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NEW RESEARCH HORIZON

Understanding pluripotency—how embryonic stem cells keep their options open

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Embryonic stem (ES) cells have the capacity to proliferate indefinitely in culture while maintaining the ability to differentiate to form any of the cells of the body. This unique combination of functions suggests that these cells could provide a potentially unlimited source of differentiated cells for the treatment of disease and aging. Understanding the molecular processes that underpin these functions in ES cells will allow us to harness their potential and develop strategies that control their differentiation. Combination of controlled differentiation with ground-breaking technologies for the reversal of somatic cells to an ES cell-like state promise the generation of patient-derived pluripotent cell lines for the treatment of disease in the future.

Keywords: pluripotency; embryonic stem cell; early primitive ectoderm-like cell; cell reprogramming

Introduction

The ability of embryonic stem (ES) cells to give rise to all cells of the embryo and adult, in culture or within the context of the developing embryo, has been termed pluripotency. Pluripotency resides for a short period of time during the embryonic development of a mammal within the cells of the inner cell mass (ICM) of the blastocyst and the subsequent epiblast of the pre-gastrulation embryo, and is preserved in the primordial germ lineage during later development and into adulthood. In culture, pluripotent cells have been derived from all three embryonic populations. ES cells are derived from the embryonic ICM and maintain their pluripotency in culture. The maintenance of pluripotency within these cells requires extrinsic factors, either added to the growth medium or provided by growth on a feeder layer of differentiated cells (Smith *et al*., 1988; Williams *et al*., 1988), thereby creating an appropriate external signalling environment. It has become apparent that these factors are likely to be ultimately responsible for the maintenance of a network of key transcription factors within the cell that controls pluripotency. This network includes the homeodomain transcription factor Oct4 (Nichols *et al*., 1998; Niwa *et al*., 2000), the variant homeodomain transcription factor Nanog (Chambers *et al*., 2003; Mitsui *et al*., 2003; Kuroda *et al*., 2005; Hough *et al*., 2006) and the high mobility group (HMG)—box transcription factor Sox2 (Avilion *et al*., 2003). The identification of the many common targets and auto-regulatory networks established by these factors provides a current focus for understanding and controlling pluripotency.

Pluripotency can also be re-established in a differentiated or non-pluripotent cell by exposure of the nucleus to external factors. The introduction of the nucleus of a somatic cell into an enucleated oocyte, termed Somatic Cell Nuclear Transfer (SCNT) results in a cell that is able to give rise to all cells of the mature organism, as demonstrated when this process was used to create Dolly the sheep and more than a dozen species of mammalian clones subsequently (Wilmut *et al*., 1997; reviewed in Gurdon and Byrne, 2004). More recently, the use of four specific transcription factors, c-myc, Sox2, Oct4 and Klf4, has been shown to induce pluripotency in both human and mouse somatic cells (Takahashi and Yamanaka, 2006; Takahashi *et al*., 2007; Lowry *et al*., 2008; Park *et al*., 2008a). These studies reveal that differentiation is reversible, and provide strong foundation for the development of technologies that will enable the generation of patient-derived pluripotent cells. Immunologically matched pluripotent cells are an ideal source for the generation of cells for transplantation, and patient-derived pluripotent cells may also be useful tools for the study of disease states and drug therapies.

Loss of pluripotency and commitment to specific cell lineages results in changes in gene expression that include the down-regulation of the key pluripotency transcription factors and the up-regulation of regulators of differentiation (our unpublished data and Ivanova *et al*., 2002; Ramalho-Santos *et al*., 2002), and is accompanied by a range of epigenetic alterations including DNA methylation (Carlone *et al*., 2005; Li *et al*., 2007b; Yeo *et al*., 2007) and chromatin modifications (Azuara *et al*., 2006; Mikkelsen *et al*., 2007). Recent identification of specific combinations of epigenetic marks associated with pluripotency (Azuara *et al*., 2006; Bernstein *et al*., 2006) has highlighted the importance of epigenetic regulation in controlling cell fate. Subsequent modifications that occur on lineage specification are likely to require removal, or erasure to some degree, for pluripotency to be successfully re-established during cell reprogramming (Armstrong *et al*., 2006b).

This review focuses on the signalling pathways required to maintain mouse and human ES cells in culture, recent advances in our understanding of the transcriptional networks maintaining pluripotency

downstream of these pathways, mechanisms for the derivation of pluripotent cell lines and an overview of the epigenetic changes underlying these processes. We also discuss, briefly, the use of related pluripotent cell populations, early primitive ectoderm-like (EPL) cells (Rathjen *et al*., 1999) and the newly isolated EpiSC epiblast stem cells (Brons *et al*., 2007; Tesar *et al*., 2007), for understanding the molecular basis of pluripotency.

ES cells—sending the right signal

ES cells require extrinsic growth factors for maintenance of pluripotency in culture, suggesting that pluripotency is an inherently unstable cell state and that ES cells are 'primed' for rapid differentiation. Historically, ES cells were cultured in the presence of an underlying feeder layer of mitotically inactivated fetal fibroblast cells which provides an environment capable of supporting pluripotency. The requisite factor for self-renewal of mouse ES cells was subsequently determined to be leukaemia inhibitory factor (LIF), a cytokine able to maintain mouse ES cells in the absence of the fibroblast cell feeder layer (Williams *et al*., 1988). LIF is not required for pluripotency of the ICM *in vivo* (Nichols *et al*., 2001) and is unable to maintain pluripotency in human ES cells suggesting that alternative mechanisms function in the maintenance of pluripotency within these contexts. Serum is also important for mouse ES cell maintenance, although bone morphogenic protein 4 (BMP4) is able to replace this requirement (Ying *et al*., 2003; Qi *et al*., 2004). In addition, Wnt signalling has been found to act synergistically with LIF to maintain pluripotency in mouse ES cells and appears to have a role in human ES cells (Sato *et al*., 2004; Ogawa *et al*., 2006). Autocrine loops of Activin/Nodal signalling have also been implicated in the maintenance of mouse ES cells (Ogawa *et al*., 2007).

Mouse and human ES cells exhibit distinct growth habits: doubling rates for human ES cells are characteristically longer than those recorded for mouse ES cells, between 30 and 40 h (Park *et al*., 2008b), human ES cells require maintenance of cell–cell contacts for propagation, and spontaneous differentiation within human ES cell colonies is initiated from central cells (Oh *et al*., 2005), whereas spontaneous differentiation of mouse ES cells occurs at the colony periphery. Human ES cells are routinely cultured on a fibroblast cell feeder layer, but their growth factor requirements also differ from those of mouse ES cells. The growth factors capable of promoting pluripotency in this system appear to be fibroblast growth factor (FGF2), produced by the feeder layer, and insulin-like growth factor (IGF), secreted by human ES cells, which setup interdependent paracrine loops (Bendall *et al*., 2007). Studies focusing on extrinsic signals required for maintaining human ES cells have found FGF2 is sufficient to support growth of these cells on Matrigel, a substrate made up predominantly of laminin and collagen but with additional unknown factors (Xu *et al*., 2005). It is likely extrinsic signals maintaining human ES cells will inhibit BMP signalling to sustain proliferation without differentiation. BMP4 has been shown to negatively regulate pluripotency and induce trophoblast-like cell formation from human ES cells (Xu *et al*., 2002). Activin/Nodal and FGF2 are capable of maintaining human ES cells in the absence of feeder layers and other exogenous factors (Vallier *et al*., 2005). One role of Activin A in the maintenance of human ES cells has been postulated as inhibition of the BMP4 signalling pathway mediators Smads 1 and 5. Activin A and Fgf2 have recently been shown to facilitate derivation and maintenance of pluripotent mouse epiblast-derived cell lines (Brons *et al*., 2007; Tesar *et al*., 2007).

Although culture conditions and extrinsic growth factors that can support pluripotent cell maintenance have been identified, how the signalling pathways controlled by these factors act to maintain the intrinsic transcription factor network required for pluripotency is poorly understood. In mouse ES cells, LIF activates JAK/STAT signalling and mitogen activated protein kinase (MapK) pathways. The choice between pluripotency and differentiation is dependent on a balance between Stat3 and extracellular signal regulated kinase (ERK) MapK activity, respectively. In mouse ES cells, BMP4 prevents differentiation through the inhibition of ERK (Qi *et al*., 2004) and induction of other inhibitors of differentiation such as Ids (Ying *et al*., 2003). Stat3 activates a number of genes that play important roles in pluripotency, including *c-myc* (Cartwright *et al*., 2005), *nanog* (Chambers *et al*., 2003; Mitsui *et al*., 2003; Darr *et al*., 2006; Suzuki *et al*., 2006), *eed* (Ura *et al*., 2008), *jmjd1a* (Ko *et al*., 2006) and *GABPα*, which appears to be required for the maintenance of Oct4 expression (Kinoshita *et al*., 2007).

Neither STAT3 nor BMP4 activity are implicated in pluripotency of human ES cells (Xu *et al*., 2002; Humphrey *et al*., 2004), while ERK activity is required for the maintenance of pluripotency (Armstrong *et al*., 2006a; Li *et al*., 2007a). Moreover, sustained activation of c-Myc in human ES cells induces differentiation and apoptosis (Sumi *et al*., 2007). Thus, consistent with the differing extrinsic requirements, the intracellular signals regulating pluripotency in mouse and human ES cells appear to share little commonality, despite the conservation of the core transcription factor networks (see below) and functional similarity of the cells.

Transcriptional regulation of pluripotency—key players in the network

A major advance in understanding the pluripotent state has come with the identification of a network of auto- and cross-regulatory control mediated by three key transcription factors—Oct4, Nanog and Sox2 (see below; Boyer *et al*., 2005; Loh *et al*., 2006). Each of these factors is required for pluripotency both *in vivo* and *in vitro*. Loss of Oct4 or Nanog results in the loss of pluripotency and the spontaneous differentiation of cells to trophectoderm and primitive endoderm respectively (Nichols *et al*., 1998; Niwa *et al*., 2000; Chambers *et al*., 2003; Mitsui *et al*., 2003; Hough *et al*., 2006). Overexpression of Nanog, but not Oct4, is able to maintain pluripotency in the absence of LIF in mouse ES cells (Chambers *et al*., 2003; Mitsui *et al*., 2003) and in the absence of a feeder layer in human and primate ES cells, although the cells appear to exhibit primitive ectoderm-like characteristics (Darr *et al*., 2006; Yasuda *et al*., 2006). Sox2 is expressed in both ICM and early primitive ectoderm (Wood and Episkopou, 1999; Avilion *et al*., 2003) and $Sox2^{-/-}$ mouse embryos arrest at a similar time to $Oct4^{-/-}$ and $Nanog^{-/-}$ embryos (Avilion *et al*., 2003).

Recently, two groups have applied chromatin immuno-precipitation (ChIP) using antibodies directed against these key transcriptional regulators to determine target genes in both mouse and human ES cells. These studies showed that many target genes were bounded by the combinations of Oct4, Nanog and Sox2 in human ES cells (Boyer *et al*., 2005) and Oct4 and Nanog in mouse ES cells (Sox2 was not examined; Loh *et al*., 2006). The results also confirmed the numerous earlier studies describing direct regulation of the key regulators by each other and auto-regulation of the factors themselves. The targets described included both actively transcribed genes and inactive genes, consistent with a role for these regulatory factors in the maintenance of pluripotency via both active and repressive mechanisms. Substantial differences were observed between Oct4/Nanog/Sox2 targets in mouse and human ES cells; these differences have been the subject of much speculation. It may be that core regulatory mechanisms are conserved although many direct target genes of the pluripotency regulating factors clearly are not. These three transcription factors bind to many hundreds of promoters in ES cells, and the transcriptional activity

of the genes varies. This suggests that, while Oct4, Sox2 and Nanog impose a general program for maintaining pluripotency, other factors act with them to control expression of individual genes.

Recent analysis of an extended Oct4, Sox2, Nanog transcription factor network in mouse ES cells included the Oct4- or Nanog-interacting proteins Rex1, Dax1, Zpf281 and Nac1 (Wang *et al*., 2006; Kim *et al*., 2008) and two pluripotency-associated factors c-Myc (Cartwright *et al*., 2005), and Klf4 (Jiang *et al*., 2008). This genome-wide screen of promoters bound by each of the factors found that many promoters (~800) were occupied by multiple, common factors. Moreover, promoters bound by few of the factors were generally inactive, whereas those bound by four or more of the factors were more likely to be active in ES cells and repressed on differentiation (Kim *et al*., 2008). The second major finding was that genes bound by Myc and Rex1 were distinct from those bound by the other factors (Kim *et al*., 2008), with Myc-association correlating more highly with expressed genes, suggesting that Myc regulation differs from that of the other pluripotency factors. This work also proposes a hierarchy of factors, where Klf4 is an upstream regulator of larger feed-forward loops containing Oct4 and Sox2, a regulator of common targets including Nanog and a potential regulator of Myc, as it binds the c-Myc promoter (Fig. 1).

Although a role for Sox2 in pluripotency has been established, the major function of this factor in the stable network appears to be maintaining Oct4 expression (Masui *et al*., 2007). However, an additional role for Sox2 in establishing a pluripotent transcriptional network cannot be overlooked (Takahashi and Yamanaka, 2006; Okita *et al*., 2007; Wernig *et al*., 2007). New work has also questioned the requirement for Nanog in maintaining pluripotency, as mouse ES cells with no detectable Nanog expression can proliferate as pluripotent stem cells (Chambers *et al*., 2007). Rather, Nanog may act to suppress differentiation and stabilize the pluripotent state. This and other work (Enver *et al*., 2005) suggest that ES cell populations are heterogeneous and cells fluctuate between states with no loss in potency.

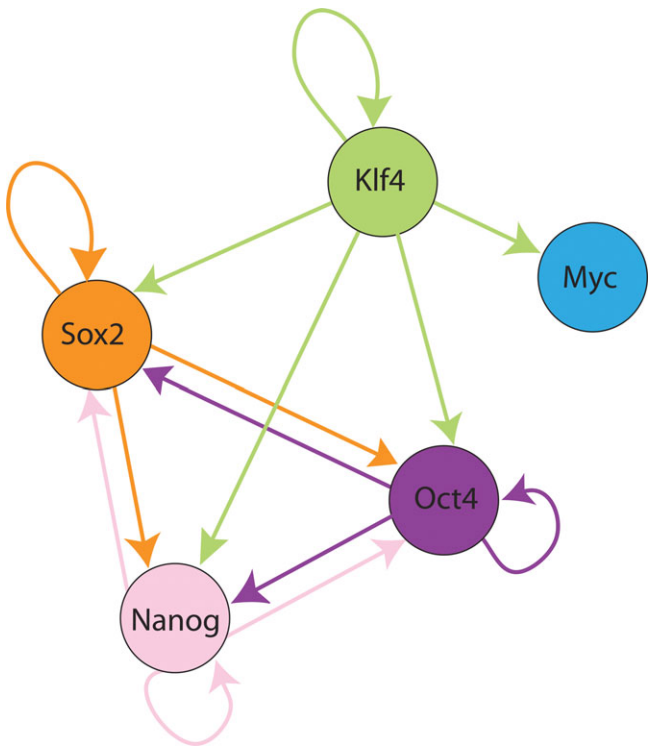

Figure 1: Proposed hierarchy of key pluripotency network transcription factors showing auto-regulatory and cross-regulatory transcriptional loops (arrows). These factors are also able to induce pluripotency in somatic cells.

Epigenetic regulation of pluripotency—poised and ready for differentiation

Examination of the epigenetic status of pluripotent cells has identified a number of pluripotent cell-specific properties that provide some insight into how pluripotent cells maintain potential while preserving the ability to respond rapidly to differentiation signals.

Although the large-scale organization of genomes at the level of chromosomes is not significantly different in ES and differentiated cells, ES cell nuclei appear to have a distinct nuclear structure at key loci involved in the maintenance of pluripotency or in the process of differentiation. For example, *NANOG* is localized significantly more centrally in the nuclei of human ES cells compared with a more peripheral location in B cells, and *OCT4* loops out from the chromosome territory in human ES cells (Wiblin *et al*., 2005). Replication timing analyses support these observations. Patterns of replication timing generally reflect global genome organization with actively transcribed genes commonly replicated early in S-phase and silenced genes usually replicated later. Global replication patterns are essentially identical between pluripotent murine ES and differentiated cells (Panning and Gilbert, 2005). However, the replication timing of certain stem cell- and lineage-specific genes has been shown to correlate with their expression. The ES cell-specific genes examined replicated early in ES cells but their time of replication was delayed after induction of neural differentiation, consistent with down-regulation of their expression (Hiratani *et al*., 2004; Perry *et al*., 2004). Conversely, several neuronal genes, including *Sox3* and *Mash1*, switch to early replication on neural induction, accompanying up-regulation of their expression (Azuara *et al*., 2006). Although a subset of lineage-specific genes change their replication timing from late to early upon activation, a large number of lineage-specific genes, such as *Ikaros*, *Myog* and *Math1*, are already early replicating in ES cells even though they are not actively transcribed (Azuara *et al*., 2006). This led to the notion that many lineage-specific genes may be 'primed' for activation in ES cells.

Pluripotent cells such as ES and P19 cells (a primitive ectoderm-like transformed cell line) also appear to have a unique chromatin state. Referred to as hyperdynamic or 'breathing' chromatin, this state is characterized by the rapid turnover of a subset of proteins associated with chromatin, including the H2B and H3 core histones when compared with non-pluripotent cells (Meshorer *et al*., 2006). Generation of a mutant form of histone H1, which bound stably to chromatin and reduced the dynamic exchange, blocked differentiation of ES cells and the cells eventually died on prolonged culture (Meshorer *et al*., 2006). These findings are consistent with a model whereby pluripotent cells have regions of unique chromatin that may be in a 'primed state' and in which the dynamic chromatin facilitates the rapid implementation of specific developmental programs and shutting down of programs that are not required in response to differentiation cues.

Further evidence for a 'primed state' existing in pluripotent cells comes from the observation that large regions of chromatin in ES cells contain epigenetic marks which are characteristically associated with both repression and activation. These 'bivalent domains' consist of regions with histone 3 lysine 4 tri-methylation (H3K4me3), an active mark, within more extensive regions of histone 3 lysine 27 tri-methylation (H3K27me3), which is a repressive mark. Bivalent domains are often associated with regions encoding developmentally important transcription factors which are not expressed, or expressed at low levels, in ES cells (Azuara *et al*., 2006; Bernstein *et al*., 2006). In addition, ~50% of bivalent domains contain OCT4, NANOG or

SOX2 binding sites (Bernstein *et al*., 2006). Almost all of the genes encoding developmental regulators that were occupied by OCT4, SOX2 and NANOG and repressed in the regulatory circuitry highlighted in Boyer *et al*. (2005) were also shown to be the targets of Polycomb Repressive Complex 2 (PRC2). A further study identified over 200 genes in human ES cells that are bound by PRC2, display H3K27me3 marks and are transcriptionally repressed (Lee *et al*., 2006), and similar results were found when mouse ES cells were examined (Boyer *et al*., 2006). Expansion of the pluripotent factor network to include Dax1 and Klf4 showed that their transcriptional targets were also enriched for bivalent marks (Kim *et al*., 2008).

PRC2 catalyses tri-methylation of H3K27. Disruption of key components of PRC2 in ES cells resulted in loss of H3K27me3, derepression of most target genes examined and inappropriate expression of developmental regulators and differentiation-specific genes (Azuara *et al*., 2006; Boyer *et al*., 2006; Pasini *et al*., 2007; Chamberlain *et al*., 2008). This suggests a role for PRC2 in repressing 'primed state' genes, which may help stabilize the pluripotent state. It is apparent, however, that H3K27me3 repressive marks are not required for the maintenance of pluripotency, but are important for subsequent differentiation (Pasini *et al*., 2007; Chamberlain *et al*., 2008).

Although maintenance of a 'primed state' may be necessary for correct implementation of differentiation programs, epigenetic modifiers have a role to play in maintaining pluripotency. The histone 3 lysine 9 (H3K9) histone demethylases *jmjd1a* and *jmjd2c* have been shown to be required for the continued expression of pluripotent regulators in mouse ES cells, with depletion of these enzymes leading to loss of self-renewal and ES cell differentiation (Loh *et al*., 2007).

The contribution of DNA methylation to the maintenance of pluripotency has also been examined. DNA methylation-deficient mouse ES cells are able to self-renew and proliferate (Tsumura *et al*., 2006), but cell death is induced on differentiation (Panning and Jaenisch, 1996). Genome-wide mapping of promoter CpG methylation patterns in mouse ES cells and correlation with gene expression, bivalent marks and Oct4, Polycomb or Nanog binding found that ~87% of genes that lack both H3K4me3 and H3K27me3 modifications are DNA methylated (Fouse *et al*., 2008). Furthermore, looking in methylase mutant ES cells, Fouse *et al*. (2008) found that ~1.7% of genes were expressed at significantly elevated levels compared with control ES cells and that tissue-specific genes were over-represented in this cohort. A similar comparison in fibroblasts showed that roughly 10% of genes showed increased expression. These findings suggest that DNA methylation is not a major contributor to pluripotency and supports the notion that ES cells use several different mechanisms to repress differentiation-associated genes. It will be interesting to see whether similar methylation patterns are observed in human ES cells.

Like DNA methylation, genomic imprinting does not appear to play an important role in pluripotency, as the successful derivation of ES cell lines from parthenogenetic human, mouse and non-human primate embryos demonstrates (Cibelli *et al*., 2002; Kim *et al*., 2007a,b; Revazova *et al*., 2007). Parthenogenetic ES (pES) cells are produced from embryos that result from artificial oocyte activation without fertilization. These entirely maternally derived ES cells lack paternal imprinting but are capable of differentiation to form a broad range of cell types, as assessed by *in vitro* differentiation, teratoma formation and contribution to tissues in chimeric mice (Allen *et al*., 1994; Cibelli *et al*., 2002; Hikichi *et al*., 2007; Revazova *et al*., 2007, 2008). However, lower levels of contribution in chimeric mice and reduced rates of *in vitro* differentiation of pES cell lines have been reported (Fundele *et al*., 1990; Allen *et al*., 1994; Hikichi *et al*., 2007), suggesting some impairment in these cells. This may be due, at least in part, to an aberrant epigenetic state, as some improvement is observed when pES cells are subjected to reprogramming by nuclear transfer and ES cell lines are then re-derived (Hikichi *et al*., 2007).

Many of the distinctive epigenetic signatures found in ES cells to date are important for the process of differentiation, probably to ensure the continued silencing of alternative fates once lineage choice has been made. The epigenetic modifications acquired as cells subsequently differentiate then act to enforce the stability of those cell fate decisions.

Primitive ectoderm-like cells—a differentiation step in the right direction

A comprehensive understanding of pluripotency and its regulation will only be gained through a broadening of research to include the primitive ectoderm, a pluripotent derivative of the ICM in the embryo. This population of cells undergoes gastrulation to form the three germ layers (progenitor cell-types) of the embryo from which differentiated tissues are subsequently derived. As such, these cells may hold the key to understanding and directing the earliest steps of cell fate choice. There are currently two *in vitro* models of primitive ectoderm: EPL cells and epiblast stem cells (EpiSCs).

EPL cells are formed from mouse ES cells and share many of the features characteristic of primitive ectoderm including the expression of specific gene markers and a reduced ability to form extra-embryonic tissues (Pelton *et al*., 1998; Rathjen *et al*., 1999, 2002; Lake *et al*., 2000). EPL cells are generated via addition of conditioned media from HepG2 cells, believed to mimic signals arising from the primitive endoderm *in vivo*, to stimulate a controlled transition of ES cells to the EPL cell type. Several lines of evidence suggest key regulators of pluripotency are controlled by distinct mechanisms in primitive ectoderm and EPL cells compared with ICM and ES cells. *Klf4* expression is down-regulated on the formation of EPL cells, suggesting major changes in the transcription factor network (our unpublished data). *Nanog* expression decreases in much of the primitive ectoderm (Chambers *et al*., 2003) and on generation of EPL cells (our unpublished data). However, a concomitant increase in *Gata6* expression is not observed (our unpublished data) as is seen on loss of Nanog in ES cells (Mitsui *et al*., 2003; Hyslop *et al*., 2005; Hamazaki *et al*., 2006; Hough *et al*., 2006).

Epiblast stem cell (EpiSC) lines, isolated from post-implantation mouse embryos (Brons *et al*., 2007; Tesar *et al*., 2007), also represent a post-ICM/ES cell pluripotent population. Initial characterization of these cell lines demonstrate that they are LIF-independent, requiring either Fgf2 or Activin A depending on the method of derivation. These cells express the core pluripotency-associated transcription factors Oct4, Nanog and Sox2, but have a gene expression profile similar to that of the late epiblast and distinct from ICM and ES cells. Similarly, some Oct4 target genes and epigenetic marks at specific loci also differed in ES cells and EpiSCs. Interestingly, these features of EpiSCs more closely resembled those of human ES cells, raising the possibility that human ES cells are more similar to epiblast-like cells than ICM.

An investigation of the differences between ES, EPL and EpiSC cells at the level of signalling, transcriptional regulation and epigenetics will provide information on how pluripotency is maintained in distinct but directly related cell types. Ultimately, a greater understanding of the ES to EPL cell transition and manipulation of the differentiation potential of EPL cells and EpiSCs will enhance our ability to direct the differentiation of pluripotent cells toward the cell types we desire.

Somatic cell reprogramming—new sources of pluripotent cells

Despite the complexity of the transcriptional network in pluripotent cells, the ability to generate ES cell lines, as well as whole organisms,

from somatic nuclei demonstrates the network can be re-established. Interestingly, it is the failure to completely re-set the epigenetic code that appears to reduce the efficiency of reprogramming (Armstrong *et al*., 2006b).

A number of methods for reprogramming differentiated cells are being investigated, including fusion of differentiated cells with pluripotent cells (Tada *et al*., 2001; Cowan *et al*., 2005; Tada and Tada, 2006), and treatment of differentiated cells with extracts from pluripotent cells (Taranger *et al*., 2005). Currently, the most effective and reproducible methods for producing ES-like cells are SCNT and the generation of induced pluripotent stem (iPS) cells using specific transcription factors.

Mouse ES cell lines have been derived by introducing nuclei from various somatic donor cell types into an enucleated oocyte and harvesting ES cells from the resulting cloned blastocyst. ES cells derived from this procedure (Fig. 2B) have been shown to express a profile of genes which is also present in ES cells derived from fertilized embryos (Brambrink *et al*., 2006). SCNT has been used successfully to form non-human primate blastocysts (Mitalipov *et al*., 2007) and, more recently, human blastocysts (French *et al*., 2008). However, currently no successful generation of a human ES cell line by this method has been reported. A well publicized claim was subsequently retracted (Hwang *et al*., 2004), and the cell line has since been shown to have been derived by parthenogenesis (Kim *et al*., 2007b).

The cytoplasm of the enucleated oocyte is able to re-establish the pluripotent state via a mechanism dependent on epigenetic changes. However, the identities of the oocyte factors (and pluripotent cell factors in the cell fusion and extracts methods) that mediate this process are largely undefined. The best insight into the molecular mediators of reprogramming comes from the generation of iPS cells. This innovative technology was borne out of a radical approach in

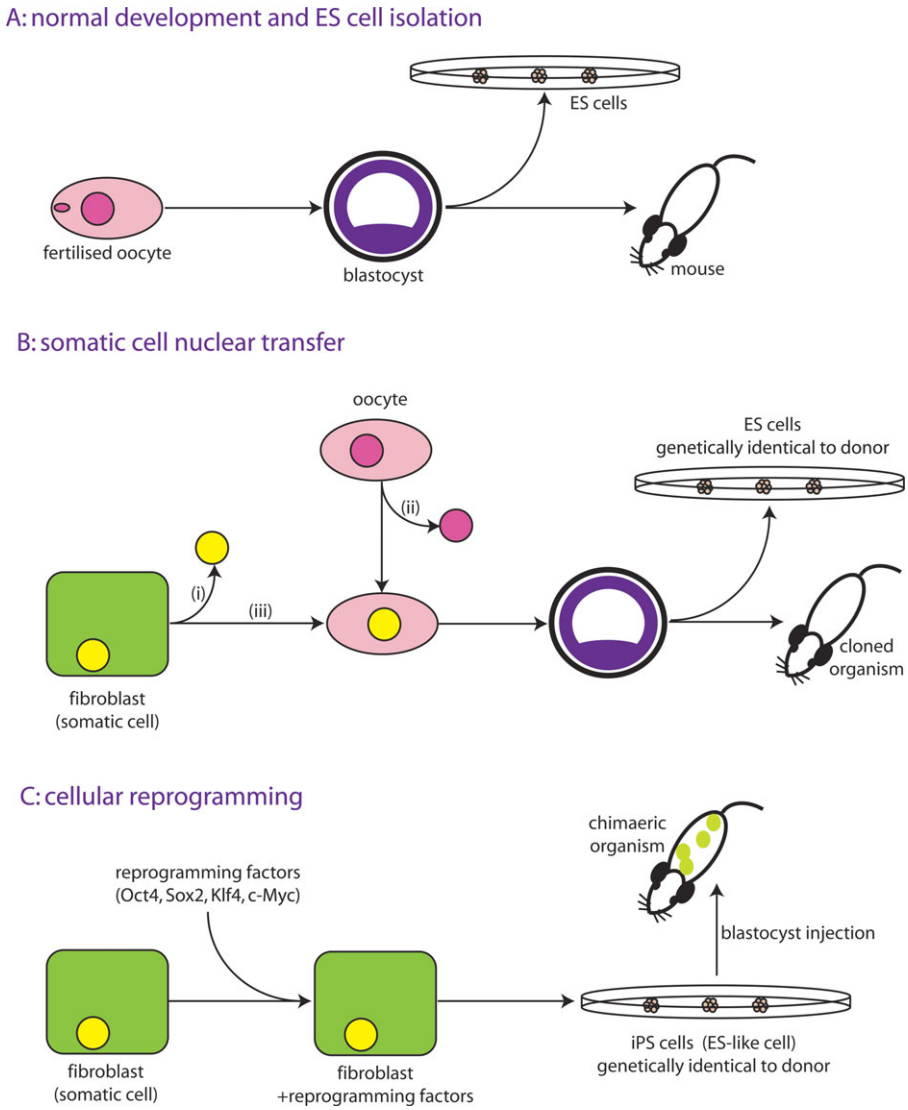

Figure 2: Methods for generating pluripotent stem cells.
(**A**) Blastocyst derivation of an ES cell line. Mouse embryonic stem cells are conventionally derived from a mouse blastocyst in culture. Blastocysts can be obtained from fertilized oocytes or from parthenogenetic activation of unfertilized oocytes. ES cells from other species are similarly derived. (**B**) Somatic Cell Nuclear Transfer (SCNT). SCNT entails: (i) enucleation of a somatic cell (green with yellow nucleus); (ii) enucleation of an oocyte (light pink with pink nucleus); (iii) introduction of the nucleus of the somatic cell into the enucleated oocyte. The resulting cell may be propagated to the blastocyst stage and ES cell lines derived in the normal manner (as described in A). Alternatively, the blastocyst can be used to produce a cloned organism. (**C**) Generation of induced pluripotent stem (iPS) cells. Transduction of somatic cells with retroviral vectors expressing four transcription factors (c-Myc, Oct4, Klf4 and Sox2) was sufficient to generate a pluripotent-like cell capable of contributing to all three germ layers and to tissues in chimeric mice when re-introduced into the blastocyst. These cells represent a pluripotent-like cell with features resembling those of ES cells.

which candidate genes associated with pluripotency were expressed in non-pluripotent cells to re-establish a pluripotent state (Takahashi and Yamanaka, 2006; Fig. 2C). Mouse fibroblast cells were transduced with a combination of retroviral vectors, each encoding cDNA for a prospective reprogramming protein, and initially assayed for the ability to activate a reporter gene that is specifically expressed in mouse ES cells. More rigorous tests followed to confirm pluripotency, including *in vitro* differentiation and teratoma formation to produce cells derived from all three germ layers, as well injection into blastocysts and subsequent contribution to chimeric mice (Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007). Takahashi and Yamanaka (2006) identified a minimal set of four cDNAs required to produce an 'ES-like' iPS cell: *Oct4, Sox2, Klf4 and c-myc*.

As discussed above, Oct4, Sox2 and Klf4 form part of a core group of pluripotency regulators that appear to work in a highly combinatorial manner to control a pluripotent-specific program of gene expression and repression (Boyer *et al*., 2005; Loh *et al*., 2006; Kim *et al*., 2008). c-Myc appears to lie outside this core group as its targets are generally distinct from those of the other factors (Kim *et al*., 2008). In addition, promoters occupied by c-Myc are likely to have the 'active' H3K4me3 mark, lack H3K27me3 and be expressed in ES cells, suggesting c-Myc binding is associated with chromatin modifications that support gene expression. c-Myc expression has now been shown to be dispensable for iPS cell formation, but its presence dramatically enhances the timing and efficiency of reprogramming (Nakagawa *et al*., 2008; Wernig *et al*., 2008a), consistent with a role in promoting changes in chromatin accessibility.

Since the iPS cell technique was first published in mid-2006, the field has flourished. Human cells have been reprogrammed successfully (Takahashi *et al*., 2007; Liao *et al*., 2008; Lowry *et al*., 2008; Nakagawa *et al*., 2008; Park *et al*., 2008a), additional and alternative reprogramming factors have been identified (Yu *et al*., 2007; Liao *et al*., 2008; Nakagawa *et al*., 2008) and mouse models of human disease treated with cells of iPS cell origin (Hanna *et al*., 2007; Wernig *et al*., 2008b).

The potential of these cells appears to match that of ES cells, with the added advantages of ease of derivation and no requirement for oocytes. However, despite the rapid pace and promise, significant hurdles remain in the race to the clinic. These include safety issues surrounding the clinical application of iPS cells due to use of genome-integrating viral vectors for the introduction and expression of the reprogramming factors. The random integration site and presence of promoter sequences increase the possibility of inappropriately inactivating or activating genes. Similarly, the use of proto-oncogenes such as *myc* and *Klf4* correlate with an increased risk of tumour formation. In addition, caveats remain over the completeness of reprogramming of both iPS cells and SCNT-derived ES cells, with the cell line variability in differentiation potential and functionality and stability of the 're-differentiated' cells still to be rigorously examined.

Conclusions

Understanding the molecular basis of pluripotency and the earliest differentiation processes will provide the knowledge needed to grow and manipulate pluripotent cells efficiently, reproducibly and in a manner appropriate for clinical applications. Despite differences in growth factor requirements, gene expression profiles and Oct4/Nanog/Sox2 targets in mouse and human ES cells, apparent conservation of epigenetic features, transcription factor networks and the ability of the same four factors to induce pluripotency imply that common mechanisms underlie pluripotency in mouse and human cells. Although considerable advances have been made in identifying the complex networks involved, we do not yet understand how these factors maintain pluripotency (repression of differentiation-specific genes does not appear to be required), how growth factors control and stabilize these networks or how these cells respond so precisely to differentiation cues. Similarly, the exciting prospect of generating pluripotent cells from any source warrants a deep understanding of the reprogramming process. This will lead to improvements in efficiency and methods of generation and ensure the safety of the cells for therapeutic use.

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