The genetics of induced pluripotency

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Abstract

The flurry of recent publications regarding reprogramming of mature cell types to induced pluripotent stem cells raises the question: what exactly is pluripotency? A functional definition is provided by examination of the developmental potential of pluripotent stem cell types. Defining pluripotency at the molecular level, however, can be a greater challenge. Here, we examine the emerging list of genes associated with induced pluripotency, with particular attention to their functional requirement in the mouse embryo. Knowledge of the requirement for these genes in the embryo and in embryonic stem cells will advance our understanding of how to reverse the developmental clock for therapeutic benefit.

Reproduction (2010) **139** 35–44

What is pluripotency?

Pluripotency can be defined in both functional and molecular terms. Functionally, pluripotency refers to the ability of a cell to give rise to cell types of all three germ layers of the embryo: ectoderm, mesoderm, and endoderm, as well as the germline. As such, the very early embryo contains a succession of cells with pluripotent capacity from blastocyst to gastrula stages, but none of these cells behave as an ongoing source of stem cells in the later embryo. Embryonic stem (ES) cells are also pluripotent, but exhibit an additional feature: unlimited proliferation in a pluripotent state. Molecular definition of pluripotency requires identification of molecules that support these functional properties. Identification of these has been a major focus in the fields of developmental and stem cell biology. Below we discuss sources of pluripotent stem cells, strategies for identifying molecular regulators of their establishment and maintenance, and the role of these genes during mouse development.

Different kinds of pluripotent stem cells exist

Pluripotent stem cells hold promise for regenerative medicine, since they theoretically provide an unlimited source of new tissue for therapeutic intervention. Additionally, pluripotent stem cells provide a unique perspective into the establishment and development of the tissues from which they originate. Understanding the molecular regulation of the origins of pluripotent stem cells can thus provide insight into normal development.

Pluripotent stem cell lines have been derived from the mouse embryo at several stages. All are capable of differentiating into cells representative of a variety of adult tissue types in various assays, including embryoid body, teratoma, and some can contribute to mouse development in chimeras (Fig. 1). However, in spite of these similarities, many differences have been noted among pluripotent stem cell types. Differences include morphology, gene expression profiles, growth factor requirements, and the ability to contribute to chimeras (Rossant 2008). The best-studied pluripotent stem cell is the ES cell, derived from mouse or human inner cell mass (ICM) at the blastocyst stage (Evans & Kaufman 1981, Martin 1981, Thomson *et al.* 1998). In addition, self-renewing, pluripotent stem cells, called epiblast stem cells, have been derived from the mouse embryo at a slightly later stage, when the ICM has become the epiblast (Brons *et al.* 2007, Tesar *et al.* 2007). Pluripotent mouse embryonic germ cells can be derived from embryo-derived primordial germ cells (PGCs; Matsui *et al.* 1992), and pluripotent cells have been derived from spontaneously arising embryonal carcinomas (EC cells). In addition, multipotent germline stem cells have been derived from neonatal and adult mouse testes cells (Kanatsu-Shinohara *et al.* 2004, Guan *et al.* 2006). Whether a common pluripotency/self-renewal pathway underlies establishment, maintenance, and regulation of all of these cell lines is an area of active research.

To this end, much can be learned through efforts to induce pluripotency and self-renewal in mature cell types by nuclear reprogramming. Initial reprogramming

Figure 1 Functional assays of pluripotency. (A) Embryoid bodies are generated by growing cells, such as ES cells, at low density on non-adherent plates in the absence of the self-renewal cytokine LIF. Alternatively, embryoid bodies can be generated in hanging medium droplets. After several days of culture, resulting embryoid bodies can be individually transferred and cultured further under conditions appropriate to coax development of various cell types. (B) Teratomas are generated by injection of cells into non-obese/severe combined immunodeficient (NOD/SCID) mice, usually intraperitoneally, intramuscularly, or under testicular or kidney capsules. After 6–8 weeks or longer, resulting teratomas can be recovered and analyzed using histological approaches, usually to examine potential of injected cells to form cell type derivative of the embryonic germ layers (ectoderm, mesoderm, and endoderm). (C) Chimeric mice are generated by injection of labeled cells into an unlabeled host embryo, which are subsequently transferred to foster mothers. Contribution of genetically labeled cells to the resulting fetus or adult mouse provides a qualitative assessment of the degree of contribution to embryonic germ layers. Contribution of labeled cells to the germline of the chimeric mouse can be assessed by breeding and tracking the inheritance of the label.

strategies relied on changing the environment of the entire mature cell nucleus, for e.g. by transplantation to an activated oocyte (Wilmot *et al.* 1997) or fusion with an ES cell (Tada *et al.* 2001). More recently, genetic strategies initially developed in the laboratory of Shinya Yamanaka have been used for nuclear reprogramming. The first efforts involved four genes (*Pou5f1* (*Oct4*), *Sox2*, *Klf4*, and *Myc* (*cMyc*)) introduced into mature cell types using retroviral vectors (Takahashi & Yamanaka 2006). Subsequent efforts have used alternate vectors or have substituted drugs for some of these genes (Maherali & Hochedlinger 2008, Feng *et al.* 2009b). Importantly, genetic reprogramming has been used to generate iPS cells in other species, including human, monkey, and rat (Takahashi *et al.* 2007, Yu *et al.* 2007, Liu *et al.* 2008, Li *et al.* 2009), arguing that genetic reprogramming is not a mouse-specific parlor trick.

The existence of different kinds of pluripotent stem cells and different reprogramming strategies raises the interesting notion that pluripotency can be achieved in different ways. Recognizing and describing these inherent differences is essential for understanding what makes cells competent to respond to pluripotency factors. Use and comparison of these different cells may thus lead to identification of new molecular regulators of development and pluripotency. Several methods have been used to identify molecular regulators of pluripotency in both stem cell lines and embryos, and these are summarized next.

Methods for identifying pluripotency factors

Several methods have been used for identifying pluripotency factors in both embryos and stem cells. Studies in embryos have relied on candidate gene analysis, with examination of phenotypes resulting from knockout alleles *in vivo*. Studies in cell lines have been either functional or descriptive, but have been more amenable to more large-scale, whole genome approaches. Large-scale functional strategies have included RNAi screens and overexpression screens (Chambers *et al.* 2003, Ivanova *et al.* 2006), while large-scale descriptive approaches have used gene expression profiling, epigenetic profiling, and protein expression profiling (Boyer *et al.* 2005, Bernstein *et al.* 2006, Loh *et al.* 2006, Wang *et al.* 2006, Walker *et al.* 2007). These approaches have rapidly expanded our knowledge of molecules associated with pluripotency. In some cases, the functional importance of new genes identified in these studies has been further validated in ES cells. However, much remains to be learned of the functions of and epistatic relationships among these genes, not just in established ES lines, but also during normal development. These efforts, in contrast with high throughput approaches, require detailed mechanistic analysis performed in the embryo using knockout mouse lines. Although *in vivo* genetic approaches can be more time consuming than high-throughput *in vitro* approaches, they provide essential knowledge for which there is no substitute.

While the list of pluripotency-associated genes continues to grow, genes involved in cellular reprogramming warrant special attention. The ability of these genes to uniquely initiate a cascade of events leading to the pluripotent stem cell state underscores their fundamental importance at the top of a pluripotency hierarchy. Much consideration has been given to how and why reprogramming even works. Initial speculation raised cautionary caveats about the role of viral integration or cell type in reprogramming. However, non-integrating vectors, excisable vectors, and even soluble proteins, have since been used for factor delivery (Okita *et al.* 2008, Stadtfeld *et al.* 2008b, Kaji *et al.* 2009, Soldner *et al.* 2009, Woltjen *et al.* 2009, Zhou *et al.* 2009).

In addition, reprogramming may not involve unique rare, reprogramming-competent cells since many different cell types have been used (Nakagawa *et al.* 2008, Shi *et al.* 2008, Silva *et al.* 2008, Stadtfeld *et al.* 2008a, Guo *et al.* 2009), although it is hard to rule out this latter possibility completely. Regardless of the origin of the parental cell, exogenous reprogramming factors are clearly required for expansion of iPS cells. A better understanding of the requirement for these genes in embryos and in ES cells is essential to discussion of how reprogramming works.

What are the reprogramming factors?

It all began with a list of ES cell associated transcripts (ECATs), a list of about 20 genes identified as enriched in mouse ES cells compared to other non-pluripotent cell types (Mitsui *et al.* 2003). In the first successful reprogramming study, pools of ECATs were systematically introduced into mouse fibroblasts, while selecting for expression of another ES cell-expressed gene. Eventually, a minimum combination of four transcription factors was identified: POU5F1 (Oct4), SOX2, KLF4, and MYC (cMyc; Takahashi & Yamanaka 2006). Later, MYC was found to be inessential (Nakagawa *et al.* 2008), although it plays a role in the efficiency and speed of reprogramming, which is about twice as slow without MYC (Nakagawa *et al.* 2008). Since this time, human fibroblasts have been reprogrammed with the same four factors (Takahashi *et al.* 2007, Wernig *et al.* 2007, Yu *et al.* 2007, Park *et al.* 2008), or with a slightly different combination of factors, including POU5F1, SOX2, NANOG, and the RNA-binding protein LIN28 (Yu *et al.* 2007). Subsequently, POU5F1 and SOX2 alone have been shown to reprogram human fibroblasts in the presence of a chemical inhibitor of the histone deacetylase valproic acid (Huangfu *et al.* 2008), suggesting that chromatin modification plays an essential role in cellular reprogramming. More recently, many other chemical regulators of chromatin-modifying

machinery have also been shown to substitute for SOX2 or KLF4 or to increase efficiency in reprogramming mouse fibroblasts (Feng *et al.* 2009b).

While reprogramming factors reset the cellular phenotype from the inside, reprogramming clearly also requires extrinsic signals provided by the cell culture milieu, since all reprogramming events have been performed in ES cell culture conditions. These conditions include growth factors, cytokines, and other signals provided by the cell culture medium, fetal bovine serum, and feeder cells. Together, intrinsic and extrinsic factors maintain pluripotency and guarantee self-renewal. How extrinsic signals are integrated with intrinsically acting factors is not entirely clear, but is an active area of investigation. Possibilities include activation of self-renewal/proliferation genes or repression of prodifferentiation genes. For example, signaling by the cytokine LIF leads to activation of the transcription factor STAT3, which may regulate a suite of ES cell genes (Sekai *et al.* 2005, Chen *et al.* 2008). Bone morphogenetic protein (BMP) or serum suppresses expression of the inhibitors of differentiation (Id) family of transcription factors (Ying *et al.* 2003). In addition, parallel signaling pathways have been found to connect LIF signaling and transcription factors maintaining pluripotency in ES cells (Niwa *et al.* 2009). While ES cell signaling pathways will not be discussed further, it is important to keep in mind that they play an essential role in supporting pluripotency. Rather, we will focus on factors delivered into the cell during reprogramming.

Early embryonic development

To understand the role of reprogramming factors in development, it is useful to begin with an overview of mouse development. Following fertilization, the zygote undergoes cleavages that increase cell number without affecting the total size of the embryo. This lasts throughout preimplantation development, and culminates with formation of the blastocyst (Fig. 2). By the

Figure 2 Origins and destination of the first three lineages of the mouse. During cleavage stages, the embryo generates inside and outside cell populations, ultimately thought to give rise to trophoblast (brown) and inner cell mass of the blastocyst. The trophoblast becomes trophoblast, then chorion and ectoplacental cone, and later placenta. The inner cell mass contains cells that become epiblast and later fetus and some extraembryonic tissues (green) and the inner cell mass also contains primitive endoderm cells (yellow) that become parietal and visceral endoderm, which becomes part of the yolk sac.

blastocyst stage, the first three lineages are established: embryo, placenta, and extraembryonic endoderm (Yamanaka *et al.* 2006). Shortly after this stage, the blastocyst hatches from its protein coat, the zona pellucida, and implants in the uterine wall. Subsequent growth, patterning, and reorganization of all three lineages produce mature tissues of the fetus and accompanying extraembryonic tissues through a series of stereotyped steps. Cells on the outside of the blastocyst or trophoblast will form trophoblast structures such as giant cells, extraembryonic ectoderm, chorion, ectoplacental cone, and eventually the placenta (Simmons & Cross 2005). Inside cells of the blastocyst's inner cell mass are fated to become either embryo or extraembryonic endoderm (Chazaud *et al.* 2006). The embryonic lineage, or epiblast, will go on to produce three germ layers during gastrulation, as well as some extraembryonic structures, while the extraembryonic endoderm will give rise to the endoderm of the yolk sac. Since ES cells are derived from the inner cell mass of the blastocyst, ES cell genes may also play an essential early role in development.

The list of genes regulating pluripotency in ES cells continues to grow at an exciting pace. However, relatively few of these genes have been demonstrated to possess the unique ability to participate to induce pluripotency, and some have been found not to promote reprogramming (Feng *et al.* 2009a). Arguably, genes sufficient to reprogram mature cell types could play a special upstream role in establishment of ES or ICM-like properties and are thus of particular interest to developmental biologists. We will therefore focus on the subset of pluripotency genes that have been shown to be dominantly capable of reprogramming mature cell types during induced pluripotency. Here, we will examine requirements for these reprogramming factors in mouse embryonic development and ES cell homeostasis.

POU5F1

POU5F1 (also known as Oct3, Oct4 and Oct3/4) is a POU-domain transcription factor encoded by the *Pou5f1* gene. Given that POU5F1 plays essential roles in both the mouse embryo and in ES cells, the involvement of POU5F1 in reprogramming was not surprising. In embryos lacking *Pou5f1*, the inner cell mass degenerates, and neither embryonic nor extraembryonic endoderm cells survive (Nichols *et al.* 1998). Not surprisingly, ES cells cannot be derived from *Pou5f1* mutants (Nichols *et al.* 1998). Moreover, genetic reduction of *Pou5f1* in established ES cell lines leads to the formation of trophoblast-like cells (Niwa *et al.* 2000). Together, these observations have led to the proposal that POU5F1 represses the trophoblast program of development in ES cells and during early lineage decisions in the embryo.

POU5F1 plays different roles in stem cells at later stages in mouse development. At later stages, *Pou5f1* is

expressed in multipotent stem cells, including PGCs (Scholer *et al.* 1990), where it is required for survival (Kehler *et al.* 2004). These observations led to the proposal that POU5F1 promotes 'stem cell-ness' in general. However, *Pou5f1* is not required for self-renewal of somatic stem cells in a wide variety of organs (Lengner *et al.* 2007). Thus, although POU5F1 is an essential reprogramming factor, enabling mature somatic cells to revert to an ES-like state, it does not appear to be required for somatic stem cell self-renewal. This observation underscores intrinsic differences in gain and loss of function assays. Notably, POU5F1 is sufficient to prevent differentiation and expand progenitor cell proliferations when overexpressed in adult epithelia, presumably due to expansion of adult stem cell populations (Hochedlinger *et al.* 2005). However, in spite of the proposed role for *Pou5f1* in maintaining ES cell fates, overexpression of *Pou5f1* in ES cells does not maintain their self-renewal in the absence of self-renewal factors such as LIF, but leads to their differentiation (Niwa *et al.* 2000). This phenotype was proposed to result from titration of other factors by overexpressed *Pou5f1* in ES cells. Thus, functional differences in response to POU5F1 exist between adult and ES cells, but the molecular basis for these differences is not understood. One testable hypothesis is that different POU5F1 cofactors exist within ES and adult stem cell populations, and this could alter the response to ectopic POU5F1.

Several key POU5F1 cofactors operating in ES cells are known. According to the current view, POU5F1 acts together with SOX2 and NANOG at the core of a transcription factor network regulating pluripotency in ES cells. These three proteins have been found to co-occupy putative enhancer elements of genes thought to promote pluripotency and repress differentiation (Boyer *et al.* 2005, Loh *et al.* 2006). POU5F1 and SOX2 bind transcriptional targets in a protein-DNA ternary complex (Yuan *et al.* 1995), and promote their own expression (Tomioka *et al.* 2002, Okumura-Nakanishi *et al.* 2005), as well as that of *Nanog* (Kuroda *et al.* 2005) in ES cells. However, many genes are not co-occupied by all three factors (Boyer *et al.* 2005, Loh *et al.* 2006), and thus each gene must have distinct targets as well. The central importance of SOX2 and NANOG to ES and iPS cell biology is recapitulated by the requirement for these genes in early embryogenesis.

SOX2

The SRY-box containing transcription factor SOX2 has been used for most genetic reprogramming efforts, excluding those in which *Sox2* was expressed endogenously in the starting cell population (Kim *et al.* 2008, 2009). As an POU5F1 cofactor, *Sox2* mutants might be expected to phenocopy *Pou5f1* mutants. However, *Sox2* mutants are lethal at a slightly later stage than *Pou5f1* mutants, suggesting that maternal SOX2 protein may

participate in early lineage decisions (Avilion *et al.* 2003). Importantly, *Sox2* mutants do exhibit defects in the embryonic lineage, or epiblast, although at a later stage than *Pou5f1* mutants. Interestingly, the placental lineage is also affected, with defects detected in the chorion. Like *Pou5f1*, *Sox2* is required for maintaining pluripotency and/or self-renewal in ES cells. ES cells cannot be derived from *Sox2* null embryos (Avilion *et al.* 2003), even though embryos survive beyond the point at which ES cells are derived. This observation highlights an intrinsic difference between formation of ES cells and embryogenesis, i.e. establishment of pluripotency must proceed by a unique genetic program, which is distinct from programs overseeing normal development.

Analysis of the requirement for *Sox2* in existing ES cell lines has been informative. Reduction of *Sox2* levels in established ES cell lines leads to formation of trophoblast-like cells (Li *et al.* 2007, Masui *et al.* 2007), supporting the notion that POU5F1 and SOX2 cooperate to repress trophoblast fates in ES cells. Notably, *Pou5f1* overexpression can rescue *Sox2* deficiency in ES cells (Masui *et al.* 2007), consistent with the proposal that activation of *Pou5f1* expression is a major role of SOX2. However, these observations also suggest that POU5F1/SOX2 dimerization is not essential for activation of pluripotency network genes, although this proposal has not been tested by direct interference of POU5F1/SOX2 binding. Similar to the consequences of *Pou5f1* overexpression, overexpression of *Sox2* also leads to rapid differentiation of ES cells (Kopp *et al.* 2008).

The role of *Sox2* as a general supporter of stem cell behavior has been examined in other stem cell contexts as well. SOX2 is also highly expressed in progenitor cells throughout the CNS. *Sox2* is not absolutely required for neural stem cell self-renewal or multipotency (Miyagi *et al.* 2008). However, genetic redundancy with other SOX factors may mask its role. Interestingly, in retinal progenitor cells, where *Sox2* is the sole SOX factor expressed, *Sox2* is required for proliferation and differentiation (Taranova *et al.* 2006). Beyond regulating proliferation, *Sox2* plays important roles in embryo patterning, such as foregut patterning (Que *et al.* 2007). Thus, *Sox2* appears to be regulated in a context-dependent manner to achieve multiple developmental outcomes.

NANOG

The homeobox transcription factor NANOG, which has been used for reprogramming human cells (Yu *et al.* 2007), plays an essential role during lineage specification in the mouse embryo. In the mouse, *Nanog* mutant exhibit defects in lineage specification sometime between the blastocyst and embryonic day 5.5, and only extraembryonic endoderm appears to survive in the absence of *Nanog* (Mitsui *et al.* 2003). Thus, *Nanog* is essential for the embryonic versus extraembryonic endoderm lineage decision.

The requirement for *Nanog* in pluripotency has been examined to some extent as well. Deletion of *Nanog* from existing ES cells does not eliminate their ability to self-renew or contribute to embryos in chimeras (Mitsui *et al.* 2003, Chambers *et al.* 2007). *Nanog* null ES cells exhibit subtle morphological differences and are more prone to differentiate than wild type ES cells (Mitsui *et al.* 2003, Chambers *et al.* 2007). Normal populations of ES cells, in fact, appear to express variable levels of NANOG, with those expressing less NANOG being more prone to differentiation and less prone to self-renewal (Hatano *et al.* 2005, Chambers *et al.* 2007). Thus, NANOG levels may act as a switch, allowing ES cells to rapidly choose between self-renewal and differentiation. Importantly, *Pou5f1* and *Sox2* continue to be expressed in ES cells forcibly lacking NANOG, as are all other ES cell markers examined (Chambers *et al.* 2007). This observation is consistent with the fact that *Pou5f1* and *Sox2* are alone sufficient to initiate the reprogramming cascade in both mouse and human cells in the absence of ectopic *Nanog* (Takahashi & Yamanaka 2006, Takahashi *et al.* 2007). Nonetheless, overexpression of *Nanog* in mouse ES cells can prevent differentiation in the absence of the critical self-renewal cytokine LIF (Chambers *et al.* 2003, Mitsui *et al.* 2003). Thus, *Nanog* is sufficient, but not necessary for ensuring self-renewal.

The role of *Nanog* in other stem cell types of the mouse has been examined to a limited extent. *Nanog* is also expressed in the germline (Hatano *et al.* 2005), and *Nanog* mutant ES cells are not detected in the germline beyond embryonic day 11.5 (Chambers *et al.* 2007), suggesting a requirement for *Nanog* in germline stem cells. Analysis of the requirement for *Nanog* in the mouse at later stages should provide interesting insight into its role in adult stem cells, and how these differ from pluripotent stem cells.

KLF4

KLF4 belongs to a large family of 17 Kruppel-like zinc finger domain transcription factors, several of which are expressed in the preimplantation mouse embryo. Loss of *Klf4* does not result in embryonic lethality, but to defects in multiple lineages post-natally, including skin (Segre *et al.* 1999) and goblet cells in the colon (Katz *et al.* 2002). Reduction of *Klf4* in ES cells does not alter self-renewal or developmental potential (Nakatake *et al.* 2006, Jiang *et al.* 2008). However, two other *Klf* factors (*Klf2* and *Klf5*) are co-expressed with *Klf4* in ES cells (Jiang *et al.* 2008). While reduction of each *Klf* alone, or each combination of two *Klfs*, did not perturb ES cell self-renewal, loss of the three *Klf* factors simultaneously led to differentiation and decreased proliferation (Jiang *et al.* 2008). Together, these results argue for genetic redundancy among the *Klf* genes during lineage specification.

KLF4 is sufficient to trigger reprogramming when combined with POU5F1 and SOX2, and supports other KLF factors during pluripotency and self-renewal. Consistent with this, overexpression of *Klf4* can reduce ES cell differentiation in some assays (Li *et al.* 2005). In addition, other KLF factors, such as KLF1, KLF2, and KLF5 can substitute for KLF4 during reprogramming, albeit with lower efficiency (Nakagawa *et al.* 2008). Genome-wide location analysis revealed that the KLF factors bind putative regulatory elements of *Pou5f1*, *Sox2*, *Nanog*, and many other pluripotency-associated genes (Nakagawa *et al.* 2008). Thus, KLF may participate in regulating a pluripotency network.

Examination of the requirement for other KLF factors has led to some insight into the connection between KLF factors in ES cells and their establishment in the early embryo. While loss of *Klf2* leads to embryonic lethality between embryonic days 12–14 owing to severe hemorrhage (Kuo *et al.* 1997), loss of *Klf5* has a much earlier phenotype. Loss of *Klf5* leads to lethality due to trophoblast defects around the blastocyst stage (Ema *et al.* 2008). ES cells cannot be derived from *Klf5* null embryos, even when trophoblast tissues are rescued by tetraploid complementation (Ema *et al.* 2008). When deleted from existing ES cells, ES cells continue to self-renew, although they are more prone to spontaneously differentiate and proliferate more slowly (Ema *et al.* 2008). This phenotype was not observed in RNAi knockdown experiments. However, it is not currently clear whether this discrepancy is due to differences between ES cell establishment and maintenance, or simply to the assay in use. Overexpression of *Klf4* can rescue *Klf5*-deficient ES cells *Klf5* (Ema *et al.* 2008), again suggesting some redundancy between these *Klf* factors.

Since *Klf4* and *Klf5* play overlapping roles in pluripotency, study of *Klf5* may provide insight into the mechanism by which *Klf4* promotes pluripotency. One mechanism proposed for KLF5-mediated maintenance of ES cell self-renewal involves the TCL1–AKT pathway. Both TCL1 levels and AKT phosphorylation are reduced in ES cells in the absence of *Klf5*, and expression of a constitutively active form of AKT partially restores the rate of proliferation to wild-type levels. These observations suggest that KLF factors may regulate self-renewal through regulation of AKT signaling. Alternatively, KLF4 may upregulate *Nanog* during reprogramming by repressing p53 (Rowland *et al.* 2005). Elucidating the precise molecular mechanisms underlying the role of KLF factors in maintaining and driving pluripotency will be an exciting avenue for future research.

ESRRB

The orphan nuclear factor estrogen-related receptor beta (ESRRB) was recently shown to be capable of reprogramming mouse fibroblasts in conjunction with POU5F1 and SOX2 (Feng *et al.* 2009a). ESRRB can thus substitute

for KLF4 in reprogramming. Consistent with this proposal, the authors found that ESRRB can also rescue self-renewal in ES cells in which all three *Klf* paralogs had been knocked down. In addition, genome-wide location analysis revealed a tendency for ESRRB-binding sites to colocalize with KLF-binding sites (Feng *et al.* 2009a). The authors suggest that ESRRB may promote reprogramming via KLF factors. However, the efficiency of reprogramming by ESRRB was lower than by KLF4 (Feng *et al.* 2009a). In addition, differences exist between the roles of these two genes in the embryo.

Both the timing and tissues affected differ between *Esrrb* and *Klf* gene family knockouts. *Esrrb* knockout embryos die around E10.5 with placental defects (Luo *et al.* 1997). Moreover, deletion of *Esrrb* strictly within the embryonic lineage rescues this phenotype (Luo *et al.* 1997), arguing for a specific requirement for *Esrrb* in the extraembryonic lineage. Genetic redundancy with the related genes *Esrra* or *Esrrg* could preclude observation of a requirement for *Esrrb* in the embryonic lineage. Interestingly, *Esrrg*, but not *Esrra* could also promote reprogramming in the presence of POU5F1 and SOX2 (Feng *et al.* 2009a). Analysis of *Esrrb* and *Esrrg* double knockout embryos would therefore be particularly interesting.

MYC

The basic helix–loop–helix/leucine zipper transcription factor MYC is neither essential for reprogramming nor for early lineage specifications. Rather, *Myc* null embryos die between embryonic days 9 and 10, with a phenotype suggesting requirements in growth and proliferation of many organ systems (Davis *et al.* 1993). However, much of this phenotype is likely to result indirectly from abnormal extraembryonic structures in these mutants, since embryo-specific loss of *Myc* enables normal development of all organs, with the exception of the hematopoietic system, up to day 12 of embryogenesis (Dubois *et al.* 2008). MYC has been independently shown to be important for regulating hematopoietic stem cell self-renewal and differentiation (Wilson *et al.* 2004). The related factor MYCN (nMyc) has been shown to be important in neural stem cells (Knoepfler *et al.* 2002), suggesting a more general role for MYC factors in proliferative populations. Whether simultaneous loss of MYC, MYCN, and/or related MYCL1 (lMyc) would result in earlier developmental or widespread stem cell defects remains an open question.

In addition to promoting proliferation and survival of somatic stem cells, MYC may play an important role in ES cell proliferation. *Myc* levels are decreased as ES cells undergo differentiation during LIF withdrawal, and overexpression of a dominant-negative MYC promotes differentiation (Cartwright *et al.* 2005). Interestingly, overexpression of stable, non-phosphorylatable MYC variant can delay differentiation (Cartwright *et al.* 2005), suggesting a potential link between proliferation and potency.

Although it is still unclear what role MYC plays during reprogramming, several models have been proposed (Knoepfler 2008). For example, MYC may simply promote proliferation, which could indirectly alter the state of the cell or activity of the reprogramming factors, i.e. MYC-stimulated proliferation could lead to chromatin remodeling, facilitating access of reprogramming factors to pluripotency targets. This hypothesis is consistent with the finding that the related protein MYCN influences chromatin structure in neural stem cells (Knoepfler *et al.* 2006). Alternatively, MYC may have its own set of pluripotency targets. MYC and the LIF signaling target STAT3 co-occupy putative regulatory elements of many pluripotency-associated genes in ES cells (Kidder *et al.* 2008). Thus, MYC may help transduce pluripotency-promoting effects of signaling pathways.

While MYC can be omitted completely during reprogramming, its inclusion greatly increases efficiency (Nakagawa *et al.* 2008). Importantly, fibroblasts express *Myc* at around 20% of levels expressed in ES cells, and this is increased during reprogramming (Nakagawa *et al.* 2008), suggesting that endogenous *Myc* still participates in reprogramming. Nonetheless, it may be safer to avoid use of exogenous *Myc*, as tumors have been observed to arise in mice generated from iPS cells bearing MYC, but not from those that did not (Takahashi & Yamanaka 2006, Takahashi *et al.* 2007), although this appears to be cell type-specific (Nakagawa *et al.* 2008). As an alternative, other MYC paralogs can substitute in the reprogramming mix. For example, MYCL1 (Nakagawa *et al.* 2008) and MYCN have been used, resulting in reduced tumorigenicity (Blelloch *et al.* 2007, Nakagawa *et al.* 2008).

The microRNA pathway

Two studies have identified roles for the microRNA (miRNA) pathway in genetic reprogramming of fibroblasts to pluripotent stem cells. One of these studies identified the miRNA processing protein LIN28 as sufficient to reprogram human fibroblasts, in conjunction with POU5F1, SOX2, and NANOG (Yu *et al.* 2007). The other study identified a subset of miRNAs that improve reprogramming efficiency in the mouse, when combined with POU5F1, SOX2, and KLF4 (Judson *et al.* 2009). Interestingly, both pathways involve MYC, although at different levels, either upstream or downstream of MYC (Fig. 3).

LIN28, originally identified in *Caenorhabditis elegans*, is an RNA-binding protein that has been shown to participate in reprogramming of human cells in combination with POU5F1, SOX2, and NANOG (Yu *et al.* 2007). *Lin28* exhibits dynamic expression patterns throughout mouse embryogenesis (Yang & Moss 2003), suggesting important and developmentally-regulated roles. *Lin28* is required for differentiation of skeletal muscle from cultured myoblasts (Polesskaya *et al.* 2007). *Lin28* knockdown in ES cells

Figure 3 Model of factor activity during reprogramming. POU5F1, SOX2, KLF4 or *Esrrb*, and MYC (mouse) or POU5F1, SOX2, NANOG, and LIN28 (human) can reprogram fibroblasts to ES-like cells. Chromatin remodeling, directly or indirectly mediated by MYC, is thought to facilitate access of downstream pluripotency and self-renewal genes to reprogramming factors. While miRNAs can influence MYC stability, a special class of miRNAs also acts downstream of MYC to promote some aspects of ES cell behavior.

limits germ cell formation both *in vivo* and *in vitro* (West *et al.* 2009). Analysis of a null allele should provide exciting insight into the role of miRNAs in early lineage specifications.

Lin28 is expressed at high levels in both mouse and human ES cells, and is downregulated during their differentiation (Yang & Moss 2003, Richards *et al.* 2004). *Lin28* has recently been found to be necessary and sufficient to inhibit processing of the miRNA *let7* in mouse ES cells (Viswanathan *et al.* 2008). However, levels of pluripotency markers were unchanged (Viswanathan *et al.* 2008). Nonetheless, *Lin28* is able to participate in cellular reprogramming in human cells (Yu *et al.* 2007) and promote proliferation of tumor cells (Guo *et al.* 2006). The explanation for this behavior may lie with the fact that LIN28 appears to be capable of regulating *Myc* through *let7*, i.e. the miRNA *let7* (*Mirlet7*), which is regulated by LIN28, can target *Myc* for degradation (Koscianska *et al.* 2007, Kumar *et al.* 2007). Consistent with this observation, *Lin28* can regulate *Myc* expression in cultured myoblasts (Polesskaya *et al.* 2007). These observations are consistent with *Lin28* substituting for *Myc* during reprogramming in human cells. Examination of the requirement for *Lin28* during mouse development should help reveal to what extent *Lin28* activity is conserved between human and mouse, and whether there are additional *Lin28* targets besides *Myc*.

More recently, a subset of miRNAs was found to increase efficiency of mouse fibroblast reprogramming by POU5F1, SOX2, and KLF4 (Judson *et al.* 2009). Importantly, these miRNAs, which include members of the *miR-290* family, led to an increased proportion of correctly reprogrammed iPS colonies, than when MYC was used. More detailed analysis revealed that the expression of these miRNAs is normally activated during conventional 3 or 4 factor-based reprogramming, but not until relatively late in the process, suggesting that these miRNAs act fairly downstream in the reprogramming process.

Members of the *miR-290* family are normally expressed in ES cells and downregulated during their differentiation (Wang *et al.* 2008). These miRNAs play an important role in cell cycle regulation in self-renewing ES cells (Wang *et al.* 2008). However, during reprogramming they may act downstream of MYC and subsequent to chromatin remodeling (Judson *et al.* 2009). The requirement for the *miR-290* cluster during embryonic development has not been reported. Partial insight into this issue may be provided by analysis of loss of *Dgcr8*, an RNA-binding protein specific to the miRNA pathway (Wang *et al.* 2007). Knockout of *Dgcr8* leads to abnormal development by E6.5, and lethality sometime prior to E10 (Wang *et al.* 2007), consistent with a possible role in early lineage specification.

Programming and reprogramming in reproduction and human health

We are only just beginning to understand how reprogramming factors induce pluripotency in differentiated cells. Systems-level analyses in iPS and ES cells have identified large lists of genes probably acting downstream of the reprogramming genes to effect cellular changes at different levels (Fig. 3). Examining the requirement of endogenous copies of these genes and targets during reprogramming will be essential for testing this and other models of induced pluripotency. For example, two studies have shown that a single factor can revert other stem cell types to ES-like cells (Guo *et al.* 2009, Kim *et al.* 2009), and these efforts were presumably successful owing to the endogenous expression of other reprogramming factors and/or targets. Reprogramming in cells lacking components of the pluripotency network should help formally test this assumption and organize lists of genes into discrete pathways promoting pluripotency.

While the list of genes sufficient to induce pluripotency in mature cell types continues to grow, so do the number of small molecules capable of substituting for one or more of them. Together, these studies will facilitate alternative and possibly safer mechanisms for cellular reprogramming, with the ultimate goal of generating patient-derived stem cells for therapeutic

treatment of a variety of human health issues. Meanwhile, examination of the role of these genes during embryonic development in the mouse will help us close the gap between induced pluripotency and fetal health.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This work was supported by grant no. FRN13426 from the Canadian Institutes of Health Research.

Acknowledgements

We are indebted to members of the Rossant Laboratory for discussion.

References

- Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N & Lovell-Badge R 2003 Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes and Development* **17** 126–140.
- Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K *et al.* 2006 A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125** 315–326.
- Blelloch R, Venere M, Yen J & Ramalho-Santos M 2007 Generation of induced pluripotent stem cells in the absence of drug selection. *Cell Stem Cell* **1** 245–247.
- Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG *et al.* 2005 Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122** 947–956.
- Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA *et al.* 2007 Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448** 191–195.
- Cartwright P, McLean C, Sheppard A, Rivett D, Jones K & Dalton S 2005 LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* **132** 885–896.
- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S & Smith A 2003 Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113** 643–655.
- Chambers I, Silva J, Colby D, Nichols J, Nijmeijer B, Robertson M, Vrana J, Jones K, Grotewold L & Smith A 2007 Nanog safeguards pluripotency and mediates germline development. *Nature* **450** 1230–1234.
- Chazaud C, Yamanaka Y, Pawson T & Rossant J 2006 Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2–MAPK pathway. *Developmental Cell* **10** 615–624.
- Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J *et al.* 2008 Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* **133** 1106–1117.
- Davis AC, Wims M, Spotts GD, Hann SR & Bradley A 1993 A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes and Development* **7** 671–682.
- Dubois NC, Adolphe C, Ehninger A, Wang RA, Robertson EJ & Trumpp A 2008 Placental rescue reveals a sole requirement for c-Myc in embryonic erythroblast survival and hematopoietic stem cell function. *Development* **135** 2455–2465.

- Ena M, Mori D, Niwa H, Hasegawa Y, Yamanaka Y, Hitoshi S, Mimura J, Kawabe Y, Hosoya T, Morita M *et al.* 2008 Kruppel-like factor 5 is essential for blastocyst development and the normal self-renewal of mouse ESCs. *Cell Stem Cell* 3 555–567.
- Evans MJ & Kaufman MH 1981 Establishment in culture of pluripotent cells from mouse embryos. *Nature* 292 154–156.
- Feng B, Jiang J, Kraus P, Ng JH, Heng JC, Chan YS, Yaw LP, Zhang W, Loh YH, Han J *et al.* 2009a Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. *Nature Cell Biology* 11 197–203.
- Feng B, Ng JH, Heng JC & Ng HH 2009b Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. *Cell Stem Cell* 4 301–312.
- Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W *et al.* 2006 Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 440 1199–1203.
- Guo Y, Chen Y, Ito H, Watanabe A, Ge X, Kodama T & Aburatani H 2006 Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma. *Gene* 384 51–61.
- Guo G, Yang J, Nichols J, Hall JS, Eyres I, Mansfield W & Smith A 2009 Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* 136 1063–1069.
- Hatano SY, Tada M, Kimura H, Yamaguchi S, Kono T, Nakano T, Suemori H, Nakatsuji N & Tada T 2005 Pluripotential competence of cells associated with Nanog activity. *Mechanisms of Development* 122 67–79.
- Hochedlinger K, Yamada Y, Beard C & Jaenisch R 2005 Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. *Cell* 121 465–477.
- Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W & Melton DA 2008 Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nature Biotechnology* 26 1269–1275.
- Ivanova N, Dobrin R, Lu R, Kotenko I, Levorse J, DeCoste C, Schafer X, Lun Y & Lemischka IR 2006 Dissecting self-renewal in stem cells with RNA interference. *Nature* 442 533–538.
- Jiang J, Chan YS, Loh YH, Cai J, Tong GQ, Lim CA, Robson P, Zhong S & Ng HH 2008 A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nature Cell Biology* 10 353–360.
- Judson RL, Babiarczyk JE, Venere M & Blueloch R 2009 Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nature Biotechnology* 27 459–461.
- Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P & Woltjen K 2009 Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 458 771–775.
- Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S *et al.* 2004 Generation of pluripotent stem cells from neonatal mouse testis. *Cell* 119 1001–1012.
- Katz JP, Perreault N, Goldstein BG, Lee CS, Labosky PA, Yang VW & Kaestner KH 2002 The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. *Development* 129 2619–2628.
- Kehler J, Tolkunova E, Koschorz B, Pesce M, Gentile L, Boiani M, Lomeli H, Nagy A, McLaughlin KJ, Scholer HR *et al.* 2004 Oct4 is required for primordial germ cell survival. *EMBO Reports* 5 1078–1083.
- Kidder BL, Yang J & Palmer S 2008 Stat3 and c-Myc genome-wide promoter occupancy in embryonic stem cells. *PLoS ONE* 3 e3932.
- Kim JB, Zaehres H, Wu G, Gentile L, Ko K, Sebastiano V, Arauzo-Bravo MJ, Ruau D, Han DW, Zenke M *et al.* 2008 Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 454 646–650.
- Kim JB, Sebastiano V, Wu G, Arauzo-Bravo MJ, Sasse P, Gentile L, Ko K, Ruau D, Ehrlich M, van den Boom D *et al.* 2009 Oct4-induced pluripotency in adult neural stem cells. *Cell* 136 411–419.
- Knoepfler PS 2008 Why myc? An unexpected ingredient in the stem cell cocktail. *Cell Stem Cell* 2 18–21.
- Knoepfler PS, Cheng PF & Eisenman RN 2002 N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. *Genes and Development* 16 2699–2712.
- Knoepfler PS, Zhang XY, Cheng PF, Gafken PR, McMahon SB & Eisenman RN 2006 Myc influences global chromatin structure. *EMBO Journal* 25 2723–2734.
- Kopp JL, Ormsbee BD, Desler M & Rizzino A 2008 Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. *Stem Cells* 26 903–911.
- Koscianska E, Baev V, Skreka K, Oikonomaki K, Rusinov V, Tabler M & Kalantidis K 2007 Prediction and preliminary validation of oncogene regulation by miRNAs. *BMC Molecular Biology* 8 79.
- Kumar MS, Lu J, Mercer KL, Golub TR & Jacks T 2007 Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nature Genetics* 39 673–677.
- Kuo CT, Veselits ML, Barton KP, Lu MM, Clendenin C & Leiden JM 1997 The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. *Genes and Development* 11 2996–3006.
- Kuroda T, Tada M, Kubota H, Kimura H, Hatano SY, Suemori H, Nakatsuji N & Tada T 2005 Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Molecular and Cellular Biology* 25 2475–2485.
- Lengner CJ, Camargo FD, Hochedlinger K, Welstead GG, Zaidi S, Gokhale S, Scholer HR, Tomilin A & Jaenisch R 2007 Oct4 expression is not required for mouse somatic stem cell self-renewal. *Cell Stem Cell* 1 403–415.
- Li Y, McClintick J, Zhong L, Edenberg HJ, Yoder MC & Chan RJ 2005 Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. *Blood* 105 635–637.
- Li J, Pan G, Cui K, Liu Y, Xu S & Pei D 2007 A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. *Journal of Biological Chemistry* 282 19481–19492.
- Li W, Wei W, Zhu S, Zhu J, Shi Y, Lin T, Hao E, Hayek A, Deng H & Ding S 2009 Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell* 4 16–19.
- Liu H, Zhu F, Yong J, Zhang P, Hou P, Li H, Jiang W, Cai J, Liu M, Cui K *et al.* 2008 Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell* 3 587–590.
- Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J *et al.* 2006 The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nature Genetics* 38 431–440.
- Luo J, Sladek R, Bader JA, Matthysen A, Rossant J & Giguere V 1997 Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. *Nature* 388 778–782.
- Maherali N & Hochedlinger K 2008 Guidelines and techniques for the generation of induced pluripotent stem cells. *Cell Stem Cell* 3 595–605.
- Martin GR 1981 Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *PNAS* 78 7634–7638.
- Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA *et al.* 2007 Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nature Cell Biology* 9 625–635.
- Matsui Y, Zsebo K & Hogan BL 1992 Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* 70 841–847.
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M & Yamanaka S 2003 The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113 631–642.
- Miyagi S, Masui S, Niwa H, Saito T, Shimazaki T, Okano H, Nishimoto M, Muramatsu M, Iwama A & Okuda A 2008 Consequence of the loss of Sox2 in the developing brain of the mouse. *FEBS Letters* 582 2811–2815.
- Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochizuki Y, Takizawa N & Yamanaka S 2008 Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nature Biotechnology* 26 101–106.
- Nakatake Y, Fukui N, Iwamatsu Y, Masui S, Takahashi K, Yagi R, Yagi K, Miyazaki J, Matoba R, Ko MS *et al.* 2006 Klf4 cooperates with Oct3/4 and Sox2 to activate the Lefty1 core promoter in embryonic stem cells. *Molecular and Cellular Biology* 26 7772–7782.
- Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H & Smith A 1998 Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95 379–391.

- Niwa H, Miyazaki J & Smith AG 2000 Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genetics* **24** 372–376.
- Niwa H, Ogawa K, Shimosato D & Adachi K 2009 A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature* **460** 118–122.
- Okita K, Nakagawa M, Hyenjong H, Ichisaka T & Yamanaka S 2008 Generation of mouse induced pluripotent stem cells without viral vectors. *Science* **322** 949–953.
- Okumura-Nakanishi S, Saito M, Niwa H & Ishikawa F 2005 Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. *Journal of Biological Chemistry* **280** 5307–5317.
- Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW & Daley GQ 2008 Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451** 141–146.
- Polesskaya A, Cuvellier S, Naguibneva I, Duquet A, Moss EG & Harel-Bellan A 2007 Lin-28 binds IGF-2 mRNA and participates in skeletal myogenesis by increasing translation efficiency. *Genes and Development* **21** 1125–1138.
- Que J, Okubo T, Goldenring JR, Nam KT, Kurotani R, Morrissey EE, Taranova O, Pevny LH & Hogan BL 2007 Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* **134** 2521–2531.
- Richards M, Tan SP, Tan JH, Chan WK & Bongso A 2004 The transcriptome profile of human embryonic stem cells as defined by SAGE. *Stem Cells* **22** 51–64.
- Rossant J 2008 Stem cells and early lineage development. *Cell* **132** 527–531.
- Rowland BD, Bernards R & Peeper DS 2005 The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nature Cell Biology* **7** 1074–1082.
- Scholer HR, Dressler GR, Balling R, Rohdewohld H & Gruss P 1990 Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO Journal* **9** 2185–2195.
- Segre JA, Bauer C & Fuchs E 1999 Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nature Genetics* **22** 356–360.
- Sekkai D, Gruel G, Herry M, Moucadel V, Constantinescu SN, Albagli O, Tronik-Le Roux D, Vainchenker W & Bennaceur-Griscelli A 2005 Microarray analysis of LIF/Stat3 transcriptional targets in embryonic stem cells. *Stem Cells* **23** 1634–1642.
- Shi Y, Do JT, Despons C, Hahm HS, Scholer HR & Ding S 2008 A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell* **2** 525–528.
- Silva J, Barrandon O, Nichols J, Kawaguchi J, Theunissen TW & Smith A 2008 Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS Biology* **6** e253.
- Simmons DG & Cross JC 2005 Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. *Developmental Biology* **284** 12–24.
- Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M *et al.* 2009 Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* **136** 964–977.
- Stadtfeld M, Brennand K & Hochedlinger K 2008a Reprogramming of pancreatic beta cells into induced pluripotent stem cells. *Current Biology* **18** 890–894.
- Stadtfeld M, Nagaya M, Utikal J, Weir G & Hochedlinger K 2008b Induced pluripotent stem cells generated without viral integration. *Science* **322** 945–949.
- Tada M, Takahama Y, Abe K, Nakatsuji N & Tada T 2001 Nuclear reprogramming of somatic cells by *in vitro* hybridization with ES cells. *Current Biology* **11** 1553–1558.
- Takahashi K & Yamanaka S 2006 Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126** 663–676.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K & Yamanaka S 2007 Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131** 861–872.
- Taranova OV, Magness ST, Fagan BM, Wu Y, Surzenko N, Hutton SR & Pevny LH 2006 SOX2 is a dose-dependent regulator of retinal neural progenitor competence. *Genes and Development* **20** 1187–1202.
- Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL & McKay RD 2007 New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448** 196–199.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS & Jones JM 1998 Embryonic stem cell lines derived from human blastocysts. *Science* **282** 1145–1147.
- Tomioka M, Nishimoto M, Miyagi S, Katayanagi T, Fukui N, Niwa H, Muramatsu M & Okuda A 2002 Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. *Nucleic Acids Research* **30** 3202–3213.
- Viswanathan SR, Daley GQ & Gregory RI 2008 Selective blockade of microRNA processing by Lin28. *Science* **320** 97–100.
- Walker E, Ohishi M, Davey RE, Zhang W, Cassar PA, Tanaka TS, Der SD, Morris Q, Hughes TR, Zandstra PW *et al.* 2007 Prediction and testing of novel transcriptional networks regulating embryonic stem cell self-renewal and commitment. *Cell Stem Cell* **1** 71–86.
- Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW & Orkin SH 2006 A protein interaction network for pluripotency of embryonic stem cells. *Nature* **444** 364–368.
- Wang Y, Medvid R, Melton C, Jaenisch R & Blelloch R 2007 DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nature Genetics* **39** 380–385.
- Wang Y, Baskerville S, Shenoy A, Babiarz JE, Baehner L & Blelloch R 2008 Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. *Nature Genetics* **40** 1478–1483.
- Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE & Jaenisch R 2007 *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448** 318–324.
- West JA, Viswanathan SR, Yabuuchi A, Cunniff K, Takeuchi A, Park IH, Sero JE, Zhu H, Perez-Atayde A, Frazier AL *et al.* 2009 A role for *Lin28* in primordial germ-cell development and germ-cell malignancy. *Nature* **460** 909–913.
- Wilmot I, Schnieke AE, McWhir J, Kind AJ & Campbell KH 1997 Viable offspring derived from fetal and adult mammalian cells. *Nature* **385** 810–813.
- Wilson A, Murphy MJ, Oskarsson T, Kaloulis K, Bettess MD, Oser GM, Pasche AC, Knabenhans C, Macdonald HR & Trumpp A 2004 c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes and Development* **18** 2747–2763.
- Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M *et al.* 2009 piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* **458** 766–770.
- Yamanaka Y, Ralston A, Stephenson RO & Rossant J 2006 Cell and molecular regulation of the mouse blastocyst. *Developmental Dynamics* **235** 2301–2314.
- Yang DH & Moss EG 2003 Temporally regulated expression of Lin-28 in diverse tissues of the developing mouse. *Gene Expression Patterns* **3** 719–726.
- Ying QL, Nichols J, Chambers I & Smith A 2003 BMP induction of *Id* proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115** 281–292.
- Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R *et al.* 2007 Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318** 1917–1920.
- Yuan H, Corbi N, Basilico C & Dailey L 1995 Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes and Development* **9** 2635–2645.
- Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y *et al.* 2009 Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* **4** 381–384.

Received 26 January 2009

First decision 23 March 2009

Revised manuscript received 18 June 2009

Accepted 15 July 2009