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Faithful reprogramming to pluripotency in mammals - what does nuclear transfer teach us?

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ABSTRACT Nuclear reprogramming toward pluripotency has been now achieved either *in vivo* by somatic cell nuclear transfer into the ooplasm of an enucleated egg, or *in vitro* by three different approaches, namely cell fusion, treatment with cell extracts and more recently, forced expression of a reduced set of defined transcription factors. This last technique has expanded our view of genome plasticity with important applied perspectives in regenerative biomedicine. Because of their ease of generation, induced pluripotent stem cells represent a major hope in the field of regenerative medicine. However, the extent to which such an *in vitro* induced pluripotency can be considered to be equivalent to embryonic-derived pluripotency remains undetermined and also largely dependent on how pluripotency is assessed. Here, we provide an overwiew of the data published in the recent literature on the ability of each of the above techniques to reprogram somatic nuclei into pluripotent embryonic-like nuclei. These data support the view that even though nuclear transfer is technically demanding, it remains a fast and efficient means for a systematic derivation of *bona fide* embryonic stem cells from somatic donor cells. We conclude that nuclear transfer has still much to teach us about faithful nuclear reprogramming to pluripotency.

KEY WORDS: *reprogramming technique, pluripotency, totipotency, epigenetic signature*

Introduction

Pluripotency refers to the ability of a cell to differentiate into any cell type of an organism. Pluripotency is operationally defined as the ability of a cell to be clonally differentiated *in vitro* and/or *in vivo* into the derivatives of the three primitive embryonic layers, (namely the ectoderm, mesoderm and endoderm) and to contribute to the germ line in chimera experiments (Smith, 2001). Pluripotency is a key developmental concept for basic biological questions related to the way embryonic cells can acquire and maintain their further ability to become committed to any types of cells either *in vivo* within an organism or *in vitro* within culture dishes. But pluripotency also emerges as a paradigm for application in biomedicine where pluripotent cells could become versatile tools for tissue replacement or regeneration using the own patient cells.

From a developmental point of view, pluripotency corresponds to the first restriction, in terms of developmental potential, of the cells issued from the totipotent egg. In mammals, this restriction is initiated after the first two to three divisions of the egg and completed at the preimplanted blastocyst stage when the differentiating cells of the trophoblastic lineage form an epithelium distinguishable from the remaining small clump of pluripotent cells called the inner cell mass (ICM). During blastocyst differentiation pluripotency is associated with the epiblast that separates from the hypoblast or primitive endoderm and remains until the onset of gastrulation and the emergence of a mesoderm streak. Although ICMs and pre-gastrulating epiblasts are both pluripotent, they are functionally distinct: only ICM cells can contribute to all lineages in chimera whereas epiblast cells do not (Gardner *et al.*, 1985). ICM and epiblast cells have been shown to differ with respect to the completion of the X inactivation process and the extent of *de novo* DNA (re)methylation (Kafri *et al.*, 1992; Monk *et al.*, 1987; Rossant *et al.*, 1986)(see Fig. 1). These epigenetic modifications taking place during implantation have been hypothesized to form an "epigenetic barrier" during embryo development (Hayashi *et al.*, 2008). As a consequence, the ICM has been defined as a "naïve" state of pluripotency, while the post-implan-

Abbreviations used in this paper: EpiSC, epiblast stem cell; ESC, embryonic stem cell; FT, fertilized; ICM, inner cell mass; iPSC, induced pluripotent stem cells; NT, nuclear transfer; SCNT, somatic cell nuclear transfer; TF: transcription factor.

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tation epiblast represents a "primed" state of pluripotency (Nichols and Smith, 2009).

The naïve state of pluripotency can be captured *in vitro,* at least in the mouse, in the form of embryonic stem cell lines (mESCs) derived from cultured ICMs (Evans and Kaufman, 1981; Martin, 1981). Conversely, the primed state of pluripotency is obtained after derivation of epiblast stem cells (mEpiSCs) from post-implantation epiblasts (Brons *et al.*, 2007; Tesar *et al.*, 2007). In the human, recent evidences suggest that the so called human Embryonic Stem Cells (hESCs) obtained from the ICM of preimplanted blastocysts (Thomson *et al.*, 1998) may actually correspond to a primed state of pluripotency (Hanna *et al.*, 2010; Xu *et al.*, 2010). Conversion of pluripotent cells from the primed state to the naïve state has been achieved in both mouse and human species (Bao *et al.*, 2009; Hanna *et al.*, 2010; Xu *et al.*, 2010; Zhou *et al.*, 2010).

Several methods have been developed to induce specialized somatic cells to reacquire a pluripotent state, in other words to reprogram differentiated nuclei into pluripotent ones. Since differentiation of cells is a developmental process that keep the nuclear DNA sequence unchanged (except for the highly specific case of immunoglobulin gene rearrangements), reprogramming to a pluripotent state implies the resetting of epigenetic modifications (Tada, 2006) and the associated mechanisms that allow a cell nucleus to change its fate and adopt another one (Solter, 2002).

Historically, there are two routes to pluripotency that have been independently pursued. One considers the reprogramming of nuclear activities using the oocyte cytoplasm to which the nucleus is exposed after nuclear transfer. The second does not use the oocyte cytoplasm but rather considers that reprogramming can be achieved by changing the microenvironment of a differentiated nucleus after its exposure *in vitro* to the whole cytoplasm or to some specific cellular factors of undifferentiated cells. Each of these approaches has proven to be at least to some extent successful in term of allowing a differentiated nucleus to re-acquire part if not all of the morphological, molecular and functional features of a pluripotent nucleus.

Here we evaluate each of these methods in term of their ability to reprogram a differentiated nucleus into a pluripotent one. We also consider the extent to which these methods allow the nucleus to recapitulate all the functional properties of a pluripotent cell, necessary for faithful reprogramming. This last point is of importance for applications since the reprogramming of somatic cells to a pluripotent state has a tremendous potential in biomedicine (Amabile and Meissner, 2009).

We will first describe the different techniques used so far to reprogram a somatic cell, and will refer to the functional and molecular evidence for pluripotency reprogramming. Then we will see how the outcomes of the different reprogramming strategies can be compared to a common standard not only for the analysis of the underlying mechanism but also for a more rigorous assessment of reprogrammed cells for applications in the biomedical field.

Reprogramming to pluripotency with an ovocyte cytoplasm by somatic cell nuclear transfer

Cloning by nuclear transfer (NT) in the mouse can be achieved by injection of the donor nucleus into the enucleated recipient oocyte (Wakayama *et al.*, 1998; Zhou *et al.*, 2000), or electrofusion of the donor nucleus with the recipient ovocyte (Ogura *et al.*, 2000b). Next, the reconstructed embryo is artificially activated by chemical treatment with strontium, and either cultured *in vitro* before transfer into a foster mother, or directly transferred.

A variety of somatic donor cells have been used to produce cloned mice such as cumulus (Wakayama *et al.*, 1998), fibroblast (Ogura and Tai, 2002), tail tip (Wakayama and Yanagimachi, 1999), sertoli (Ogura *et al.*, 2000a), and even lymphocyte natural killer T cells (Inoue *et al.*, 2005). Adult animals of healthy appearance have now been obtained in 13 mammalian species (Barlow *et al.*, 2008) thereby demonstrating that the synthesis activities of a nucleus obtained from a differentiated donor cell can be fully reprogrammed. Evidence that the lifespan of clones can match that of controls have also been established in the mouse species (Kishigami *et al.*, 2006b, Wakayama, 2007; Wakayama *et al.*, 1998).

How "normal" adult and fertile clones can be considered remains, however, a matter of debate. Even if clones that develop into adults have been assumed to possess a normalized epigenome that corresponds to their normal phenotype (Lanza *et al.*, 2001; Senda *et al.*, 2007), it remains generally considered

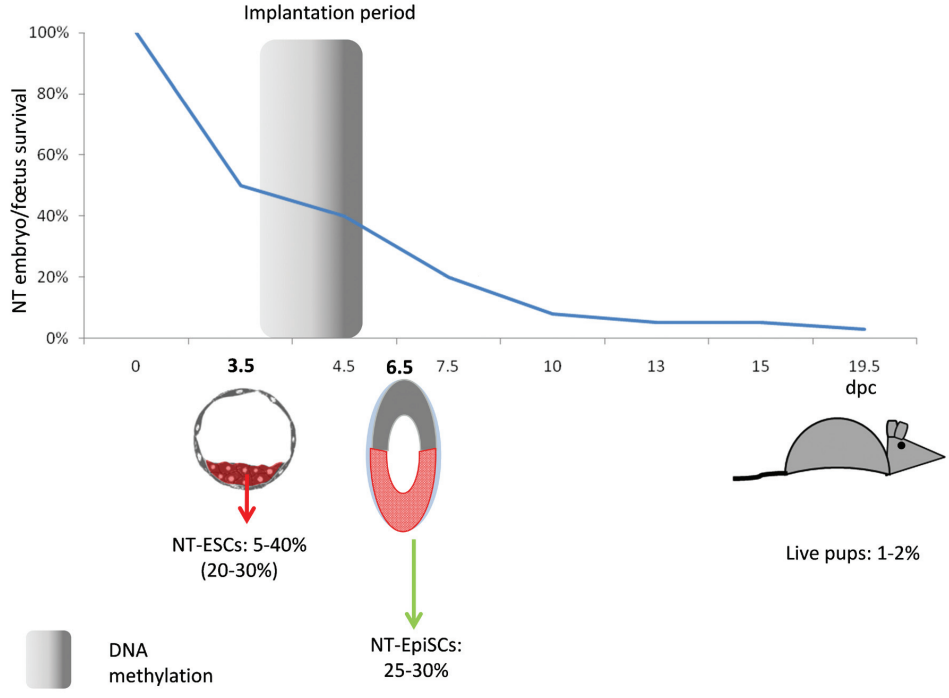

Fig. 1. Survival of nuclear transfer derived embryo/fœtus during *in vivo* development

that those animals are survivors of the poorly efficient process of full reprogramming to totipotency (Jouneau and Renard, 2003; see Fig. 1). The subtle gene expression abnormalities and/or epigenetic altered marks they may exhibit are considered as reprogramming errors (Jaenisch and Wilmut, 2001; Rideout *et al.*, 2001; Yang *et al.*, 2007). More recent studies of monozygotic twins have, however, highlighted the important role of epigenome variations as the basis for differences in heritable complex traits (Kaminsky *et al.*, 2009). To which extent the unexpected epigenetic plasticity of healthy adult clones recently observed in cattle (de Montera *et al.*, 2010) is the consequence of epigenetic errors or that of normal individual variations between closely resembling, if not identical, genomes remains to be documented. In this view, genome-wide sequence-specific epigenetic modification analyses using MeDIP-Seq and CIP-Seq techniques will be useful tools. They will help to determine whether subtle gene expression differences between adult reprogrammed genomes should be considered as either abnormalities not severe enough to affect the health of the animals or variations resulting from the dynamic interplay of chromatin and DNA sequence during development and the built-up of the complex traits of an individual (Johannes *et al.*, 2008).

If development to term after NT is achieved with low efficiency, reprogramming to the blastocyst stage is much more efficient - with 30-50% success, versus 80-90% for controls (Boiani *et al.*, 2002; Ogura *et al.*, 2000c, Wakayama *et al.*, 1998; Yang *et al.*, 2007) - and amenable to routine experimentation (see Fig. 1). However, the expression of pluripotency markers such as OCT4 (Nichols *et al.*, 1998) is perturbed in cloned blastocysts (Boiani *et al.*, 2002; Kishigami *et al.*, 2006a) and this result has been extended to a larger set of genes (Jincho *et al.*, 2008; Mann *et al.*, 2003).

Perturbed gene expression profiles of cloned embryos are correlated to epigenetic errors (Dean *et al.*, 2003; Niemann *et al.*, 2008). DNA methylation at the *Oct4* promoter for instance is insufficiently removed during the preimplantation development of SCNT mouse embryos (Yamazaki *et al.*, 2006). In addition, perturbed DNA methylation patterns are observed for several imprinted genes in most NT-blastocysts (Mann *et al.*, 2003). In cloned bovine embryos the methylation status of the ICM is relatively normal, when compared with - *in vivo* or *in vitro* - fertilized controls but the trophoblast cells show abnormal hypermethylation (Yang *et al.*, 2007). Histone marks of the somatic genome are also perturbed after nuclear transfer, as evidenced by the study of H3K4me and H3K27me, otherwise described as bivalent domains in ESCs (Bernstein *et al.*, 2006; Gan *et al.*, 2007). The dynamic patterns of the active mark H3K4me2 are different between control and SCNT embryos (Shao *et al.*, 2008), while the inactive mark H3K27me3 is absent from the ICM of most SCNT blastocysts (Zhang *et al.*, 2009a).

Despite the fact that blastocyst stage cloned embryos are plagued with gene expression and epigenetic errors, pluripotent embryonic stem cells can be relatively easily obtained from them. The efficiency of isolation, although variable (Kawase *et al.*, 2000; Munsie *et al.*, 2000; Wakayama *et al.*, 2001), is similar to that obtained with fertilized blastocysts (Boiani *et al.*, 2005; Kishigami *et al.*, 2006b, Mombaerts, 2003). Moreover, embryonic stem cells derived from nuclear transfer blastocysts (NT-ESCs) are morphologically similar to those obtained from fertilized blastocysts (FT-ESCs). Both express pluripotency markers such as OCT4, NANOG and SOX2, and are able to differentiate *in vitro* into derivatives of the three embryonic lineages (Munsie *et al.*, 2000; Rideout *et al.*, 2002; Wakayama *et al.*, 2006). Germline contribution has also been evidenced in diploid and tetraploid chimeras generated by injection of cells into blastocysts (Brambrink *et al.*, 2006; Wakayama *et al.*, 2006; Wakayama *et al.*, 2001). Therefore pluripotent NT-ESCs can be efficiently derived after reprogramming of a somatic genome by nuclear transfer and development of the resulting embryo up to the blastocyst stage.

Later in development, important losses happen with 20% to 60% of NT-blastocysts actually implanting (Ono *et al.*, 2001; Wakayama and Yanagimachi, 2001). From implantation to gastrulation, about 50% of the remaining embryos are lost and about 2/3 of post-implantation embryos display gross morphological abnormalities (Jouneau *et al.*, 2006; Maruotti *et al.*, 2010). There is currently a lack of studies reporting in depth analysis of NT post-implantation embryos on the transcriptomic and epigenetic level. Nevertheless we have successfully managed to derive NT-EpiSC lines from the epiblast of morphologically normal and abnormal post-implantation cloned embryos with the same efficiency as when cell lines are derived from the epiblast of fertilized embryos (FT-EpiSCs) (Maruotti *et al.*, 2010). We found that all the derived NT- lines are similar to FT-EpiSCs both in terms of morphology, expression of pluripotent markers and ability to differentiate *in vitro* into derivatives of the three embryonic lineages (Maruotti *et al.*, 2010). We also found contribution of NT-EpiSCs to chimera to be very poor (Maruotti J., personal observation), but this has also been reported for FT-EpiSCs (Brons *et al.*, 2007; Tesar *et al.*, 2007). All these data lead to the provisional conclusion that reprogrammed pluripotent cell lines can also be derived from the epiblast of post-implantion nuclear transfer embryos. Consequently, reprogramming of a somatic genome through nuclear transfer leads to the rather efficient derivation of both naïve (NT-ESCs) and primed (NT-EpiSCs) pluripotent cell lines in mouse.

Since the nuclear transfer technique remains a technically challenging method requiring large amounts of oocytes, a biological material whose access is greatly restrained in human, alternative techniques have been developed in order to reprogram a differentiated cell without the requirement for an oocyte cytoplasm.

Reprogramming without an oocyte cytoplasm

By cell fusion

Fusion between a differentiated and an undifferentiated mammalian cell was the first method considered, far before the emergence of nuclear transfer, to analyze the mechanisms operating in the differentiation of nuclear functions (Davidson *et al.*, 1966).

Cytoplasmic fusion between a pluripotent and a differentiated cell can occur spontaneously at very low rates, around 0.0005% (Terada *et al.*, 2002), but the efficiency can be markedly increased by using various chemical treatments such as poly-ethyleneglycol (Cowan *et al.*, 2005; Mise *et al.*, 1996; Yu *et al.*, 2006) or electrofusion (Tada *et al.*, 2003; Tada *et al.*, 1997; Tada *et al.*, 2001) to reach in the better cases a rate of about 5% (Wong *et al.*, 2008). In the fused cells, nuclei remain separated during the next 48h and form heterokaryons (Gurdon and Melton, 2008; Pereira

et al., 2008). Nuclear fusion occurs thereafter and hybrid cells are obtained. Selection for the hybrid cells that result from fusion between a differentiated cell and a pluripotent one can be done by dual drug selection when each of the genome carries a different resistance gene (Cowan *et al.*, 2005) or by FACS for dual staining when each of the cell type is labeled with a different dye (Pereira *et al.*, 2008).

In early experiments, mouse thymocytes were first fused with pluripotent embryonal carcinoma (EC) cells. The resulting tetraploid hybrid cells acquired an a EC cell-like morphology (Miller and Ruddle, 1977) and were able to differentiate into derivatives of the three embryonic lineages after injection into immunodeficient mice. This provided a good indication that EC nuclear pluripotency is not abolished in the presence of a differentiated genome (Miller and Ruddle, 1976). Differentiation into derivatives of the ectoderm, mesoderm and endoderm was later confirmed when teratoma and embryoid bodies were derived from hybrid cells made from somatic and ESCs (Ambrosi *et al.*, 2007; Cowan *et al.*, 2005; Tada *et al.*, 2003; Terada *et al.*, 2002; Yu *et al.*, 2006). When hybrid cells resulting from the fusion of thymocytes with either embryonic germ cells (EGCs) or ESCs were injected into mouse embryos they contributed to the three lineages of the resulting chimera at the embryonic stage (Tada *et al.*, 1997; Tada *et al.*, 2001). Similar observations were obtained in adult chimeras, using hybrids between neuronal stem cells and ESCs (Ying *et al.*, 2002).

Using this approach, and because in fused cells both the genome of an undifferentiated and of a somatic differentiated cells become surrounded by the same cytoplasm, it is of importance to determine whether the somatic genome has actually been reprogrammed to a pluripotent state, and not merely remains as a silent counterpart.

After fusion with ESCs, reprogramming of the somatic genome can be evidenced when marker genes of a pluripotent nuclear activity, such as *Oct4* (Nichols *et al.*, 1998) or *Rex1* (Rogers *et al.*, 1991), are expressed by the differentiated genome (Cowan *et al.*, 2005; Kimura *et al.*, 2004; Pereira *et al.*, 2008; Tada *et al.*, 2001; Yu *et al.*, 2006), while somatic cell specific genes are repressed (Cowan *et al.*, 2005; Kimura *et al.*, 2004; Miller and Ruddle, 1977; Pereira *et al.*, 2008). Upon induction of differentiation, genes specific to the three germ layers are also expressed by the somatic genome of the hybrid cells (Cowan *et al.*, 2005; Kimura *et al.*, 2004; Pereira *et al.*, 2008; Tada *et al.*, 2003). From these experiments it can be concluded that fusion with a pluripotent cell is capable of reprogramming a somatic genome to a pluripotent state.

Epigenetic reprogramming of the somatic genome has been further evidenced by the reactivation of the inactive X-chromosome (Takagi *et al.*, 1983). Reversal of X-inactivation in the somatic genome has later been linked to partial *de novo* DNA methylation of the unmethylated *Xist* allele, resulting in its repression (Mise *et al.*, 1996). In contrast, DNA demethylation is observed, sometimes as early as 24 to 48h after cell fusion, at the promoter and enhancer regions of *Oct4*, resulting in its up-regulation (Cowan *et al.*, 2005; Han *et al.*, 2008; Kimura *et al.*, 2004; Pereira *et al.*, 2008). Similar results have been obtained with *Nanog* (Han *et al.*, 2008), another pluripotency-specific gene (Chambers, 2003; Mitsui, 2003). DNA demethylation of the somatic genome after cell fusion with ESCs is a targeted process that does not alter genes whose methylation status is similar in the pluripotent and somatic genome, such as the imprinted genes *H19* and *Igf2r* (Pereira *et al.*, 2008; Tada *et al.*, 2001). Besides, it is an active process that takes place in the absence of cell division or DNA replication (Bhutani *et al.*, 2010; Pereira *et al.*, 2008) and requires the presence of activation-induced cytidine deaminase (AID) (Bhutani *et al.*, 2010).

In addition to DNA methylation changes, histone modifications are also observed in intersubspecies hybrids between thymocytes and ESCs. The reprogrammed somatic genome of the hybrid cells become hyperacetylated at histone H3 and H4, while globally hyper-di– and hyper-tri–methylated at lysine 4 (K4) of H3, similarly to the ESC genome (Kimura *et al.*, 2004).

Pluripotency is therefore gained by a somatic genome upon fusion with a pluripotent cell and epigenetic remodeling of its chromatin. In established pluripotent hybrid cell lines, suppression of *Oct4* expression from the mouse ESC genome does not induce differentiation (Pereira *et al.*, 2008), nor does the elimination of a pair of ESC-derived chromosome 6, which are key chromosomes for maintaining pluripotency (Matsumura and Tada, 2008). Consequently, reprogramming by fusion with pluripotent cells induces an epigenetically stable (and heritable) resetting of gene expression in the somatic nucleus (Pereira *et al.*, 2008).

By cell extract

Cell fusion experiments demonstrate that factors contained within a pluripotent cell can induce reprogramming of a somatic genome. However, since the resulting hybrids are tetraploid, this strategy is not pertinent to the generation of customized cells for transplantation therapy (Hochedlinger K. and Jaenisch R., 2006). Reprogramming strategies based on the use of cellular extracts have therefore been devised.

In order to prepare such extracts, pluripotent cells (ECCs or ESCs) are first lysed and sonicated. Following permeabilization by streptolysin O, somatic cells are incubated for about an hour in pluripotent cell extract, before membrane resealing in $CaCl_2$ containing media (Taranger *et al.*, 2005).

Treatment of differentiated epithelial cells (293T) with an extract of EC cells induces long lasting changes in morphology with the differentiated population forming tightly packed colonies characteristic of the EC cells (Freberg *et al.*, 2007; Taranger *et al.*, 2005). After retinoic acid induction, extract-treated cells can differentiate to some extent into derivatives of the ectoderm and mesoderm lineages (Freberg *et al.*, 2007; Taranger *et al.*, 2005).

Enhanced expression of key pluripotency genes such as *Oct4*, *Nanog* and *Sox2* is observed in the treated cells during the weeks following exposure to cell extracts, while differentiation-specific genes are down-regulated (Freberg *et al.*, 2007; Taranger *et al.*, 2005; Xu *et al.*, 2009).

Up-regulation of the pluripotency markers *Oct4* and *Nanog* has been associated with epigenetic changes such as DNA demethylation at their promoter regions (Bru *et al.*, 2008; Freberg *et al.*, 2007; Taranger *et al.*, 2005). Additionally, the repressive histone marks H3K9me and H3K27me are lost, while acetylation at H3K9 increases (Bru *et al.*, 2008; Freberg *et al.*, 2007).

In summary, treatment of a somatic cell with extracts from pluripotent cells elicits some level of epigenetic reprogramming. The reminiscence of morphological changes typical of undifferentiated cells for up to 12 weeks is an indication that the reprogram-

ming of a somatic nucleus can remain quite stable (Taranger *et al.*, 2005). However, data regarding their *in vivo* development potential are still absent, currently limiting the use of this method to biochemical and kinetic analysis of reprogramming (Hochedlinger K. and Jaenisch R., 2006).

By defined factors

In the late 80's, the instructive role of transcription factors (TF) in lineage specification was demonstrated by Davis and colleagues when forced expression of MyoD induced myotube formation in fibroblasts (Graf and Enver, 2009). While results from cell fusion experiments suggested that only nuclear factors contained in ESCs had the ability to reprogram toward a pluripotent state (Do and Scholer, 2004), studies on ESCs and the developing mouse embryo further allowed for the identification of TFs involved in pluripotency regulation (reviewed in (Ralston and Rossant, 2010). The flow of research on cell reprogramming was revivified after the demonstration that cloning was biologically possible. It led to the landmark discovery that forced expression of a few select transcription factors could turn differentiated cells into pluripotent ES-like cells, thus leading to the term "induced pluripotent stem cells" (iPSCs) (Takahashi and Yamanaka, 2006).

iPSCs were initially obtained by ectopic expression of *Oct4*, *Sox2*, *Klf4* and *c-Myc* after retroviral infection of differentiated cells, followed by selection for expression of pluripotency markers such as *Oct4* and *Nanog* or by selection based on pluripotent morphology (Okita *et al.*, 2007; Takahashi *et al.*, 2007; Wernig *et al.*, 2007). Later reprogramming strategies involved different TF mixes such as *Oct4*, *Sox2*, *Nanog* and *LIN28* (Yu *et al.*, 2007), the use of chemical compounds to replace some of the TFs (Maherali and Hochedlinger, 2009; Shi *et al.*, 2008) or to increase reprogramming efficiency (Huangfu *et al.*, 2008), and generation of iPSCs free of viral integration (Kim *et al.*, 2009; Okita *et al.*, 2008; Stadtfeld *et al.*, 2008b; Woltjen *et al.*, 2009; Yakubov *et al.*, 2010; Yu *et al.*, 2009; Zhou *et al.*, 2009; Warren *et al.,* 2010).

Besides mouse (Okita *et al.*, 2007; Wernig *et al.*, 2007), iPSCs have been generated from different species such as Human (Lowry *et al.*, 2008; Park *et al.*, 2008; Takahashi *et al.*, 2007; Yu *et al.*, 2007), Rat (Li *et al.*, 2009; Liao *et al.*, 2009) and Swine (Ezashi *et al.*, 2009; Wu *et al.*, 2009). To date, a variety of cells have been used to generate iPSCs including fully differentiated ones such as mature B lymphocytes or pancreatic beta cells (Hanna *et al.*, 2008; Stadtfeld *et al.*, 2008a).

The ectopic expression of defined factors in somatic cells leads to gradual changes in morphology and generation of iPSCs that look like ESCs (Araki *et al.*, 2010). iPSCs readily differentiated into derivatives of the three lineages *in vivo* and *in vitro* (Okita *et al.*, 2007; Takahashi *et al.*, 2007; Wernig *et al.*, 2007; Yu *et al.*, 2007). Germline transmission has also been observed after injection of miPSC into diploid and tetraploid host embryos (Boland *et al.*, 2009; Okita *et al.*, 2007; Wernig *et al.*, 2007; Zhao *et al.*, 2009). Inducing the expression of defined factors in differentiated cells can therefore reprogram them into a fully pluripotent state.

The reprogramming process is gradual and slow. Ectopic expression of the defined factors in somatic cells first leads to gradual changes in morphology (Araki *et al.*, 2010). Then, the expression of genes specific to somatic cells such as *Thy1* are down-regulated, before the gradual up-regulation of pluripotency markers such as alkaline phosphatase and *SSEA-1* (Brambrink *et al.*, 2008; Mikkelsen *et al.*, 2008). Up to several weeks later, expression of endogenous *Oct4* and *Nanog* indicates completion of the reprogramming process (Araki *et al.*, 2010; Brambrink *et al.*, 2008; Mikkelsen *et al.*, 2008).

X-chromosome reactivation, a hallmark of epigenetic remodeling, is observed in mouse and human iPSC lines (Lagarkova *et al.*, 2010; Maherali *et al.*, 2007; Silva *et al.*, 2008). After reprogramming by defined factors, DNA demethylation at the promoter region of key markers of pluripotency such as *Oct4* and *Nanog* is observed (Okita *et al.*, 2007; Takahashi *et al.*, 2007; Wernig *et al.*, 2007), as well as for other pluripotency related genes (Mikkelsen *et al.*, 2008; Okita *et al.*, 2007; Takahashi *et al.*, 2007). Genome-wide methylation profiling have confirmed that promoter elements of differentiation specific genes were methylated following reprogramming whereas pluripotency-related gene promoters were hypomethylated similar to levels observed in ESCs (Lagarkova *et al.*, 2010).

Compared to their differentiated ancestors, iPSCs are enriched for H3K4 methylation at the promoter region of pluripotency genes (Takahashi *et al.*, 2007; Wernig *et al.*, 2007), while displaying ESC characteristic bivalent domains carrying both "active " H3K4 methylation marks and "inactive" H3K27 methylation marks (Bernstein *et al.*, 2006; Pan *et al.*, 2007) at the promoter region of non-expressed developmental regulators (Maherali *et al.*, 2007; Mikkelsen *et al.*, 2008; Takahashi *et al.*, 2007; Wernig *et al.*, 2007).

All the above data convincingly show that epigenetic reprogramming of a differentiated cell to a pluripotent state can be induced by ectopic expression of a set of defined TFs. Importantly, silencing of transgene expression in iPSC lines (Lowry *et al.*, 2008; Okita *et al.*, 2007; Takahashi *et al.*, 2007; Wernig *et al.*, 2007) and maintenance of such lines without exogenous supply of the defined factors (Kim *et al.*, 2009; Yakubov *et al.*, 2010; Yu *et al.*, 2009; Zhou *et al.*, 2009) indicate that reprogramming of the somatic nuclei is stable and heritable.

Different strategies have successfully been applied to reprogram differentiated cells into functionally pluripotent ones (Fig. 2). However to what extent the functional properties of the pluripotent cell lines, generated by either method, are similarly reprogrammed remains a question of importance (Baker, 2009). In the same manner that cloned mammals are scrutinized in details to answer the question of their normality in regard to their fertilized counterparts, it is necessary to determine whether cell lines obtained without the assistance of an oocyte cytoplasm are "normally" pluripotent. In other words, how faithful is the reprogramming of their somatic genome to a pluripotent state?

Matching different cellular reprogramming strategies to a common standard

Reprogramming efficiencies vary according to the *in vitro* method. If they are typically less than 0.001% after cell fusion (Cowan *et al.*, 2005; Tada *et al.*, 2001), they can be improved up to several hundred times by over-expression of pluripotency specific genes such as *Nanog* (Silva *et al.*, 2006) or *Sall4* (Wong *et al.*, 2008) in the hybrid cells. In the case of reprogramming with defined factor, efficiencies typically range around 0.05 to 0.1% (Takahashi *et al.*, 2007; Wernig *et al.*, 2007), but can be increased

up to 10% when small-molecule compounds are added (Huangfu *et al.*, 2008), matching those reported for the generation of NT-ESCs.

In order to compare cell lines resulting from different reprogramming strategies, a well-known and well-studied standard for pluripotency is required. During the last three decades, naïve and primed pluripotent embryo-derived stem cells and their differentiated progenies have been obtained and thoroughly investigated in major models including Mouse (Brons *et al.*, 2007; Evans and Kaufman, 1981; Martin, 1981; Tesar *et al.*, 2007), Monkey (Thomson *et al.*, 1995) and Human (Thomson *et al.*, 1998). We therefore propose to match the various cell lines described in the previous parts to their embryo-derived alter-ego, as a golden standard. From a practical standpoint, faithful reprogramming can be considered as achieved when a given type of cell lines and their embryo-derived counterparts are undistinguishable at the functional and molecular level.

Faithful reprogramming of somatic nuclei to a naïve but not a primed pluripotent state can be achieved by nuclear transfer

In the mouse, several studies indicated that NT-ESCs could differentiate *in vitro,* as efficiently as FT-ESCs, into functional tissues such as dopaminergic neurons (Wakayama *et al.*, 2001), hematopoietic cells (Huang *et al.*, 2009b) or pancreatic Beta-cells (Jiang *et al.*, 2008). Myogenic derivatives of NT-ESCs have also been obtained at a similar frequency to control FT-ESCs (Munsie *et al.*, 2000). In rhesus monkeys, efficient differentiation into beating cardiomyocytes and neuronal derivatives has been observed for the 2 NT-ESC lines analyzed (Byrne *et al.*, 2007). In addition, viable mice were generated after injection of NT-ESCs into tetraploid host embryos with efficiencies similar to FT-ESCs (Brambrink *et al.*, 2006; Wakayama *et al.*, 2006).

That NT-ESCs are reprogrammed as FT-ESCs is further supported by the comparison of their transcriptomic profiles: cluster analysis segregates ESC lines according to their genetic background, but not according to their origin (FT or NT) (Brambrink *et al.*, 2006; Wakayama *et al.*, 2006). Similarly, transcriptomic comparison of a limited set of FT and NT-lines in the monkey suggests no significant differences between the two kinds (Byrne *et al.*, 2007). At the epigenetic level, DNA methylation profiles do not differ significantly between FT and NT-ESCs in the mouse (Wakayama *et al.*, 2006) and the monkey (Cohen *et al.*, 2009; Sparman *et al.*, 2009). Finally micro RNA signatures of mouse NT-ESC lines have been shown to be identical to their FT counterparts, a result which has been extended to their proteomic profiles (Ding *et al.*, 2009).

Strikingly, NT-ESCs can even be derived from blastocysts with abnormal morphology, perturbed transcriptional activities and epigenetic status. They can even be obtained from NT-embryos with no full-term potential (Wakayama and Yanagimachi, 2001).

In view of these results, the similarity of NT-ESCs and FT-ESCs is intriguing and suggests that either the defects in the NT-ICM do not affect the derivation of ES cells *in vitro* or that a normal pattern of gene expression is acquired during the *in vitro* culture period required to establish ESC lines. Alternatively, but not exclusively, it can be hypothesized that NT-ESCs might be derived from a subset of correctly reprogrammed NT-ICM cells.

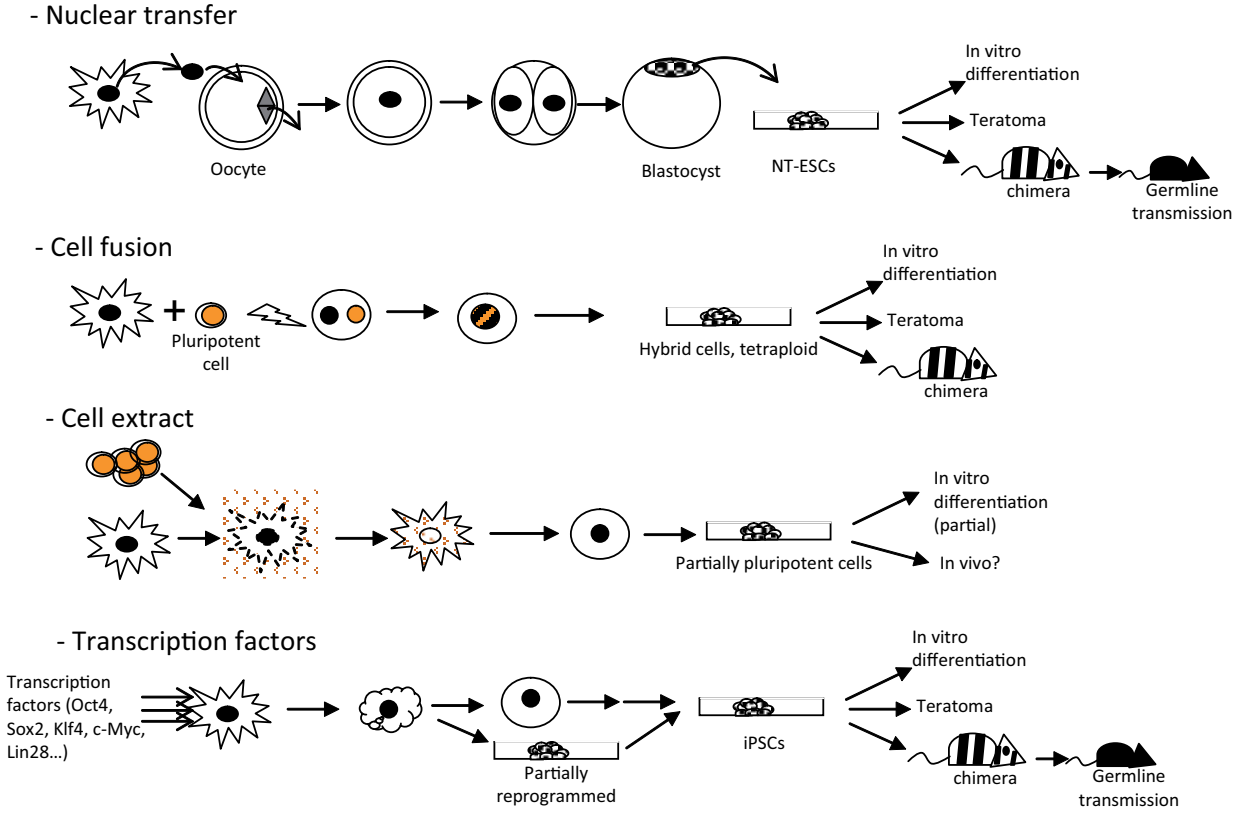

Fig. 2. Different roads to reprogramming to pluripotency.

In sharp contrast to the data obtained with ESCs, we recently found that NT-EpiSC and FT-EpiSC lines are distinguishable based on their transcriptomic profiles and that gene expression differences are associated with epigenetic reprogramming errors after NT (Maruotti *et al.*, 2010).

Therefore, in the mouse, generation of faithfully reprogrammed pluripotent cell lines by NT seems to be achievable only prior to implantation. Afterward, memory of NT induced epigenetic alterations occurring during the *in vivo* establishment of the epigenetic barrier in the epiblast would be retained.

Can faithful reprogramming of somatic nuclei be obtained after cell fusion or induction by defined factors?

Given the lack of data regarding comparisons between cell lines obtained after extract treatment and ESCs, we will only focus on the results achieved after cell fusion and use of defined factors.

Cell fusion

Reports documenting efficiencies to generate a given cell type after *in vitro* oriented differentiation of pluripotent hybrid cells are scarce. To date, myeloid precursors were obtained as efficiently from hybrid lines as from hESC (Yu *et al.*, 2006). In chimeras, the contribution of the hybrid cells to the embryonic tissues was limited compared to ESCs (Tada *et al.*, 1997; Tada *et al.*, 2001; Ying *et al.*, 2002), possibly due to competition between tetraploid hybrid cells and diploid host embryo cells (Tada *et al.*, 1997). Importantly, because of their tetraploid genome, hybrid cells were unable to contribute to the germline.

Genome-wide transcriptional profiling in two different studies indicate that cell fusion could lead to hybrid lines which are closely related, yet distinguishable, to the ESC lines used to reprogram their somatic nuclei. Thus, sets of genes are expressed at similar levels in hybrid and somatic cells, but differently in ESCs, suggesting incomplete resetting of the somatic genome (Ambrosi *et al.*, 2007; Cowan *et al.*, 2005).

Taking into account these data, we consider that cell fusion can reprogram, to some level, somatic nuclei to a pluripotent state. However, the resulting hybrid cell lines still display clear differences on the functional and transcriptional level compared to the ESC lines used to induce reprogramming.

Defined factors

In the mouse, full differentiation of pluripotent cells can easily be obtained by tetraploid complementation, making it a very efficient tool to assess the differentiation ability of miPSC lines. Since viable and fertile mice have been obtained by tetraploid complementation using miPSCs, it can be inferred that iPSCs maintain a developmental potential very close to mESCs (Boland *et al.*, 2009; Zhao *et al.*, 2009). However, caution is now required regarding this conclusion, since more recent reports indicate that miPSC at early passage retain the epigenetic memory of their cell type of origin, impairing their differentiation toward specific lineages (Kim *et al.*, 2010; Polo *et al.*, 2010), while further reprogramming during long term *in vitro* culture seems to relieve these limitations (Polo *et al.*, 2010).

In the human, understanding the developmental potential of iPSC lines is of crucial importance for biomedical applications. Because tetraploid complementation is not available, hiPSC pluripotency was initially assessed by teratoma formation. An early study reported that some cell lines displayed marked differences regarding generation of neuronal tissues, possibly owing to a failure to down-regulate transgene expression of pluripotency regulators upon differentiation (Yu *et al.*, 2007).

In order to further assess iPSC developmental potential, stud-

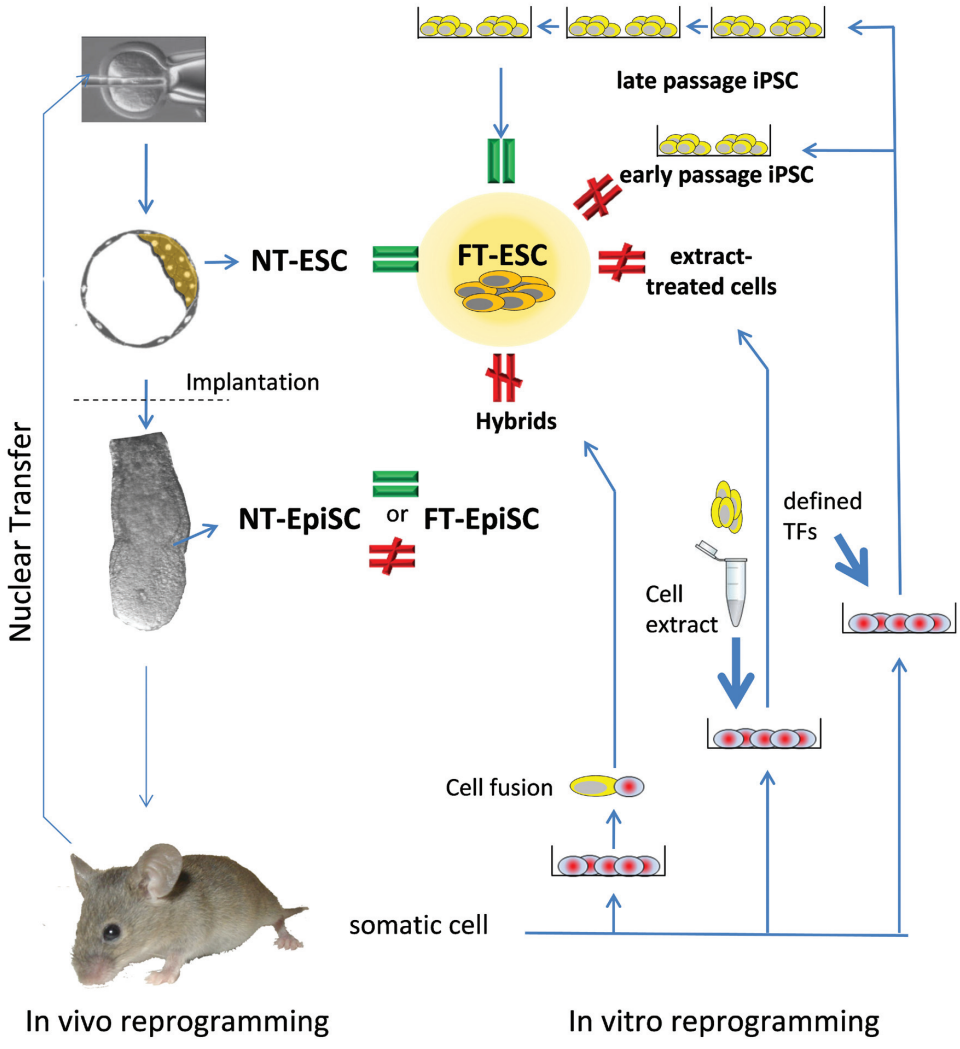

Fig. 3. Comparison of the different reprogramming techniques.

ies involving oriented differentiation were performed. Signaling pathways that control hESC and hiPSC specification into extraembryonic tissues, neuroectoderm and mesendoderm have been found very similar, although hiPSCs display more variability in their answers to extrinsic clues as well as a higher BMP4 activity, requiring specific inhibition (Hu *et al.*, 2010; Vallier *et al.*, 2009). Several groups have similarly observed some variability among hiPSC lines in their relative abilities to undergo directed differentiation (Choi *et al.*, 2009; Karumbayaram *et al.*, 2009; Zhang *et al.*, 2009b). Nonetheless, differences in differentiation propensity have also been described among hESC (Osafune *et al.*, 2008). It has been recently shown, however, that hemangioblast derivatives from hiPSC are functionally impaired with early senescence and limited expansion (Feng *et al.*, 2010). Importantly, in this latter study, reprogramming transgenes were integrated into the iPSC genome and could have caused disruption of pluripotency if inappropriately re-expressed during differentiation (Yamanaka, 2009). The issue of improper transgene expression was highlighted by the finding that excision of reprogramming transgenes in miPSC significantly improved activin mediated endoderm differentiation, as well as mesoderm and ectoderm formation (Sommer *et al.*, 2010). However, in hiPSC, if generation of functional neurons has been achieved without regards to the reprogramming strategy, efficiencies can vary greatly among cell lines and are not found to depend on the presence or absence of transgenes (Hu *et al.*, 2010).

Consequently, miPSCs and mESCs seem very similar regarding *in vivo* differentiation abilities, while hiPSCs display more variability in their aptitude to produce a given type of differentiated progeny. It has therefore been suggested that differences between hiPSCs and hESCs could be more important than between miPSC and mESCs (Feng *et al.*, 2010). Such differences between iPSCs and ESCs have been further studied on a whole genome scale, looking at gene expression and miRNA.

Several reports have suggested that iPSCs are nearly identical to ESCs on the transcriptomic level in mouse (Wernig *et al.*, 2007) and human (Guenther *et al.*, 2010; Lowry *et al.*, 2008; Newman and Cooper, 2010; Park *et al.*, 2008; Yu *et al.*, 2009; Yu *et al.*, 2007). This was balanced by another study which reported about 1200 genes to be differentially expressed (>5-fold) between hiPSCs and hESCs (Takahashi *et al.*, 2007). Even in the absence of transgene integration, significant gene expression differences are observed between hiPSCs and hESCs with persistence of donor cell gene expression in hiPSC and failure to induce hESC specific genes. These differences indicate incompleteness in reprogramming (Ghosh *et al.*, 2010; Marchetto *et al.*, 2009). Interestingly, long term *in vitro* culture seems to further reprogram miPSCs (Polo *et al.*, 2010) and bring hiPSC lines transcriptionally closer to hESCs (Chin *et al.*, 2009; Chin *et al.*, 2010).

Recently, the comparison of transcriptomic data from several laboratories suggested that a gene signature characteristic of the iPSC state existed in mouse and human lines (Chin *et al.*, 2009; Chin *et al.*, 2010), however, a consensus hasn't yet been reached regarding this matter (Guenther *et al.*, 2010; Newman and Cooper, 2010).

In a further effort to characterize faithfully reprogrammed cell lines, Stadtfeld and colleagues have compared genetically matched miPSCs and mESCs. They have discovered that silencing of a cluster of imprinted genes - *Dlk1–Dio3*- in miPSCs is responsible for their impaired ability to generate mice after tetraploid complementation (Stadtfeld *et al.*, 2010). Expanding such results to human would be of great interest, although a preliminary evaluation did not seem to indicate a similar aberrant regulation for the *Dlk1-Dio3* locus in hiPSCs compared to hESCs (Stadtfeld *et al.*, 2010)

Similarly to gene expression profiles, miRNA comparisons have also demonstrated differences between iPSCs and ESCs in mouse and human (Chin *et al.*, 2009; Stadtfeld *et al.*, 2010).

Whether in the mouse or in the human species, iPSCs can usually be distinguished from their embryo-derived counterparts based on transcriptomic or miRNA data. Differential binding of the reprogramming factors to the promoter region of their target genes in iPSCs and ESCs could constitute one possible explanation for the expression differences observed (Chin *et al.*, 2009; Huang *et al.*, 2009a).

Because of their importance in regulating gene expression, epigenetic modifications could also account for the gene expression differences observed between iPSCs and ESCs.

Indeed, DNA methylation analysis show that hiPSC and hESC lines have subtle yet noticeable differences, with hiPSCs being more methylated (Deng *et al.*, 2009; Doi *et al.*, 2009), and overt-methylation linked with gene silencing in miPSCs (Stadtfeld *et al.*, 2010). Although comparison of histone modifications, namely H3K37me and H3K4me, on a global scale by ChIP-chip only identified very minor differences between hiPSC and hESC lines (Chin *et al.*, 2009; Guenther *et al.*, 2010). Significant differences were observed for histone acetylation and H3K4me between miPSCs and mESCs (Stadtfeld *et al.*, 2010).

Therefore, transcriptomic and epigenetic data show that iPSC can be more or less closely related to ESCs with, in some cases, a faithful epigenetic reprogramming. In line with variable extents of reprogramming by TFs, iPSC lines seem to display a higher degree of variability upon differentiation compared to ESCs. These results should highlight the necessity to use the ESC as a standard against which iPSCs should be screened in order to identify those which have recapitulated full nuclear reprogramming. On the other hand, identification of a common gene signature accross many different iPSC lines may indicate that the iPSC state should be considered as a novel pluripotent state (Chin *et al.*, 2009). Therefore, another path to improve differentiation outcomes would be to adapt current ESC protocols to this new pluripotent state.

Conclusion and prospects

Nuclear reprogramming has been defined as the molecular dominance of one distinct cell type over another, resulting in the transformation of the pliant nucleus to the dominant type (Western and Surani, 2002). This definition applies to the various strategies presented in this paper that have proven to be able to reprogram a differentiated cell into a pluripotent one.

Only the induction by defined factors and nuclear transfer are able to reprogram somatic cells to a state of pluripotency compatible with the most stringent tests (*in vivo* differentiation into chimeras and germline transmission). Moreover, according to a recent study, nuclear transfer is the most efficient way to bring cells to a state almost identical to fertilization-derived ESCs (Kim *et al.*, 2010) without the need for further reprogramming *in vitro* by

long term culture, as it is the case for iPSCs. On the other hand, even though gene expression and epigenetic status are perturbed in a majority of NT-blastocysts, they can still be used to efficiently generate faithfully reprogrammed NT-ESC lines. Selection of the subset of correctly reprogrammed cells within a NT-ICM, and/or further reprogramming during *in vitro* derivation have been proposed to explain the equivalence between NT- and FT-ESCs. With the advent of single cell "omics" (Tang *et al.*, 2010; Wang and Bodovitz, 2010), following the fate of each cell, from the NT-ICM to the NT-ESC line, may help answer this question. A more precise understanding of these processes could eventually bring up new approaches for the generation of faithfully reprogrammed iPSC at a higher frequency.

With nuclear transfer, cell fusion or cell extract, epigenetic modifications and re-expression of pluripotency markers are achieved within a limited number of cell cycles. Contrastingly, in the iPSC system, *Oct4* and *Nanog* re-expressions are only observed after several weeks and thus a much higher number of replication events (Hanna *et al.*, 2009). When the Yamanaka factors are used to reprogram somatic cells in conjunction with *Nanog* over-expression, reprogramming kinetics are somewhat accelerated in a cell-division-rate-independent way (Hanna *et al.*, 2009). Studying the fast paced reprogramming mechanisms after NT, cell fusion or cell extract could therefore help identifying new factors, such as AID (Bhutani *et al.*, 2010), whose over-expression might also increase the speed of reprogramming by defined factors.

Results from nuclear transfer experiments in the mouse indicate that faithfully reprogrammed pluripotent cell lines are not as efficiently obtained with primed NT-EpiSCs as compared with naïve NT-ESCs. Recently, hiPSC lines in a naïve pluripotent state have been obtained from fibroblasts after ectopic expression of 5 defined factors (Buecker *et al.*, 2010), or ectopic expression of 3 TFs and addition of small molecules (Hanna *et al.*, 2010). Since primed hESCs can also be reverted to a naïve state of pluripotency similar to mESCs (Hanna *et al.*, 2010; Xu *et al.*, 2010), it would be of interest to study the extent of reprogramming by defined factors in this new context and compare naïve hiPSCs to naïve hESCs. Faithful reprogramming to pluripotency might actually be more efficiently achieved when iPSCs are conserved in their naïve state.

Obtaining iPSC lines equivalent to ESC lines would be especially interesting in the biomedical field. For instance, hiPSCs and their differentiated progeny are considered as a very valuable tool for toxicological studies or to model disease *in vitro* (Yamanaka, 2009). The relevance of the hiPSC based models will, however, ultimately depend on how closely they can compare with hESCs.

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