REVIEW

Pluripotent stem cell lines

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The derivation of human embryonic stem cells 10 years ago ignited an explosion of public interest in stem cells, yet this achievement depended on prior decades of research on mouse embryonic carcinoma cells and embryonic stem cells. In turn, the recent derivation of mouse and human induced pluripotent stem cells depended on the prior studies on mouse and human embryonic stem cells. Both human embryonic stem cells and induced pluripotent stem cells can self-renew indefinitely in vitro while maintaining the ability to differentiate into advanced derivatives of all three germ layers, features very useful for understanding the differentiation and function of human tissues, for drug screen and toxicity testing, and for cellular transplantation therapies. Here we review the family of pluripotent cell lines derived from early embryos and from germ cells, and compare them with the more recently described induced pluripotent stem cells.

Pluripotent stem cells of different origins

Embryonal carcinoma (EC) sells

The field of pluripotent stem cells began with the study of teratocarcinomas in the 1950s. Teratocarcinomas are malignant germ cell tumors that comprise an undifferentiated EC component and a differentiated component that can include all three germ layers. Although teratocarcinomas had been described centuries ago, the rarity of these tumors made them difficult to study. The discovery that male strain 129 mice had a high incidence of testicular teratomas (or more accurately, teratocarcinomas; ~1%) (Stevens and Little 1954) made them amenable to experimental analysis for the first time. The persistence of the EC cell component allows teratocarcinomas to be serially transplanted between mice, as these undifferentiated cells function as stem cells for the other tumor components. In 1964 Kleinsmith and Pierce (1964) demonstrated that a single EC cell is capable of both unlimited self-renewal and multilineage differentiation, establishing the existence of a pluripotent stem cell and also providing the intellectual framework for both mouse and human embryonic stem (ES) cells. This was also the first experimental demonstration of a cancer stem cell, predating the current intense interest in cancer stem cells by several decades. Mouse EC cell lines that could be stably propagated in vitro were established in the early 1970s (Kahan and Ephrussi 1970) and were widely studied as "in vitro caricatures of development," as they could be cultured in sufficient quantities to perform experiments that would have been impossible with intact mammalian embryos.

The similar developmental properties of EC cells and early embryonic cells naturally led to a search for an in vivo counterpart of these cells. Mammalian development starts from a single cell that can give rise to all cells required for a new life, but through subsequent differentiation events, developmental potential becomes increasingly restricted. As the one-cell embryo divides, it forms a morula, a "mulberry"-like cluster of undifferentiated cells. The first differentiation event occurs when the outer layer of cells of the morula differentiates to the trophectoderm, forming the blastocyst stage embryo. The cells inside the blastocyst (inner cell mass, or ICM) give rise to all cells of the adult body and some extraembryonic tissues, while the trophectoderm gives rise to the outer layer of the placenta. Mouse EC cells express antigens and proteins that are similar to cells present in the ICM (Gachelin et al. 1977; Solter and Knowles 1978), which led to the concept that EC cells are an in vitro counterpart of the pluripotent cells present in the ICM (Martin 1980). Indeed, some EC cell lines are able to contribute to various somatic cell types upon injection into mouse blastocysts (Brinster 1974). However, most EC cell lines have limited developmental potential and contribute poorly to chimeric mice, likely due to the accumulation of genetic changes during teratocarcinoma formation and growth (Atkin et al. 1974).

Human EC cells were subsequently derived (Hogan et al. 1977), and these cells proved to be significantly different from mouse EC cells. For example, SSEA-1, a cell-surface marker specifically expressed on mouse EC cells, is absent on human EC cells, while SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 are absent on mouse EC cells but are present on human EC cells (Andrews et al. 1982,

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1984; Kannagi et al. 1983). Also, in contrast to mouse EC cells, human EC cells are highly aneuploid, which likely accounts for their inability to differentiate into a wide range of somatic cell types, and which limits their utility as an in vitro model of human development. Whether the cell surface and morphological differences of human and mouse EC cells reflected species-specific differences in early embryology or reflected some peculiarity of the neoplastic transformation of human germ cells was an open question until the derivation of human ES cells.

Mouse ES cells

Pluripotent cells are present only transiently in embryos in vivo, as they quickly differentiate into various somatic cells through development. However, if early mouse embryos are transferred to extrauterine sites, such as the kidney or testis capsules of adult mice, they can develop into teratocarcinomas (Solter et al. 1970; Stevens 1970). The high frequency of teratocarcinoma formation, even in strains that do not spontaneously have an elevated incidence of germ cell tumors, suggested that this process was not the result of rare neoplastic transformation events. These embryo transplantation experiments demonstrated that the intact embryo has a cell population that can give rise to pluripotent stem cell lines, and this key discovery led to the search for culture conditions that would allow the direct in vitro derivation of pluripotent stem cells from the embryo, without the intermediate need for teratocarcinoma formation in vivo.

The first mouse ES cell lines were derived from the ICM of mouse blastocysts using culture conditions (fibroblast feeder layers and serum) previously used for mouse EC cells (Evans and Kaufman 1981; Martin 1981). ES cell cultures clonally derived from a single cell could differentiate into a wide variety of cell types in vitro and form teratocarcinomas when injected into mice (Martin 1981). More importantly, unlike EC cells, these karyotypically normal cells can contribute at a high frequency to a variety of tissues in chimeras, including germ cells, thus providing a practical way to introduce modifications to the mouse germline (Bradley et al. 1984). The efficiency of mouse ES cell derivation is strongly influenced by genetic background. For example, ES cells can be easily derived from the inbred 129/ter-Sv strain but less efficiently from the C57BL/6 strain (Ledermann and Burki 1991). However, mouse ES cells can be derived from some nonpermissive strains using modified protocols (McWhir et al. 1996). Mouse ES cells have also been derived from cleavage stage embryos and even from individual blastomeres of two- to eight-cell stage embryos (Chung et al. 2006; Wakayama et al. 2007).

Mitotically inactivated cell feeder layers were first used to support difficult-to-culture epithelial cells (Puck et al. 1956) and were later successfully adapted for the culture of mouse EC cells (Martin and Evans 1975) and mouse ES cells (Evans and Kaufman 1981). Medium that is "conditioned" by coculture with various cells was found to be able to sustain ES cells in the absence of feeders, and fractionation of conditioned medium led to the identification of leukemia inhibitory factor (LIF), a cytokine that sustains ES cells (Smith et al. 1988; Williams et al. 1988). LIF and its related cytokines act via the gp130 receptor (Yoshida et al. 1994). Binding of LIF induces dimerization of LIFR/gp130 receptors, which in turn activates the Janus-associated tyrosine kinases (JAK)/the latent signal transducer and activator of transcription factor (STAT3) (Yoshida et al. 1994), and Shp2/ERK mitogen-activated protein kinase (MAPK) cascade (Takahashi-Tezuka et al. 1998). STAT3 activation alone is sufficient for LIF-mediated self-renewal of mouse ES cells in the presence of serum (Matsuda et al. 1999). Activation of ERK, however, appears to impair mouse ES cell proliferation. In contrast, suppression of the ERK pathway by the addition of MEK inhibitor PD098059 promotes ES cell self-renewal (Burdon et al. 1999). Thus the proliferative effect of LIF on mouse ES cells requires a finely tuned balance between positive and negative effectors.

In serum-free medium, LIF alone is insufficient to prevent mouse ES cell differentiation, but in combination with BMP (bone morphogenetic protein, a member of the TGFβ superfamily), mouse ES cells are sustained (Ying et al. 2003). BMPs induce the expression of Id (inhibitor of differentiation) proteins through the Smad pathway. The overexpression of Id could indeed promote mouse ES cell proliferation in the presence of LIF alone without the need for either BMPs or serum. However, BMPs might also act through inhibition of the MAPK pathways independent of Smads. The latter is supported by the facts that ES cells can be derived from blastocysts lacking Smad4 (the common partner for all Smads) (Sirard et al. 1998) and that inhibition of p38 MAPK by SB203580 allowed derivation of ES cells from blastocysts lacking BMP type I receptor Alk-3, which were previously refractory to ES cell derivation (Qi et al. 2004). In normal development, however, there is no apparent requirement for LIF, gp130 or STAT3 prior to gastrulation (Escary et al. 1993; Yoshida et al. 1996; Takeda et al. 1997), and homozygous Alk-3 mutant mouse embryos can develop normally to early post-implantation stage (Mishina et al. 1995). Thus the pluripotent ICM/epiblast could employ alternative signaling pathways for undifferentiated proliferation.

More recently, pluripotent stem cell lines (epiblast stem cells or EpiSCs) have been established from epiblasts isolated from E5.5 to E6.5 post-implantation mouse embryos that differ significantly from mouse ES cells but share key features with human ES cells (see below) (Brons et al. 2007; Tesar et al. 2007). For example, EpiSCs derivation failed in the presence of LIF and/or BMP4, the two factors required for the derivation and self-renewal of mouse ES cells. In contrast, similar to human ES and iPS cells, FGF and Activin/Nodal signaling appear critical for EpiSC derivation. Gene expression by EpiSCs closely reflects their post-implantation epiblast origin and is distinct from mouse ES cells. Nevertheless, EpiSCs do share the two key features characteristic of ES cells: prolonged proliferation in vitro and mul-

tilineage differentiation. The ability to derive pluripotent stem cells from early post-implantation embryos is consistent with early extrauterine embryo transplantation experiments where transplanted mouse embryos at stages ranging from one cell to egg cylinders (E8) are able to form teratocarcinomas (Stevens 1968, 1970; Solter et al. 1970), but this ability is quickly lost with further development.

Pluripotent cell lines derived from germ cells

Despite the evidence that teratocarcinomas were derived from primordial germ cells (PGCs) (Stevens 1962), it was not until 1992 that pluripotent stem cells (embryonic germ cells or EG cells) were successfully derived from PGCs directly in vitro (Matsui et al. 1992; Resnick et al. 1992). In contrast to mouse ES cells, the initial derivation of mouse EG cells requires a combination of stem cell factor (SCF), LIF, and FGF in the presence of a feeder layer. In culture, EG cells are morphologically indistinguishable from mouse ES cells and express typical ES cell markers such as SSEA-1 and Oct4. And similar to ES cells, upon blastocyst injection, they can contribute extensively to chimeric mice including germ cells (Labosky et al. 1994; Stewart et al. 1994). Unlike ES cells, however, EG cells retain some features of the original PGCs, including genome-wide demethylation, erasure of genomic imprints, and reactivation of X-chromosomes (Labosky et al. 1994; Tada et al. 1997), the degree of which likely reflects the developmental stages of the PGCs from which they are derived (Shovlin et al. 2008).

Pluripotent stem cells (multipotent germline stem cells or mGSCs) more recently have been derived from both neonatal and adult mouse testis (Kanatsu-Shinohara et al. 2004; Guan et al. 2006). mGSCs share a similar morphology with mouse ES cells, express typical mouse ES cell-specific markers, differentiate into multiple lineages in vitro, form teratomas, and contribute extensively to chimeras including germline cells upon injection into blastocysts. However, mGSCs have an epigenetic status distinct from both ES cells and germline stem cells (Kanatsu-Shinohara et al. 2004). The mouse testis contains different subpopulations of germline stem cells (Izadyar et al. 2008). The origin of mGSCs is still somewhat unclear, though it might be possible that in vitro culture of germline stem cells reprograms a minority of these cells to resume an ES cell-like state. For example, culture of GPR125$^+$ (c-Kit$^-$) spermatogonial progenitor cells (GSPCs) were able to convert these cells into pluripotent stem cells (multipotent adult spermatogonia-derived stem cells, or MASCs), which could differentiate into derivatives of all three primary germ layers both in vitro and in vivo (Seandel et al. 2007). The MASCs, however, have a gene expression pattern distinct from either GSPCs or ES cells.

The derivation of human EG cells was reported in 1998 (Shamblott et al. 1998), but in spite of efforts by several groups, their long-term proliferative potential appears to be limited (Turnpenny et al. 2003). Early passage human EG cells have been reported to differentiate into multiple lineages in vitro, but this has yet to be demonstrated from a clonally derived, stable cell line, nor to date have any human EG cell lines been reported to form teratomas. Besides having different growth factor requirements from human ES cells, human EG cells have a very distinct morphology and express SSEA-1, a cell-surface marker absent on human ES cells but present on early human germ cells. Human EC cells are also germ cell-derived and share markers and the basic morphology of human ES cells, so these differences suggest that a final step in converting these human germ cell lines to a proliferative cell comparable with human ES/EC cells is still missing. The properties of the human EG cell lines reported to date suggest fundamental species-specific differences between the early germ cell biology of mice and humans and suggest that a human counterpart truly comparable with mouse EG cells has yet to be derived.

Human ES cells

There was a considerable delay between the derivation of mouse ES cells in 1981 and the derivation of human ES cells in 1998 (Thomson et al. 1998), in spite of several earlier attempts at human ES cell derivation. This delay was primarily due to species-specific ES cell differences and suboptimal human embryo culture media. For example, the isolation of ICMs from human blastocysts had been previously reported (Bongso et al. 1994), but subsequent culture in media supplemented with LIF and serum, conditions that allow the derivation of mouse ES cells, resulted only in differentiation, not in the derivation of stable pluripotent cell lines. In the mid-1990s, ES cell lines were derived from two nonhuman primates: the rhesus monkey and the common marmoset (Thomson et al. 1995, 1996). Experience with those primate ES cell lines and concomitant improvements in culture conditions for human IVF embryos (Gardner et al. 1998) permitted the subsequent derivation of human ES cell lines (Thomson et al. 1998). Human ES cells are karyotypically normal and, even after prolonged undifferentiated proliferation, maintain the developmental potential to contribute to advanced derivatives of all three germ layers, even after clonal derivation (Amit et al. 2000). Similar to mouse ES cells, human ES cells have been derived from morula, later blastocyst stage embryos (Stojkovic et al. 2004; Strelchenko et al. 2004), single blastomeres (Klimanskaya et al. 2006), and parthenogenetic embryos (Lin et al. 2007; Mai et al. 2007; Revazova et al. 2007). It is not yet known whether pluripotent cell lines derived from these various sources have any consistent developmental differences or whether they have an equivalent potential.

Mitotically inactivated fibroblast feeder layers and serum-containing medium were used in the initial derivation of human ES cells, essentially the same conditions used for the derivation of mouse ES cells prior to the identification of LIF (Thomson et al. 1998). However, it now appears largely to be a lucky coincidence that fibroblast feeder layers support both mouse and human ES cells, as the specific factors used to sustain mouse ES cells do not support human ES cells. LIF and its related

cytokines fail to support human or nonhuman primate ES cells in serum-containing media that supports mouse ES cells (Thomson et al. 1998; Daheron et al. 2004; Humphrey et al. 2004). Consistent with this observation, human ES cells do not express or express at very low levels of critical components of the LIF pathway—LIFR, gp130, and JAK 1 and 2 (Brandenberger et al. 2004), and in conditions that do support human ES cells, STAT3 is minimally activated (Daheron et al. 2004). Components of the BMP pathway are all present in human ES cells (Rho et al. 2006), but unlike mouse ES cells, BMPs added to human ES cells in conditions that would otherwise support self-renewal, cause rapid differentiation (Xu et al. 2002).

In contrast to mouse ES cells, FGF and TGFβ/Activin/Nodal signaling are of central importance to the self-renewal of human ES cells, making human ES cells similar to the recently described mouse epiblast-derived stem cells (Brons et al. 2007; Tesar et al. 2007). Basic FGF (bFGF) allows the clonal growth of human ES cells on fibroblasts in the presence of a commercially available serum replacement (Amit et al. 2000). At higher concentrations, bFGF allows feeder independent growth of human ES cells cultured in the same serum replacement (Wang et al. 2005; C. Xu et al. 2005; R.H. Xu et al. 2005; Levenstein et al. 2006). The mechanism through which these high concentrations of bFGF exert their functions is incompletely known, although one of the effects is suppression of BMP signaling (R.H. Xu et al. 2005). Serum and a widely used commercially available serum replacement have significant BMP-like activity, which is sufficient to induce differentiation of human ES cells, and conditioning this medium on fibroblasts reduces this activity. At moderate concentrations of bFGF (40 ng/mL), the addition of noggin or other inhibitors of BMP signaling significantly decreases background differentiation of human ES cells. At higher concentrations (100 ng/mL), bFGF itself suppresses BMP signaling in human ES cells to levels comparable with those observed in fibroblast-conditioned medium, and the addition of noggin no longer has a significant effect. Suppression of BMP activity by itself is insufficient to maintain human ES cells (R.H. Xu et al. 2005), thus additional roles for bFGF signaling exist. Evidence suggests that bFGF up-regulates the expression of TGFβ ligands in both feeder cells and human ES cells, which, in turn, could promote human ES cell self-renewal (Greber et al. 2007). Human ES cells themselves produce FGFs, which appear insufficient for low-density cell culture but can maintain high-density cultures for variable periods. Inhibition of FGFRs by SU5402 causes differentiation of human ES cells (Dvorak et al. 2005), suggesting the involvement of FGFRs. The required downstream events, however, are still not well understood, but some evidence implicates activation of the ERK and PI3K pathways (Kang et al. 2005; Li et al. 2007).

Both Activin and TGFβ have strong positive effects on undifferentiated proliferation of human ES cells in the presence of low or modest concentrations of FGFs, and based on inhibitor studies, it has been suggested that TGFβ/Activin signaling is essential for human ES cell self-renewal (Beattie et al. 2005; James et al. 2005; Vallier et al. 2005). However, when TGFβ/Activin signaling is inhibited with SB431542, there is a concomitant rise in BMP signaling activity (Beattie et al. 2005; James et al. 2005; Vallier et al. 2005), so it has been unclear whether signaling through TGFβ/Activin is merely acting to inhibit the sister BMP pathway, or whether TGFβ/Activin signaling has other, independent roles. Recent studies have revealed multiple interactions between the FGF, TGFβ, and BMP pathways in human ES cells. Activin induces bFGF expression (Xiao et al. 2006), and bFGF induces Tgfβ1/TGFβ1 and Grem1/GREM1 (a BMP antagonist) expression and inhibits Bmp4/BMP4 expression in both fibroblast feeders and in human ES cells (Greber et al. 2007). This reciprocity of induction between the FGF and TGFβ/Activin pathways likely explains why at high doses of bFGF, exogenous TGFβ or Activin has only very modest effects on undifferentiated human ES cell proliferation (Ludwig et al. 2006) and, similarly, at sufficient doses of Activin, the beneficial dose of exogenous FGF is greatly reduced (Vallier et al. 2005; Xiao et al. 2006).

Although other growth factors have been reported to have a positive effect on human ES cell growth including Wnt (Sato et al. 2004), IGF1 (Bendall et al. 2007), heregulin (Wang et al. 2007), pleiotrophin (Soh et al. 2007), sphingosine-1-phosphate (S1P), and PDGF (Pebay et al. 2005), there are clearly additional important pathways yet to be identified. For example, although compounds have been found that do increase the efficiency of clonal human ES cell culture such as the Rock inhibitor Y-27632 (Watanabe et al. 2007), such efficiencies for low passage cells, nonetheless, remain poor. This low efficiency is not merely due to cell damage associated with cell dissociation, as individualized human ES cells do survive at a high frequency if seeded at a sufficient density, suggesting that important ES cell–ES cell interactions have yet to be elucidated.

Induced pluripotent stem cells

The cloning of Dolly demonstrated that *trans*-acting material in mammalian oocytes is sufficient to change the epigenetic status of a differentiated nucleus to a totipotent state (Wilmut et al. 1997). This discovery completely changed the mindset of developmental biologists who previously thought this was impossible in mammals (McGrath and Solter 1984). Immediately following the derivation of human ES cells, it was suggested that somatic cell nuclear transfer (SCNT) might be used to create patient-specific stem cells. Yet applying SCNT to human material has proven challenging, and has only very recently been achieved in nonhuman primates (Byrne et al. 2007). In these studies, two primate ES cell lines were derived from 304 oocytes using SCNT from fibroblasts. Thus, although SCNT is biologically possible in primate material, the current efficiencies would make it impractical for widespread human clinical use, and the recent rapid progress with reprogramming using

defined factors suggests that other approaches will be more practical.

The success of SCNT demonstrated that reprogramming could be mediated by transacting factors. Through cell–cell fusion, EC cells, ES cells, and EG cells were also capable of reverting the differentiated state of somatic cells to that of pluripotent stem cells (Miller and Ruddle 1976, 1977a,b; Tada et al. 1997, 2001; Cowan et al. 2005; Yu et al. 2006), suggesting the presence of similar reprogramming activities in these pluripotent stem cells. But neither SCNT nor cell–cell fusion experiments offer any hints at how many or which factors are required. Two groups successfully conducted screens of factors for their ability to reprogram somatic cells. The Yamanaka group (Takahashi and Yamanaka 2006) conducted their screens with mouse somatic cells and identified *Oct4*, *Sox2*, *c-Myc*, and *Klf4* as sufficient to reprogram mouse fibroblasts to cells closely resembling mouse ES cells. These results were rapidly confirmed and extended in mouse material (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007) and eventually successfully applied to human material (Takahashi et al. 2007; Lowry et al. 2008; Park et al. 2008). Our own group independently pursued a similar screen using only human material, starting with the observation that human ES cells reprogram hematopoietic cells in cell–cell fusions (Yu et al. 2006) and overexpressing combinations of factors highly enriched in human ES cells in human somatic cells. This second screen yielded *OCT4*, *SOX2*, *NANOG*, and *LIN28* as sufficient to reprogram human cells, with *OCT4* and *SOX2* appearing essential and the other two factors either strongly (*NANOG*) or modestly (*LIN28*) influencing the efficiency of reprogramming (Yu et al. 2007). *OCT4*, *SOX2*, and *NANOG* are clearly sufficient to reprogram fetal, neonatal (Yu et al. 2007), and adult (J. Yu and J. Thomson, unpubl.) human cells in the absence of *LIN28*. *c-Myc* and *KLF4* were not included in our candidate gene list, as both genes are not enriched in human ES cells. Thus the screens represent two different samplings of potential reprogramming factors. Since the initial sets of genes were chosen by both groups to include genes previously shown to have a role in pluripotency, the first sets of successful genes tested by the Yamanaka group (Takahashi and Yamanaka 2006) (24 genes) and our group (14 genes) were extensively over-lapping. Thus, the two screens clearly do not represent a truly independent, random sampling of potential reprogramming factors. It remains to be seen whether other factors can be substituted for *OCT4* and *SOX2*, though it does seem likely that other combinations will be able to substitute for the positive effects of *KLF4*/*c-Myc* and *NANOG*/*LIN28*.

Mouse iPS cells are remarkably similar to mouse ES cells. Although the initial mouse iPS cells did not contribute to the germline in chimeras (Takahashi and Yamanaka 2006), subsequent modification of the procedure to select iPS cells based on the reactivation of *Oct4* or *Nanog* promoter resulted in iPS cells that more closely resembled mouse ES cells (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007), including the ability to contribute to germlines. Genetic selection applied during reprogramming, however, was later shown to be unnecessary for obtaining iPS cells closely resembling ES cells, as such cells could be selected based on colony morphology alone (Blelloch et al. 2007; Meissner et al. 2007). Despite the high similarity between mouse iPS and ES cells, tumor formation in iPS cell chimeric mice was high, presumably due to the expression of c-Myc in iPS cell-derived somatic cells (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007). More recently, it has been shown that *OCT4*, *SOX2*, and *KLF4* are sufficient to allow reprogramming of both mouse and human somatic cells, albeit at a much lower efficiency than when *c-Myc* is included (Nakagawa et al. 2008).

Human iPS cells, produced either by expression of *OCT4*, *SOX2*, *c-Myc*, and *KLF4* or by *OCT4*, *SOX2*, *NANOG*, and *LIN28* are also remarkably similar to human ES cells. These cells are morphologically similar to human ES cells, express typical human ES cell-specific cell surface antigens and genes, differentiate into multiple lineages in vitro, and form teratomas containing differentiated derivatives of all three primary germ layers when injected into immunocompromised mice. Indeed, these new pluripotent cell lines satisfy all the original criteria proposed for human ES cells (Thomson et al. 1998), except that they are not derived from embryos. Reprogramming by *Oct4*, *Sox2*, *Nanog*, and *Lin28* has not been reported for mouse cells, although *Nanog* was reported not to influence the frequency of reprogramming mouse cells in mouse ES cell culture conditions (Takahashi and Yamanaka 2006). Curiously, Nanog expression does greatly increase the ability of mouse ES cells to reprogram neural stem cells through cell–cell fusion (~200-fold) (Silva et al. 2006). In consideration of the recent finding that mouse EpiSCs share key features with human ES cells, including a dependence on bFGF and TGFβ/Activin and a lack of dependence on LIF (Brons et al. 2007; Tesar et al. 2007), it will be important to attempt reprogramming mouse cells in EpiSC/human ES cell culture conditions.

There is already extensive literature on *Oct4*, *Sox2*, and *Nanog* as key regulators of pluripotency, but the mechanism by which such a limited number of transcription factors orchestrate the erasure of the differentiated state is only beginning to be studied. *Oct4*, a member of the POU family of transcription factors, is essential for both the derivation and maintenance of ES cells (Pesce et al. 1998). The expression of Oct4 in mouse is restricted to early embryos and germ cells (Scholer et al. 1989), and homozygous deletion of this gene causes a failure in the formation of the ICM (Nichols et al. 1998). For mouse ES cells to remain undifferentiated, the expression of Oct4 must be maintained within a critical range. Overexpression of this protein causes differentiation into endoderm and mesoderm, while decreased expression leads to differentiation into trophoblast (Niwa et al. 2000). The expression of Oct4 is also a hallmark of human ES cells, and its down-regulation leads to differentiation and expression of trophoblast markers (Matin et al. 2004). *Sox2*, a member of the Sox family of HMG box transcription factors, also plays a key role in the self-renewal and pluripotency of ES cells. Similar to

Oct4, the expression of Sox2 in ES cells needs to be kept within a critical range. Expression outside that range led to differentiation of ES cells (Fong et al. 2008; Kopp et al. 2008). *Nanog* is a homeodomain-bearing transcription factor. Its expression, similar to Oct4, decreases rapidly as ES cells differentiate (Chambers et al. 2003; Mitsui et al. 2003). However, unlike Oct4, overexpression of Nanog in mouse ES cells allows their self-renewal to be independent of LIF/STAT3, though Nanog appears not to be a direct downstream target of LIF/STAT3 pathway (Chambers et al. 2003). Nanog overexpression also enables feeder-independent growth of human ES cells and improves their cloning efficiency (Darr et al. 2006), both properties likely contributing to successful iPS clone recovery. In both mouse and human ES cells, reduced expression of Nanog predisposes to differentiation to extraembryonic lineages (Chambers et al. 2003; Mitsui et al. 2003; Hyslop et al. 2005), but the expression of Nanog is not absolutely required for the pluripotency of ES cells, as ES cells can retain the ability to self renew in its absence (Chambers et al. 2007). Through a genome-wide location analysis, the DNA-binding sites of Oct4, Sox2, and Nanog have been studied extensively (Boyer et al. 2005). In addition to regulating their own transcription (Catena et al. 2004; Kuroda et al. 2005; Okumura-Nakanishi et al. 2005), these three transcription factors can also activate or repress the expression of many other genes, including developmentally important transcription factors.

The roles of *c-Myc*, *Klf4*, and *Lin28* in pluripotency are less studied, and their roles in reprogramming are even less clear. Overexpression of c-Myc in mouse ES cells allows their LIF-independent self-renewal (Cartwright et al. 2005), but overexpression of c-Myc in human ES cells leads to cell death and differentiation (Sumi et al. 2007). Thus, it is somewhat surprising that c-Myc expression improves the efficiency of human iPS cell derivation, and the difficultly in achieving critical levels of c-Myc in both the starting fibroblasts and the resulting iPS cells is a reasonable explanation for why it proved so challenging to apply the mouse reprogramming factors to human cells. Recent studies in mouse cells suggest that reprogramming is a rather slow process characterized by a gradual up-regulation of ES cell-specific marker genes and requires the expression of reprogramming genes for at least 12 d (Brambrink et al. 2008), suggesting that cells need to progress through multiple cell divisions. One possibility is that *c-Myc* helps in reprogramming human somatic cells through an ability to drive cell division, which has been shown to be important in somatic cell reprogramming in both SCNT and cell–cell fusion experiments (Fulka et al. 1996; Sullivan et al. 2006). Klf4 has recently been shown to share many DNA targets with Nanog in mouse ES cells, and its down-regulation with other *Klf* members leads to differentiation (Jiang et al. 2008). LIN28 has recently been shown to block the processing of differentiation-inducing microRNAs (Viswanathan et al. 2008). The *Lin28* locus was identified as a binding site for Oct4, Sox2, and Nanog in a genome-wide location analysis (Boyer et al. 2005), suggesting that these three reprogramming factors might induce its expression and, with appropriate induction levels, allow reprogramming in its absence. Lin28 expression levels are also critical in reprogramming experiments, as at high expression levels, it is toxic (J. Yu and J. Thomson, unpubl.).

The efficiency of reprogramming adult fibroblasts remains low (<0.1%), but whether this frequency reflects the need for the precise timing, balance, and absolute levels of expression of the reprogramming genes, or selection for rare genetic/epigenetic changes either initially present in the somatic cell population or acquired during prolonged reprogramming culture remains unresolved. It appears that retroviral integration into specific sites in the somatic cell genome is not required (Aoi et al. 2008). Additionally, iPS cells are not likely derived from rare pluripotent stem cells already present in somatic cell culture, as liver-derived iPS cells were shown to have an origin of albumin-expressing cells (Aoi et al. 2008), and iPS cells have been derived recently from pancreatic β cells (Stadtfeld et al. 2008) and mature B cells (Hanna et al. 2008). Inclusion of additional factors, such as TERT, T genes, and down-regulation of somatic cell-specific transcription factors (e.g., down-regulation of Pax5 in mature B cells), can improve the reprogramming efficiency (Hanna et al. 2008; Mali et al. 2008). However, since reprogrammed clones can be consistently recovered and expanded with the existing gene combinations, for practical applications, the current low reprogramming efficiency itself is really not an issue, unless reprogramming selects for abnormal genetic or epigenetic events that are stably propagated in the resulting iPS cell lines. To help resolve these issues, and for potential clinical applications, methods to induce iPS cells that leave the genome unaltered are essential and are being actively pursued by several groups.

Conclusion

Since the first demonstration that the EC cell component of teratocarcinomas is a pluripotent stem cell, the family of pluripotent stem cell lines that can form teratomas has grown to include ES, EpiSCs, EG, mGSCs, and now iPS cells. Yet, even with more than 40 years of research on pluripotent stem cells, it is still not possible to write a simple paragraph describing why these special cells can differentiate to all other cell types but others cannot. Our lack of understanding of the pluripotent state is highlighted by the fact that although our group has now shown that *OCT4*, *SOX2*, and *NANOG* are sufficient to reprogram human cells, we did not predict this ability ahead of time but instead carried out a very time consuming screen that eventually narrowed the list down to these three factors. However, although these specific three factors were already considered to be the key factors in pluripotency, even with hindsight and with the recent explosion of data on their genome-wide protein–DNA and protein–protein interactions, it is still impossible to explain why these genes (or the other combination successfully used by several other groups) are

sufficient to induce reprogramming, or to predict what additional combinations of factors might also be successful in reprogramming somatic cells.

Cellular transplantation based on iPS cells has tremendous clinical potential, but a truly regenerative medicine would direct endogenous cells to participate in the repair of damaged tissues that cannot normally regenerate itself, and such regeneration would require nonphysiological changes in differentiation status. Now that such a dramatic change as the conversion of a somatic cell to a pluripotent stem cell has been achieved, clearly similar screens will be carried out to test whether other nonphysiological transitions can be induced between other cell types by a limited number of factors. However, given the huge combinatorial space of factors that could be tested for any given pair of cell types, progress in predicting which factors, if any, could mediate such transitions will be essential. Just as the cloning of Dolly inspired researchers to look for defined factors that could mediate reprogramming to an undifferentiated state, the derivation of iPS cells by such a small set of factors will inspire researchers to attempt similar inductions between other cell types, as it now seems much more plausible that such experiments will work. Should those experiments prove successful, the legacy of iPS cells may well be the birth of a truly regenerative medicine.

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