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 prospecção 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

PLURIPOTENCY AND CELLULAR REPROGRAMMING

Mammalian development commences after fertilization and syngamy of
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 sperm 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 functional level, blastomeres of early embryos are totipotent, namely, have the potential to form all fetal and placental tissues. This fact was formerly
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 accumulate liquid and forms a cavity denominated blastocoele. 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 segregate into two distinct populations: hypoblast or primitive endoderm and the epiblast (Rossant, 2008). Hypoblast 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 established 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 stringent 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 constraints, 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 first 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, Salh1*, *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, Salh1*), 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 rely 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 suppression 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 demonstrate 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 successful at deriving much anticipated 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 uncovering cellular mechanisms by which these genes contribute to the maintenance 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 understanding 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 rely 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 morphological, 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 undifferentiated 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 accompanied 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 useful 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.

![Cellular reprogramming in mammals.](image)

**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, transplantation 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 similar to a zygote. Not surprisingly, cloning by NT is an inefficient 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 rely 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 modifiers, 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 challenging, 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 combinatorial 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 amenable 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 reprogramming protocols rely 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 useful 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 heterogeneity 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 acquire 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 unsuccessful (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 unpractical 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 pursued (Hochedlinger & Jaenisch, 2006).

The road to cellular reprogramming without oocytes seemed very long: reprogramming mechanisms were poorly investigated; the understanding of pluripotency and epigenetic mechanisms 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 counterparts 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 amenable 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 modifiers, 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 \textit{in vivo} and subsequent amelioration of hyperglycaemia in mice (Zhou \textit{et al.}, 2008). Mouse and human fibroblasts were also efficiently converted to neurons, and murine neuronal cells recapitulate a neurodegenerative disease phenotype \textit{in vitro} (Son \textit{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, maintenance of genome integrity, among others. This focus on methodological aspects is driven by promising opportunities 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 \textit{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 \textit{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 cellular 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 useful curiosity is needed in order to fully understand these remarkable biological processes at the molecular level.

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