PLURIPOTENCY AND CELLULAR REPROGRAMMING

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RESUMO

PLURIPOTÊNCIA E REPROGRAMAÇÃO CELULAR

O desenvolvimento em mamíferos começa após a fecundação e singamia dos genomas haplóides. O recém formado zigoto passa por múltiplas divisões celulares e torna–se um embrião composto de células pluripotentes, que darão origem a todos os tecidos encontrados nos animais adultos. As linhagens de células–tronco embrionárias (CTE) são cultivos *ex vivo* de células pluripotentes provenientes de embriões. As CTE oferecem novas oportunidades para investigar o desenvolvimento em mamíferos, para criar novos modelos de doenças humanas, e possivelmente para oferecer células para transplantes. O nascimento de animais clonados pela transferência nuclear de células somáticas para ovócitos demonstrou a reversibilidade da diferenciação celular, um processo denominado reprogramação celular. Outros métodos para obtenção de células reprogramadas indiferenciadas a partir de núcleos somáticos foram descritos, baseados na fusão celular ou na expressão exógena de determinados genes. A reprogramação celular permitiu o isolamento de células pluripotentes de pacientes para investigação da etiologia de doenças humanas e prospeção de novos medicamentos. Apesar do progresso monumental no entendimento do desenvolvimento inicial em mamíferos, a complexidade da pluripotência e reprogramação celular ainda são pouco entendidos. Esta revisão tem como objetivo descrever as principais descobertas nestes campos científicos, citar limitações técnicas e biológicas destas linhas de pesquisas, e prover possíveis soluções para contornar tais dificuldades.

Termos para indexação: células tronco, epigenética, clonagem.

ABSTRACT

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Mammalian development commences after fertilization and syngamy of

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haploid genomes. The newly formed zygote faces multiple cell divisions and becomes an embryo composed of pluripotent cells, that will give rise to all cell types found in adult animals. Embryonic stem (ES) cell lines are *ex vivo* cultures of pluripotent cells from early embryos. ES cells offer new possibilities to dissect mammalian development, to model human disease, and to potentially provide cells for transplantation. The birth of cloned animals after somatic cell nuclear transfer to oocytes demonstrated the reversibility of cellular differentiation, a process denominated cellular reprogramming. Other methods to obtain reprogrammed undifferentiated cells from somatic nuclei have been described, based on cell fusion and ectopic expression of key genes. Cellular reprogramming allowed derivation of patient–specific pluripotent cells for investigations of disease etiologies and prospection for new therapeutics. Despite monumental progress in understanding on the nature of mammalian early development, the complexity of pluripotency and cellular reprogramming are still poorly understood. This review aims to describe major findings in these research fields, outline technical and biological limitations to this research, and provide possible alternatives to overcome them.

Index terms: stem cells, epigenetics, cloning.

1. Embryonic Development

The process of fertilization marks the encounter of two highly specialized cell–types: sperm cells and oocytes. After the sperm cell enters the oocyte cytoplasm, its nucleus is engaged in an intense event of remodelling, in order to convert the highly condensed, transcriptionally inactive spem cell nucleus into a transcriptionally and replication competent haploid genome. Since the genome at the DNA level remains unchanged, reprogramming is not genetic, but epigenetic in nature (e.g. histone marks and DNA methylation). This remodelling is achieved by replacing protamines by acetylated histones and genome–wide DNA demethylation (Oswald *et al.*, 2000; Mayer *et al.*, 2000; Feng *et al.*, 2010). Remarkably, the oocyte genome is protected from epigenetic remodelling by trans–acting proteins *STELLA* (Nakamura *et al.*, 2007), *ZFP57/KAP1* (Quenneville *et al.*, 2011), and possibly other unidentified factors.

After completion of DNA replication on both male and female pro–nuclei, they fuse and the first embryonic cell division takes place (Figure 1) (Li *et al.*, 2010). Several rounds of cell division are followed, accompanied by continuous genome–wide reprogramming, but in a passive fashion (Feng *et al.*, 2010; Inoue & Zhang, 2011). At the funcional level, blastomeres of early embryos are totipotent, namely, have the potential to form all fetal and placental tissues. This fact was formely

demonstrated in several species by live births from isolated blastomeres (Moore *et al*., 1968; Willadsen, 1980). Although the genome is facing an intense structural remodeling during these initial cell divisions, transcription is not detected from most genes (Li *et al*., 2010). Development is controlled at this point by the oocyte cytoplasm, and when the embryonic genome becomes active, the embryo faces a progressive transition period of development control that ends around the time of implantation (Stitzel & Seydoux, 2007; Li *et al*., 2010).

After embryonic genome activation, the embryo embarks in the first process of cellular differentiation (Figure 1). The whole embryo begins to compact at the morula stage: outer cells become flat and polarized, while the inner cells remain unchanged (Li *et al*., 2010; Cockburn & Rossant, 2010). Shortly after, the embryo starts to acumulate liquid and forms a cavity denominated blastocele. At this time point, the embryo now denominated blastocyst, holds two morphologically and functionally distinct cell types: trophoblast (will form all placenta tissues) and the inner cell mass (ICM), a pluripotent cell population. Pluripotency is defined as the ability to give rise to all cell types that make up the body of the adult animal (Cockburn & Rossant, 2010). Moreover, the ICM will segragate into two distinct populations: hypoblast or primitive endoderm and the epiblast (Rossant, 2008). Hyploblast cells form the yolk sac, and epiblast cells will give rise to fetal somatic cell types and primordial germ cells (PGC). The proliferating PGC pool migrates to genital ridges of the midgestation fetus, and differentiate to sperm cells or oocytes, depending on embryo gender (Figure 1).

2. Embryonic Stem Cells

Embryonic stem (ES) cells are pluripotent cell lines stablished from ICM cultures under specific *in vitro* conditions (Figure 1) (Evans & Kaufman, 1981; Martin, 1981; Thomson *et al*., 1995, 1998; Solter, 2006). If properly cultured, ES cell lines can be propagated indefinitely, without signs of senescence or differentiation (Smith, 2001). When introduced into blastocysts, mouse and rat ES cells demonstrate their capacity to colonize somatic tissues and the germline (Bradley *et al*., 1984; Smith, 2001; Buehr *et al*., 2008; Li *et al*., 2008). A more instrigent version, the tetraploid embryo complementation assay, further demonstrated the capacity of ES cells to solely form the mouse fetus (Nagy *et al*., 1993; Smith, 2001; Eggan *et al*., 2004; Stadtfeld *et al*., 2010). Due to ethical constrains, human ES cells pluripotency *in vivo* is assayed by injection of ES cells in immuno–compromised mice, also known as

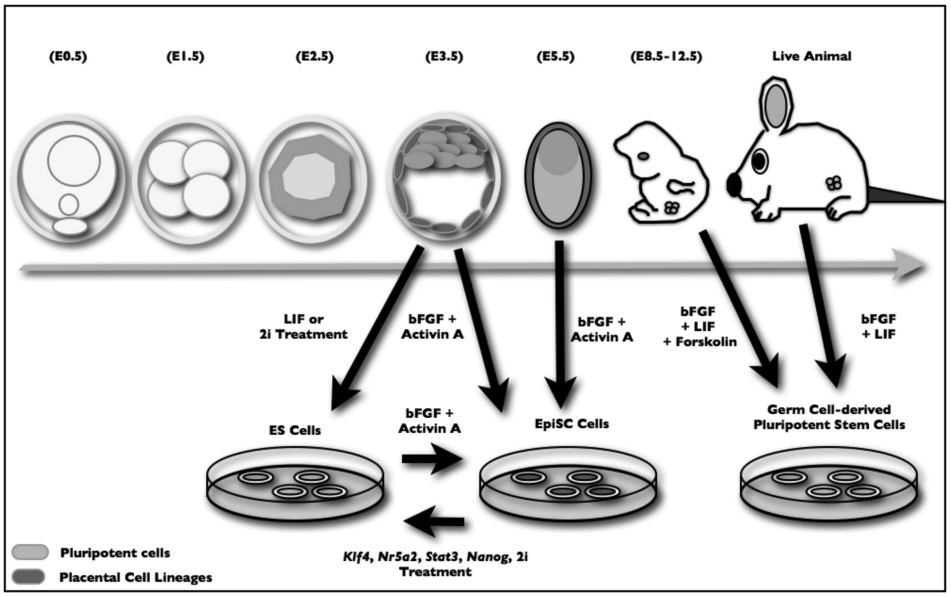

Figure 1. — Mouse development and pluripotent stem cell derivation.

the teratoma assay (Lensch *et al.*, 2007). Moreover, this tumor can be inferred if it contains ES–derived tissues of all three embryonic germ layers.

Concomitant with firts reports of ES cell derivation in the mouse, several groups succeeded using homologous recombination to manipulate the mouse genome at precise *loci* (Capecchi, 1989). When combined, these two technologies became an efficient approach to modify the mouse genome in order to investigate gene function (Capecchi, 1989, 2005; Adams & Van Der Weyden, 2008).

Several genes, most notably transcription factors, have been described as pluripotency genes: *Oct4*, *Sox2*, *Stat3*, *Nanog*, *Sall4*, *Ronin*, *Nr5a2*, among others (Nichols *et al.*, 1998; Niwa *et al.*, 2000; Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Welstead *et al.*, 2008). Transcription factors are DNA–binding proteins that modulate transcription of their target genes by recruitment of activation and/or repression complexes to regulatory sequences such as promoters and enhancers (Lee & Young, 2000; Hobert, 2008). Pluripotency genes are divided in two subclasses: genes that maintain the undifferentiated state in embryos and ES cells (*Oct4*, *Sox2*, *Nanog*, *Sall4*), and self–renewal genes (*Stat3*, *c–Myc*, *Klf4*). Self–renewal is defined as the ability of an ES cell to divide and form two daughter ES cells. In the mouse, ES self–renewal is activated by addition of leukemia inhibitory factor (*LIF*) to culture media, which induces Janus Kinase / *Stat3* signalling (Williams *et al.*, 1988; Niwa *et*

al., 1998; Burdon *et al*., 2002; Boiani & Schöler, 2005). However, human and monkey ES cells relie on *ERK* signalling activated by *bFGF* to avoid differentiation in culture (Amit *et al*., 2000). These discoveries led to a widely accepted hypothesis that ES cells were an *in vitro* culture artifact, and their functional correspondence to a defined cell population in embryos as an unlikely possibility (Hansson *et al*., 2007). Remarkably, the supression of differentiation stimuli (*ERK* and *GSK3* signalling pathways – 2i condition) allowed mouse ES cells to self–renewal in absence of *LIF/Stat3* signalling activation (Ying *et al*., 2008). These facts demonstrates that pluripotent cells in early embryos and ES cells hold an intrinsic ability to self–renewal, but are guided for differentiation during development (Ying *et al*., 2008; Nichols *et al*., 2009). Furthermore, use of 2i condition was successfull at deriving much antecipated rat ES cell lines (Buehr *et al*., 2008; Li *et al*., 2008).

The determination of binding sites of pluripotency genes such as *Oct4*, *Sox2*, and *NANOG* at genomic level are undercovering cellular mechanisms by which these genes contibute to the maintainace of pluripotency (Boyer *et al*., 2005; Loh *et al*., 2006; Jaenisch & Young, 2008; Kim *et al*., 2008a). Alternatively, physically associated proteins to pluripotency genes such as *NANOG* have been identified, and further characterized the pluripotency gene repertoire (Wang *et al*., 2006). Collectively, these reports described the cooperation between multiple genes in order to establish pluripotency and avoid activation of differentiation inducing factors (Boyer *et al*., 2005, 2006; Wang *et al*., 2006). Due to the fast growing amount of genome–wide projects, systems biology approaches will be extremely useful to integrate fast–growing number of data sets and provide a broader understading of cellular states and their transitions (Lu *et al*., 2009; Macarthur *et al*., 2009).

The epigenetic basis of pluripotency is focus of intense research (Jaenisch & Young, 2008). DNA methylation is dispensable for ES cells self–renewal (Tsumura *et al*., 2006), but is required for their differentiation (Jaenisch, 1997). ES cells relie on histone methylation (H3k9me2/3 and H3K27me3) for gene repression (Boyer *et al*., 2006a; Lee *et al*., 2006). Transcription factors with important roles in development are marked in ES cells by both activating histone methylation (H3K4me3) and repressive marks (H3k9me2/3 and H3K27me3), a chromatin state termed bivalent domain (Azuara *et al*., 2006; Bernstein *et al*., 2006). This discovery was surprising, because chromatin states were always captured as carrying "activating" or "repressive" epigenetic marks, but never both types. The interpretation is that bivalent domains poise genes for rapid activation or repression following instructive

cellular differentiation signals (Boyer *et al*., 2006b). In accordance with this model, after differentiation, bivalent domains found in ES cells are generally resolved as fully repressed genes or actively transcribed genes (Mikkelsen *et al*., 2007). Other epigenetic components of bivalent domains have been found, suggesting a more complex regulation of this chromatin state. Bivalent domains were also found in early embryos and somatic cells (Mikkelsen *et al*., 2007; Vastenhouw *et al*., 2010), demonstrating the potential of ES cells to underscore epigenetic regulatory mechanisms.

It became evident over the years that pluripotency is not one stable cellular state. Variations at molecular and functional levels have been observed across pluripotent cultures, within ES cell lines, and even in individual cells (Chambers *et al*., 2007; Hayashi *et al*., 2008; Toyooka *et al*., 2008; Han *et al*., 2010). Despite intrinsic functional fluctuations in pluripotent states, a pronounced difference in developmental potency was observed between mouse and human ES cells (Nichols & Smith, 2009). An important finding came from mouse pluripotent cultures established using human ES–specific conditions. These lines, coined Epiblastic stem cells (EpiSC), hold morphpological, transcriptional, and functional properties that resembled more closely human ES cells than mouse ES counterparts (Brons *et al*., 2007; Tesar *et al*., 2007). This lead to the concept that pluripotency states are to some degree dependent on culture conditions, that guide undifferentated cells to cycle in a determined cellular state (Nichols & Smith, 2009; Hanna *et al*., 2010a). Moreover, our growing understanding of these differences are coming from demonstrations that precise changes in culture conditions (2i culture) and ectopic expression of pluripotency genes (e.g. *Stat3*, *Nr5a2*, *Klf4*) can convert mouse EpiSC in ES cells (Hanna *et al*., 2010a), and human ES or iPS into "Naive" pluripotent cells, that resemble mouse ES cells (Buecker *et al*., 2010; Hanna *et al*., 2010b).

Genome–wide epigenetic and transcriptional analysis of pluripotent cell subpopulations with contrasting functional properties (Chambers *et al*., 2007; Hayashi *et al*., 2008; Toyooka *et al*., 2008; Han *et al*., 2010), may aid dissection of molecular determinants of pluripotency and further describe molecular signatures for these discrete pluripotent states.

3. Cellular Reprogramming by Nuclear Transfer

Nuclear transfer (NT) was developed in order to test if cellular differentiation is acompanied by loss of genetic content (Briggs & King, 1952; Solter, 2000). The

introduction of somatic cells into enucleated oocytes was capable of recapitulating development to term, with some clones surviving to adulthood, demonstrating nuclear equivalency between adult cells (Figure 2) (Gurdon *et al*., 1958; Wilmut *et al*., 1997). More recently, cloning technology became an usefull technology for a broad range of applications: for scientific research (Eggan *et al*., 2004; Hochedlinger *et al*., 2004), to generate transgenic animals (Schnieke *et al*., 1997; Kuroiwa *et al*., 2004), to replicate desired genomes (Loi *et al*., 2001), to produce advantageous mouse models (Kirak *et al*., 2010), among other applications.

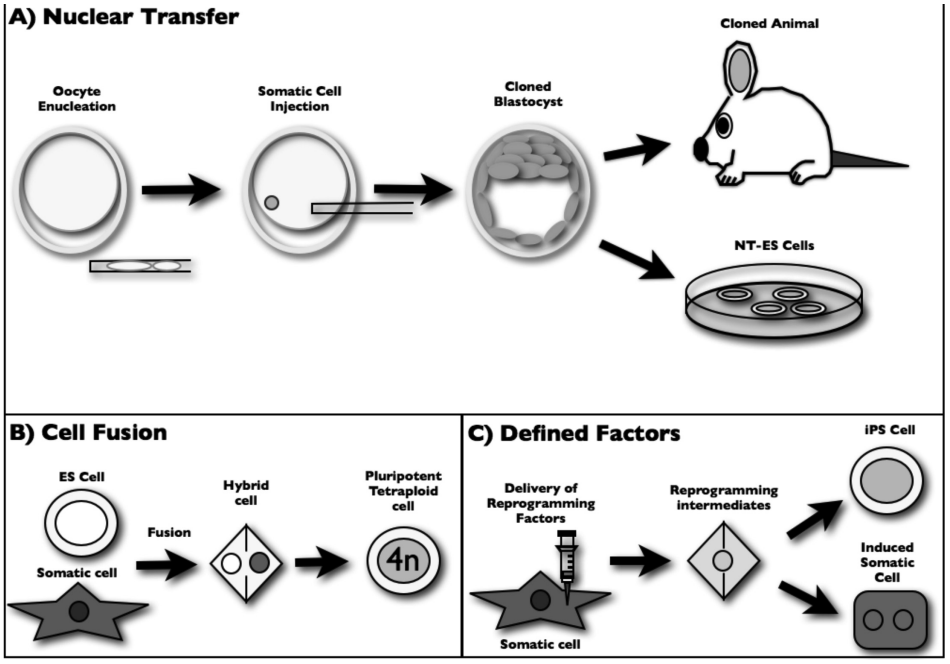

Figure 2. — Cellular reprogramming in mammals.

The therapeutic potential of NT was demonstrated by isolation of ES cells from cloned embryos (NT–ES cells), directed differentiation of these cells to a specific cell type, tranplantation in animal models, followed by amelioration of disease symptoms (Rideout *et al*., 2002; Lanza *et al*., 2004; Barberi *et al*., 2003). This approach, known as therapeutic cloning, has potential to generate unlimited numbers of genetically matched cells from patients for transplantation, avoiding graft immune rejection (Solter, 2006). These patient–specific cells can also be useful to establish *in vitro* disease models and drug screening (Solter, 2006). However, human and non–human primate NT research has progress so far at a slower pace than other mammalian

species, and only rhesus monkey NT–ES cell lines have been described (Byrne *et al.*, 2007).

Cellular reprogramming by NT represents an enormous biological challenge: the oocyte needs to remodel a somatic nucleus to an epigenetic state similiar to a zygote. Not surprisingly, cloning by NT is an inneficient process (Moura *et al.*, 2008; Moura, 2011). No more than 5% of cloned embryos develop to term, and around 50% of cloned infants reach adulthood (Wilmut *et al.*, 2002). Similar cloning efficiencies were obtained from vertebrate and invertebrate animals, ruling out species–specific technical or biological limitations (Gurdon *et al.*, 1958; Wilmut *et al.*, 1997, 2002; Lee *et al.*, 2002; Haigh *et al.*, 2005). Some investigations have found genetic instability after NT (Simerly *et al.*, 2003; Shi *et al.*, 2004; Mizutani *et al.*, 2012), but incorrect epigenetic resetting of the genome accounts for the majority, if not all causes of development losses (Kang *et al.*, 2001; Santos *et al.*, 2003). Moreover, it can not be ruled out that these genetic alterations in animal cloning have an epigenetic basis (Gaudet *et al.*, 2003).

The remodelling or reprogramming of sperm cells by oocytes is a precise, and well orchestrated process. Mammalian sperm genomes are mostly packed by protamines and DNA methylation (Miller *et al.*, 2010). Curiously, around 4% of nucleosomes found in somatic cells are found in human spermatozoa and are not randomly distributed across the genome: coding sequences of imprinted genes, microRNA, and hox genes are enriched for nucleosome–bound DNA (Hammoud *et al.*, 2009). In contrast, somatic cells relie on coorporation between DNA methylation and repressive histone methylation in order to stably maintain their cellular phenotype (Cedar & Bergman, 2009). Collectively, somatic cells lack instructive signals for remodelling (such as protamines), carry multiple regulatory mechanisms to avoid activation of pluripotency genes (Feldman *et al.*, 2006), and possess mechanisms that reinforce their somatic cell fate in cloned embryos (e.g. histone variant H3.3) (Ng & Gurdon, 2008). These biological barriers to somatic nuclei remodelling are supported by several evidences of partial reprogramming: incomplete reactivation of pluripotency genes (Boiani *et al.*, 2002; Bortvin *et al.*, 2003), precocious activation of donor cell–especific genes (Ng & Gurdon, 2005, 2008), DNA and histone hypermethylation in cloned embryos or somatic and extra–embryonic tissues of cloned fetuses (Dean *et al.*, 2001; Reik *et al.*, 2001; Rideout *et al.*, 2001; Yang *et al.*, 2007).

The spectrum of placentation problems is considered a particular bottleneck

for mammalian cloning, due to the fact that most pregnancies are lost around the time of maternal recognition and implantation (Yang *et al.*, 2007). However, the generation of mouse embryos with contribution of reprogrammed cells limited to placental lineages resulted in conflicting results (Miki *et al.*, 2009; Lin *et al.*, 2011). Similar functional assessments of NT–derived placentas on larger species (e.g. sheep, bovine) might solve this question. Remarkably, mouse NT–ES cells are indistinguishable from ES cells derived from fertilized embryos at both molecular and functional levels (Brambrink *et al.*, 2006; Wakayama *et al.*, 2006).

Multiple procedures have been tested for improving cloning efficiency: embryo re–cloning (Galli *et al.*, 1999), serial cloning (Polejaeva *et al.*, 2000), two–step cloning (Hochedlinger & Jaenisch, 2002), NT embryo aggregation (Boiani *et al.*, 2003), and treatment of donor cells (chromatin modifyers, heating, or cellular extracts) (Loi *et al.*, 2002; Sullivan *et al.*, 2004). The overall limited increase in reprogramming efficiency by these "intuitive" methods call for research on targeted approaches to raise cloning efficiency. The substantial increase in mouse cloning efficiency by ablation of *Xist* expression in both male and female somatic cells or NT embryos is in accordance with this hypothesis (Inoue *et al.*, 2010; Matoba *et al.*, 2011).

The phenomenon of cellular reprogramming has been extensively explored using cloning technology, but the mechanism at the molecular level remains largely enigmatic (Hochedlinger & Jaenisch, 2006). This scenario is mostly due to limited suitability of NT reprogramming system for biochemical analysis: oocytes are scarce, difficult for genetic manipulation; NT is labor intensive, technically challeging, and yields embryos at small numbers.

Future research should focus on genome–wide studies using recently developed protocols for small biological samples (Adli *et al.*, 2010), since research to scale–up NT embryo production have met little success (Moura, 2011). The analysis of DNA methylation and histone modification dynamics at high resolution during embryonic development and after NT will be extremely informative (Smith *et al.*, 2012). Attention should be focused on early reprogramming events, when intense chromatin dynamics are found (Kikyo *et al.*, 2000). Direct comparison of chromatin remodelling using different templates (sperm cell, oocyte, and somatic nucleus) should shed some light on limiting factors to reprogramming, core reprogramming components, and combinatory interaction between epigenetic mechanisms. More detailed information on epigenetic reprogramming by oocytes will refine future approaches to increase reprogramming efficiency.

4. Cellular Reprogramming by Cell Fusion

Based on the notion that oocytes contain factors to reprogram somatic cells to totipotency, ES cells were tested as an alternative source to convert differentiated nuclei into pluripotent cells (Miller & Ruddle, 1976; Tada *et al.*, 2001). After cell fusion and progression through mitosis, ES cell nuclear factors interact with the somatic cell genome, resulting in a tetraploid undifferentiated cell (Tada *et al.*, 2001; Egli *et al.*, 2008) (Figure 2). This strategy was used to demonstrate that human somatic cells are also ameneable to cellular reprogramming (Cowan *et al.*, 2005; Yu *et al.*, 2006). Cell fusion has also gained attention as an attractive method to identify cellular components required for reprogramming (Hochedlinger & Jaenisch, 2006; Silva *et al.*, 2006; Ma *et al.*, 2008; Pereira *et al.*, 2008, 2010; Bhutani *et al.*, 2010).

Some technical and biological issues limit potential applications of cell fusion for addressing reprogramming: low fusion yields, presence of an extra genome (ES cell nucleus), and possible epigenetic and functional heterogeneity in cells under reprogramming (Pralong *et al.*, 2006; Skelley *et al.*, 2009). Cell fusion–mediated reprograming protocols relie on chemical membrane fusion or electrofusion of cell suspensions to generate somatic–ES cell hybrids (Tada *et al.*, 2001; Cowan *et al.*, 2005). Low fusion rates are generally due to low and random cell–cell pairing for fusion. The development of a microfluidic device allowed high cell pairing and fusion between different cell types, and reprogrammed cells could be obtained at large numbers (Skelley *et al.*, 2009). The presence of an extra genome in hybrid cells complicates the investigation of epigenetic remodelling of somatic cells after cell fusion. An usefull approach would be to use genetically distinct genomes to provide somatic and ES cell populations for fusion, and by DNA sequencing, identify the source of analysed DNA or chromatin samples (Cowan *et al.*, 2005; Noggle *et al.*, 2011). The epigenetic heterogenety of cells undergoing reprogramming is probably due, at least in part, to increasing stochastic noise as cells progressively lose stable somatic cell transcriptional program and acquires a pluripotency transcriptional signature (Pujadas & Feinberg, 2012). These hybrid cell populations could be fractioned by selective markers (e.g. cell surface markers), in order to identify different stages or cell populations during reprogramming. Alternatively, single–cell analysis could also be used to estimate this reprogramming heterogeneity.

The main functional limitation of reprogrammed cells by fusion is the extra chromosome set in resulting cells (Pralong *et al.*, 2006). Although 4n ES–like cells

may hold some therapeutic potential (Sullivan & Eggan, 2006), removal of ES genome will still be necessary to demonstrate complete reprogramming after cell fusion (Yamanaka, 2007). Investigations aiming to enucleate ES cells, mainly by centrifugation, have been unsucessful (Do & Schöler, 2004; Hasegawa *et al.*, 2010). Some reports have claimed that reprogramming–competent enucleated ES cells were obtained after centrifugation, but evidence of complete somatic cell reprogramming is still lacking (Pralong *et al.*, 2005; Strelchenko *et al.*, 2006; Du *et al.*, 2011). The targeted deletion of single chromosomes was achieved in ES cells (Matsumura *et al.*, 2007), but the method seems impractical for enucleation of several chromosomes. Alternatives methods based on chemical enucleation may hold potential to enucleate ES cells while maintaining reprogramming ability (Moura, 2011).

An important biological issue should be resolved before further ES cell enucleation efforts: the determination of reprogramming kinetics following cell fusion (Egli *et al.*, 2008; Han *et al.*, 2008). If multiple cell divisions are required for completion of reprogramming, than current ES cell enucleation methods may not be suitable, and approaches to deliver additional loads of reprogramming factors after ES cell enucleation may need to be designed.

Technical variations for cell fusion reprogramming were developed aiming to avoid the induction of tetraploidy: use of mitotic ES cell nuclear/cytoplasmic extracts incubation with permeabilized somatic cells or whole transcriptome transfer to differentiated nuclei (Taranger *et al.*, 2005; Sul *et al.*, 2009). However, these alternate methods still need to demonstrate that cells obtained are truly reprogrammed.

5. Cellular Reprogramming by Defined Factors

The enthusiasm brought by therapeutical evidence of both reprogramming and ES cell technologies in animal models was somewhat diminished by ethical, logistical, and technical hurdles found in human NT research. Even if successful, therapeutic cloning would probably be an umpractical technology in clinical settings. Based on these facts, it became a concensus that methods to reprogram cells to a pluripotent state without the use of oocytes should be persued (Hochedlinger & Jaenisch, 2006).

The road to cellular reprogramming without oocytes seemed very long: reprogramming mechanisms were poorly investigated; the understanding of pluripotency and epigenetic mechanims were far from complete.

A major advance came from one of the first attempts to reprogram cells without

oocytes: ES–like cells were obtained by exogenous expression of 24 pluripotency genes in mouse fibroblasts (Takahashi & Yamanaka, 2006). It became evident from this report that only four genes were responsible for this astonishing cellular conversion: *Oct4*, *Sox2*, *Myc*, and *Klf4* (Figure 2). However, molecular and functional evaluations demonstrated that these cells, coined induced pluripotent stem (iPS) cells, were not fully reprogrammed (Takahashi & Yamanaka, 2006). Notwithstanding, these evidences proved the potential of cellular reprogramming by defined factors.

Refinements in selection methods resulted in mouse iPS cells that better resembled ES couterparts in morphology, growth characteristics, gene expression and epigenetic profiles, differential potential (*in vitro* and *in vivo*), and germline transmission competence (Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007; Yamanaka & Blau, 2010). Human cells from healthy donors or patients were also ameneable to reprogramming by defined factors (Takahashi *et al*., 2007; Yu *et al*., 2007; Dimos *et al*., 2008; Lowry *et al*., 2008; Park *et al*., 2008a,b; Cherry & Daley, 2012).

Several reports have demonstrated that mouse iPS–derived cells rescue animal disease models (Hanna *et al*., 2007; Wernig *et al*., 2008; Xu *et al*., 2009). Human patient–specific iPS cell lines can differentiate to disease relevant cells at unlimited numbers (Dimos *et al*., 2008), establish human *in vitro* disease models, and recapitulate disease phenotypes (Saha & Jaenisch, 2009; Cherry & Daley, 2012). The most promising iPS–based disease models to date are monogenic diseases with high penetrance, with early childhood onset or during development (Cherry & Daley, 2012). An evident exception is the recapitulation of schizophrenia associated neuronal phenotypes (Brennand *et al*., 2011). A caution note came from the demonstration that iPS cells from autologous skin can generate immunogenic differentiated progeny, probably due to incomplete reprogramming (Zhao *et al*., 2011).

A major current research objective is to determine if there are any molecular or functional differences between ES and iPS cells. The birth of mice derived from iPS using the tetraploid embryo complementation assay demonstrates that these cells are compatible with the most rigorous functional test for pluripotency (Boland *et al*., 2009; Kang *et al*., 2009; Zhao *et al*., 2009). However, several differences at the molecular level have been identified: DNA methylation profiles (Kim *et al*., 2010; Lister *et al*., 2011), mutation rates (Gore *et al*., 2011; Hussein *et al*., 2011), skewed *in vitro* differentiation (Kim *et al*., 2010; Polo *et al*., 2010; Rizzi *et al*., 2012). Further research is need in order to determine if these molecular differences affect the

potential of iPS for research or therapeutic applications.

The rationality of iPS technology came at a high expense: reprogramming efficiency is extremely low, ranging from 0.001% to 5% (Kiskinis & Eggan, 2010), and transgene integration may lead to oncogenic transformation (Okita *et al.*, 2007). Several combinations of transcription factors, chemicals (e.g. chromatin modifyers, kinase inhibitors), microRNAs, donor cells, and non–integrating methods have been used to increase reprogramming efficiency and to generate transgene–free iPS cells (Yu *et al.*, 2007; Aoi *et al.*, 2008; Ichida *et al.*, 2009; Judson *et al.*, 2009; Heng *et al.*, 2010; Kiskinis & Eggan, 2010; Efe & Ding, 2011; Li *et al.*, 2011; Moura, 2011).

The nature of cellular reprogramming by defined factors seems to be different than reprogramming by NT or cell fusion (Jullien *et al.*, 2011). Nuclear remodelling by ES cells or oocytes is expected to operate by instructive, deterministic processes (Jullien *et al.*, 2011). In contrast, reprogramming by defined factors is stochastic, where reversal of cellular differentiation and induction of pluripotency occurs in random sequences of events (Hanna *et al.*, 2009; Yamanaka, 2009; Jullien *et al.*, 2011). Some somatic cells become trapped in an intermediate reprogramming state, and may require additional factors to reach the pluripotent state (Mikkelsen *et al.*, 2008; Ichida *et al.*, 2009). The exogenous expression of reprogramming factors induces global chromatin remodelling, induction of proliferation genes, and repression of somatic cell transcriptional program (Mikkelsen *et al.*, 2008; Koche *et al.*, 2011). DNA demethylation is a late event in the process of iPS generation, and occurs in a passive manner (Mikkelsen *et al.*, 2008). Moreover, several components of pluripotency and epigenetics machineries have been identified as mediators of iPS reprogramming (Singhal *et al.*, 2010; Maekawa *et al.*, 2011; Onder *et al.*, 2012).

The development of iPS cells impelled investigations to test if somatic cells could be converted directly to another cell type by defined factors for several reasons. Pluripotent cells have important limitations to potential medical applications: tumorigenic potential, genetic instability in culture, and current limited understanding of developmental cues to efficiently differentiate ES–iPS *in vitro* toward specific cell types. Cell lineage conversion or transdifferentiation has potential to produce unlimited numbers of disease relevant cells and overcome outlined hurdles associated with pluripotency. Neurons, pancreatic β–cells, cardiomyocytes, and other cell types have been obtained by direct reprogramming of development–related cells or readily available skin cells (Zhou *et al.*, 2008; Graf & Enver, 2009; Ieda *et al.*, 2010; Son *et al.*, 2011).

In a therapeutic perspective, a remarkable example was the direct conversion of pancreatic exocrine cells into β–like cells *in vivo* and subsequent amelioration of hyperglycaemia in mice (Zhou *et al*., 2008). Mouse and human fibroblasts were also efficiently converted to neurons, and murine neuronal cells recapitulate a neurodegenerative disease phenotype *in vitro* (Son *et al*., 2011).

Extensive work has explored several technical difficulties found in reprogramming by defined factors: delivery of reprogramming genes to donor cells, selection of reprogramming events, maintainance of genome integrity, among others. This focus on metodological aspects is driven by promising oportunities of this technology for regenerative medicine. However, accumulating evidence is suggesting that direct reprogramming will require other improvements in order to produce patient–specific differentiated that more closely resemble *in vivo* counterparts. Further refinements will probably emerge from investigations of the extension of reprogramming and its underlying mechanisms. The stochastic nature of direct reprogramming represents an important roadblock for addressing this phenomenon, and calls for single–cell analysis. Integrative genomics of representative discrete cell populations undergoing reprogramming could also be extremely informative (Mikkelsen *et al*., 2008).

6. Conclusion

The understanding that development starts from a single cell, and following several stepwise, cell fate lineage decisions, builds into complex organisms with hundreds of cell types is a well established concept. From the last century to this day, several striking technologies (such as NT, ES cell derivation, and iPS cells) were developed based on ingenuous ideas. These new technologies have led to great discoveries on how cell fate decisions are made and reversed at the celullar level. Molecular analysis of pluripotency and cellular reprogramming using low resolution and genome–wide approaches are expanding our understanding of these events at a very fast pace. However, several technical and biological challenges remain. More creativity, and usefull curiosity is needed in order to fully understand these remarkable biological processes at the molecular level.

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