-renewal of Nanog-expressing cells, implying that the two function in parallel (Chambers et al., 2003). While Nanog is indispensible for establishment of the pluripotent state in the early embryo, it is not absolutely required for maintenance of pluripotent self-renewal (Chambers et al. 2007). Although genetic deletion of Nanog increases the likelihood of differentiation, long-term self-renewal capacity is not lost, and the cells also retain their ability to contribute to all germ layers in chimeras. Therefore loss of Nanog expression does not induce differentiation, but destabilises the pluripotent self-renewing state (Chambers et al. 2007).
Ivanova et al. (2006) used a short hairpin RNA (shRNA) loss-of-function screen to identify additional transcriptional regulators that positively contribute to ESC self-renewal. The nuclear receptor Estrogen-related receptor b (Esrrb) acts as a reprogramming factor, and interacts with Nanog to induce Oct4 expression (van den Berg et al., 2008; Feng et al., 2009; Zhang et al., 2008). Constitutive expression of Esrrb renders ES cell self-renewal LIF-independent (Zhang et al., 2008). Nanog directly induces Esrrb transcription, and Esrrb is able to functionally substitute for Nanog (Festuccia et al., 2012). Constitutive expression of Esrrb restores stable self-renewal in Nanog-null ES cells, even in absence of LIF. T-box transcription factor 3 (Tbx3) stabilises ESC self-renewal by positively regulating Nanog downstream from the LIF signal (Niwa et al., 2009). Expression of T-cell leukemia oncogene 1 (Tcl1) is directly induced by Oct4, and it promotes proliferation of ESCs (Matoba et al., 2006).

Krüppel-like factor 4 (Klf4) is a target of LIF signalling and its forced expression confers partial independence of LIF (Bourillot et al., 2009; Hall et al., 2009; Niwa et al., 2009). While Klf4 expression is dispensable for ES cell self-renewal, its simultaneous deletion in cells that are also null for the related factors Klf2 and Klf5, leads to differentiation (Jiang et al., 2008). The Klf transcription factors positively regulate Nanog expression, and also share some of its targets (Jiang et al., 2008).

The pluripotency transcription factors each regulate the expression of the others, and together constitute the pluripotency transcription network (Fig.1.3).
While Sox2 and Oct4 are absolutely required for maintenance of pluripotency, their consecutive expression does not confer stable self-renewal on ES cells. The other pluripotency factors stabilise pluripotent self-renewal and many of them confer signal-independence, but they are individually dispensable under normal culture conditions (reviewed in Nichols & Smith 2012). However, loss of expression of some of these core factors predisposes ES cells to differentiation.

Figure 1.3. Schematic of the core pluripotency gene regulatory network and the impact of external stimuli. The transcription factors cross-regulate expression of one another. The maintenance of the naïve pluripotent self-renewing state absolutely depends on sustained Oct4 levels, while Klf2, Klf4, Esrrb, Nanog and Tbx3 are individually dispensable, but loss of their expression predisposes cells to differentiation. LIF signals through Stat3 and upregulates Klf4 expression. Fgf4 is secreted by ESCs and directly or indirectly inhibits expression of Nanog. Tcf3 suppresses Esrrb expression. Multiple pathways that stabilize β-catenin attenuate the action of Tcf3.
1.4.2.2 Extracellular signals

LIF

LIF is a member of the IL6 family of cytokines. It signals through the heterodimeric transmembrane receptor complex that consists of gp130, which is shared between all IL6 family members, and the LIF receptor (LIFR). LIF signals through three pathways: Jack/STAT3, Grb2/ERK, and PI3K/Akt (reviewed in Graf et al. 2011).

STAT3 forms homodimers that translocate into the nucleus in response to LIF signal, where they function as transcription factors. Described targets of STAT3 that are associated with self-renewal and pluripotency include the transcription factor c-Myc (Cartwright et al., 2005), Klf4 and 5 (Bourillot et al., 2009; Hall et al., 2009; Niwa et al., 2009), and Pramel7 (Casanova et al., 2011). STAT3 and Nanog are thought to cooperate in regulation of many pluripotency- and self-renewal-promoting targets (Bourillot et al., 2009; Chen et al., 2008). Constitutive expression of c-Myc and Pramel7 render self-renewal independent of LIF. Activation of ERK is known to promote differentiation, and Pramel7 stabilises pluripotent self-renewal through its inhibitory impact on signalling through ERK (Casanova et al., 2011).

PI3K activation by LIF contributes to the robustness of pluripotent self-renewal through multiple pathways. It inhibits ERK signalling, activates Tbx3, a pluripotency-related transcription factor, which in turn regulates expression of
Nanog and Sox2, and it attenuates Gsk3 signalling (Ling et al., 2013; Niwa et al., 2009; Paling et al., 2004).

**BMP**

LIF is unable to support self-renewal of ES cells in absence of serum or bone morphogenetic protein (BMP). BMP4 suppresses neural differentiation, but in absence of LIF and presence of BMP ES cells differentiate into non-neural lineages. BMP signals through SMADs, which induce expression of Inhibitor of DNA binding proteins (Id) (Nakashima et al., 2001; Ying et al., 2003). LIF and BMP together support maintenance of the unstable pluripotent self-renewing state by blocking differentiation along opposing lineages (reviewed in Chambers and Smith 2004). LIF stabilizes the pluripotency circuit by positively regulating Klf4 and Tbx3 expression through STAT3 and PI3K respectively, while at the same time contributing to its destabilisation by stimulation of ERK signalling.

**MAPK and ERK1/2 signalling**

ERKs are mitogen-activated protein kinases (MAPK) that transmit signals through transmembrane receptors to their downstream effectors through a phosphorylation-cascade (reviewed in Katz et al. 2007). ERKs activate both cytoplasmic targets and nuclear transcription factors through phosphorylating them (Marais et al., 1993).

The negative impact on pluripotency of extracellular signal-regulated protein kinase-1 and -2 (ERK1/2) was first described by Burdon et al. (1999), when they
showed that LIF signalling through Shp2 and ERK1/2 is not only dispensable for ESC self-renewal, but in fact inhibitory. Dysfunction in the pathway leading to ERK1/2 activation prevents differentiation of ES cells (Kunath et al., 2007; Stavridis et al., 2007). Autocrine Fgf4 signalling in, the major inducer of ERK1/2 phosphorylation, enables, but does not itself induce differentiation of ES cells (Kunath et al. 2007). Fgf4-null ES cells resist induction of both non-neural and neural differentiation, and responsiveness to differentiation-inducing signals is restored by supplementation with Fgf4 protein. Concurrently and in agreement with the role of ERK1/2 phosphorylation as a commitment-enabling signal, Stavridis et al. (2007) showed that a period of Fgf-induced Erk1/2 activation is essential for neural differentiation. Fgf signalling through ERK1/2 therefore has similar effects on pluripotent cells in vitro and in vivo. ES cells that lack Fgf4 or in which the Fgf-MEK-ERK pathway is inhibited fail to differentiate along neural or mesodermal lineages, and differentiation can be rescued by addition of exogenous Fgf4 (Kunath et al., 2007; Stavridis et al., 2007). ERK signalling is therefore also required to for differentiation along embryonic lineages. Fgf4 signalling through ERK directly or indirectly represses Nanog expression (reviewed in Lanner & Rossant 2010) (Fig. 1.3).

**Canonical Wnt signalling**

Canonical Wnt signalling has been implicated in the regulation of ES cell maintenance and differentiation, but the evidence was conflicting (Haegele et al., 2003; Otero et al., 2004). Constitutively active β-catenin was found to support self-renewal (Ogawa et al., 2006), and it was later found that β-catenin directly
interacts with Tcf3 and abolishes its negative regulatory impact on the expression of pluripotency-associated genes, including that of Esrrb (Martello et al., 2012; Wray et al., 2011) (Fig. 1.3).

1.4.3 Models of pluripotency

At the centre of the prevailing model of pluripotency is the network of pluripotency transcription factors that, through feedback loops, collectively maintain expression of the network components (reviewed in Ng & Surani 2011). The core circuit functions as a bistable switch, and pluripotency is lost when it is switched off by extrinsic signals (Chickarmane et al., 2006). Pluripotency factors are assumed, in the manner of master regulators, to positively regulate ‘stem cell genes’ and to repress expression of ‘differentiation genes’ (Chickarmane et al., 2006; Young, 2011).

This view is supported by chromatin immunoprecipitation (ChIP) analysis of genomic target sites of the core transcription factors. Nanog and Oct4 seem capable of both positively and negatively regulating expression of their targets (Loh et al., 2006). Targets of Nanog, Oct4 and Sox2 that are active in ES cells tend to be those with positive impact on pluripotency, while the targets that are inactive are enriched for known developmental regulators (Boyer et al., 2005). Mapping the protein interaction network around the core pluripotency factors revealed that they associate with members of multiple known cofactor pathways involved in transcriptional repression, often through mediating chromatin
modifications. Oct4 for instance interacts with polycomb components, and Nanog with the histone deacetylase NuRD (Wang et al., 2006).

It is unclear how explaining the function of the core pluripotency circuit by its role in activation of ‘stemness’ genes sheds any further light on how pluripotent cells function, besides perhaps by collective positive reinforcement of pluripotency factor expression. Repression of ‘differentiation genes’ by pluripotency transcription factors can set up cross-antagonistic interactions with lineage-specifying factors. The detectable presence of lineage-specific factors in ES cells and in the uncommitted cells of the early embryo points in this direction. It is possible that pluripotency factors themselves induce low-level priming of expression of lineage-mediators (Boyer et al., 2005; Teo et al., 2011). The observation that excess Oct4 seems to drive differentiation into primitive endoderm (Niwa et al., 2000) suggest that this assumption may be correct. However in the case of primitive endoderm, as we have seen, the pluripotency factors seem to initiate expression of Gata6 in a less direct manner, by driving Fgf4 production, which non-cell autonomously induces expression of the lineage-associated factor (Frankenberg et al., 2011).

Loh & Lim (2011) proposed that pluripotency transcription factors themselves specify different lineages, and that their simultaneous and balanced expression constitutes the pluripotent state. The fact that overexpression of pluripotency factors, rather then inhibiting differentiation, often drive it supports this model. Oct4 induces primitive endoderm and mesoderm differentiation and suppresses
ectoderm differentiation (Niwa et al., 2000; Szabo et al., 2010; Thomson et al., 2011; Wang et al., 2012). Sox2 promotes neural ectoderm- and inhibits mesoderm differentiation (Kopp et al., 2008; Thomson et al., 2011; Wang et al., 2012). Nanog participates in specification of definitive endoderm in human ESCs (Teo et al., 2011). Other pluripotency factors have also been reported to participate in lineage determination (reviewed in Loh & Lim 2011). Pluripotency factors can act as lineage specifiers by driving expression of lineage-affiliated factors. Consistently with this, Nanog directly drives expression of the definitive endoderm specifier *Eomes* in hESCs, while Tbx3 directs epigenetic remodeling of the *Gata6* promoter to allow transcription (Lu et al., 2011; Teo et al., 2011).

Further support for this emerging model of pluripotency comes from the recent discovery that mouse somatic cells can be reprogrammed to a pluripotent state using lineage specifying transcription factors that are not associated with maintenance of pluripotency (Shu et al., 2013). The initial success of reprogramming through the forced expression of the pluripotency-associated transcription factors Oct4, Sox2, Klf4 and cMyc (Takahashi and Yamanaka, 2006) pointed to the powers of master regulators to enforce cell state transitions that are illegitimate under normal conditions. Various combinations of pluripotency-associated transcription factors, and even expression of epigenetic regulators have been shown to be able to drive reprogramming to pluripotency through secondary induction of the endogenous pluripotency master regulators (reviewed in Papp & Plath 2013). Shu et al. (2013) were able to replace *Oct4* with mesoderm or endoderm specifying transcription factors Gata3, Gata6,
SOX7 or PAX1 in the reprogramming cocktail. Similarly, Sox2 could be replaced with specifiers of ectoderm Sox1, Sox3, RCOR2 or GMNN. GATA3 and GMNN together completely replaced Oct4 and Sox2 respectively in the reprogramming cocktail in presence of Klf4 and c-Myc. The factors substituting for Oct4 promote expression of endo-mesodermal factors and also repress suppress ectoderm-associated expression, while the factors that are able to replace Sox2 promote ectodermal-expression and inhibit mesodermal gene expression. Montserrat et al. (2013) similarly used mesodermal lineage specifiers to replace Oct4 and ectoderm specifiers to replace Sox2 in reprogramming human fibroblasts to a pluripotent state. In essence, establishment of a state of mutual inhibition and self-induction between lineages by expression of opposing lineage specifying transcription factors forms a platform for re-establishment of the pluripotent state.

1.4.4 Naïve and primed pluripotent states

As discussed earlier, mouse embryonic stem cells (mESCs) are derived from the inner cell mass (ICM) of the pre-implantation embryo, and share their gene expression, epigenetic and many of their functional features. More recently pluripotent cell lines have been established from the post-implantation epiblast, and are known as epiblast stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007). EpiSCs have in vitro multi-lineage differentiation potential, and have the capacity to contribute to post-implantation chimeras, but not to pre-implantation ones (Guo et al., 2009; Huang et al., 2012; Tesar et al., 2007). Unlike ESCs, and
similarly to human embryonic stem cells (hESCs), long-term EpiSC self-renewal depends not on LIF, but Fgf and Activin signalling. They express Oct4, Sox2 and Nanog, but their overall gene expression profile is closer to that of hESCs than of mESC. In contrast with mESCs, one of the copies of the X chromosome of XX cells is silenced in both EpiSCs and hESCs. ESCs can be differentiated into EpiSCs, which in turn can be reprogrammed to the ESC state by transfection with the transcription factor Klf4 (Guo et al., 2009). Their similarity to mouse EpiSCs suggests that human embryonic stem cells also represent the developmental stage of the post-implantation epiblast. While human ES cells are derived from the ICM, they inevitably seem to progress to epiblast stage in culture. ES cells and EpiSCs/hESCs are often referred to as ‘naïve’ and ‘primed’ pluripotent states respectively (Nichols and Smith, 2009).

1.4.5 Dynamic heterogeneity in embryonic stem cell populations

Promiscuous and variable expression of lineage-affiliated factors was first described in the haematopoietic system, and formed the basis for the proposal that that lineage commitment is a probabilistic threshold event that depends on heterogeneous lineage priming (Enver and Greaves, 1998; Hu et al., 1997). Given that cell-to-cell variability spontaneously arises in the ICM and plays a key role in lineage segregation, we might expect to see a similar phenomenon in ES cells. Indeed, dynamically heterogeneous expression of pluripotency- and lineage-specific factors and associated lineage biases have been described in mouse ES cells (reviewed in Graf and Stadtfeld 2008). At least three
dynamically interconverting subpopulations have been identified in ESC cultures: one biased towards epiblast, another biased toward primitive endoderm, and a third characterised by low differentiation propensity, described as residing in an early ICM-like state (Fig. 1.4).

Rex1 and Stella both mark the ICM; their expression is lost both in the epiblast and in the primitive endoderm (Clark et al., 2004; Payer et al., 2006). Expression of both of these factors is heterogeneous in serum-based ES cell cultures. While subpopulations with different levels of expression interconvert in self-renewing conditions, cells expressing high Rex1 or Stella have higher chimera- and embryoid body-forming capacity respectively, but differentiate into embryonic lineages inefficiently in vitro (Hayashi et al., 2008; Toyooka et al., 2008). Stella and Rex1 expression mark an ICM-like state of pluripotent self-renewal. ES cells that do not express Stella or Rex1 are predisposed to differentiate into epiblast.

Canham et al. (2010) were able to isolate a subpopulation of Oct4-positive undifferentiated ES cells with very low expression of Hex1, an early marker of the primitive endoderm. Hex1-positive cells are in an early, pre-commitment stage of primitive endoderm specification, but in self-renewal conditions cells transition between biased and non-biased state. Hex1-expressing ES cells are unable to contribute to embryonic tissues in chimeras; rather they contribute to extra-embryonic tissues. Analogously, they tend to form the outer rather than the inner cell layers in embryoid bodies. In contrast, Hex1 negative ES cells, which
are in a non-biased ESC state, efficiently contribute to the embryonic lineages of chimeras and tend to form the inner cell layers corresponding to embryonic tissue in embryoid bodies.

**Figure 1.4. Dynamic heterogeneity in ESC populations.** Prior to commitment ES cells fluctuate between primitive endoderm (PrEnd)-biased state, an epiblast (Epi)-biased state and an ICM-like stably self-renewing state. ICM-like cells are characterised by high levels of Nanog, Stella and Rex1, while the biased cells express less of these pluripotency markers. (Figure adapted from Canham et al. (2010))

Nanog expression is also heterogeneous in serum-based ESC cultures, characterized by a bimodal distribution, and inversely correlation with Gata6 expression (Chambers et al., 2007; Singh et al., 2007). Cells with low Nanog expression are characterised by a higher likelihood of differentiation, but they are not committed, and can transition back into a state with high Nanog expression and stable self-renewal phenotype. The inverse correlation between Nanog and Gata6 expression and the associated functional differences in ESC cultures recapitulate the ‘salt and pepper’ pattern of heterogeneity in the blastocyst prior to primitive endoderm and epiblast segregation (Chazaud et al.,
Heterogeneity in Nanog expression also propagates and drives variability in the expression of other pluripotency factors (MacArthur et al. 2012).

Kalmar et al. (2009) proposed two possible mechanisms that could produce the observed bimodal distribution in Nanog levels, both through system-level dynamics of small gene regulatory circuits. Positive auto-regulation of Nanog is central to the “stochastic state transitions” scenario. The Oct4-Sox2 complex maintains basal expression, while induction of its own expression stabilizes the state with high Nanog expression. Either stochastic fluctuations in Nanog expression or signals that directly induce or repress its expression can cause transitions between the two ‘metastable’ states of Nanog expression. An alternative mechanism that could underlie Nanog bimodality considered by the authors is that of oscillatory dynamics due to negative feedback regulation. Nanog is assumed to induce expression of a factor X, which in turn represses Nanog. Such a system exhibits oscillatory dynamics.

Nanog expression is repressed by Fgf4 signaling through ERK (Hamazaki et al., 2006). Heterogeneity in Nanog and Tbx3 expression is reduced in presence of inhibitors of ERK activation and in ERK2-null cells, suggesting that Fgf4 signaling through ERK contributes to heterogeneity, perhaps due to cell-to-cell variability in perception or transduction of the signal (Hamilton et al., 2013; Ying et al., 2008). This idea is supported by the finding that N-sulfation of the proteoglycan co-receptor of Fgf4, a process that is essential for signaling
through ERK, stabilizes high Nanog, Klf4 and Tbx3 levels and reduces heterogeneity (Lanner et al. 2010).

Nanog heterogeneity has also been shown to reflect variability in the expression of other transcription factors with which it interacts, notably Esrrb. The two transcription factors form a positive regulatory loop, and show correlated, mosaic expression (van den Berg et al., 2008; Festuccia et al., 2012; Loh et al., 2006).

Consecutive Nanog expression reduces cell-to-cell variation in expression of pluripotency-associated transcription factors and suppresses expression of lineage-affiliated genes (MacArthur et al., 2012). It has been identified as a central node in the feedback-rich regulatory network of transcription factors, whose balanced and self-sustaining co-expression defines and maintains the pluripotent self-renewing state (MacArthur et al., 2012). Depletion of Nanog initially results in stochastic divergence of gene expression among the cells, which eventually predisposes them to differentiation, likely by compromising the feedback structure of the pluripotency transcription network (MacArthur et al., 2012). The destabilising effect of loss of Nanog is likely related to its position in the self-sustaining gene regulatory circuit that underlies the naïve pluripotent state.

The degree of cell-to-cell variability in Oct4 levels pales in comparison to heterogeneity in expression of other pluripotency transcription factors, but it has
recently been shown to be critical for establishment of the molecular and functional heterogeneity in ES cell cultures (Karwacki-Neisius et al., 2013). Diversification of ES cells into Nanog-low and Nanog-high fractions depends on the presence of ES cells with relatively high Oct4 expression. Nanog-negative cells are absent from populations of ES cells that lack an Oct4 allele (Oct4+/−) and therefore the capacity for high Oct4 expression. Lack of capacity for high Oct4 expression stabilizes the pluripotent self-renewing state. Cells show enhanced self-renewal capacity and resistance to differentiation. They are also more sensitive to LIF than wild-type cells, show enhanced Wnt signalling, and resistance to the destabilising effect of ERK activation by Fgf4. Further evidence for a role of Oct4 in attenuating expression of Nanog comes from mouse embryos with conditional deletion of Oct4, which show markedly elevated Nanog expression in the presumptive inner cell mass (ICM) (Le Bin et al., 2014). The same study also concluded that eventual primitive endoderm differentiation depends on expression of Oct4 from as early as the 8-cell stage.

1.4.6 Ground state pluripotency

ES cells can be propagated as a remarkably homogenous population with respect to expression of key pluripotency-associated transcription factors and with minimal spontaneous differentiation (Marks et al., 2012; Wray et al., 2010; Ying et al., 2008). This is achieved by culturing cells with two small molecule inhibitors (2i), one of which inhibits Fgf4 signalling through ERK1/2, while the other inhibits Gsk3 signalling, relieving inhibition of Esrrb expression by Tcl3
(Fig. 1.3). 2i medium is a completely defined serum-free medium supplemented with small molecule inhibitors of MEK and Gsk3 signalling (2 inhibitors or 2i). In 2i ES cells can be propagated in absence of LIF and BMP (Ying et al. 2008). ES cells in 2i culture form tight, compact, strongly alkaline phosphatase-positive colonies. This highly stable pluripotent self-renewing state has been termed the ‘ground state’ of mammalian pluripotency. Ying et al. concluded that the naïve pluripotent state of ES cells is intrinsically stable and requires no extrinsic signal to support its stable self-renewal. Recently reprogramming of human ES cells to ground state pluripotency has also been achieved (Takashima et al., 2014).

Autocrine Fgf4 signalling through the ERK pathway promotes transition of ES cells into a state that permits lineage commitment and differentiation, but it does not induce differentiation per se (Kunath et al., 2007; Stavridis et al., 2007). Its expression being under the positive control of Oct4-Sox2 and Nanog, Fgf4 expression is both intrinsic to the embryonic stem cell state and the initiator of its demise (Boyer et al., 2005; Yuan et al., 1995). MEK inhibition block phosphorylation of ERK and sustains cells in an inherently stable pluripotent state that is refractory to differentiation (Ying et al., 2008).

Gsk3 inhibition restores to normal the low proliferation and metabolic capacities of ES cells cultured with inhibitors of ERK signalling (Ying et al., 2008). Gsk3 inhibition also contributes to stabilisation of pluripotent self-renewal through stabilisation of β-catenin, which abrogates inhibition of Esrrb expression by Tcf3 (Martello et al., 2012; Wray et al., 2011; Yi et al., 2011).
Inhibition of MEK and Gsk3 signalling maintains ES cells in the ground state by preventing repression of auxiliary pluripotency factors, transcriptional regulators that are not absolutely required for maintenance of the pluripotent state, but which collectively sustain serum- and LIF-independent self-renewal (Nichols and Smith, 2012). The ‘outer circuit’ of pluripotency factors includes Esrrb, Tbx3, Klf2 and Klf4 (Nichols and Smith, 2012). Inhibitors of MEK and Gsk3 act by blocking the repression of the outer circuit transcription factors. In contrast, LIF/Stat3 and BMP signalling act by inducing expression of pluripotency factors and blocking differentiation. This strategy is apparently insufficient to sustain homogeneous high expression across the population in the face of repressive Fgf4 and Gsk3 signalling.

The functional homogeneity and lack of lineage priming in 2i argues against the hypothesis that heterogeneity is integral to pluripotency and required for lineage commitment (Marks et al., 2012; Nichols and Smith, 2012). It is entirely conceivable that lineage-inducing signals could mediate homogeneous differentiation of ground state ES cells. Rather, it is the population-autonomous symmetry breaking capacity of pluripotent ES cells that likely depends on heterogeneity and lineage priming. Analogously to the process of epiblast and primitive endoderm segregation in the ICM, discussed in section 1.3.2 of this chapter, initiation and reinforcement of cell-to-cell variability has the capacity to initiate differentiation of ES cells along different lineages in absence of outside sources of symmetry breaking.
ES cell populations in serum-based culture exist in a dynamic heterogeneous equilibrium of ICM-like, EPI-primed and PE-primed cells, reflecting already broken symmetry and population poised to diverge in the event of destabilisation of the pluripotent state. Biased cells are ‘metastable’ and are more likely to spontaneously differentiate even under self-renewing conditions, but early ICM-like stably self-renewing cells are also present. Unlike the ICM in vivo, which exist only for a short time prior to commitment, ES cells can be propagated indefinitely, allowing time for the different subpopulations to dynamically interconvert. Small initial cell-to-cell differences are amplified by the gene regulatory network and through cell-to-cell interactions, including Fgf4 signalling through ERK. In 2i culture the emergence of primed subpopulations is inhibited, likely by the inhibition of Fgf4 signalling, and pluripotent self-renewal of individual cells and of the population as a whole is stabilised.

Recently Takashima et al., (2014) succeeded at reprogramming human embryonic stem cells, which normally reside at the epiblast stage of development, to naïve, ground state pluripotency. Transient forced expression of Nanog and Klf2 was able to reset hESCs to ground state, which can then be maintained by inhibition of ERK and protein kinase C (PKC). Reset hESCs share key functional properties, their gene expression and metabolic profile with ground state (2i) mouse ES cells (Fig. 1.5).
1.5 Energy metabolism

Evidence is accumulating in favour of the notion that aspects of energy metabolism actively regulate stem cell self-renewal and differentiation (Folmes et al., 2012; Shyh-Chang et al., 2013; Zhang et al., 2012). A complete picture of the mechanisms that underlie stem cell maintenance and lineage commitment will therefore include understanding of the energy metabolic variables involved and their interactions with the gene regulatory circuits that are also central these processes.

1.5.1 Pluripotent stem cells are more glycolytic than their differentiated progeny

Pluripotent cells both in the early embryo and in culture have low mtDNA copy number and immature mitochondrial cristae, and, in contrast with their differentiated progeny, mouse and human ESCs and iPSCs rely on glycolysis for ATP production to a greater extent, even in aerobic conditions (Chung et al., 2007; Folmes et al., 2011; Panopoulos et al., 2012; Varum et al., 2011; Zhang et al., 2011). During early embryonic development from zygote to blastocyst, there is shift from oxidative ATP production to glycolysis in the epiblast, where conditions are hypoxic, arguing that glycolytic ATP production may be simply a prerequisite for survival in the early embryo (Van Blerkom, 2009). High glycolytic flux seems to be necessary to support high proliferation rate required for both pluripotent self-renewal and nuclear reprogramming to pluripotency (Hanna et al., 2009; Ruiz et al., 2011). Diversion of substrates from the prospect of complete breakdown through mitochondrial oxidative ATP production and
into the glycolytic pathway, and subsequent shunting of intermediates into pentose phosphate pathway contributes to meeting the anabolic requirements of highly proliferative cells (Folmes et al., 2012). In human ESCs, for example, uncoupling protein 2 (UCP2) shifts energy metabolism towards glycolysis by shunting substrate away from mitochondrial oxidative phosphorylation and into the glycolytic pathway (Zhang et al., 2011).

Suppression of OXPHOS in favour of glycolytic ATP production also reduces production of reactive oxygen species (ROS), protecting cells from oxidative damage. Increasing ROS production with a shift toward oxidative metabolism during differentiation also plays a role in mediating lineage commitment in stem cells (Cho et al., 2006; Owusu-Ansah and Banerjee, 2009; Tormos et al., 2011; Zhang et al., 2012).

1.5.2 Active glycolysis enhances nuclear reprogramming to pluripotency both in human and mouse

Consistently with the regulatory role of mitochondrial function in cell fate determination, high mitochondrial membrane potential and a shift toward glycolytic metabolism facilitate nuclear reprogramming. Mouse embryonic fibroblasts sorted from the high end of the mitochondrial membrane potential (MMP) distribution show markedly higher efficiency in reprogramming to iPSCs (Liu et al., 2013). ES cells have strikingly higher MMP than mouse embryonic fibroblasts (MEFs), and the mitochondrial membrane becomes polarised in the process of nuclear reprogramming along with elevation of
glycolytic enzyme expression. Mitochondrial polarisation and increase in glycolysis preceded the shift of gene expression to the pluripotent profile in reprogramming embryonic fibroblasts to pluripotency (Folmes et al., 2011). Stimulation of glycolysis by activation of PDK1 or by supplying D-fructose-6-phosphate increases reprogramming efficiency, while inhibition of glycolysis by the glucose analogue 2-deoxyglucose (2-DG), by inhibition of hexokinase 2 or PDK1, has the opposite effect (Folmes et al., 2011; Panopoulos et al., 2012; Zhu et al., 2010). A shift towards glycolysis is a crucial and active step in nuclear reprogramming of terminally differentiated cells into iPSCs, and energy metabolic variables likely play regulatory roles (Panopoulos et al., 2012; Suhr et al., 2010; Varum et al., 2009).

1.5.3 Hypoxia modulates energy metabolism, self-renewal and differentiation

Hypoxia has been credited with stabilising the stem cell state and self-renewal by shifting the cell away from mitochondrial oxidative metabolism towards glycolysis (Fehrer et al. 2007; Jang and Sharkis 2007). Hypoxia prevents spontaneous differentiation and supports self-renewal of human ESCs, and increases expression of pluripotency markers and transcription factors in these cells (Forristal et al., 2010; Närvä et al., 2013; Prasad et al., 2009). Increased expression of hypoxia inducible factors (HIFs), which in turn regulate transcription, induction of Myc, and activation of Activin/Notch signalling, play various roles in orchestrating cellular response of hESCs to hypoxia. In contrast with its pluripotent self-renewal stabilising impact in hESCs, hypoxia promotes
differentiation in mouse ESCs (Jeong et al., 2007; Katsuda et al., 2013; Schieke et al., 2008; Simon and Keith, 2008).

1.5.4 EpiSCs and hESCs are metabolically distinct from mESCs and reset naïve hESCs

Because both mouse and human ESCs and iPSCs have higher glycolytic flux than their oxidative differentiated progeny, the opposing impact of hypoxia on mouse and human PSCs is puzzling at first glance. However, given the relative shift towards reliance on glycolysis in the early embryo, metabolic differences between pluripotent cells representing two different stages of development would not be entirely unexpected (Van Blerkom, 2009; Folmes et al., 2012). Indeed, direct comparison of energy metabolism of mouse ES cells with that of human ESCs and EpiSCs revealed significant differences (Zhou et al., 2012). Human ESCs and EpiSCs rely almost exclusively on glycolysis for ATP production and have very low oxidative capacity, while mouse ESCs use both glycolysis and OXPHOS. This difference is also reflected in the higher mitochondrial membrane potential exhibited by mouse ES cells (Fig. 1.5). Interestingly, metabolic shift precedes detectable increase in EpiSC-specific marker expression during differentiation of ESCs into EpiSC, suggesting that metabolism plays a regulatory role in the process.

Zhou et al. were able to induce ESC differentiation into EpiSC by forced expression of HIF1α. The factor mediates energy metabolic shift toward glycolysis. It also plays a role in activation of Activin/Nodal signalling, which is
a requirement to sustain EpiSC/hESC self-renewal. Activin/Nodal signalling has in turn been reported to downregulate mitochondrial metabolism (Li et al., 2009). Glycolytic energy metabolism, HIF1α and Activin/Nodal signalling seem to form a feedback loop that initiates and reinforces and distinguishes EpiSC identity. Hypoxia therefore may induce progression of ESCs to the epiblast stem cell stage. Because ES cells and EpiSCs/hESCs require different signalling environments to maintain pluripotent self-renewal, hypoxia-induced EpiSCs would be expected to differentiate in mESC culture, accounting for the opposing impact of hypoxia on EpiSCs/hESCs and mESCs.

Electron transport chain (ETC) complex IV cytochrome c oxidase (COX) family genes were downregulated in EpiSCs compared with ESCs, resulting in reduced COX activity and defective ETC function, explaining the reduced mitochondrial function observed in these cells (Zhou et al., 2012). Low COX expression in EpiSCs compared with mESCs reflects a similar in vivo difference between epiblast and ICM cells.

The recent success of Takashima et al. (2014) in resetting human ES cells to naïve pluripotency, the same developmental stage as that of mouse ES cells, allowed energy metabolic comparison of the two pluripotent stem cell types in a second species (Fig. 1.5). In line with the differences between mouse ESCs and EpiSCs, the authors found that reset cells have higher basal respiration rate and higher oxygen consumption capacity than conventional hES cells, indicative of active mitochondrial respiration. Electron transport chain complex IV
cytochrome c oxidase (COX) gene family expression is elevated in reset cells, and they are also distinguished by higher mitochondrial membrane potential.

Figure 1.5. EpiSCs and hESCs are metabolically distinct from mESCs and reset naïve hESCs. Mitochondrial metabolism is more active, as evidenced by higher membrane potential (MMP) and oxygen consumption rate (OCR), in pluripotent stem cells that are in earlier stages of development, and declines as cells progress to the epiblast stage. These metabolic differences have been described both in mouse (Zhou et al., 2012) and human (Takashima et al., 2014) pluripotent stem cells. Solid arrows represent cell state transitions that can be achieved by change of culture conditions, while dashed arrows represent cell state transitions that can be achieved through forced expression of transcription factors.

1.6 Challenges

Maintenance of the naïve pluripotent state depends on the stable co-expression of a set of pluripotency transcription factors. Temporary loss of expression of some pluripotency factors defines metastable cell states and predisposes cells to differentiation. Fluctuations in the levels of many of these factors propagate through the gene regulatory interactions between these transcription factors and through paracrine Fgf signalling, reinforcing and perpetuating cell-to-cell variability in gene expression, lineage bias and differentiation propensity. Cell-autonomous initiation of this heterogeneity likely underlies multilineage
differentiation of pluripotent cell populations, with no need for heterogeneous extrinsic input to break the symmetry. However, the sources of cellular variability that underlie the heterogeneity are not yet known. The aim of this project is to explore the mitochondrial cell-to-cell variation as source of gene expression noise and functional heterogeneity in mouse embryonic stem cells.
Chapter 2: Materials and Methods

2.1 Cell culture

Media and supplements

GMEM Complete Medium: Glasgow Minimal Essential Medium (GMEM)

- Foetal Calf Serum, 10% (ESC culture tested)
- Non-essential amino acids (Gibco 11140-050)
- Sodium Pyruvate, 1mM (Gibco 11360-070)
- 2-mercaptoethanol
- LIF (tested)
- Pen-Strep, 1:100 (Gibco 15070-063)

LIF: Conditioned media was harvested 4 days after transient transfection of COS7 cells with a plasmid encoding human LIF.

2i:

- 1:1 Neurobasal : DMEM:F-12 (Gibco)
- L-Glutamine, 2mM (Gibco 25030-081)
- N2, 1:200
- B27, 1:100 (Gibco 17504-044)
- 2-mercaptoethanol
- Pen-Strep, 1:100 (Gibco 15070-063)
- PD0325901, 1µM in DMSO
- CHIR99021, 3µM in DMSO
N2 (100x): Insulin (Sigma I-1882) 2.5mg/ml
Apo-transferrin (Sigma T-1147) 10mg/ml
BSA (Gibco 15260-037) 7.5mg/ml
Progesterone (Sigma P8783) 0.002 mg/ml
Putrescine (Sigma P5780) 1.6mg/ml
Na Selenite (Sigma S5261) 3µM
Dissolve in DMEM:F12 (Gibco 21331-020)

**Other tissue culture reagents**

Bovine gelatine (0.1% in PBS)

Trypsin: 0.25% Trypsin (Gibco 15090-046)

EDTA, 1mM

In PBS

**Tissue culture routine**

All cell lines were maintained at 37°C in humidified incubators with 5% CO₂.

Mouse embryonic stem cells were routinely maintained on gelatine-coated tissue culture flasks or plates in GMEM complete medium or in 2i. Plates/flasks were covered with 0.1% gelatine in PBS for a minimum of 5 minutes at room temperature, excess gelatine removed, and cells plated immediately. Medium
was changed daily and cells were passaged when approaching confluency (every 2 to 3 days). To passage, cells were washed in PBS and incubated with Trypsin at room temperature until the cell detached. Cells cultured in 2i were pipetted up and down in trypsin prior to resuspension in min. 5 times trypsin volumes of media. Cells were pelleted by centrifugation (1300rpm for 3-5 minutes), and replated.

Freezing
Cell lines were stored in liquid nitrogen. Cells were pelleted by centrifugation and resuspended in GMEM complete medium with 10% dimethyl-sulphoxide (DMSO). 1mL aliquots of cell suspension were placed in cryovials (Nunc) and placed immediately at -80C in freezing pots. After overnight storage vials were transferred to liquid nitrogen for long-term storage.

Making COS7 conditioned medium (LIF) by FuGENE transfection

10^6 Cos7 cells were plated onto 10 cm tissue culture plates 24 hours prior to transfection. Cells were transfected with plasmid using FuGENE 6 transfection reagent according to the manufacturer’s instructions. Medium was replaced after overnight incubation or max. 24 hours post-transfection to prevent carry-over of FuGENE. Media was harvested and filtered after 3 days of further culture. LIF was tested using colony-forming assays followed by fixation and alkaline phosphatase staining.
Colony forming assay
Cells were plated at single cell density to assess the ability of single mES cells to form undifferentiated colonies. Wells of 6-well tissue culture plates were coated with 0.1% gelatine. Live cells were counted using haemacytometer and Trypthan Blue, and diluted to the final concentration of 250 cells per mL medium. 2 mL of the cell suspension was plated into each well to give a total of 500 cells per well. Cells were cultured for 6 days, after which they were fixed and stained for alkaline phosphatase activity (see molecular biology methods), and scored for the frequency of alkaline phosphatase (AP) positive and negative colonies.

Cells were sorted into FACS tubes, washed and counted prior to plating onto 6-well plates, or directly sorted into growth medium in 6-well plates and immediately placed into an incubator. Both methods gave similar results.

ATP depletion
ESCs were incubated with or without 20mM 2-DG and 10mM sodium azide in PBS 10% FBS as a cell suspension for one hour, after which 10mM final BrU was added. Intracellular ATP was measured prior to addition of BrU. Control cells were incubated in PBS 10% FBS, but without 2-DG and sodium azide.

BrU incorporation assay
Cells were trypsinized and washed in growth medium prior to counting. 5x10^6 cells were placed in FACS tubes, pelleted, and resuspended in growth medium with 10mM 5’-bromo-uridine (BrU) (Sigma). The cells were incubated at 37C
for 1 hour, adding mitochondrial membrane potential-specific dye where required 30 minutes into the incubation time. Cells were subsequently washed and immediately fixed. Fixed and permealibised cells were incubed with conjucated BrdU mouse monoclonal antibody clone MoBU-1 (Invitrogen, B35140).

**Transcription inhibition**
D-ribofuranosylbenzimidazole (DRB) (Sigma) was used at 50µg/mL concentration
α-amanitin (Sigma) was used at 1µg/mL concentration to completely inhibit transcription, and at 0.1µg/mL for attenuation of Pol II transcription elongation.

**Flow cytometry**
Beckman Coulter Gallios machine was used for flow cytometric analysis. The data was analyzed using the Kaluza software.

**Fluorescence activated cell sorting (FACS)**
BD Aria III machine was used for fluorescence activated cell sorting. Cells were collected in tubes with growth medium.

**Measuring mitochondrial membrane potential**
*MitoTracker Red CMXRos* (Invitrogen) was used at 50nM final concentration in cell culture medium. Cells were stained as a cell suspension at 5x10^5 cells per mL density. Cell suspensions were incubated at 37C for 20 to 30 minutes. Cells
were washed with growth medium and resuspended in growth medium for FACS analysis.

*DilC1(5)* (Invitrogen) was used at 50nM final concentration in cell culture medium. Cells were stained as a cell suspension at $5 \times 10^5$ cells per mL density. Cell suspensions were incubated at 37°C for 20 to 30 minutes. Cells were washed with growth medium and resuspended in growth medium for FACS analysis.

*Carbonyl cyanide 3- chlorophenylhydrazone (CCCP)* (Invitrogen) was used at 100µM final concentration. For controls, CCCP was added to the cell suspension prior to adding the mitochondrial membrane potential-sensitive dye.

**Apoptosis Assay**

BD Biosciences PE Apoptosis Kit was used for Annexin V staining according to the manufacturer’s instructions.

**Cell fixation and permeabilisation**

$5 \times 10^5$ cells were washed first in growth medium, then in PBS/10%FCS. To fix the cells, the pellet was resuspended in 100µl 1.6% paraformaldehyde (PFA) in PBS, and incubated at room temperature in the dark for 10 minutes. Cells were washed with 1ml PBS/10%FCS, and the pellet loosened by vortexing. Cells were permeabilized by adding 0.3ml ice-cold 90% methanol (10% PBS), and incubated on ice for 30 minutes. Cells were washed with 10ml PBS/10%FCS.
DAPI staining

Fixed and permeabilised cells were resuspended in 100µl 0.5µg/mL DAPI stain in PBS and incubated on ice for 40 minutes. After washing in 1ml PBS/10%FCS, cells were resuspended in PBS/10%FCS and analysed by flow cytometry.

Measurement of Oxygen Consumption Rate (OCR)

SeaHorse XF24 tissue culture microplates were treated with 0.1% bovine gelatine prior to plating 5x10^5 sorted cells in each well. Cell density titrations were performed using bulk, unsorted culture to determine optimal seeding density. Cells were allowed to attach and were cultured overnight in GMEM and serum-based ESC medium. Wells were washed twice and media were replaced with SeaHorse XF base medium supplemented with 5mM Sodium Pyruvate, 5.5mM glucose and 100x Glutamax. Selective inhibitors of mitochondrial function were injected during the experiment to achieve the final concentrations of oligomycin (1µg/mL), FCCP (2µM) and antimycin A (2.5µM).

2.2 Cell lines

E14Tg2a

ES cell line derived from 129/Ola mice.

Oct4GFP

Targeted Oct4 GFP; green fluorescent protein inserted at the AUG codon of the Oct4 gene. A gift from Austin Smith (University of Cambridge).
Rex1GFP
Targeted Rex1 GFP; green fluorescent protein inserted at the AUG codon of the Rex1 gene. A gift from Austin Smith.

TNGA
Targeted Nanog GFP clone A; green fluorescent protein inserted at the AUG codon of the Nanog gene. Described in (Chambers et al., 2007). A gift from Austin Smith.

FUCCI
FUCCI reporter E14Tg2a cells were a gift from Marta Roccio. They were generated by infecting cells with FUCCI lentiviral vectors. Reporters: Cdt1-KO2 (red), Gem-AG (green).

After thawing FUCCI cells were put through two rounds of sorting prior to use. First cells positive for Cdt1-KO2 were sorted and put back into culture prior to sorting Gem-AG-positive cells. This was to ensure that all cells used in experiments retained both reporters through the freeze-thaw cycle.

2.3 Molecular biology techniques

RNA extraction
0.5 million cells were harvested after FACS sorting, washed once in medium, then in PBS and lysed in buffer RLT (Quiagen RNeasy Mini Kit). Lysates were
stored at -80°C or used directly for RNA extraction. Lysate was homogenised by spinning in Quiashredder column (Quiagen) and RNA was extracted using the Quiagen RNeasy Mini Kit according to the manufacturer’s instructions, and included an on-column DNase digest step (Quiagen) to remove genomic DNA. RNA concentration was determined by Nanodrop.

**cDNA synthesis**

Invitrogen Superscript III First-Strand Synthesis kit was used to synthesize cDNA according to the manufacturer’s instructions and using random hexamers.

**PCR program:**

- 25 °C for 5 minutes
- 50 °C for 45 minutes
- 70 °C for 15 minutes

cDNA was diluted 1:5 in water prior to use in qRT-PCR reactions.
qRT-PCR

Real-time quantitative PCR was performed using Applied Biosystems 7500 Fast Real-Time PCR System machine, using TaqMan probes and TaqMan Universal PCR Master Mix (Applied Biosystems).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Probe ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanog</td>
<td>Mm02384862_g1</td>
</tr>
<tr>
<td>Dppa3 (Stella)</td>
<td>Mm01184198_g1</td>
</tr>
<tr>
<td>Zfp42 (Rex1)</td>
<td>Mm03053975_g1</td>
</tr>
<tr>
<td>Klf4</td>
<td>Mm00516104_m1</td>
</tr>
<tr>
<td>Klf5</td>
<td>Mm00456521_m1</td>
</tr>
<tr>
<td>Esrrb</td>
<td>Mm00442411_m1</td>
</tr>
</tbody>
</table>

ATP luciferase assay

The ATP Lite Luminescence Assay System (PerkinElmer) was used according to the manufacturer’s instructions. Cells were counted and all cell suspensions diluted to the same concentration prior to deposition in wells for ATP luciferase assay.

Alkaline Phosphatase Staining

Fixative: 65% Acetone

25% Citrate (solution from kit)

8% Formaldehyde
Stain: Alkaline Phosphatase (AP) kit (Sigma 86R-1KT) according to the manufacturer’s instructions.

Tissue culture plate well with adherent mES cells was washed once with PBS and fixed for 45 seconds with fixative. Wells were then washed with dH2O. Staining solution was added and plates were incubated for 45 minutes in the dark, washed in dH2O and dried. Stained colonies were scored by eye using a dissecting microscope.

2.4 Statistics

Two-tailed, two-sample Student’s t-test, assuming unequal variances, was used to determine the significance of all observed differences. The number of independent repeats of each experiment (n) is indicated in the figures legends.
Chapter 3: Mitochondrial membrane potential is an axis of functionally relevant cell-to-cell variability in mouse embryonic stem cells

3.1 Introduction

3.1.1 Heterogeneity in pluripotent populations

It is reasonably clear that cell fate in the inner cell mass (ICM) of the blastocyst is not instructively determined by signals outside the ICM (reviewed in Zernicka-Goetz et al. 2009). Rather, small cell-to-cell differences in the expression of key transcription factors are amplified by the gene regulatory circuit that governs the cell fate choice and through signalling interactions between cells of the ICM. The reinforcement and amplification of initially small and likely stochastic cell-to-cell differences eventually lead to crossing the threshold of commitment and cells differentiate along divergent lineages. Pre-commitment heterogeneity represents cell-to-cell variation in lineage bias, which contributes a stochastic component to commitment, while the interactions between differently primed cells act to reinforce cell fate divergence. Pre-commitment heterogeneity is therefore integral to population-autonomous multilineage differentiation.

Heterogeneity reminiscent of that seen in vivo is present in embryonic stem cell cultures. Epiblast-biased and ICM-like states co-exist and inter-convert under
conditions that support self-renewal, and have been isolated based on the expression of ICM-specific markers (reviewed in Graf & Stadtfeld 2008). ICM-like cells are characterised by stable self-renewal, which is demonstrated by their high capacity to form alkaline phosphatase-positive ESC colonies at single cell density and high tumorigenicity. In contrast, biased cells are ‘metastable’ with higher likelihood of differentiation, reflected in their lower colony forming capacity.

In vivo small cell-to-cell variability may be present at the birth of ICM cells (Guo et al., 2010a), which then can be amplified or reversed by input from neighbouring cells until commitment of cells of this transient cell type. In vitro however ES cells can be propagated indefinitely, and cells move between different biased metastable and ICM-like states. Isolated metastable or ICM-like fractions reconstitute the whole spectrum of the original population when returned to culture. The observed dynamic heterogeneity is likely driven by noise in cellular processes that control lineage bias and self-renewal. Given that it is a crucial component of the natural process of multilineage diversification in pluripotent cells, knowledge of the drivers of cell-to-cell variability will be integral to understanding this process.

The stability of the pluripotent self-renewing state varies along multiple axes of transcription marker and pluripotency marker variability, including Oct4, Nanog, Rex1 and Stella expression. Nanog and Esrrb are both dispensable for self-renewal, but absence of either factor increases the probability of differentiation,
reflecting a destabilisation of the embryonic stem cell state (Chambers et al., 2007; Martello et al., 2012).

3.1.2 Metabolism as a regulator of self-renewal and commitment

As discussed in the introduction to this thesis, energy metabolism in stem cells and the changes that occur during differentiation are increasingly suspected to play regulatory roles in mediating self-renewal, lineage choice and differentiation (reviewed in Folmes et al. 2012). Energy metabolism undergoes a shift from active mitochondrial respiration to almost complete reliance on glycolysis with progression from naïve, inner cell mass (ICM) stage to primed, epiblast stage pluripotency (Takashima et al., 2014; Zhou et al., 2012). The metabolic shift may have the power to initiate the transition, we know at least that it is an integral part of the regulatory network driving it (Zhou et al., 2012).

In addition to heterogeneous transcriptional priming, cell-to-cell variation in energy metabolism, if present, could also contribute to functional heterogeneity in naïve pluripotent stem cell populations. Metabolic priming might then impact the differentiation propensity of ES cells. Indeed, Schieke et al. (2008) described metabolic heterogeneity in mouse ES cell populations, and showed that cells with higher metabolic rate, including higher mitochondrial respiration, show higher tumorigenicity, a characteristic of naïve pluripotency.
In this chapter I explore the possibility that similarly to Rex1 and Nanog expression, mitochondrial membrane potential is a functionally relevant axis of cell-to-cell variability in embryonic stem cell populations. I use mitochondrial membrane potential (MMP) as the indicator of metabolic state as it is easily measurable in live cells, and because naïve pluripotent stem cells are distinguished from primed by high MMP in both mouse and human (Takashima et al., 2014; Zhou et al., 2012).

3.2 Results

3.2.1 Mitochondrial membrane potential is associated with the stability of pluripotent self-renewal

Measurement of mitochondrial membrane potential and sort strategy

Mitochondrial membrane potential is measurable by flow cytometry and fluorescence microscopy using fluorescent lipophilic cationic dyes that equilibrate across the mitochondrial inner membrane, with the amount of dye accumulating in the mitochondrial matrix proportional to the polarisation across the membrane. I use MitoTracker Red CMXRos, a fixable dye detectable in the PE channel of flow cytometers, and DiIC1(5) detected in the APC channel. The fluorescence intensity detected by the flow cytometer is a measure of the integrated mitochondrial membrane potential; it depends both on the mitochondrial mass present in the cell and the membrane potential of that mass. Mitochondrial membrane potential is specifically dissipated by carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a mitochondrial uncoupling agent that
discharges the proton gradient. A shift in fluorescence intensity in response to CCCP confirms the specificity of MMP dyes (Fig. 3.1A). Titrations of both mitochondrial membrane potential-specific dyes (CMXRs and DiIC1(5)) were performed and the minimal concentrations selected for use, such that separation from unstained control is obtained and the signal is abolished by CCCP.

Figure 3.1. Flow cytometric measurement of mitochondrial membrane potential (MMP) and sorting strategy. A) Flow cytometric analysis of mES cells stained with the mitochondrial membrane potential-specific dye MitoTracker CMXRs. CCCP uncouples mitochondria and dissipates mitochondrial polarisation. B) Undifferentiated ES cells were selected based on expression of Oct4GFP reporter (gate p5), followed by sorting of MMP low (gate P6) and MMP high (gate P7) cells.

**Colony forming capacity as a measure of the stability of the pluripotent self-renewing state**

A hallmark of ES cells with stable pluripotent self-renewal capacity is their ability to form alkaline phosphatase (AP)-positive colonies. Cells that have lost their ESC identity and are undergoing differentiation do not fail to form AP-positive colonies. Cells that are still identifiable as ES cells at the time of
plating, but whose state is destabilised, have a higher probability of failure to initiate AP-positive colonies. For example, while Nanog is not absolutely required for pluripotent self-renewal, it stabilizes the state. As a result, Nanog-expressing mES cells form significantly more colonies than do the Nanog-negative cells from the same population (Chambers et al., 2007). Similarly, Nanog-/- populations as a whole retain long-term in vitro self-renewal capacity at high density, but form fewer colonies at single cell density than do wild-type ES cells (Chambers et al., 2007). Colony forming capacity at single cell density is a measure of the stability of the pluripotent self-renewing state, and it is used in this work to assess any association between variation in mitochondrial membrane potential and the stability of the embryonic stem cell state.

Mitochondrial membrane potential is associated with the stability of pluripotent self-renewal

Self-renewal capacity of mouse embryonic stem cells that have low or high mitochondrial membrane potential was assessed by colony forming assay. Embryonic stem cells with an Oct4GFP reporter cultured in GMEM complete medium with serum and LIF were stained for mitochondrial membrane potential in single cell suspension in culture medium using the MitoTracker CMXRos dye, followed by fluorescence activated cell sorting (FACS). Live cells were selected based on forward and side scatter characteristics and also Hoechst 33258 exclusion, followed by doublet discrimination based on scatter properties. Cells not expressing the Oct4GFP reporter were excluded from the sort to eliminate cells that have undergone spontaneous differentiation. Finally, 6-8% of
Oct4GFP positive cells exhibiting the lowest and highest mitochondrial membrane potential were sorted, and plated at single-cell density (500 cells/10cm² well of 6-well plates). Sorted cells were cultured for 6 days in GMEM complete medium prior to fixation and staining for alkaline phosphatase (AP) activity, a marker of undifferentiated embryonic stem cells (Fig. 3.1B).

Oct4GFP-positive ES cells with high mitochondrial membrane potential (MMP high) showed remarkably higher colony forming potential than those with low mitochondrial membrane potential (MMP low) (Fig. 3.2). MMP high cells initiated about five times more alkaline phosphatase-positive colonies that are either composed entirely of ES cells or contain ES cells at their core with some differentiated cells surrounding them. Both fractions initiated very few completely differentiated colonies, but MMP low cells consistently gave a higher number of these than did MMP high cells.
Figure 3.2. Oct4GFP-positive ES cells with high mitochondrial membrane potential (MMP) form significantly more alkaline phosphatase (AP)-positive colonies than do those with low MMP. A) Bar chart showing the numbers of alkaline phosphatase (AP)-positive and differentiated colonies initiated by five hundred MMP low or high cells plated. MMP high cells initiated a significantly higher number of total (t-test, p<0.001, n=3) and AP-positive colonies (t-test, p<0.001, n=3). (Error bars represent standard deviation.) B) Representative wells of colony forming assay. Bright spots are AP-positive colonies.

3.2.2 MMP reconstitution and culture at high density

Oct4GFP positive cells with low and high mitochondrial potential were plated at higher density (2x10^4 cells/cm^2) after sorting, and cultured in GMEM complete medium in the presence of serum and LIF. By the fourth day in culture, the two fractions exhibited very similar distributions of mitochondrial membrane...
potential, demonstrating the capacity of each to reconstitute the original population distribution of mitochondrial membrane potential (Fig. 3.3).

![Graph showing distribution of MMP after 4 days in high-density culture of cells sorted as MMP low and high](image)

**Figure 3.3. Sorted MMP low and high cells reconstitute the parental MMP distribution.** Distribution of MMP after 4 days in high-density culture of cells sorted as MMP low and high (FACS data from a representative experiment).

Dissipation of mitochondrial membrane potential is associated with apoptosis (reviewed in Ly et al. 2003). Apoptotic status of MMP low cells would clearly interfere with their colony forming ability, necessitating assessment of apoptotic status of these cells.

To assess the association between mitochondrial membrane potential and apoptotic status in ESCs, cells were co-stained with a mitochondrial membrane potential dye and with fluorochrome-labelled Annexin V. Translocation of membrane phosphatidylserine (PS) to the surface of the cell membrane is an early event in apoptosis. Annexin V is phospholipid-binding protein with high affinity for PS, and its fluorochrome-conjugate can be used to detect apoptotic cells by flow cytometry (Vermes et al., 1995). Gating strategy was identical to that used in other experiments, including doublet discrimination and exclusion of dead cells based on both scatter characteristics and Hoechst 33258 uptake. An Annexin V-positive apoptotic subpopulation was clearly distinct, and these cells
tended to have mid to low mitochondrial membrane potential (Fig 3.4A). Of the
cells routinely gated as ‘MMP low’ in the experiments presented in this thesis,
8-10% were Annexin-V positive, compared with 0.8-2% of ‘MMP high’ cells
(Fig. 3.4B). Apoptotic cells then account for 6-9% of the reduction in colony
number in MMP low cells compared to MMP high cells. The observed
difference in colony forming efficiency was considerably greater than what the
presence of apoptotic cells in the MMP low fraction could have accounted for.

Figure 3.4. Only a slow proportion of MMP low cells have high levels of an apoptosis indicator.
ES cells were stained with Hoechst 33258 to enable exclusion of non-viable cells, with the
mitochondrial membrane potential (MMP)-specific DiIC1(5) dye and PE-conjugated Annexin V to
assess apoptotic status. A) Representative FACS scatter plot with MMP fluorescence intensity (A.U.)
on the x-axis and Annexin V fluorescence intensity (A.U.) on the y-axis. (Colours indicate cell
density.) B) Representative distributions of Annexin V fluorescence of cells gated as MMP high and
low (7% of cells with highest and lowest MMP). Percentage of Annexin V-positive cells is shown.
3.2.3 The MMP low fraction of ESC populations is enriched in epiblast-biased, metastable cells

Oct4 continues to be expressed in the epiblast \textit{in vivo} and in epiblast stem cells (EpiSCs) \textit{in vitro}. It therefore does not exclusively mark the ICM and embryonic stem cells, and the Oct4GFP-positive fraction may therefore include cells that have proceeded to the EpiSC stage of differentiation, and also uncommitted ES cells that are nevertheless biased towards epiblast fate. Indeed, because EpiSCs have lower mitochondrial membrane potential compared with ESCs, sorting Oct4GFP-positive cells from heterogeneous culture may be expected to enrich for EpiSCs if they are present, and for epiblast-biased ESCs (Zhou et al., 2012). Conversely, the MMP high fraction may be enriched for ICM-like ESCs, which would account for their high colony forming capacity irrespective of mitochondrial membrane potential.

Rex1 is a specific marker of the ICM; its expression is extinguished by the epiblast stage of development. Its expression is dynamically heterogeneous in embryonic stem cell cultures, and cells not yet committed, but biased towards the epiblast and embryonic differentiation, are marked by low Rex1 expression (Toyooka et al., 2008).

Flow cytometric analysis of a knock-in Rex1GFP reporter cell line enabled analysis of association between Rex1 expression and mitochondrial membrane potential in a large number of individual cells. The majority of cells with low
MMP lack Rex1, while its expression distribution is bimodal within the MMP high fraction (Fig. 3.5A).

RT-qPCR analysis of confirmed the difference in Rex1 expression between Oct4-positive MMP low and high cells (Fig. 3.5B). Additionally, the pluripotency-associated factors Esrrb, Klf4, Klf5, Nanog and Dppa3 (Stella) are also more highly expressed in MMP high cells (Fig. 3.5B). Consistently with the hypothesis that the MMP low fraction is enriched in epiblast-biased cells, these factors are enriched in the Rex1 expressing, ICM-like cells (Toyooka et al. 2008). The gene expression profile of the MMP high fraction is also more similar to that of unbiased, stably self-renewing ground mESCs achieved in the presence of inhibitors of Gsk3 and Erk signalling (2i) (Ying et al., 2008).
3.2.4 High mitochondrial membrane potential predicts stable pluripotent self-renewal beyond its association with Rex1 expression

To assess the association between mitochondrial membrane potential and colony forming potential without the confounding effect of the presence of metastable biased cells, epiblast-biased cells were excluded based on their lack of Rex1 expression prior to sorting MMP low and high cells. Rex1GFP-positive cells with either low or high mitochondrial membrane potential initiated a greater...
number of AP-positive colonies than did Oct4GFP-positive cells that had low MMP. The difference between MMP low and high cells is reduced, to about a 1.5 fold difference, but it remains significant (Fig. 3.6). This result indicates that high mitochondrial membrane potential is predictive of colony forming capacity beyond its association with expression of markers of the stable early ICM-like state of pluripotent self-renewal.

Figure 3.6. Rex1GFP-positive ES cells with high mitochondrial membrane potential (MMP) form significantly more alkaline phosphatase (AP)-positive colonies than do those with low MMP, ES. A) Bar chart showing the numbers of alkaline phosphatase (AP)-positive and differentiated colonies initiated by five hundred MMP low or high cells plated. MMP high cells initiated a significantly higher number of total (t-test, p<0.001, n=3) and AP-positive colonies (t-test, p<0.001, n=3). (Error bars represent standard deviation.) B) Representative wells of colony forming assay. Bright spots are AP-positive colonies.
3.2.5 High mitochondrial membrane potential indicates more active mitochondrial respiration in mESCs

Mitochondrial membrane potential largely consists of the proton gradient across the inner mitochondrial membrane. The electron transport chain oxidises NADH to NAD$^+$ and proceeds to pass the electrons along the chain in a series of redox reactions to oxygen, the terminal electron acceptor. This process is coupled to the transfer of protons across the membrane, generating the gradient. ATP synthase harvests the energy of the proton gradient by coupling release of protons back into the mitochondrial matrix to catalysis of phosphorylation of ADP to produce ATP. Mitochondrial membrane potential is therefore usually assumed to reflect mitochondrial respiratory activity.

Measurement of oxygen consumption rate (OCR) was used to compare the respiratory activity of mouse ES cells with low and high MMP. Oct4GFP-positive, MMP high and low ES cells were sorted, cultured overnight in a Seahorse XF24 cell culture microplate, and analysed using the Seahorse XF24 Extracellular Flux Analyzer. Cells with high MMP exhibited higher basal oxygen consumption rate than those with low MMP, indicating that high MMP indeed reflects higher mitochondrial respiratory activity in these cells (Fig. 3.7. prior to addition of Oligomycin).

Oligomycin is a phosphorylation inhibitor and blocks the functioning of ATP synthase. The proton pumps are unable to operate in the face of already strong
proton gradient. Eventually NAD+ is depleted and the citric acid cycle fails. Its addition abolished oxygen consumption, confirming that it was due to oxidative phosphorylation. FCCP disrupts the proton gradient and uncouples electron transport chain activity and therefore oxygen consumption from ATP synthesis. Addition of FCCP caused a higher increase in OCR in MMP high cells, indicating that these have higher electron transport chain (ETC) activity. Antimycin A inhibits the electron transport activity of Complex III, disrupting the formation of the proton gradient across the mitochondrial inner membrane. As expected, exposure of the cells to Antimycin A abolished oxygen consumption in both the MMP low and the high fractions.

**Figure 3.7.** High MMP is associated with more active mitochondrial respiration. Basal oxygen consumption rate (OCR) is higher in MMP high cells than in MMP low cells (minute 0 to minute 60). Addition of the solvent DMSO by itself does not affect OCR. Oligomycin abolishes oxygen consumption, confirming that the measured basal OCR was due to oxidative phosphorylation. The uncoupler FCCP causes a larger increase in OCR in cells with high MMP, indicating that these have more active electron transport chain complexes. As expected, exposure of cells to Antimycin A, an inhibitor of ETC complex III, abolishes oxygen consumption. (Error bars indicate standard deviation of data collected from four wells within a single experiment.)
3.2.6 Cell cycle asynchrony does not mediate the difference in colony forming capacity between cells with low and high MMP

To assess the contribution of cell cycle asynchrony to the observed cell-to-cell variability in mitochondrial membrane potential, Oct4GFP ES cells were incubated with an MMP-specific dye, and following fixation and permeabilisation, co-stained with the DNA dye 4’,6-diamidino-2-phenylindole (DAPI), and analysed by flow cytometry. DAPI fluorescence intensity is proportional to the amount of DNA in the cell, which makes it a useful indicator of cell cycle stage with cells in G1 exhibiting half the fluorescence intensity of cells in the G2/M that have completed genome replication (Fig. 3.8A). ES cells are highly proliferative, lacking G0 phase and with a short G1 phase, resulting in a high proportion of cells being in the S and G/M phases of the cell cycle.

Mitochondrial mass is expected to double on average between cell division events. Because the intensity of fluorescence emitted by a cell stained with a mitochondrial membrane potential-sensitive dye depends both on mitochondrial mass and the membrane potential of that mass, the expected cell-to-cell variation of MMP due to cell cycle asynchrony is also about two-fold. Accordingly, the observed mean fluorescence intensity indicative of MMP is about two-fold higher in cells in the G2/M phases than it is in cells in the G1 phase, but there is large cell-to-cell variability of MMP values within each cell cycle stage (Fig. 3.8B).
Because some of the observed cell-to-cell variability in MMP is due to cell cycle asynchrony, the fraction of cells sorted as ‘MMP low’ is enriched in G1 cells, while G2/M cells are overrepresented in the ‘MMP high’ fraction. To control for the impact of cell cycle stage, I sought to assay the colony forming potential of cells with low and high MMP in cells purified based on their cell cycle status.

![Figure 3.8](image)

**Figure 3.8. Cells in later stages of the cell cycle are both larger and have higher mitochondrial membrane potential.** A) DAPI staining and cell cycle stage distribution of Oct4GFP-positive mES cells. B) Mitochondrial membrane potential distributions of cells in the G1, S and G2/M stages of the cell cycle.

Because of the low resolution of cell cycle stages that I was able to achieve by staining live cells with DNA dyes, Fluorescence Ubiquitination Cell Cycle Indicator (FUCCI) E14 mES cells were used to sort cells in the G1 and the S/G2/M stages of the cell cycle (Sakaue-Sawano et al., 2008). The FUCCI system uses the fusion of a red (mKO2) and a green (mAG) fluorescent protein to two cell cycle stage-specific proteins (Cdt1 and Gemini) to indicate the cell
cycle stage of single cells (Fig. 3.9A). Mitochondrial membrane potential was detected using the DiIC1(5) mitochondrial membrane potential-specific dye fluorescence intensity. Use of this dye was necessitated by the large spectral overlap of the MitoTracker Red CMXRos dye with one of the fluorescent reporter proteins of the FUCCI system. Cells with the lowest and highest 7% of DiIC1(5) fluorescence intensity were sorted within each cell cycle fraction. Note that as a result, G1 cells sorted as MMP low and high had lower MMP than did S/G2/M MMP low and high cells respectively.

Both in the G1 and S/G2/M cell cycle stages cells with high MMP exhibited higher capacity to form AP-positive colonies than cells with low MMP (Fig. 3.9). FUCCI cells formed no AP-negative, differentiated colonies. While MMP high cells in G1 and S/G2/M formed a similar number of colonies, cells with low MMP that were in G1 phase consistently formed a fewer colonies than those in the later stages. This difference likely reflects the reportedly higher propensity of ES cells in the G1 phase of the cell cycle to differentiate in response to inducing signals (Coronado et al., 2013; Roccio et al., 2013).
Figure 3.9. ES cells within the different cell cycle stages with high mitochondrial membrane potential (MMP) form significantly more alkaline phosphatase (AP)-positive colonies than do those with low MMP. A) Cells in the G1 and S/G2/M cell cycle stages were sorted separately based on FUCCI reporter fluorescence. B) Bar chart showing the numbers of alkaline phosphatase (AP)-positive colonies initiated by five hundred MMP low or high cells plated. MMP high cells initiated a significantly higher number of colonies whether the cells were in the G1 phase (t-test, p<0.01, n=3) or the G2/M stage (t-test, p<0.001, n=3). (Error bars represent standard deviation.) C) Representative wells of colony forming assay. Bright spots are AP-positive colonies.


3.3 Discussion

Pre-commitment heterogeneity in pluripotent cell populations is proposed to be central to the process of symmetry breaking and population-autonomous lineage segregation. Cell-to-cell variation in the expression of transcription factors and signalling components represent axes of heterogeneity in ESC cultures that are relevant to this process (reviewed in Graf & Stadtfeld 2008). In this chapter I presented evidence that mitochondrial membrane potential is highly variable in ESC populations, and that the stability of pluripotent self-renewal varies along this axis. Given that metabolic variables regulate stem cell self-renewal and can act as drivers of lineage commitment and differentiation (Agathocleous and Harris, 2013; Folmes et al., 2012; Rafalski et al., 2012; Zhang et al., 2012), I hypothesize that cell-to-cell variability in energy metabolism plays a role in population-autonomous lineage segregation.

3.3.1 Mitochondrial membrane potential is associated with differentiation propensity

Oct4-positive mouse ES cells that have high mitochondrial membrane potential (MMP) are in a more stable pluripotent self-renewing state than are those with low MMP, as evidenced by the difference in their colony forming potential under conditions that support self-renewal (Fig. 3.2). Single cell colony forming capacity is a hallmark of stable self-renewing pluripotent states. Loss of Nanog expression for example does not cause differentiation per se, but destabilises
pluripotent self-renewal, which is reflected in the reduced colony forming capacity of Nanog-negative ES cells (Chambers et al., 2007).

The difference in colony forming capacity between ES cells with low and high mitochondrial membrane potential is not accounted for by differences in viability or apoptotic status (Fig. 3.4). The two fractions are morphologically indistinguishable when plated and cultured at high density, and both reconstitute the whole spectrum of MMP within days in self-renewing culture (Fig. 3.3).

The cell’s mitochondrial complement doubles on average between division events. Consistent with this, the mean MMP of ES cells late in the cell cycle (G2/M) is twice as high as the mean MMP of those in the early phase (G1) (Fig. 3.7). The total cell-to-cell variation in MMP is larger than what would be expected if the variation in MMP simply reflected its co-variation with cell cycle stage.

Nevertheless, the fraction of cells sorted as MMP low is enriched in cells in the G1 stage of the cell cycle compared with the MMP high fraction, which largely consists of cells in the G2/M phases (Fig. 3.8). Both mouse and human embryonic stem cells are more sensitive to differentiation signals and have higher differentiation propensity during the G1 stage of the cell cycle, necessitating controlling for cell cycle stage when assessing the association of MMP with colony forming capacity (Calder et al., 2013; Coronado et al., 2013; Pauklin and Vallier, 2013; Roccio et al., 2013). ES cells with high MMP
exhibited significantly higher colony forming capacity than MMP low cells when G1 and S/G2/M cells were assayed separately, confirming that cell cycle staging does not account for the observed differences between MMP low and high cells (Fig.3.9).

3.3.2 ES cells identified as epiblast-primed metastable have lower MMP

Mitochondrial membrane potential variability associates with cell-to-cell differences in gene expression. Namely, many pluripotency-associated transcription factors are more highly expressed in the cells that have MMP (Fig. 3.5 B). In particular, ES cells with low mitochondrial membrane potential tend to exhibit low expression of Rex1 (Fig. 3.5A).

In contrast with Oct4, which continues to be expressed in the epiblast in vivo and in epiblast stem cells (EpiSCs) in vitro, Rex1 is a selective marker of the inner cell mass (Eiges et al., 2001; Rogers et al., 1991). Oct4-expressing ES cells in serum-based culture transition between epiblast-primed and ICM-like states characterised by low and high Rex1 expression respectively, while retaining their ES cell identity (Toyooka et al., 2008) (Fig. 1.4). Similarly to what I see in MMP low cells, the expression of some pluripotency transcription factors is reduced in ES cells with low Rex1 expression. Based on these results I hypothesize that the metastable epiblast-like and the more stable ICM-like subpopulations in ES cell cultures are characterised by distinct metabolic states, which may contribute to the functional differences between them.
Rex1-expressing ICM-like ES cells with low MMP have higher colony forming capacity than that of Oct4-positive cells with low MMP (Fig.3.2 and Fig. 3.6). The significant rescue of colony forming potential of MMP low cells by exclusion of Rex1-negative metastable cells is consistent with the idea that low mitochondrial membrane potential associates with a metastable, lineage-biased ES cell state. Consistent with this, Schieke et al. (2008) found that mouse ES cells with low and high mitochondrial membrane potential exhibit more efficient in vitro mesodermal differentiation and higher tumorigenicity respectively, suggestive of respective epiblast-biased metastable and ICM-like identities.

ES cells in the identified distinct metastable states owe their lineage bias and high differentiation-proneness to transcriptional priming of lineage-specifying transcription factors (reviewed in Graf and Stadtfeld 2008; Huang 2009). Could low mitochondrial membrane potential also represent a shift towards the epiblast fate? If this were the case, one would expect significant energy metabolic differences between ICM- and epiblast stage pluripotent cells, and that the cell-to-cell differences exhibited by ES cells parallel these. Multiple recent reports suggest that this is indeed the case.

Energy metabolism shifts from active mitochondrial respiration in the inner cell mass (ICM) of the blastocyst to heavy reliance on glycolysis in the late epiblast (Van Blerkom, 2009; Folmes et al., 2012). Analogously, mitochondrial respiration is more functional and active in mouse ES cells and reset, naïve
human ES cells than it is in EpiSCs and human ES cells, which rely on glycolysis for ATP production (Takashima et al., 2014; Zhou et al., 2012). HIF1α expression is sufficient to drive EpiSC differentiation from mESCs (Zhou et al., 2012). In contrast with its self-renewal supporting impact on hES cells, hypoxia enhances differentiation of mES cells and increases spontaneous differentiation in self-renewing conditions (Jeong et al., 2007; Schieke et al., 2008; Simon and Keith, 2008), perhaps by driving differentiation into EpiSCs. Culture conditions suitable for mESC maintenance cannot support self-renewal of EpiSCs, which require Fgf and Activin. The ICM and epiblast stages of pluripotency are then characterised not only by differences in gene expression and cell culture requirements, but also by distinct energy metabolic profiles, shift of which can drive differentiation of ESCs into EpiSCs. Significantly, mouse ES cells and reset, naïve human ES cells have higher mitochondrial membrane potential than EpiSCs and epiblast-stage human ES cells (Takashima et al., 2014; Zhou et al., 2012). I found that high mitochondrial membrane potential in ESCs is indicative of high mitochondrial respiration (Fig. 3.7). Considering this result in light of the known differences between ESCs and EpiSCs provides further indication that low MMP is indicative of an epiblast-biased cellular state. In line with these known energy metabolic differences between ESCs and

If Rex1-negative epiblast-biased metastable ES cells are excluded from the analysis, cells with high mitochondrial membrane potential still exhibit significantly higher colony forming potential under self-renewal supporting conditions. Therefore, mitochondrial membrane potential is indicative of the
stability of the pluripotent self-renewing state beyond its association with expression of markers of the early ICM.
Chapter 4: Mitochondrial variability as a source of global transcription rate variability in ES cells

4.1 Introduction

Dynamic molecular and functional heterogeneity is very likely an essential property of stem cell populations (Graf and Stadtfeld, 2008). Beyond cell surface marker and transcription factor expression, more unexpected axes of functionally relevant heterogeneity are being described. High side scatter, a measure of intracellular granularity for example is associated with higher colony forming potential in pluripotent stem cells (Ramirez et al., 2013). In chapter 3 of this thesis I presented evidence that high mitochondrial membrane potential is a marker of stable self-renewal capacity in mouse and human embryonic stem cells.

das Neves et al. (2010) made the fascinating observation that seemingly homogeneous populations of eukaryotic cells transcribe RNA at markedly different rates. The authors found global transcription rate to be sensitive to intracellular ATP concentration, which in turn varied with cellular mitochondrial content and activity, and more generally with the energy state of the cell in HeLa cells. Ultimately the authors traced part of the origin of the observed transcription rate variability to stochastic partitioning of mitochondria at cell division, which produces cell-to-cell variation in mitochondrial complement.
These findings pointed to mitochondrial variability as a source of extrinsic noise in gene expression.

Using the data in the study by das Neves et al, Johnston et al. (2012) formalised in a mathematical model the processes of stochastic partitioning of mitochondria at cell division, the impact of the mitochondrial complement on cell cycle progression and cell growth, using the data in the study by das Neves et al. Using a model of expression of a single gene that incorporates time-varying rate of transcription that depends on mitochondrial status of the cell, Johnston et al. found that mitochondrial variability can be a significant source of extrinsic gene expression noise. The implication is that mitochondrial differences between cells may initiate cell-to-cell variation in gene expression. In pluri- and multipotent cell populations, mitochondrial variability might contribute to population-autonomous symmetry breaking and initiation of lineage divergence.

In this chapter I first present results obtained using small gene regulatory circuit models, suggesting that global transcription rate modulates the stability of multipotent states. My next aim is to explore the possibility that transcription rate variability partially underlies the functional differences between ES cells with low and high mitochondrial membrane potential. To this end, I set out to test whether the connection between cell-to-cell mitochondrial and transcription rate variability described in HeLa by das Neves et al. (2010) is also present in mouse embryonic stem cell cultures. I present evidence that cells in mouse embryonic stem cell populations transcribe RNA at different rates and that this
variation is associated with cell-to-cell variability in mitochondrial membrane potential. Finally, I present evidence that direct pharmacological manipulation of global transcription rate of RNA polymerase II impacts the stability of the self-renewing state of mouse ES cells.

4.2 Results

4.2.1 Transcription rate variability modulates the stability of the multipotent state in models of gene regulatory circuits that function as cell fate switches

Lineage-specifying transcription factors play significant roles in directing differentiation, and also interact and form regulatory circuits with transcriptional regulators of alternative lineages. Mathematical modelling of these gene circuits has led to an understanding of multipotency as a metastable state, in which specifying transcription factors of alternative lineages are co-expressed. This is consistent with the observation that lineage-specific transcription factors and markers are often co-expressed in multipotent cells (Hu et al., 1997).

The canonical cell fate switch for instance consists of two transcription factors, each of which positively regulates its own expression and negatively regulates that of the other. The system has three stable steady states (attractors). Two steady states correspond to the alternative differentiated cell types characterized by high expression of one transcription factor and repression of the other. The third steady state corresponds to the multipotent cell, in which both transcription
factors are expressed. Differentiation corresponds to transition from the multipotent attractor to one of the differentiated ones.

Due to noise in gene expression or in external influences, the gene expression state of individual multipotent cells deviates from the steady state. Cells are ‘dispersed’ in the basin of attraction of the multipotent steady state (Enver et al., 2009; Huang, 2009). The result is cell-to-cell gene expression variability in multipotent stem- and progenitor populations (reviewed in Graf & Stadtfeld 2008). The stochastic component of cell fate choice has been understood to be a consequence of this gene expression heterogeneity, the asymmetric expression of lineage-affiliated transcription factors, which manifests as variability in lineage bias, and which can also result in spontaneous differentiation (Enver et al., 2009; Huang et al., 2007; MacArthur et al., 2009; Palani and Sarkar, 2009). The transcription rate variability associated with mitochondrial variability could be one of the sources of this expression level heterogeneity in multipotent cell populations (Johnston et al., 2012).

We investigated the impact of transcription rate variability on the dynamics of a small gene regulatory model of the Nanog – Gata6 switch that orchestrates the primitive endoderm versus epiblast cell fate choice of the inner cell mass. We used a model proposed by Huang et al. (2007), which describes the canonical cell fate switch with mutual repression between two transcription factors and autoinduction of each of the factors. The model consists of two coupled ordinary differential equations (ODEs) that give the rate of change in NANOG
(x_1) and GATA6 (x_2) protein levels (Equations 1 and 2). The parameters $a_1$ and $a_2$ represent auto-induction rates of NANOG and GATA6, $b_1$ and $b_2$ represent the cross-inhibition rates, and $k_1$ and $k_2$ are the degradation rates. The regulatory interactions are modelled as sigmoidal functions, where $\theta$ represents the inflection point and $n$ is the Hill coefficient, determining the steepness of the function. The levels of $x_1$ and $x_2$ define the state space, and the points in state space from which equations 1 and 2 dictate return of the system to the multipotent steady state constitute the multipotent attractor basin. The attractor basins that correspond to the differentiated states take up the rest of state space.

\[
\frac{dx_1}{dt} = a_1 \frac{x_1^n}{\theta_{a1}^n + x_1^n} + b_1 \frac{\theta_{b1}^n}{\theta_{b1}^n + x_2^n} - k_1 x_1 \quad (1)
\]

\[
\frac{dx_2}{dt} = a_2 \frac{x_2^n}{\theta_{a2}^n + x_1^n} + b_2 \frac{\theta_{b2}^n}{\theta_{b2}^n + x_1^n} - k_2 x_2 \quad (2)
\]

Transcription elongation rate is implicit in all the terms of these equations that represent expression. We therefore modelled variation in global transcription rate by symmetrically varying the parameters $a$ and $b$. Transcription rate variation is modelled by change in the multiplicative factor $\lambda$, so that $a$ becomes $\lambda a$ and $b$ is $\lambda b$. The default parameters are $a_i = b_i = k_i = 1$, $n = 4$, $\theta_{ai} = \theta_{bi} = 0.5$ for $i = 1,2$. We assume that differences in global transcription rate directly translate into not only differences in transcript levels, but also in protein levels.
We found that when we increase global transcription rate ($\lambda$), the multipotent attractor basin expands, it takes up more of state space (Fig. 4.1). When the multipotent attractor basin is small, even small fluctuations in expression of NANOG ($x_1$) and GATA6 ($x_2$) are sufficient for crossing the attractor basin boundaries (commitment boundaries) and result in spontaneous differentiation. In contrast, a large attractor basin allows larger gene expression fluctuations without differentiation, conferring higher robustness on the multipotent state. Cell-to-cell differences in global transcription rate therefore mean that individual cells with different expression levels of NANOG and GATA6 not only represent different points in state space, but the topology of the attractor landscape that determines their thresholds of lineage commitment also differs. Mitochondrial heterogeneity could therefore result in cell-to-cell variability in the stability of multipotent states, and may partially explain why some cells spontaneously differentiate while others continue to stably self-renew.
Figure 4.1 Global transcription rate impacts the stability of the pluripotent state and the commitment threshold. NANOG and GATA6 each induce their own expression and repress that of the other, forming a multistable system that functions as a cell fate switch. The plot shows the structure of the attractor landscape given by equations 1 and 2 for different global transcription rates ($\lambda$) in NANOG – GATA6 expression state space. The multipotent basin widens with increasing global transcription rate.

### 4.2.2 BrU incorporation assays and RNAP II selectivity

5\textsuperscript{'}-bromo-uridine (BrU) is a uridine nucleoside with a bromine atom (Br) on the nucleobase. BrU is taken up by cells from culture medium and processed through the nucleoside salvage pathway into the nucleotide 5-Bromouridine 5\textsuperscript{'}-triophosphate (BrUTP). BrUTP is recognized as UTP, and is incorporated into nascent RNA. Some antibodies raised against 5-Bromo-2\textsuperscript{'}-Deoxyuridine 5\textsuperscript{'}-Triphosphate (BrdUTP), which incorporates into DNA and is used to detect DNA synthesis, are cross-reactive and can be used to detect Br-labelled RNA.
(Br-RNA). Immunofluorescent detection of Br-RNA requires cell fixation and permeabilization. For many cell types fixation with PFA and permeabilization with the triton or saponin works best, but I found that in the case of embryonic stem cells the assay requires PFA fixation followed by permeabilization using methanol.

I used BrU incorporation into nascent RNA to directly measure global transcription rate, and detected the Br-RNA signal using flow cytometry. Incubation of cells with 10mM BrU for 30 minutes or more produces a clear shift in fluorescence signal compared to control incubated in the absence of BrU. Both the concentration of BrU and the time of exposure were determined through titration and time-course experiments. To measure global transcription rate rather than equilibrium RNA content of cells, the values were chosen to reflect Br-RNA signal before it reaches a plateau. The anti-BrdU antibody used for detection of Br-RNA was also titrated. The Br-RNA signal is abolished by RNase I treatment, confirming that it is due to incorporation of BrU into RNA (Fig. 4.2 A).

BrU might be expected to incorporate into all nascent RNA, including rRNAs. Ribosomal RNAs are transcribed by RNA polymerase I, with the exception 5S rRNA, and account for over 50% of RNAs synthesized in the cell. (A specific Pol I inhibitor is CX-5461). RNA Pol III transcribes 5S rRNA, tRNA and other small RNAs. RNA Pol II transcribes all mRNA, most snRNA and microRNA. The components of gene regulatory networks that embody cell types and
transitions between them are produced by Pol II, which highlights it as the polymerase of primary interest for this work.

Multiple lines of evidence to suggest that the Br-RNA signal in the BrU incorporation assay primarily reflects RNA polymerase II transcription activity. Iborra et al. (1996) showed that permabilised cells incorporate BrUTP at around 80% of the normal rate in complete absence of UTP, suggesting that RNA polymerase II activity is largely unaffected by the substitution. In contrast, nucleoli, the site of rRNA transcription, have been observed to incorporate BrU poorly (Iborra et al., 1998). 45S RNA contains only 16% U, while extranucleolar transcripts are much more U rich. Iborra et al. (1998) also observed that RNA polymerase I preferentially incorporates UTP rather than Br-UTP. When cells were incubated with a fixed combined concentration of BrU and U, but decreasing ratio of BrU to U, the decrease in detected Br-RNA in the nucleous is a hundred fold higher than the decrease in the rest of the nucleus, suggesting that RNA polymerase I incorporates much more U in preference to BrU, when available, while RNA polymerase II has much lower such bias.

To confirm that the BrU incorporation assay specifically reflects RNA polymerase II activity in embryonic stem cells, I used two potent and selective inhibitors of RNA polymerase II to dissect the Br-RNA signal produced by RNAP II transcription from that produced by the other polymerases. 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) stalls RNAP II and blocks initiation of transcription elongation through its inhibitory effect on the kinase
activity of the CDK9 subunit of the positive transcription elongation factor P-TEFb (Marshall et al., 1996; Sims et al., 2004). α-amanitin inhibits transcription elongation of RNA polymerases II and III, but Pol II is inhibited at 1µg/mL concentration, while Pol III is not affected under 10µg/mL. Treatment with DRB and α-amanitin is expected to eliminate Br-RNA signal that is due to RNAP II activity, but not any signal due to RNAP I and RNAP III activity. Mouse ES cells incubated with 50µg/mL DRB and 1µg/mL α-amanitin for 15 minutes prior to addition of BrU and also during BrU incorporation showed reduced Br-RNA signal comparable to the signal observed for samples treated with RNase, demonstrating that Br-RNA signal is completely abolished (Fig. 4.2 B, C).

**Figure 4.2.** Measurement of global transcription rate using BrU incorporation into RNA and flow cytometry. A) Analysis of Br-RNA signal of ES cells incubated with BrU for 1 hour. RNase-treatment of permeabilized cells abolishes the Br-RNA signal. B) Representative histogram showing the abolishment of Br-RNA signal by DRB and α-amanitin. C) Mean Br-RNA signal is reduced by DRB and α-amanitin-treatment to a similar level as that of the RNase-treated control. No significant difference in Br-RNA signal was observed between DRB and α-amanitin–treated cells and those treated with RNase after BrU incorporation (t-test, p>0.1, n=5). Mean fluorescence was normalized to controls incubated without BrU to enable comparison of data collected on different days. (Error bars represent standard deviation.)
4.2.3 Cell-to-cell variation in global transcription rate is associated with mitochondrial membrane potential heterogeneity

As mentioned in the introduction to this chapter, global transcription rate partially depends on mitochondrial content and membrane potential in HeLa cells (das Neves et al., 2010). I sought to test whether the same relationship also exists in mouse embryonic stem cells.

The global transcription rate of a population of Oct4GFP reporter E14 mouse embryonic stem cells was assessed using BrU incorporation assay. The cells were also stained with MitoTracker Red CMXRos to measure mitochondrial membrane potential. Flow cytometry analysis permitted simultaneous assessment of global transcription rate and mitochondrial membrane potential in a large number of Oct4GFP-positive cells. To allow analysis of BrU incorporation data collected on different days, all fluorescence intensity values were normalised to that of a control sample prepared and analysed on the same day by incubation without BrU, but with the anti-BrdU antibody.

To assess the global transcription rate difference between cells sorted as mitochondrial membrane potential (MMP) ‘low’ and ‘high’ in the colony forming assays described in Chapter 4, Br-RNA signal of the top and bottom 6% of the mitochondrial membrane potential distribution were compared. Oct4GFP positive ES cells with high mitochondrial membrane potential showed significantly higher mean Br-RNA signal than those with low mitochondrial membrane potential (Fig. 4.3 A,B). The cell-to-cell variation in Br-RNA signal
is larger in the MMP low fraction, with a substantial overlap in Br-RNA signal between the MMP low and high fractions. In particular, many MMP low cells exhibit high Br-RNA signal, while cells with low Br-RNA signal are absent from the MMP high population (Fig. 4.3 A). Comparison of the smallest and largest 6% of cells, as assessed by forward scatter signal, showed that BrU incorporation also increases with cell size (Fig. 4.3 C,D). However, the difference in BrU incorporation rate is larger between MMP low and high cell than it is between small and large cells.

Figure 4.3. Mouse embryonic stem cells with high mitochondrial membrane potential (MMP high) tend to exhibit higher global transcription rate than those with low mitochondrial membrane potential (MMP low). A) A representative histogram showing the distributions of Br-RNA signals in cells with the lowest and highest 6% MMP, obtained using flow cytometry (logarithmic scale, A.U.). B) Bar chart comparing the mean Br-RNA signal in MMP low and MMP high cells (t-test, p<0.01, n=6). (Error bars represent standard deviation.) C) A representative histogram showing the distributions of Br-RNA signals in cells with the lowest and highest 6% forward scatter signal (Small and Large respectively), obtained using flow cytometry (logarithmic scale, A.U.). D) Bar chart comparing the mean Br-RNA signal in MMP low and MMP high cells (t-test, p<0.05, n=6). (Error bars represent standard deviation.)
4.2.4 Cell cycle effects

Mitochondrial compliment and membrane potential increase with cell cycle progression. As discussed in chapter 3, cell cycle asynchrony contributes some of the mitochondrial cell-to-cell variability. To control for the confounding effect of cell cycle stage, I considered cells in different stages of the cell cycle separately, and asked whether cells with higher MMP still show higher rate of BrU incorporation.

Cell cycle status was assessed by DAPI staining of DNA in Oct4GFP reporter ESCs. Prior to fixation the cells were incubated with BrU and MitoTracker Red CMXROS to facilitate quantification of transcription rate and mitochondrial membrane potential. The cells were analysed using flow cytometry. Cells in each stage of the cell cycle (G1, S, G2/M) were selected based on DAPI fluorescence intensity. The 6% of cells with highest MMP showed significantly higher mean global transcription rate than did the 6% of cells with the lowest mitochondrial membrane potential in within each cell cycle phase (Fig. 4.4 A-C).
4.2.5 Intracellular ATP content as a mediator of the connection between mitochondrial membrane potential and global transcription rate

das Neves et al. (2010) presented evidence that in HeLa the relationship between mitochondria and global transcription rate might be mediated through intracellular ATP content. I find evidence that energy status is similarly associated with global transcription rate in ES cells, and that its manipulation modulates global transcription rate.
Intracellular ATP of sorted MMP low and high ES cells was measured using a plate-based luciferase assay in a set number of cells. The intracellular ATP content of ESCs with high mitochondrial potential was significantly higher than that of those with low mitochondrial potential (Fig. 4.5 A).

The association of ATP content with MMP was also assessed separately in cells in different cell cycle stages. FUCCI cells were used to sort cells in the G1 and those in the S/G2/M stages of the cell cycle. Each compartment was further sorted into MMP low and high fractions based on DiIC1(5) fluorescence, followed by luciferase ATP assay after counting the cells. Cells with low mitochondrial membrane potential had lower intracellular ATP levels than those with high mitochondrial membrane potential within the subpopulations in different cell cycle stages (Fig. 4.5 B).

![Figure 4.5. Mitochondrial membrane potential is associated with intracellular ATP content. A) Comparison of normalised luminescence indicative of intracellular ATP in Oct4-GFP positive MMP low and high sorted ES cells. Cells with high MMP have higher ATP content than those with low MMP (t-test, p<0.05, n=3). (Error bars represent standard deviation.) B) Bar chart showing the normalised luminescence indicative of ATP levels in mESCs with low and high MMP and in different stages of the cell cycle. Cells with high MMP have higher ATP levels both in the G1 (t-test, p<0.01, n=3) and in the S/G2/M stages (t-test, p<0.05, n=3) of the cell cycle. Cell cycle stage was determined using the FUCCI reporters. (Error bars represent standard deviation.)](image-url)
Next I sought to manipulate intracellular ATP content to further investigate whether ATP concentration might mediate the relationship between mitochondria and global transcription rate in ES cells. ATP was depleted by incubation of ES cells with both 2-Deoxy-D-glucose (2-DG) and sodium azide. 2-DG is an analog of glucose that is sequestered inside the cell by hexokinase, but cannot be further metabolized, and inhibits the glycolytic pathway. Azide inhibits complex IV (cytochrome oxydase) of the electron transport chain, attenuating mitochondrial respiration and ATP production.

Incubation of mouse ES cells in PBS/10%FBS with 20mM 2-DG and 10mM sodium azide for 1 hour depleted intracellular ATP by 75% (Fig. 4.6 A). As expected, BrU incorporation into RNA was significantly reduced (by 46%) in ES cells after depletion of intracellular ATP compared with controls that were incubated in PBS/10%FCS in absence of the inhibitors (Fig. 4.6 B).

![Figure 4.6. Depletion of intracellular ATP reduces the rate of BrU incorporation into RNA in ESCs. A) Incubation of ES cells with 2DG and azide resulted in a 75% reduction in intracellular ATP (t-test, p<0.001, n=3). (Error bars represent standard deviation.) B) ATP-depletion decreases the mean Br-RNA signal by 45% (t-test, p<0.05, n=3). Mean fluorescence was normalised to mean fluorescence of RNase-treated samples to enable use of data collected on different days. (Error bars represent standard deviation.)](image-url)
4.2.6 Direct inhibition of RNA polymerase II transcription elongation increases the differentiation propensity of ES cells

Seeking direct evidence of the causal relationship between global transcription rate and functional properties of ES cells, I used α-amanitin to manipulate global transcription rate. At low concentrations α-amanitin is a specific inhibitor of RNA polymerase II transcription elongation. It allows polymerase engagement on DNA, but slows elongation by attenuating the movement of the polymerase bridge helix, which is required for translocation along DNA (Bushnell et al., 2001). Mouse ES cells are able to survive and proliferate in the presence of low concentrations of α-amanitin.

ES cells growing at normal density were treated with 0.1µg/mL α-amanitin for 48 hours. Viability, as indicated by Hoechst 33258 exclusion by viable cells, was unaltered by treatment (Fig. 4.7 A). α-amanitin did not alter the proportion of Oct4GFP expressing cells (Fig. 4.7 B) or mitochondrial membrane potential (Fig. 4.7 C) during this period. BrU incorporation assay was performed after 48 hours, and confirmed that α-amanitin-treatment decreased the rate of Br-RNA production (Fig. 4.7 D). In parallel, cells were plated at single-cell density for colony forming assay and cultured for 6 days in the continued presence of α-amanitin. Control cells were cultured in normal conditions without α-amanitin and maintained in the same control medium during the colony forming assay.

α-amanitin significantly reduced the number of colonies formed. Interestingly, α-amanitin-treated cells also formed an increased number of colonies with no
alkaline phosphatase (AP) activity and flattened, spread-out morphology characteristic of differentiated cells (Figure 4.7 E).

**Figure 4.7.** α-amanitin slows transcription and increases differentiation in ES cells. A) Hoechst 33258 exclusion profile as an indicator of viability after 48 hours of α-amanitin treatment of cells grown at normal cell culture density Gates N and R include non-viable cells. B) Representative Oct4-GFP profile of cells after 48 hours of α-amanitin-treatment. C) Representative mitochondrial membrane potential profile of cells after 48 hours of α-amanitin-treatment. Exposure to α-amanitin does not change MMP (t-test, p>0.1, n=3). D) α-amanitin reduces the rate of BrU incorporation into RNA (t-test, p<0.01, n=4). (Error bars represent standard deviation.) E) α-amanitin-treatment reduces the efficiency with which ES cells form alkaline phosphatase (AP)-positive colonies (t-test, p<0.01, n=4), and increases the number of differentiated colonies formed (t-test, p<0.001, n=4). (Error bars represent standard deviation.)

### 4.3 Discussion

In chapter 3 I provided evidence that mitochondrial heterogeneity of mouse embryonic stem cells is associated with functional cell-to-cell variation. Specifically, ES cells with high mitochondrial membrane potential (MMP) are in a more stable self-renewing state than those that have low mitochondrial membrane potential. Previous work showed that cell-to-cell mitochondrial variation is linked with global transcription rate variation in HeLa cells (das
This raised the possibility that as a source of extrinsic noise, mitochondrial variation of ES cells impacts gene expression and the stability of the self-renewing state.

das Neves et al. (2010) found that RNA polymerase II transcribes slower in HeLa cells with low mitochondrial mass and membrane potential compared with those with high MMP. The evidence suggested high sensitivity of Pol II elongation to intracellular ATP concentrations, variability in which is linked to mitochondrial heterogeneity. In this chapter I used flow cytometric detection of BrU incorporation into nascent RNA to quantify the global rate of RNA polymerase II transcription in order to explore the connection between energy metabolic status and global transcription rate is mouse embryonic stem cells. My objective was to provide evidence for evaluation of the hypothesis that cell-to-cell variability in transcription rate contributes to the differences in differentiation propensity between cells with low and high mitochondrial membrane potential described in chapter 3 of this thesis.

Similarly to the case in HeLa cells, I find that transcription rate variability is associated with mitochondrial membrane potential variation in mouse embryonic stem cells. ES cells that have high mitochondrial membrane potential (MMP) tend to transcribe at higher speeds than those with low MMP (Fig. 4.3 A,B). Intracellular ATP content is higher in cells with high mitochondrial membrane potential (Fig. 4.5 A), and depletion of ATP slows Pol II transcription (Fig. 4.6),
suggesting that ATP is likely at least one mediating factor between mitochondrial membrane potential and transcription rate.

Total mitochondrial membrane potential increase with progression of the cell cycle (Fig. 3.7). It was therefore crucial to confirm that the relationship between mitochondria, intracellular ATP and transcription rate still exist when cells in different stages of the cell cycle are considered separately (Fig. 4.5 B). The effect of heterogeneity in cell cycle stage is not negligible, but does not entirely account for the observed relations. Global RNA transcription rate reportedly increases with cell size (Zhurinsky et al., 2010). While I also find that Br-RNA signal is higher in large cells than in small cells, mitochondrial membrane potential variation is a stronger predictor of global transcription rate difference in ES cells.

To provide more direct evidence of the destabilising impact of slow global transcription on the ESC state, I used α-amanitin, a specific inhibitor of Pol II transcription elongation, to experimentally slow transcription. It was the choice of inhibitor because the large cell-to-cell variation in transcription rate in HeLa cells is due to variation in the speed of transcription elongation (das Neves et al. 2010). α-amanitin had little effect on cell viability, but caused marked decrease in the capacity of ES cells to form alkaline phosphatase-positive ESC colonies under culture conditions that support self-renewal. α-amanitin-treated cells had lower colony forming capacity and formed a high number of colonies with distinct differentiated cell morphology and no alkaline phosphatase activity (Fig.
4.7). This result supports the idea that low mitochondrial membrane potential destabilizes the embryonic stem cell state and results in an increased likelihood of spontaneous differentiation by slowing global transcription elongation. The observation that α-amanitin did not alter mitochondrial membrane potential (Fig. 4.7 C) suggests that the observed correlation between mitochondrial membrane potential may be due to increased mitochondrial activity causing increased global transcription rate, but not the reverse.

Our study of small circuit models of cell fate choice predicts that variation in global transcription rate impacts the robustness of multipotent and pluripotent states by impacting the structure of the attractor landscape (Fig. 4.1). At the same time, because transcription and translation rates impact noise in expression of individual genes, mitochondrial heterogeneity may also impact the level of intrinsic noise at the level of individual genes (Thattai and van Oudenaarden 2001; Fraser et al. 2004). Transcriptional bursting is a major source of gene expression noise in eukaryotes (Raj et al. 2006; Raser and O’Shea 2004; Kim and Marioni 2013). Multiple RNA molecules are synthesised when the promoter is active, punctuated by periods of transcriptional inactivity and it is thought to greatly contribute to expression noise in eukaryotes in general, and in mouse embryonic stem cells specifically. Promoter activation dynamics and efficiency of transcription when the promoter is active together determine the noise in RNA production, which impacts fluctuations at the protein level. For example, infrequent large busts of transcription from a gene results in large differences in the copy number of the corresponding mRNA between cells and large
fluctuations in individual cells over time (Ozbudak et al., 2002; Raj and van Oudenaarden, 2008). Transcription elongation rate is an element of transcriptional efficiency, and therefore has the potential to impact the level of noise in expression of individual genes.

Slow RNA pol II are known to transcribe less common splice variants (Dujardin et al., 2014; Howe et al., 2003; de la Mata et al., 2003). Alternative splicing is an underexplored layer of complexity in the regulation of pluripotent cell self-renewal, but Oct4, Sall4 and Tcf3 for example are known to produce alternative transcripts (reviewed in Cheong & Lufkin (2011)). Cell-to-cell variation in global transcription rate may then be expected to result in heterogeneity in the expression of alternative splice variants, which could impact differentiation propensity and self-renewal through this mechanism. Comparison of gene expression using RNA sequencing of ES cells transcribing at different rates will reveal any differences in the presence and proportions of splice variants.
Chapter 5: General Discussion

5.1 Stochastic cell fate and cellular noise

The stochastic nature of stem cell self-renewal, lineage choice and commitment had been noted half a century ago (Nakahata et al., 1982; Ogawa et al., 1983; Till et al., 1964). McAdams and Arkin (1997 and 1998) proposed that gene expression noise and the resulting cell-to-cell variability underlie the stochastic component of cellular decisions. The description of heterogeneous multilineage priming, the variable co-expression of genes associated with multiple alternative lineages in haematopoietic progenitor cells gave us the first glimpse at this pre-commitment cell-intrinsic variability (Enver et al., 1998; Hu et al., 1997). Priming is proposed to modulate the likelihood of commitment of multipotent cells, both under selective and instructive signalling (Enver and Greaves, 1998).

More recently ES cells have also been found to exist in multiple, dynamically interchanging metastable states (Enver et al., 2009; Silva and Smith, 2008). Heterogeneous expression of pluripotency- and lineage-affiliated factors is associated with cell-to-cell variation in lineage bias, self-renewal and differentiation propensity (reviewed in Graf & Stadtfeld 2008). Fluctuations in gene expression likely contribute to the spontaneous differentiation that is observed in ES cell cultures (Smith, 2009). The second cell fate choice in the embryo has been proposed to be initiated by spontaneously emerging cell-to-cell variability in the inner cell mass (Chazaud et al., 2006; Ohnishi et al., 2014).
Therefore, understanding the nature and origins of heterogeneity of pluripotent cell populations is integral to understanding lineage segregation, but also a necessity for efficient control of cell behaviour.

Yet the precise sources of cellular noise that underlie the stochastic component of cell fate choice is one of the outstanding mysteries in stem cell biology. It is years since evidence started coming out of the Iborra lab suggesting that mitochondrial variability might be a significant source of noise in immortal mammalian cells (Johnston et al., 2012; das Neves et al., 2010). Now I present evidence that mitochondrial cell-to-cell variation is a significant source of noise and functional heterogeneity in mouse embryonic stem cells.

5.2 Mitochondrial variability is an axis of functional heterogeneity

Dynamic cell-to-cell variation in the level of pluripotency-associated transcription factors cause variation in the robustness of the naïve pluripotent self-renewing state. Notably, temporary loss of Nanog expression destabilises ES cells, a manifestation of which is the diminished colony forming capacity of Nanog-low cells (Chambers et al., 2007). Similarly, in chapter 3 I find that ES cells with high mitochondrial membrane potential (MMP) have strikingly higher colony forming capacity than those with low mitochondrial membrane potential (Figs. 3.2 and 3.6). The functional differences between these fractions are not due to differences in viability, apoptotic status or cell cycle stage (Figs. 3.3, 3.4 and 3.9). We thus have reason to believe that mitochondrial heterogeneity is a
functionally relevant axis of heterogeneity, and high mitochondrial membrane potential is associated with a stably self-renewing pluripotent state.

5.3 How might mitochondrial heterogeneity originate and how might it impact pluripotent self-renewal and commitment?

das Neves et al. (2010) showed that some of the observed mitochondrial heterogeneity is due to stochastic partitioning of mitochondrial mass at cell division. However, mitochondrial biogenesis and quality control are interlinked with many cellular processes, and variability in these likely also contributes to the observed mitochondrial heterogeneity (Chan, 2006; McBride et al., 2006; Twig et al., 2008). Mitochondrial polarisation and function vary even within individual cells, contributing an additional source of variation (Collins, 2003; O’Reilly et al., 2003). Additionally, the cell’s mitochondrial DNA (mtDNA) complement also impacts mitochondrial function (Strauss et al., 2013).

Primed pluripotent stem cells (mouse EpiSCs and human ESCs) exhibit lower mitochondrial membrane potential than naïve pluripotent stem cells (mouse ESCs and human reset ESCs) (Takashima et al., 2014; Zhou et al., 2012). I hypothesized that the low mitochondrial membrane potential of ES cells reflects a reversible shift towards epiblast fate. Consistent with this, I find that the expression of key pluripotency factors, particularly that of Rex1, in MMP low cells resembles their expected expression in epiblast-primed cells (Fig. 3.5). The difference in mitochondrial membrane potential between naïve and primed
pluripotent stem cells reflects a dramatic metabolic difference between these cells, which is paralleled by a similar shift in metabolism during differentiation of inner cell mass cells into epiblast (Van Blerkom, 2009; Folmes et al., 2012). While mESCs and reset hESCs use both OXPHOS and glycolysis for ATP production, EpiSCs and normal hESCs lack a fully functional electron transport chain and rely heavily on glycolysis (Takashima et al., 2014; Zhou et al., 2012).

In line with these known differences between ICM- and epiblast stage pluripotent cells, I find that ES cells with high mitochondrial membrane potential have higher oxygen consumption rate, indicative of more active mitochondria. This finding is also further evidence that MMP low cells are in an epiblast-biased state (Fig. 3.7). During Activin-induced differentiation of ESCs into EpiSCs energy metabolism-related gene expression changes precede changes of EpiSC lineage markers (Zhou et al., 2012), suggesting that shift in metabolism may be a prerequisite of differentiation. The association between transcriptional priming of epiblast and a shift in energy metabolism towards the epiblast raises the question of which drives which and whether these two aspects reinforce each other.

5.4 Mitochondrial variability is associated with transcription rate variability is ES cells

Changes in anabolic products- and energy output may directly impact the process of gene expression. Indeed, cell-to-cell differences in expression capacity, measured as the variation of the expression level of strong, constitutively active genes, is reportedly a source of gene expression noise in
both prokaryotes and eukaryotes (Colman-Lerner et al., 2005; Maheshri and O’Shea, 2007). das Neves et al. (2010) linked mitochondrial variation and consequent variation in ATP levels to cell-to-cell differences in global transcription elongation rate. The suggestion is that variation in cellular energy directly result in cell-to-cell differences in gene expression (Johnston et al., 2012; das Neves et al., 2010). Therefore, energy metabolic variation may directly impact gene regulatory network dynamics.

I hypothesized that one of the reasons why embryonic stem cells with low and high mitochondrial membrane potential differ in their self-renewal capacities is that they transcribe at different speeds, which impacts the dynamic behaviour of the pluripotency gene regulatory network. I used flow cytometry to measure the rate of BrU incorporation into nascent RNA as a means of quantifying global transcription rate. I presented evidence that the variability in mitochondrial membrane potential is associated with variability in global transcription rate in embryonic stem cells (Fig. 4.3). I find evidence that the association between mitochondrial membrane potential heterogeneity and global transcription rate variation is partially mediated by intracellular ATP levels (Figs. 4.5 and 4.6). I find that direct inhibition of RNA Polymerase II transcription elongation reduces the colony forming capacity of ES cells without impacting viability or mitochondrial membrane potential, supporting the hypothesis that low global transcription rate destabilises the naïve pluripotent self-renewing stem cell state (Fig. 4.7).
5.5 **How might low transcription rate contribute to the destabilisation of the naïve pluripotent self-renewing stem cell state?**

Heterogeneity in global transcription rate would be expected to contribute to cell-to-cell variation in the expression level of individual genes by acting as an extrinsic source of gene expression noise. Cells with high transcription rate would produce more transcripts from individual genes. However, regulatory constraints mean that expression of some genes will be diminished in response to upregulation of their negative regulators in fast-transcribing cells.
Variation in global transcription rate may also impact intrinsic noise levels (Johnston et al., 2012). Transcriptional bursting is a major source of gene expression noise in eukaryotes (Raj et al. 2006; Raser and O'Shea 2004; Kim and Marioni 2013). Due to slow promoter activation dynamics there are periods during which many RNA molecules are synthesized in close succession, punctuated by transcriptional silence. Infrequent large bursts of transcription from a gene result in large differences in the copy number of the corresponding mRNA between cells and large fluctuations in individual cells over time (Ozbudak et al., 2002; Raj and van Oudenaarden, 2008). High transcription elongation rate can increase the burst size, and therefore has the potential to impact the level of noise in expression of individual genes.

Using two alternative variants of the model of the canonical cell fate switch, we show that variation in global transcription rate is expected to alter the stability of the multipotent state by impacting the size of the corresponding attractor basin (Fig. 4.1). Therefore, we expect that mitochondrial heterogeneity contributes to not only cell-to-cell variation in gene expression levels, but also causes cells to have different commitment thresholds due to variation in attractor landscape topology. Given my findings described in chapter 3 that ES cells with high mitochondrial membrane potential have higher colony forming capacity and the evidence I presented in chapter 4 that slow global transcription elongation destabilises naïve pluripotent self-renewal, I expect that the attractor corresponding to the ESC cell type may be stabilised by high global transcription rate.
Variability in global transcription rate may result in differences between cells in the frequency of alternative splice variants. Mutant slow RNA Pol II tends to transcribe less common splice variants (Dujardin et al., 2014; Howe et al., 2003; de la Mata et al., 2003). Slow transcription elongation allows inclusion of alternative exons by providing more time for recognition of alternative splice sites, and by promoting exon skipping by recruitment of negative splicing factors (Dujardin et al., 2014). Oct4, Sall4 and Tcf3 are known to have alternative transcripts, but the role of alternative splicing in regulation of pluripotent self-renewal is underexplored (reviewed in Cheong and Lufkin, 2011).

I expect that gene expression analysis of ES cells sorted directly based on their global transcription rates will provide clues about how global transcription rate impacts gene regulatory network dynamics in these cells. Single cell quantitative RT-PCR using the Fluidigm system has been successfully used to distinguish different metastable sub-states in human embryonic stem cell cultures (Hough et al., 2014). Single cell gene expression analysis may identify different pluripotent sub-states characterised by distinct gene expression profiles, and also be informative of the impact of global transcription rate on the level of gene expression noise.

In summary, I presented evidence suggesting that mitochondrial heterogeneity in embryonic stem cells is linked with variation in global transcription rate, and that
this variation impacts the stability of the naïve pluripotent self-renewing state. As a source of extrinsic gene expression noise, mitochondrial variability may contribute to symmetry breaking in naïve pluripotent stem cells and perhaps even in the early mammalian embryo (Fig. 5.1).
References


Feng, B., Jiang, J., Kraus, P., Ng, J.-H., Heng, J.-C.D., Chan, Y.-S., Yaw, L.-P., Zhang, W., Loh, Y.-H., Han, J., et al. (2009). Reprogramming of fibroblasts into


Jang, Y., and Sharkis, S.J. (2007). STEM CELLS IN HEMATOLOGY A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. 110, 3056–3063.


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