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REVIEW

Delineating nuclear reprogramming

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ABSTRACT

Nuclear reprogramming is described as a molecular switch, triggered by the conversion of one cell type to another. Several key experiments in the past century have provided insight into the field of nuclear reprogramming. Previously deemed impossible, this research area is now brimming with new findings and developments. In this review, we aim to give a historical perspective on how the notion of nuclear reprogramming was established, describing main experiments that were performed, including (1) somatic cell nuclear transfer, (2) exposure to cell extracts and cell fusion, and (3) transcription factor induced lineage switch. Ultimately, we focus on (4) transcription factor induced pluripotency, as initiated by a landmark discovery in 2006, where the process of converting somatic cells to a pluripotent state was narrowed down to four transcription factors. The conception that somatic cells possess the capacity to revert to an immature status brings about huge clinical implications including personalized therapy, drug screening and disease modeling. Although this technology has potential to revolutionize the medical field, it is still impeded by technical and biological obstacles. This review describes the effervescent changes in this field, addresses bottlenecks hindering its advancement and in conclusion, applies the latest findings to overcome these issues.

KEYWORDS nuclear reprogramming, naïve pluripotency

INTRODUCTION

Nuclear reprogramming is depicted by the transition between distinct cell types introduced by switches in gene patterns. The conception of this phenomenon evolved through the establishment of several experimental milestones and has received heightened interest in the past decades (Fig. 1).

Cellular plasticity is exemplified in nature by a fertilized egg. The notion of its ability to develop into three germ layers and form an entire organism was conceived and described by von Baer, Rathke, and Pander in the 1820s (Pander, 1817; von Baer, 1828). Exploration of these ideas led to the conclusions by Weismann and Roux that development occurred in a unilateral fashion where germ cells are the only cells capable of hereditary potential and somatic cells received permanent genetic modifications, rendering them wedged to perform limited functions. To support his claim, Roux showed that ablation of one cell of the two-cell frog embryo resulted in incomplete development (Roux, 1888). This was quickly confounded by Driesch and Spemann who demonstrated that isolation of single cells from sea urchin (Driesch, 1894) and newt embryos (Spemann, 1928) culminated in the development of complete larvae. However, this did not invalidate the proposal that cells accumulate mutations during differentiation.

The paradigm shift began in 1952 when Briggs and King successfully transplanted a nucleus from a *Rana pipiens* embryo into an enucleated oocyte to produce adult organisms (Briggs and King, 1952). However, a later study by the pair revealed that endoderm nuclei from an early tail-bud stage resulted in arrest at blastula gastrula and early-neurula stages (King and Briggs, 1955). Surprisingly, when similar experiments were carried out in *Xenopus laevis*, nuclei from terminally differentiated intestinal epithelial cells were successfully transplanted into irradiated oocytes and developed into fertile male and female frogs (Gurdon, 1962b). This seminal discovery ignited a series of studies and sculpted the views of nuclear reprogramming as we know it today. In this review, we give a historical perspective on the metamorphosis of this field through the description of various approaches to nuclear reprogramming, and subsequently focus on the landmark discovery of transcription factor driven induced

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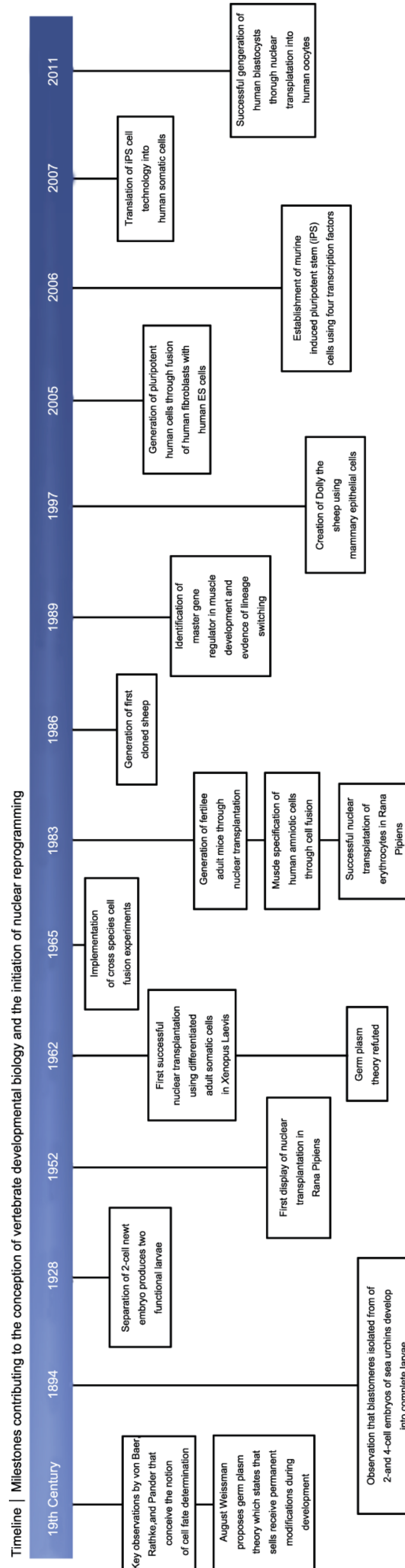

Figure 1. Timeline showing milestones contributing to the conception of vertebrate developmental biology and the initiation of nuclear reprogramming.

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pluripotency. We will highlight the dynamic evolution of this branch of cell manipulation and examine mechanistic insights into this phenomenon. In conclusion, we aim to discuss potential applications of this new technology, coupled to its future direction.

SOMATIC CELL NUCLEAR TRANSFER

The ability to transplant a nucleus from a differentiated cell into an oocyte to produce viable fertile offspring triggered huge repercussions as it portrayed similar genomic content between oocytes and somatic cells. Fraught with scepticism at the beginning, the experiments were successfully repeated across species. Rising in popularity, this process of transplanting a donor nucleus into an oocyte has now been termed somatic cell nuclear transfer (SCNT).

As described above, the first instance of SCNT was exhibited in *Xenopus laevis* (Gurdon et al., 1958; Gurdon, 1962a, 1962b). Intestinal epithelium cells of feeding tadpoles which correspond to terminally differentiated endoderm cells gave rise to normal feeding tadpoles at a frequency of 1.5% when transplanted into unfertilized oocytes. This was noticeably lower than nuclear transfers from the blastula and gastrula endoderm which led to a proportion of 36% feeding tadpoles. Interestingly, serial transplantations of intestine nuclei which promoted abnormal development after the first transfer could support the formation of normal feeding tadpoles, suggesting that disparities in efficiencies between differentiated and embryonic nuclei were partially explained by non-genetic causes such as technical limitations and incompatible mitotic properties. Collectively, first and serial transplantations, in concert with grafts, generated functional tadpoles at a frequency of 30% (Gurdon, 1962b; Byrne et al., 2003).

Shortly after came reports of successful transplantations of nuclei from a spectrum of cell origins, such as kidney, lung and skin (Laskey and Gurdon, 1970; Gurdon et al., 1975). Non-dividing erythrocytes from *Rana pipiens* were demonstrated to produce normal larvae upon serial transplantations (DiBerardino and Hoffner, 1983). Nuclear transfer from myotomes could also effectively produce swimming tadpoles at a frequency of 2% (Gurdon et al., 1984). Transcription of muscle differentiation markers ceased upon entry into the oocyte and re-activated once gastrulation occurred. Taken together, these studies suggest that the chemical milieu in the oocyte is able to perturb the transcriptional machinery of the donor cell and reverse its differentiation status.

Alternatively, SCNT can be performed using oocytes in their first meiotic prophase (Byrne, 2003). This manipulation results in the swelling of the cell due to a stark increase in mRNA synthesis. DNA replication is reversely synchronized and pauses, contrary to the use of oocytes in the second meiotic metaphase which leads to multiple rounds of cell division and little transcription. Although mechanistically distinct, this reaffirms the intrinsic ability of the oocyte to influence the donor cell nucleus.

Concurrent to the described experiments, other groups ventured away from amphibians. Using one-cell mouse embryos as donor and host, adult mice were generated at a success ratio of 13% (McGrath and Solter, 1983). In contrast, transplantation of nuclei from tail-tip fibroblasts plunged the reprogramming efficiency to 0.5%. Mirroring this phenomenon in sheep, Willadsen demonstrated the ability to produce cloned lambs by transplanting a nucleus of an 8-cell embryo to an enucleated oocyte (Willadsen, 1986). Mammary gland cell nuclei were also competent to produce adult fertile female sheep, albeit at a low efficiency of 0.2% (Wilmut et al., 1997).

Several attempts have been made with the use of human oocytes. Injection of donor nuclei into enucleated oocytes gave rise to blastocysts at low frequencies (Stojkovic et al., 2005; Hall et al., 2007; French et al., 2008). Low reprogramming efficiency was alleviated by leaving the nuclei of the oocyte intact (Noggle et al., 2011), leading to the successful development of blastocysts. The inner cell mass was stable in culture and triploid in karyotype, with one set of diploid chromosomes from the somatic nucleus and one set of haploid chromosomes from the oocyte. Competent in producing teratomas consisting of cell types representative of all three germ layers, these cells display similar transcriptional patterns to pluripotent stem cells derived from *in vitro* fertilized blastocysts.

The findings from SCNT experiments present evidence that somatic cells encompass the capacity to return to pluripotency and can be evaluated in two ways. First, cells which have veered away from pluripotency require strict conditions for reprogramming. Second, the egg naturally contains factors which dominate the performance of the somatic nucleus. Stripping the composition of the egg would allow us to comprehend the intricacies that trigger rapid dedifferentiation. To this end, histones 1 and B4 and the Tet proteins have been illustrated to play roles in the oocyte and male pronucleus, highlighting the importance of epigenetic remodeling interactions during reprogramming (Teranishi et al. 2004; Gao et al., 2004; Becker et al., 2005; Jullien et al., 2010; Maki et al., 2010; Inoue and Zhang, 2011; Wu and Zhang, 2011).

EXPOSURE TO CELL EXTRACTS AND CELL FUSION

Re-illustrating the innate ability of the chemical milieu within an oocyte to instigate transcriptional alteration, introduction of cytoplasmic contents from *Xenopus laevis* oocytes and early embryos have been described to elicit the expression of pluripotency markers in human somatic cells (Hansis et al., 2004). This capacity extended till late blastula-stages where extracts were now inhibitory to reprogramming. Human embryonic kidney cells incubated with embryonic carcinoma cell extracts for a mere hour also resulted in transcriptional acti-

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vation and partial demethylation of pluripotency genes within a week (Flasza et al., 2003; Taranger et al., 2005; Freberg et al., 2007).

Corroborating these observations, cell fusions between human lymphocytes and mouse embryonic stem (ES) cells have been competent in triggering the reactivation of Oct4. However, the level of transcriptional induction was < 1% compared to human ES cells (Pereira et al., 2008). The capacity of cell fusion induced reprogramming has been further exemplified through the combination of male ES cells and female thymocytes. Resulting heterokaryons expressed pluripotency genes and reactivated the silent X chromosome (Tada et al., 2001). In addition, spontaneous fusion between mouse neural progenitors and mouse ES cells led to the silencing of neural markers (Ying et al., 2002). Replicated in human cells, human ES cells fused to human foreskin fibroblasts generated hybrid pluripotent cells that exhibited the capacity to develop into three germ layers (Cowan et al., 2005). In general, cell fusion experiments displayed trends analagous to SCNT. First, terminally differentiated cells were more demanding to transform. Second, cell fusion leads to rapid reprogramming, where genes are expressed within two days and independent of DNA replication (Pereira et al., 2008; Do and Scholer, 2010). Despite success in generating pluripotent stem cells, reprogramming through cell fusion is inefficient (~1%), and creates a bottleneck during the purification of stable hybrids for analysis. Introduction of selection markers in the two starting populations can overcome this problem, but does not alleviate the eventual low population of reprogrammed cells.

Apart from establishing pluripotency, other cell types have also been successfully reprogrammed through cell fusion. Stemming from early experiments, heterokaryons between erythrocytes from a mature hen and Hela cells have been described to swell in size and prompt transcriptional activity in terminally differentiated erythrocytes, reminiscent of the observations in SCNT (Harris, 1965; Harris et al., 1966). Surprisingly, the combination of human amniotic cells and mouse muscle cells triggered the expression of genes specific to muscle cells, albeit the naïve nature of amniotic cells (Blau et al., 1983). This draws attention to inherent factors in somatic cells that safeguard the integrity and lineage of the cell. Besides sharing similarities to SCNT, it is useful to note that cell fusion encompasses unique qualities such as the ability to generate heterokaryons using multiple cell-nuclei. As a result, gene dosage influences the reprogramming efficiencies and the presence of the dominant nuclei in more than two fold will delay transcriptional activation (Pavlath and Blau, 1986).

TRANSCRIPTION FACTOR INDUCED LINEAGE SWITCH

It is evident from cell fusion experiments that there are specific components within each branch of cell lineage, shielding it from external influences and guarding its designed function. These intrinsic cell specific properties can be harnessed to redirect differentiation signals. Using observations that DNA demethylating agent, 5-azacytidine (AZA) enables efficient reprogramming into muscle lineage (Chiu and Blau, 1985), Weintraub and colleagues identified MyoD as a master regulator of myogenesis, and its ectopic expression forced recipient non-muscle cells to convert into myocytes (Weintraub et al., 1989). Although cells from three germ layers were successfully reprogrammed, starting material originating from the mesoderm was effectively converted, whereas cells from endoderm and ectoderm lineages retained their original gene signatures. This suggests the necessity of additional factors to induce a complete reform of the pre-existing gene expression profile.

To reinforce this notion, transdifferentiation has been described in a natural setting. Glucagon-producing α-cells have been reported to transform into pancreatic insulin-producing β-cells upon a diphtheria toxin insult (Thorel et al., 2010) and pigmented epithelial cells in a newt can regenerate its eye lens when removed (Tsonis and Del Rio-Tsonis, 2004). Time-lapse observation of cultured endothelial sheet colonies revealed their capacity to disengage their tight junctions, round up and begin to display erythroid and monocytic hematopoietic antigens (Eilken et al., 2009).

Intuitively, forced expression of transcription factors that naturally occur during developmental programs can incite lineage switches. As described above, expression of MyoD can redirect non-muscle cells into the muscle lineage (Weintraub et al., 1989). In addition, overexpression of adipocyte-specific nuclear hormone receptor, peroxisome proliferator-activated receptor (PPAR)γ2, triggers adipogenesis in fibroblasts (Tontonoz et al., 1994). Although single genetic factors have been described to control cell fate in adipocytes and myocytes, dermal fibroblasts require a combination of Gata4, Mef2c and Tbx5 to be converted into cardiomyocytes (Ieda et al., 2010). Likewise, ectopic expression of separate sets of transcription factors has successfully triggered transdifferentiation of dermal fibroblasts into neuron-like, blood progenitors, insulin secreting β-cells and brown adipose cells (Zhou et al., 2008; Kajimura et al., 2009; Szabo et al., 2010; Vierbuchen et al., 2010). Further exploration in the realm of lineage switching has placed emphasis on the importance of spatial, temporal and quantitative control of transcription factor expression in the generation of an array of cell types (Kulessa et al., 1995; Xie et al., 2004; Iwasaki et al., 2006; Laiosa et al., 2006).

Lineage switching mimics the acquisition of pluripotency via SCNT and cell fusion closely. First, cells which have climbed the differentiation ladder face more roadblocks during reprogramming (Chickarmane et al., 2009). Second, lineage switch occurs without the need for cell division. Markers of progenitor cells are often not detected, suggesting that reprogramming may occur in a direct fashion without the

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presence of an undifferentiated intermediate (Thorel et al., 2010). However, this does not exclude the possibility of a transient intermediate state.

TRANSCRIPTION FACTOR INDUCED PLURIPOTENCY

The ability of transcription factors to impose a lineage switch suggests that the right cocktail of factors would be able to erase all differentiation marks in somatic cells, driving the cell to pluripotency. In 2006, a seminal discovery by Takahashi and Yamanaka illuminated the capacity of four transcription factors, Oct4, c-Myc, Klf4, and Sox2, to transform mouse embryonic fibroblasts into cells that were similar to ES cells and disparate from their original somatic identity. These cells were able to differentiate into three germ layers when injected into immune-compromised mice, portraying its pluripotent potential.

Shortly after, the phenomenon was replicated in human fibroblasts (Takahashi et al., 2007). Concurrently, an independent study reported the generation of human ES-like cells through a discrete set of genes, Oct4, Sox2, Nanog, and Lin28 (Yu et al., 2007). These groundbreaking discoveries indicated that mere four genes can elicit pluripotency, igniting a surge of interest in this sphere of reprogramming (Fig. 2). For the ease of nomenclature, these cells have since been termed as induced pluripotent stem (iPS) cells.

Several refinements have been made to the generation of iPS cells since its inception. This includes the use of surrogate gene delivery tools, encompassing polycistronic lentiviruses, piggyBac mediated transposition and non-integrating approaches such as adenoviruses, Sendai viruses, episomal DNA, repeated transfections of mRNA or plasmid DNA and purified proteins (Okita et al., 2008; Stadtfeld et al., 2008; Carey et al., 2009; Fusaki et al., 2009; Kim et al., 2009a; Sommer et al., 2009; Woltjen et al., 2009; Yu et al., 2009; Yusa et al., 2009; Zhou et al., 2009; Warren et al., 2010). Albeit the low efficiency of producing iPS cells, the employment of non-integrating methods allows the generation of colonies which are completely free of exogenous material. Additional selection markers have also been established to allow the distinction between fully and partially reprogrammed cells (Okita et al., 2007; Maherali et al., 2007; Wang et al., 2011b).

To illustrate the robustness of the protocol, somatic cells originating from the three germ layers have been described to be pliable to reprogramming (Aasen et al., 2008; Aoi et al., 2008; Hanna et al., 2008; Shi et al., 2008). In addition, iPS cells from an array of organisms, including pigs, monkeys and rats, have also been achieved (Liu et al., 2008; Esteban et al., 2009; Liao et al., 2009). Akin to SCNT and cell fusion experiments, terminally differentiated cells have been proven difficult to reprogram (Hanna et al., 2008). Cell types which possess high intrinsic expression of certain factors negotiate the need for the complete set of reprogramming cocktail (Giorgetti et al., 2009; Kim et al., 2009b, 2009c; Tsai et al., 2010). Overall, these studies display the inherent ability of a spectrum of somatic cell types to revert to its pluripotent state. However, this technology is still in its infancy and harbours many unknowns. To address our lack of knowledge on the reprogramming process, it is crucial to begin elucidating the mechanisms behind the induction of pluripotency. There are several approaches which lead us to unravel its molecular circuitry and will be discussed below.

Molecular mechanisms behind the initial set of four reprogramming factors

Before we endeavour to expand our knowledge of the reprogramming process, it is crucial to understand the roles undertaken by each of the four transcription factors. Naturally present in ES cells, these transcription factors instigate a cascade of molecular events that preserve the functional integrity of the ES cells (Nichols et al., 1998; Avilion et al., 2003; Loh et al., 2006).

Oct4 and Sox2 are principal regulators of the transcriptional network in ES cells and act in concert with Nanog to activate pluripotency-associated elements (Boyer et al., 2005; Masui et al., 2007; Kim et al., 2008). Under tight regulation, deregulation of Oct4 and Sox2 expression levels will lead to differentiation of ES cells (Nichols et al., 1998; Niwa et al., 2000; Tomioka et al., 2002; Chew et al., 2005; Kopp et al., 2008).

Klf4 belongs to the family of Kruppel-like factors which display functional redundancy among family members. Occupying genomic sites similar to the Oct4-Sox2-Nanog cluster (Jiang et al., 2008), the Klf family has been described to act downstream of Oct4, p53 and leukemia inhibitory factor (LIF)/Stat pathways (Rowland et al., 2005; Hall et al., 2009).

c-Myc is a helix-loop-helix/leucine zipper transcription factor that mediates pleiotropic cellular functions including metabolism, cell cycle, splicing, translation, and oncogenic transformation (Klein, 1983; Kim et al., 2008; Sridharan et al., 2009). It has been implicated in the transcriptional elongation in ES cells (Rahl et al., 2010) and shares similar genetic targets with other pluripotency associated transcription factors (Chen et al., 2008; Kim et al., 2008; Dejosez et al., 2008). In ES cells, c-Myc resembles Klf4, acting in the LIF/Stat pathway (Cartwright et al., 2005) and opposing anti-proliferative properties of p21Cip1 (Claassen and Hann, 2000). c-Myc also recruits histone acetylase complexes (Bouchard et al., 2001; Frank et al., 2001), unfolds the chromatin structure in somatic cells and enables under-privileged sites to undergo remodelling.

The four transcription factors participate in the maintenance of the pluripotent state, where Oct4, Sox2 and Klf4 constitute the core apparatus and c-Myc manipulates a wider scope of cellular functions. Although the genomic targets of

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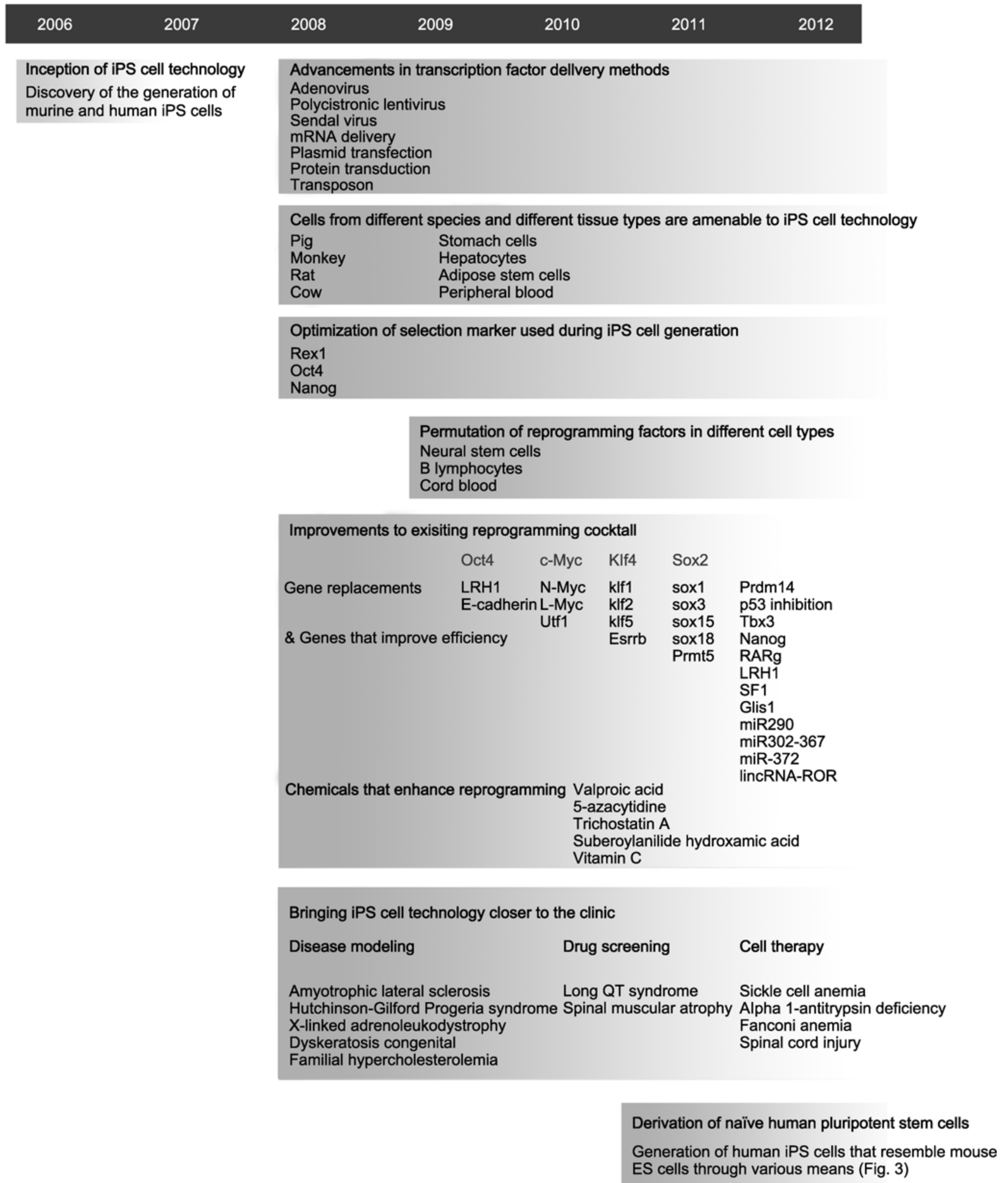

Figure 2. Timeline showing advancements in iPS cell technology since its inception. iPS, induced pluripotent stem.

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each transcription factor act as jigsaw pieces to the pluripotency network puzzle, they do not necessarily reflect the molecular changes coupled to reprogramming *in vivo*. To acquire a mechanistic view of reprogramming, additional components that participate in the process have been unraveled.

Identification of additional factors and pathways involved in reprogramming

To peel away layers of complexity behind induced pluripotency, several directed genetic screens have been endeavoured. A handful of genes such as Glis1, Tbx3, and Prmt5 were discovered to improve the original protocol of generating iPS cells (Han et al., 2010; Maekawa et al., 2011; Nagamatsu et al., 2011).

Transcription factors which competently act as substitutes for the prescribed set of reprogramming factors illuminate the role of each gene in the context of reprogramming. Inspection of functional redundancy among family members reveals that relatives of Klf4, Sox2, and c-Myc could successfully operate as substitutes, whereas Oct1 and Oct6 were unable to replace Oct4 (Nakagawa et al., 2008). Subsequent findings denote the ability of Nr5a2, Esrrb and Utf1 to replace Oct4, Klf4, and c-Myc, respectively (Zhao et al., 2008; Feng et al., 2009; Heng et al., 2010). In the absence of Sox2, Prmt5 and Nanog function as substitutes (Ichida et al., 2009; Nagamatsu et al., 2011), with the latter attributed to its synergistic function with Sox2 (Rodda et al., 2005).

Using an unbiased piggyBac-assisted approach, we show that RARγ and NR5A2 (LRH-1) co-operate to augment the transcriptional activity of Oct4 and trigger rapid dedifferentiation (Wang et al., 2011b and unpublished data). Although implication of the retinoic acid pathway in the reprogramming process is surprising, it is not completely unexpected (Ben-Shushan et al., 1995; Barnea and Bergman, 2000). As the dedifferentiation process is distinct to the maintenance of ES cell pluripotency, this study may shed light from a new perspective on the molecular mechanisms behind reprogramming.

Aside from transcription factors, microRNAs have also been addressed (Judson et al., 2009; Liao et al., 2011; Subramanyam et al., 2011). Two independent studies reported that microRNAs alone could reprogram both mouse and human somatic cells (Anokye-Danso et al., 2011; Miyoshi et al., 2011). Extending this discovery, a new class of large intergenic non-coding RNAs (lincRNAs) was portrayed to be involved in pluripotency. Of 10 lincRNAs identified to be differentially expressed in iPS and ES cells, deregulation of lincRNA-RoR in human fibroblasts has been described to affect iPS cell formation (Loewer et al., 2010).

Chemicals that enhance reprogramming

The route to pluripotency is multi-faceted and complementing chemical screens can illuminate potential approaches to improve the reprogramming process.

Transcription factor induced pluripotency occurs at sub-optimal frequencies of 0.01%–0.2% (reviewed by Hochedlinger and Plath, 2009). Forced re-expression of reprogramming factors in secondary fibroblasts resulted in 20% of cells displaying early pluripotency markers but a mere 1.2% of these cells re-activating their endogenous Nanog locus after 16 days (Mikkelsen et al., 2008). Amalgamating this to the knowledge that primitive cells which encompass fewer somatic epigenetic marks are more pliable to dedifferentiation and iPS cells which harbour incomplete erasure of silencing modifications were unable to contribute to the mouse germline (Takahashi and Yamanaka, 2006), it is tempting to speculate that overcoming the epigenetic barrier would enhance reprogramming.

To address this, a DNA methyltransferase inhibitor, AZA, was described to propel partially reprogrammed cells into pluripotency and improved the efficiency of producing iPS cell colonies by 4-fold (Huangfu et al., 2008a; Mikkelsen et al., 2008). Given that c-Myc regulates histone deacetylation and greatly improves reprogramming efficiencies, histone deacetylase inhibitors, such as valproic acid, trichostatin A, and suberoylanilide hydroxamic acid, have been proven to be efficacious in increasing the kinetics and efficiencies of generating iPS cells (Huangfu et al., 2008a, 2008b). Moreover, valproic acid ablates the need for c-Myc and Klf4, producing ES-like cells solely in the presence of Oct4 and Sox2.

As reprogramming leads to the accumulation of reactive oxygen species, antioxidant vitamin C was described to increase the efficiency of generating iPS cells (Esteban et al., 2010). This is triggered by vitamin C-dependent H3K36me2/3 demethylation and the repression of p53/p21 and Ink4/Arf loci (Wang et al., 2011a).

Besides the addition of chemicals, altering environmental conditions during reprogramming can also benefit the generation of iPS cells. In a natural setting, development of embryos occurs in physiologically hypoxic conditions. Recapitulating low oxygen concentrations during reprogramming experiments increases the efficiency of obtaining iPS cells (Yoshida et al., 2009). Furthermore, resultant female human iPS cells exhibit two activated copies of X chromosomes, indicating the attainment of ground state pluripotency (Lengner et al., 2010). Possible explanations behind this phenomenon include decreased accumulation of chromosomal abnormalities (Forsyth et al., 2006) and protection against spontaneous differentiation (Ezashi et al., 2005; Prasad et al., 2009).

Cellular processes associated with reprogramming

Although the search for genes and chemicals that enhance the creation of iPS cells has yielded a considerable amount of

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information, other avenues of studying the dedifferentiation process have also provided us with much insight. Two complementary approaches were used to determine the importance of mesenchymal-to-epithelial transition (MET) during the reprogramming process (Li et al., 2010; Samavarchi-Tehrani et al., 2010). Molecular dissection of the process identified bone morphogenetic proteins (BMP) as a key mediator, leading to a later discovery that BMP can replace Klf4 in the reprogramming process, and its expression with Oct4 alone is sufficient to generate iPS cells (Chen et al., 2011). These findings were corroborated by two independent studies describing the essential roles of E-cadherin (Redmer et al., 2011) and the TGFβ pathway (Ichida et al., 2009) in driving partially reprogrammed cells to pluripotency.

Secondary cells isolated from chimeras act as a homogenous platform to study the mechanics behind induced pluripotency. Silencing p53 or p21, or over-expressing Lin28 in secondary B-cells improved reprogramming efficiencies in a cell division dependent manner (Hanna et al., 2009). This validated an initial finding that silencing p53 in primary fibroblasts improved reprogramming conditions (Zhao et al., 2008). In addition five studies also illustrated the role of DNA damage response and immortalization in reprogramming (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009a; Marión et al., 2009; Utikal et al., 2009).

The importance of cell division in generating iPS cells is disparate to the mechanics behind SCNT and cell fusion, insinuating that the current recipe of transcription factors is unable to replicate natural reprogramming events. Interestingly, delivery of Nanog into secondary B-cells increased the number of reprogrammed colonies, independent of cell division (Hanna et al., 2009). Nanog is known to play essential roles in reprogramming and cell fusion (Silva et al., 2006; Chambers et al., 2007; Yu et al., 2007; Silva et al., 2009), hence it is possible that it functions in a discrete role, providing a new dimension to the process.

Functional applications of iPS cells

The discovery of iPS cells was accompanied with the promise of personalized cell therapy and the creation of avenues for drug screening and disease modeling. Since the advent of this technology, several studies have been conducted to serve as proof-of-principle for future applications.

Foremost, it is important to demonstrate that biopsies retrieved from patients can be competently reprogrammed and recapitulate the disease when differentiated. To this end, dermal fibroblasts, primary hepatocytes, blood, and keratinocytes have been shown to be effectively reprogrammed (Aasen et al., 2008; Dimos et al., 2008; Giorgetti et al., 2009; Loh et al., 2009; Liu et al., 2010). Extending these findings, the first notion of disease modeling was established through the generation of iPS cells from patients with a series of human disorders (Park et al., 2008). This was further exemplified in amyotrophic lateral sclerosis where reprogrammed cells could effectively differentiate into motor neurons (Dimos et al., 2008), displaying its capacity to model the disease. A plethora of diseases have since been recapitulated, including familial hypercholesterolemia, X-linked adrenoleukodystrophy, dyskeratosis congenital, Hutchinson-Gilford Progeria syndrome, and Down's Syndrome (Rashid et al., 2010; Agarwal et al., 2010; Jang et al., 2011; Zhang et al., 2011; Shi et al., 2012).

iPS cells also act as a suitable platform to perform drug screening tests, as portrayed in Long QT syndrome type I (Moretti et al., 2010; Itzhaki et al., 2011). Cardiomyocytes differentiated from patient derived cells demonstrated the classical electrocardiographic aberration of a long QT interval and were susceptible to catecholamine-induced tachyarrhythmia, whereas the addition of β-adrenergic receptor blockers could rescue this phenotypic abnormality. Drug screening abilities of iPS cells have also been illustrated in the contexts of spinal muscular atrophy and familial dysautonomia ((Ebert et al., 2009; Lee et al., 2009).

The inception of iPS cell technology highlights the potential of cell therapy and has been addressed in an autologous mouse model displaying sickle cell anemia (Hanna et al., 2007). This was followed by the notion of human iPS cell therapy in the treatment of spinal cord injuries and Fanconi anemia (FA) (Raya et al., 2009; Nori et al., 2011). More recently, targeted gene correction in human iPS cells was demonstrated through the application of piggyBac transposons and zinc-finger nucleases (Yusa et al., 2011), surmounting potential silencing modifications of rescue cassettes delivered by viruses (Raya et al., 2009).

Although the use of iPS cells in the clinic is regarded attractively, there are several anomalies which have to be addressed. Cells derived from patients suffering from FA and Fragile X (FX) syndrome are not easily amenable (Raya et al., 2009; Urbach et al., 2010), where additional genetic modifications are necessary to produce FA iPS cells, and FX iPS cells do not recapitulate the disease phenotype. These bottlenecks impede disease modelling and amendments to the existing protocol have to be taken into consideration.

Current pitfalls in reprogramming

In addition to technical difficulties, there are several concerns about the authenticity of iPS cells, in relation to ES cells. Using available techniques, rigorous comparisons have been performed between ES cells and iPS cells. Comprehensive studies of phosphoproteomic and transcriptomic components revealed minute differences that were statistically disregarded (Phanstiel et al., 2011). However, epigenomic analysis at high resolution revealed subtle differences between human iPS cells and ES cells (Lister et al., 2011). Differentially methylated regions (DMRs) associated with CG islands

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and genes were observed and these differences can be catalogued to represent persistent methylation patterns from the parental cell and *de novo* signatures specific to iPSCs. Additionally, aberrant silencing of the imprinted Dlk1-Dio3 gene cluster on chromosome 12qF1 in mice has also been correlated to poor chimera contributions and low germline competence (Stadtfeld et al., 2010). Overall, these studies point out that there are epigenetic discrepancies between iPS cells and ES cells.

Given these differences, it is understandable that functional disparities, such as epigenetic memory, exist between iPS cells and ES cells (Kim et al., 2010; Polo et al., 2010; Ohi et al., 2011). Blood-derived iPS cells displayed higher competence in differentiating into blood cells than neural progenitor-derived iPS cells, but at similar competence to nuclear-transfer-derived ESCs (Kim et al., 2010). However, differentiation of non-blood cells into blood prior to a second round of reprogramming markedly increases their blood forming capability, reiterating the notion of epigenetic footprints corresponding to the somatic state prior to reprogramming.

Another concern which has risen recently is the immunogenicity of autologous iPS cells. Injection of allogenic ES cells or highly autologous iPS cells into immune competent C57BL/6 mice elicited an immune response (Zhao et al., 2011). In contrast, autologous C57BL/6 ES cells were successfully implanted and generated teratomas efficiently. Reasons behind this phenomenon remain unclear but it is crucial that these observations are reproduced in independent studies before they can be generalized.

Given the differences between iPS cells and ES cells, it is pivotal to refine our methods in reprogramming. Prolonged maintenance of iPS cells has been suggested to erase sustained epigenetic marks (Polo et al., 2010); however continuous *in vitro* culture of these cells may incur genomic aberrations, as suggested by several independent groups (Gore et al., 2011; Hussein et al., 2011; Lister et al., 2011). Alternatively, the stoichiometry of the reprogramming factors has been suggested to affect the epigenetic status of the reprogrammed cells, strongly influencing their pluripotency (Carey et al., 2011).

Ground state or naïve pluripotent stem cells

To date, several stages of pluripotency have been recapitulated on a Petri dish (Fig. 3). ES cells derived from the inner cell mass of the mouse blastocyst (Evans and Kaufman, 1981) depict a naïve state of pluripotency and display germline competence when reintroduced into the mouse blastocyst. In culture, ES cells require LIF and BMP for maintenance. The addition of GSK3β and MEK inhibitors (2i) shields these pluripotent cells from differentiation-inducing stimuli and select for a homogenous population of primitive cells, designated as ground state pluripotency (Ying et al., 2008). Introduction of 2i to early mouse embryos does not impede blastocyst formation and results in high uniform expression of Nanog within the inner cell mass (Nichols et al., 2009). This proposes the presence of ground state pluripotency within the early mouse embryo, as denoted by the Nanog-expressing cells.

Epiblast stem cells (EpiSCs) were established from late epiblast layers and represent a primed pluripotent state (Brons et al., 2007; Tesar et al., 2007). In contrast to ES cells, EpiSCs are maintained in fibroblast growth factor (FGF) and Activin. Grown as flat compact colonies, they are morphologically distinct from domed mouse ES cell colonies, and can rarely generate chimeras when injected into immune-compromised mice. EpiSCs can be converted into ES cells through the addition of LIF or the ectopic expression of Klf2, Klf4, or Nr5a2 and the addition of 2i and LIF (Bao et al., 2009; Guo et al., 2009; Guo and Smith, 2010).

Human ES cells have been successfully isolated from human blastocysts (Thomson et al., 1998). Unlike mouse ES cells, the human ES cells require FGF and/or Activin for sustenance and the presence of LIF is insufficient to maintain them in an undifferentiated state. These pieces of evidence suggest that human ES cells resemble EpiSCs to a higher degree than their mouse counterparts (Tesar et al., 2007), indicating the existence of an unexplored naïve human pluripotent state. This notion is strengthened by the recent finding that Nanog-expressing cells within the human embryo do not respond to the inhibition of FGF and extracellular signal-regulated kinases (ERK) signals (Kuijk et al., 2012; Roode et al., 2012).

Given that conventional human iPS cells display a close relationship to human ES cells, the establishment of a stable primitive state may overcome the bottlenecks experienced by traditional human iPS cells. To address this, several studies have attempted to isolate immature human iPS cells. These strategies revolve around the ectopic constitutive expression of expanded sets of transcription factors and the addition of a spectrum of chemicals (Li et al., 2009b; Buecker et al., 2010; Hanna et al., 2010). Resultant cells resemble ES cells morphologically and display X-reactivation in female cells.

More recently, we have demonstrated that a cocktail of RARγ, Nr5a2 and the conventional set of reprogramming factors, can create naïve human pluripotent cells (Wang et al., 2011b). Coined as Sanger human iPS (SH-iPS) cells, these cells are stable over 50 passages, display dependency on the JAK/Stat pathway and generate teratomas when injected into immune-compromised mice. Using female cells as a starting material, SH-iPS cells exhibit two active X-chromosomes. Interestingly, exposure to FGF and Activin triggered their conversion to human ES-like cells, resembling the conversion of mouse ES cells to EpiSCs in similar conditions. This affirms the existence of a stable naïve state in human induced pluripotency and its competence to be maintained in culture.

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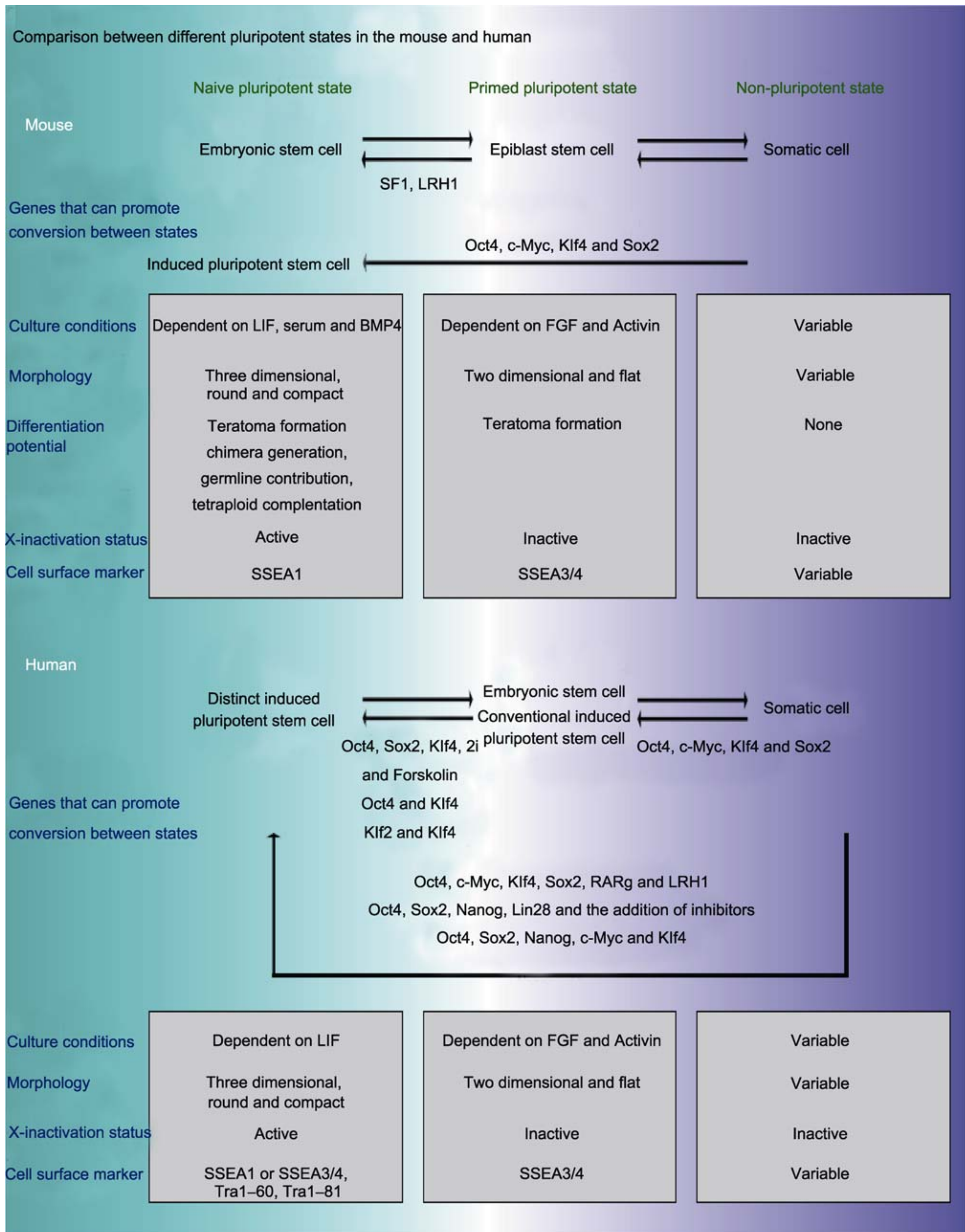

Figure 3. Comparison between different pluripotent states in the mouse and human.

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The discovery of this primitive state in human cells has immense implications. FGF-dependent human ES cells and iPS cells are technically difficult to maintain in culture. Susceptible to harsh dissociation, these cells are unable to survive devoid of cell–cell interation and exhibit genetic instability (Brimble et al., 2004; Buzzard et al., 2004; Lefort et al., 2008; Spits et al., 2008; Mayshar et al., 2010; Amps et al., 2011). In contrast, naïve human iPS cells do not display these properties and can be manipulated with ease. Resistant to tougher handling techniques, these cells are possibly more amenable to homologous recombination, creating a huge potential for genetic modification and correction. Naïve human iPS cells are also genetically and epigenetically stable, overcoming hurdles for its prospective use in the clinic. Despite these advantages, current protocols to derive naïve human iPS cells are not optimal. Further development is necessary to improve reprogramming efficiencies to levels comparable to or better than the generation of conventional FGF-dependent iPS cells. Nevertheless, since the inception of transcription factor induced pluripotency, the wealth of knowledge has amassed exponentially and continuation on this journey will inch us towards the translation of this technology to the bedside.

ABBREVIATIONS

EC cells, embryonic carcinoma cells; ES cells, embryonic stem cells; EpiSCs, Epiblast stem cells; ERK, extracellular signal-regulated kinases; FGF, fibroblast growth factor; iPS cells, induced pluripotent stem cells; LIF, leukemia inhibitory factor; SCNT, somatic cell nuclear transfer; 2i, two inhibitors

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