Chapter 11

Multiple Paths to Reprogramming

Antonio Lo Nigro and Catherine M. Verfaillie

Additional information is available at the end of the chapter

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1. Introduction

For a long time, differentiation was considered a "one way process"; Conrad Waddington, in the 1950s, described cellular differentiation and development as a ball rolling towards different one-way ramified valleys, giving rise to specific cell fates, irreversibly [1]. However, in the last decades, a series of studies have shown that somatic cells and stem cells are more plastic than previously believed. Using different technical approaches, the epigenetic barriers imposed during development in differentiated cells can be erased, and cells can re-acquire pluripotency through a process, known as "reprogramming".

The first evidence came at the end of the 1950's from the pivotal experiments performed by J.B. Gordon in the zoology department at Oxford University [2]. At that time, embryologists, not aware of epigenetic regulation, i.e. the role of chromatin and its crucial modifications in cell fate determination, wondered whether development and cellular differentiation arise upon specific restriction of the genetic information contained in their nuclei. To answer this basic but intriguing question, Gordon used a technique, now known as somatic cellular nuclear transfer (SCNT) in *Xenopus laevis laevis*. For these experiments, nuclei from intestinal epithelial cells of albino tadpoles were transferred into unfertilized and enucleated wild-type frog oocytes. This resulted in the development of normal albino frogs, which in some cases were also fertile. They concluded from these studies that adult nuclei contain the genetic information necessary for the development of a frog. Moreover, cellular differentiation, during development, does not occur through loss of genetic information. These findings were exciting for the scientific community but at the same time controversial for two reasons: I) the efficiency with which a 'cloned' frog reached the adult stage was around 1% and II) the same technique did not work with mammalian cells [3].

For forty years, the scientific community was not able to use SCNT in other species. Finally, in 1997, by using the same technique, Ian Wilmut and colleagues, at the Roslin Institute in

Edinburgh, Scotland, succeeded in generating the sheep named "Dolly" by SCNT, further confirming that genetic modifications, leading to cellular differentiation, are not irreversible. Two key improvements in his technical strategy led to the first cloning of a mammal: unfertilized oocytes were used as recipients and donor cells were induced to exit from the normal cellular cycle, by serum withdrawal [4, 5].

One year later, Wakayama and colleagues [6] reported that SCNT also allowed the cloning of the most used animal model, the mouse. Again, another technical advance led to this progress: the use of an enucleation pipette, which allowed for the removal of the nucleus from the oocytes. This advance also allowed the conclusion that reprogramming factors are not oocyte-specific, meaning that SCNT can be done also using zygotes and fertilized eggs [7], and that the molecules responsible for reprogramming were present in the cell cytoplasm. In general, nuclear transfer (see Figure 1) involves two steps: a) de-differentiation of a somatic donor cell to an embryonic state and the *in vitro* maturation till the blastocyst stage (also known as therapeutic cloning); b) the further development of the cloned blastocyst, after the implantation in the maternal uterus (reproductive cloning).

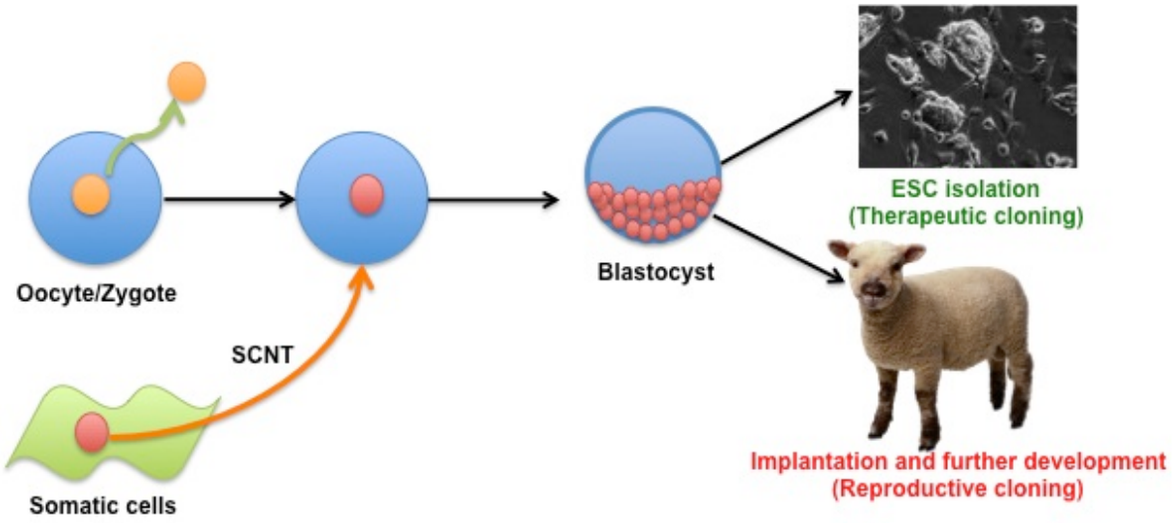

Figure 1. SCNT, Therapeutic and Reproductive cloning

Therapeutic cloning permits the derivation of nuclear transfer derived embryonic stem cells (ntESCs). Recently, the efficiency of isolation of ntESCs drastically increased, at least in mice, from 1% to 20% [8]. It has also been possible to derive similar cells in cats, dogs, wolves, goats and monkeys. Although the isolation of human ntESCs has been reported, this paper has been retracted later on [9]. Thus, the possibility of therapeutic cloning with human cells needs to still be demonstrated. However, the therapeutic cloning remains a promising technology for regenerative medicine, considering that ntESCs, from other species, were able to differentiate into all the cell types of an adult body.

Reproductive cloning is technically more difficult than therapeutic cloning, as it involves the further development *in vivo* in a pseudopregnant female. Embryos, derived after SCNT,

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develop till blastocyst stage with a good efficiency (20-50% depending on the species), but most of them die in the post-implantation stage, without reaching birth (1-5% of survival rate to birth, depending on the species).

Analysis of the cloned animals also showed several abnormalities: increased telomere shortening (which may have caused the premature death of Dolly), altered gene expression during development, prolonged gestation, fetal or placental edema, increased risk of obesity and cancer. The reasons for these pathologies remain not fully understood. The defects may be due to infidelity of the reprogramming: residual epigenetic memory of the donor cell may be present an/or imprinting of important developmental genes may be altered. Nevertheless, reproductive cloning remains attractive and may have potential implications in agriculture and industrial biotechnology. However, as it relates to humans, cloning (also therapeutic) remains controversial as theoretically, it may allow the cloning of a human being.

Evidence that differentiation is reversible also comes from another technique, known as cell fusion [10]. In cell fusion experiments, two or more cells can be fused together (by using polyethylene glycol (PEG) or electrofusion) to generate a single cell, called heterokaryon or hybrid. The larger or more dividing cell type is the "dominant" one, and the "recessive" cell will convert its gene expression profile to the one imposed by the dominant cell type. Obviously, alteration in the ratio of the two cell types during fusion will affect the final fate of the fused cells [11].

A heterokaryon, produced by inhibiting cell division, is a fused cell that becomes multinucleated and survives only short-term. If the cell cycle is not blocked, the fused cells will form a hybrid, because upon the first division the two different nuclei will become a single nucleus, having 4n chromosomes (see Figure 2). Its karyotype can be: 1) euploid, when fused cells are from same species (the two cell types have the same number of chromosomes, thus, their segregation will be balanced); 2) aneuploid, when cells fused are from different species (the two cell types have a different number of chromosomes, thus, they will be lost and/or rearranged).

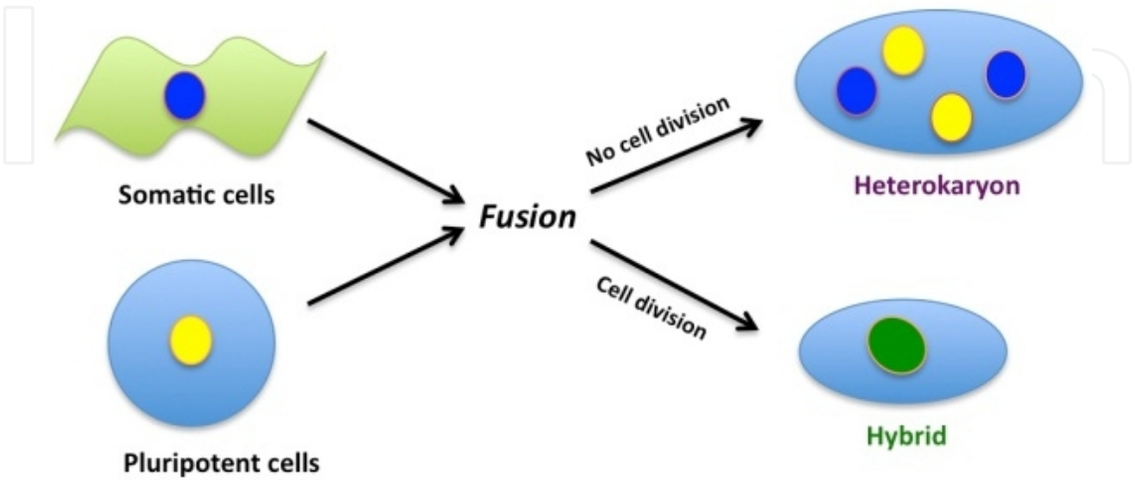

Figure 2. Cell fusion

Cell fusion experiments advanced medical knowledge on cell plasticity. In 1969, Harris et al. fused tumor cells with normal cells and demonstrated that there are trans-acting oncosuppressor genes. Upon fusion, malignancy was suppressed and this was not due to the loss of an oncogene, as after prolonged *in vitro* culture malignancy re-emerged [12]. Fibroblasts can be induced to produce albumin or melanin if fused with hepatocytes [13, 14] or melanocytes [15, 16], respectively.

In 1983, Blau et al. [17] produced for the first time heterokaryons from diploid human amniocytes fused with differentiated mouse muscle cells. She demonstrated that the heterokaryons express many human muscle-related genes and that this activation was mediated by factors present in the cytoplasm (as non-dividing heterokaryons have distinct nuclei). Similar heterokaryons with muscle cells can be produced not only by fusing them with amniocytes but also with cells of the three embryonic lineages (mesoderm, ectoderm, endoderm) [18].

In 1997, Surani, Tada and colleagues demonstrated, by producing proliferative hybrids that cell fusion not only "switches" the fate of different cell types but also "reprograms" them to a pluripotent state. Thymocytes from adult mice were fused to embryonic germ cells, pluripotent stem cells derived from primordial germ cells (PGCs). By using DNA sensitive restriction enzymes, they demonstrated that the genome of the somatic cell underwent a general demethylation, with reactivation of imprinted and non-imprinted genes, resembling the reprogramming events occurring in germ cell development [19].

They also fused female thymocytes, derived from Oct4-GFP mice, with mouse ESCs [17]. Two days after fusion, expression of GFP, from the thymocyte, was detected. The X chromosome, normally silenced in adult female cells, was reactivated. Moreover, hybrids had developmental potential, like ESCs, as they contributed to the three germ layers of chimeric animals, upon blastocyst aggregation [18]. Using the same approach, in 2005, Cowan succeeded in creating hybrids between human somatic cells and human ESCs [19].

This further elucidated that the differentiation state of cells is plastic and reversible; both SCNT and cell fusion experiments clearly demonstrated that it is possible to reset the epigenetic landscape of somatic cells. Despite all these studies were already present in the literature, the field of reprogramming only became jumpstarted in 2006 when Takahashi and Yamanaka [20] demonstrated that the overexpression of pluripotency-related transcription factors (TFs) can dedifferentiate adult fibroblasts to induced pluripotent stem cells (iPSCs), iPSCs strongly resemble ESCs. iPSC technology is an inefficient process, but differently from SCNT or cell fusion, may have in the near future therapeutic applications, including human disease modeling, drug screening and patient-specific cell therapy (see Figure 3).

After this publication [20], several studies demonstrated the potential of epigenetic reprogramming. Indeed, there is now evidence that use of different "cocktails" of TFs allows not only to redirect fibroblasts to an ESC-fate but also to a lineage-specific cell types/precursors, like cardiomyocytes, neuronal precursors, hepatocytes and blood cells, from a tissue different than the tissue from which the somatic cell was isolated [21-23].

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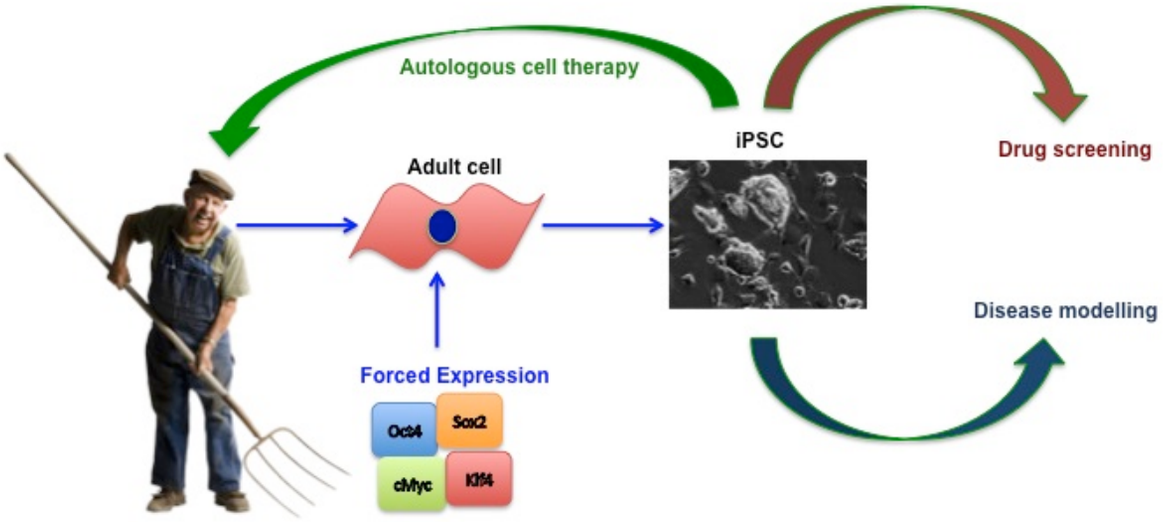

Figure 3. iPSC technology and applications

Finally, It has also become clear that cell plasticity and reprogramming may be partially achieved or enhanced by the culture microenvironment. An increasing number of studies is showing how small molecules, including epigenetic modifiers and signaling pathway modulators, play a crucial role in cell-fate determination [24]. All together, these studies highlight that culture media influences the epigenetic-state of the cells in which they are cultured and thus their features [25]. In this chapter, we will discuss:

1. *Reprogramming to the pluripotency-state*, describing transcription factors used in mouse and human, different methodologies and potentiality of iPSC technolology
2. *Lineage conversion*, illustrating the differentiated cell types/precursors obtained and the differences of this approach with the iPSC technology
3. *Culture mediated reprogramming*, providing the published data which highlight the influence of culture media and small molecules on stem cells fate and features

2. Reprogramming to the pluripotency-state

In 1987, two key discoveries highlighted how crucial the role is of some "master" TFs in cell fate determination. During *Drosophila Melanogaster* development, the gene *Antennopedia (Antp)* specifies the formation of the thoracic segment, which will then form the legs of the adult fly. Heat-induced overexpression of *Atnp,* at specific larval stage, led to the formation of additional legs instead of antennae [26]. Similarly, ectopic expression of *eyeless* (the homologous of *Pax6* in mice) caused the development of functional eyes on the wings, antennae and legs of drosophila [27].

Twenty years after these pivotal experiments, Shinya Yamanaka's group used the same TF-based technology to reprogram adult fibroblasts to pluripotent state [20]. Mouse embryonic

and adult fibroblasts, transduced with retroviral vectors encoding for Oct4, Sox2, Klf4 and c-Myc and cultured in ESC-medium, erased their differentiated epigenetic state and reestablished the pluripotent state; these cells were named induced pluripotent stem cells (iPSCs). Murine iPSCs exhibited morphological and growth properties of ESCs, and expressed alkaline phosphatase and SSEA1. No differences, if compared with ESCs, could be detected in their methylation status, X activation status, embryoid body (EB) formation, *in vitro* differentiation capacity (ectodermal, mesodermal and endodermal), teratoma formation and *in vivo* developmental potential (contribution to the three germ layers of chimeric animals).

Noteworthy, pluripotent stem cells (PSCs) possess mechanisms that lead to the silencing of the integrated transgenes. Therefore, the expression of the four TFs is necessary for generating iPSCs but dispensable for maintenance of the iPSC fate; hence, pluripotency and selfrenewal capacity rely on the trans-activation of the endogenous genes, suggesting a true and complete reprogramming. Mouse iPSCs, like ESCs, were germline competent [28] and supported the development of a mice in tetraploid complementation assay [29]. In this assay, embryonic cells at the two cell-stage are fused together. This results in a tetraploid blastocyst in which just the extraembryonic tissues will further develop; by complementing the tetraploid embryo with normal diploid PSCs, it is possible to generate an individual, completely derived from the diploid PSCs. Interestingly, the same combination of TFs [30] or a somewhat different one (Oct4, Sox2, Nanog and Lin28) can be used for the reprogramming of human cells [31].

This discovery is groundbreaking because with iPSC technology, PSCs can now be induced/derived for autologous cell transplantation, avoiding immunological problems and ethical issues related to the use of human ESCs. In addition, iPSCs from patients carrying a disease can be derived and used to better understand the biological problem leading to the disease as well as for drug-screening.

2.1. Rationale behind iPSCs

The rationale for the selection of the genes for this "reprogramming" cocktail was obviously based on the studies, done in the preceding decade, aimed at understanding the network of TFs responsible for ESC pluripotency and selfrenewal.

The Oct4 (also known as Pou5f1) gene encodes for a TF that belongs to the POU homeodomain DNA binding domain family [32]. It plays a key role in the development and in maintenance of both ESCs self-renewal and pluripotency. Misregulation of its levels triggers loss of the ESC fate; a 50% loss of expression drives ESCs to trophectoderm while a 50% greater expression induces primitive endoderm or mesoderm [33]. Knockout (KO) experiments in mouse demonstrated lethality at the preimplantation stage *in vivo* and failure of ESC derivation *in vitro* [34].

Sox2 belongs to a family of TFs, having the high mobility group (HMG) DNA-binding domain, identified for the first time in the SRY (sex determining Y region) protein, which is the testis determining factor. In general, the genes from the Sox family are involved in different and crucial steps of mammalian development [35, 36]. Sox2 KO embryos die immediately after

implantation [37]. Moreover, it is well known that Oct4 and Sox2 form a complex together, which regulates synergistically the trascription of among others, Fbx15, Fgf4 and Utf1 [38-41].

Nanog, (from Tír na nÓg, the Land of Ever-Young in the old Irish mythology) is another homeobox TF essential for pluripotency. Nanog expression can be detected in the late morula, in the ICM of the blastocyst and in the epiblast. Nanog knockout mice are lethal and the ICM fails to further progress to the epiblast-stage [42-44]. In contrast to Oct4, Nanog overexpression maintains ESC selferewal and pluripotency in a feeder-free and LIF-independent way.

Lin28 is a negative regulator of micro (mi)RNA processing. It blocks the posttranscriptional processing of several primary miRNA transcripts (pri-miRNAs). It is responsible for miRNA biogenesis in both cancer cells and ESCs; so it plays a key role in tumorigenesis and development. Lin28 KO mice have decreased weight at birth and increased postnatal lethality [45].

Different from the above genes, Klf4 and c-Myc are not ESC-specific but are required for their direct or indirect effect on cell proliferation. Of note, iPSCs can also be produced without c-Myc and this is clinically relevant, considering the oncogenic features of c-Myc [46].

Although the combination of Oct4, Sox2, Klf4 and c-Myc (OSKM) consistently allows the reprogramming, this it is not an efficient process (0.01-0.1%). For this reason, subsequent studies were focused on improving iPSC efficiency and since the first iPSC publication, many papers have reported several other genes which enhance the efficiency of iPSC generation.

Inclusion of Utf1, another TF involved in ESC pluripotency, together with the inhibition of p53, increases iPSC generation by 200-fold [47]. Similarly, other factors (like the SV40 large T antigen, SV40LT; the telomerase reverse transcriptase, TERT) and microRNAs (miRNAs) controlling cell proliferation, senescence and apoptosis also affects the efficiency and the speed of reprogramming [48-52]. Other studies have reported important roles for Sall4 [53], Esrrb (which can replace Klf4] [54] and Tbx3 (which improves the germline contribution) [55].

2.2. The donor cell type and epigenetic memory

The starting cell, used for reprogramming, is a key parameter that influences the kinetics, the efficiency and the quality of the iPSCs. Fibroblasts are the most commonly used somatic cells because they can be easily isolated. In mouse studies, embryonic fibroblasts (MEFs) are commonly used as iPSCs can be generated in 10-12 days; MEF-derived iPSC generation is therefore recommended for studies aimed at understanding the mechanisms underlying iPSC generation, as well as the TFs and the chemicals that may enhance this process.

To generate iPSCs from human foreskin fibroblasts (HFFs), three weeks are required and the efficiency is 100 fold less compared with human primary keratinocytes, in which reprogramming also occurs faster [56]. When using $CD133^+$ cord blood cells, iPSCs can be produced by overexpression of only Oct4 and Sox2. As cord blood banks exist, it is believed that this cell source may be useful to make an iPSC bank representing a wide panel of haplotypes for regenerative medicine [57].

Another crucial parameter is the differentiation status; Hematopoietic stem cells and progenitors have a higher efficiency of reprogramming than terminally differentiated B- and T-

lymphocytes [58]. Similarly, $Sox2^+$ neural progenitor cells form iPSCs just by forced overexpression of Oct4 [59]. Many other cell types, such as adipose stem cells [60], dental pulp cells [61], oral mucosa cells [62] and peripheral blood cells [63] can also be used to generate iPSCs.

iPSCs from different origins have a similar, if not identical, gene expression profile in their pluripotent state. However, it has become clear that some genomic regions are differentially methylated [64]; they retain an epigenetic memory of the cell of origin and this is reflected in their differentiation capacity. For example, iPSCs generated from blood poorly differentiated into neuronal cells but had a higher capacity to differentiate into hematopoietic cells [65].

The cell of origin to be used for iPSC generation, also has impact on safety issues; iPSC lines, generated from tail tip fibroblasts, have shown a higher propensity to form teratomas than lines obtained from stomach, hepatocyte or MEF, due to the persistence of undifferentiated cells even after iPSC differentiation [66].

2.3. Methods for iPSC generation

The method of transgene delivery is a crucial factor in determining the efficiency but also the clinical relevance of iPSCs. Although initial reports were based on retroviral vectors, later publications described several other methods, which allow the generation of iPSCs. They can be divided into two main groups: integrative and non-integrative methods [67, 68]. Integrative methods are in general more efficient but they are less safe than the non-integrative methods, which are, however, inefficient.

When choosing the strategy of reprogramming, it is important to consider the aim of the study; integrative methods, the most efficient ones, should be used for elucidating mechanisms underlying iPSC generation, and TFs and chemicals that may enhance this process, while, non-integrative methods will be required to generate clinical-grade iPSCs.

2.3.1. Integrative method

2.3.1.1. Viral vector-based delivery

Mouse and human iPSCs were initially produced by transduction of Moloney murine leukemia virus (MMLV)-derived retroviral vectors. Vectors, based on this system, allow cargoes of up to 8Kb fragments, can efficiently infect (although only dividing cells) and are generally silenced in pluripotent cells [69].

Lentiviral vectors, derived from HIV, have also been used. Differently from the retroviral vectors, the latter have a higher cloning capacity (up to 10Kb of DNA) and can infect both dividing and non-dividing cells. However, transgenes introduced using lentiviral vectors are less-silenced and this can result in a more laborious identification of bona fide iPSC clones (having the transgenes silenced). The lentiviral vector system allowed the Tet-inducible expression of the transgenes in a tightly controlled way [70]. Subsequently, polycistronic lentiviral vectors, having the OSKM cDNA under the control of a unique promoter were used

[71]. This was possible by including in between the different cDNAs, the 2A self-deleting peptide. This permits the continuous translation of downstream cDNA after the release of the previous protein [72]. In general, viral vector-based methods are quite efficient and reproducible (>0.1% in mouse cells, <0.01 in human cells). However, clones generated by viral delivery are not clinically safe. The transgenes may be reactivated during iPSC differentiation; moreover long terminal repeats (LTR) may activate proto-oncogenes increasing their tumorigenicity [28, 73].

2.3.1.2. Transposon delivery

Another possible strategy for iPSC generation is the transient delivery of OSKM by Piggyback (PB) transposon [74]. This system consists of a donor vector, containing the cassette (OSKM), flanked by a 5′ and a 3′ inverted repeat, and a helper plasmid, expressing the PB transposase. When cotransfected, the cassette of the donor plasmid is pasted into the TTAA sequences present in the genome, but can be remobilized after the reprogramming [75, 76]. The PB transposon-mediated generation of iPSCs occurs with high efficiency and, among the integrative methods, this is the only one that allows a precise deletion of the cassette. However, alterations at the integration sites were found; therefore, sequences at the integrations sites must be verified.

2.3.2. Non-integrative method

2.3.2.1. Viral vector-based delivery

Adenoviral vectors do not integrate into the host genome and can, thus, be used for making iPSCs [77, 78]. Adenoviral vectors can carry up to 36kB and can infect both dividing and non-dividing cells. However, the efficiency of iPSC generation is extremely low (0.002 to 0.0001%), probably because the premature dilution of adenoviral vectors during cell replication.

A more efficient alternative has been reported by Fusaki and colleagues [79], using F-deficient Sendai viral vectors. Sendai viruses are minus strand RNA virus, which replicate their genome in the host cytoplasm. Because these viral vectors replicate ubiquitously, their efficiency of reprogramming is similar to retroviral vectors. However, to obtain viral vector-free iPSCs, elimination of the vector using temperature sensitive mutant or antiviral compounds is required.

2.3.2.2. Episomal and minicircle vectors

OriP/Epstain-Barr nuclear antigen-1-based (OriP/EBNA1) vectors can be transfected into host cells and maintained stably episomically (because they replicate during cell divisions through their oriP element) using a drug in the culture medium [80]. Yu and colleagues [81] used the combination of three OriP/EBNA1 vectors (having a combination of 10 reprogramming factors) to generate iPSCs from HFF. By removing the drug selection, episomal vectors are eliminated from proliferating iPSCs.

Another alternative are the minicircle vectors, that differently from the above vectors, are non-replicative [82]. These vectors have a better transfection efficiency than OriP/EBNA1, due to their reduced length (they lack the bacterial origin of replication and the antibiotic resistance gene). However, both strategies have a three-fold lower efficiency (<0.001%) than retroviral vector-based reprogramming.

2.3.2.3. Protein/RNA based delivery

Previous studies have shown that proteins can be directly delivered into cells by fusing them with peptides [83], which mediates their transduction, such as poly-arginine or the HIV transactivator of transcription (TAT). Zhou et al. [84] produced recombinant OSKM proteins fused with poly-arginine in *Escherichia coli* and generated iPSCs from Oct4-GFP MEF, including valproic acid (a histone deacetylase inhibitor) in the medium. Kim et al. [85], succeeded in reprogramming human neonatal fibroblasts by producing OSKM, fused to a Myc tag and nine arginines.

Similarly, *in vitro* transcribed ssRNA, modified by phosphatase treatment and by substituting cytidine and uridine for 5-methylcytidine and pseudouridine, can be delivered into different human cells [86]. This method also requires a recombinant B18R protein, which improves cell viability and protein stability. Differently from protein delivery, the latter strategy has a fast kinetics and a higher efficiency [0.01-0.1%, depending on the cell type). However, also in this case, a careful screening for mutations in different iPSC clones will be needed before an eventual clinical application.

Type of vector	Method	Genomic Integration	Efficiency reported
Viral	Retrovirus	+	4X
Viral	Lentivirus	+	3X
Viral	Adenovirus	-	1X
Viral	Sendai Virus	-	4X
DNA	Transposon	-	2X
DNA	Minicircle	-	X
DNA	Episomal plasmid	-	X
RNA	Recombinant RNA	-	3X
PROTEIN	Recombinant protein	-	X

4x= >0.1%; 3X= <0.1%; 2X= <0.01%;1X= <0.001%

Table 1. Comparison of different strategies for iPSC generation

2.4. iPSC technology, unsolved questions and emerging technologies

iPSCs were reproducibly derived from most, if not all, somatic tissues; however the efficiency reported is always less than 1%. It is the consensus of scientific community, that many more than 1% of the transfected/transduced cells start the reprogramming process. Using a live cell imaging approach, it was demonstrated [87] that almost all the transduced cells undergo symmetric divisions within 48 hours, retaining a fibroblast-morphology. At later stages, reprogramming cells undergo asymmetric divisions: one descendant becomes an iPSC while the other one undegoes cell death. Still unknown, stochastic and clonal events appear to control this process at later stages; in fact most of the cells do not complete the initiated process. Several studies have demonstrated key roles for demethylation [88], telomerase length [89] and mesenchymal to epithelial transition [90], during the reprogramming. A better understanding, especially of the later stochastic mechanisms, is still needed to fully understand and improve the efficiency of iPSC technology [91].

Another important question has been whether or not ESCs and iPSCs are similar and if not, whether differences are functionally important for their application. Conclusions from different studies are conflicting. Several papers have reported that there are remarkable differences in gene expression and DNA methylation [92, 93], while other studies, which included a large number of ESC and iPSC lines, concluded that it is quite difficult to distinguish between ESCs and iPSCs [94-96]. Considering that there are differences among different ESC lines [97], it is now believed that iPSCs clones have a higher variability than ESCs but that at least some iPSC clones are indistinguishable from ESCs.

Furthermore, the recent work of Young and colleagues [98] demonstrated that most of the genetic variability in between different iPSC clones is already present in the starting cell line and is thus not caused by the reprogramming process.

Interestingly, in the last five years, several studies have clearly demonstrated the potential of iPSC technology in regenerative medicine. Hanna et al [99] generated iPSCs from a humanized model of sickle cell anemia. After correcting the hemoglobin gene, by gene targeting, iPSCs were able to generate hematopoietic stem cells and to rescue the disease. Similarly, the potential of iPSCs for cell therapy was demonstrated for macular degeneration [100], Parkinson's disease [101], platelet deficiencies [102] and spinal cord injury [103, 104].

iPSCs derived from patients with specific diseases have been used for studying the mechanisms involved in these diseases and for drug screening [105, 106]. *In vitro* disease modeling is not only possible for monogenic disease but also for more complex polygenic diseases, having a late onset, like schizophrenia [107] and Alzheimer [108, 109].

As a result of the success with reprogramming of somatic cells to a pluripotency-state, lineage reprogramming (trans-differentiation) between different somatic cell types has also become a burgeoning field of research (see next section).

In conclusion, iPSC technology will, in the near future, have a drastic impact on science, regenerative medicine and business. Precise selection of "clean" clones, through the evaluation of their genomic and epigenetic integrity, as well as their gene expression profile, will be crucial

for downstream applications. Despite these remaining hurdles, it is believed that clinical applications for iPSCs are not far off.

3. Lineage conversion

The discovery of iPSCs, together with previous experiments involving SCNT and cell fusion, clearly showed that differentiation is a reversible process and that cells are more 'plastic' than previously believed. Therefore, a new field, called lineage reprogramming, emerged rapidly in the last five years. Recent attempts have demonstrated that, the forced overexpression of TFs can also convert one cell type to another of the same or of other somatic germ layers. Lineage reprogramming depends on the capacity of certain TFs to overcome the existing epigenetic barriers and to rapidly initiate the new identity-specific gene network [110-113].

Examples of direct lineage conversion were described already in 1986; Davis, Lassar et al. [114, 115] converted different fibroblast lines into myogenic cells by overexpression of MyoD, a basic helix-loop-helix transcription factor, in combination with the demethylating agent, 5-azacytidine. Subsequent studies confirmed that myogenic conversion, as shown by presence of desmin and myosin heavy chain, could be achieved *in vitro* starting from a variety of cell types (adipose, melanoma, neuroblastoma and liver cell lines). However, the complete downregulation of the 'original' tissue-specific genes was only seen when starting with mesodermal cells and not with endodermal or ectodermal cell lines [116, 117].

A similar transdifferantiation was also seen in the blood system. The deletion of Pax5 in pro-B cells resulted in their switch into the T-cell lineage [118, 119]. Later on, the same group investigated this transdifferentiation more extensively; mature B cells were isolated from Pax5 knockout mice and transplanted back into a lymphocyte deficient recipient. Surprisingly, they could detect in the reconstituted mice donor pro B cells, which then gradually converted into T cells [120]. This demonstrated that lost of Pax5 led to a T cell phenotype through de-differentiation rather than direct transdifferentiation. Another example of direct conversion came from the work done by Graf and colleagues [121]; overexpression of C/EBPα *or* C/EBPβ, a basic leucine zipper TF binding CCAAT enhancers, induced a macrophage phenotype (as shown by Mac1 expression) in bone marrow or spleen-derived B cells. In the induced macrophages almost all the B cell genes analyzed were downregulated and cells acquired phagocytic function *in vitro*.

The above examples describe experimental conversions but there were also cases in which this conversion occurs naturally. Jarriault et al. [122], demonstrated that the epithelial rectal cell 'Y', migrates anterodorsally from the rectum to become a 'PDA' motor neuron. This conversion from Y to PDA is not direct but occurs through a de-differentiation state, in which the initial (Y-cell) and the final (PDA-cell) identity are not present [123]. In this section, we will describe the relevant cell types, recently, obtained by lineage reprogramming.

3.1. Conversion into mesodermal types

Seale and colleagues have recently found that Myf5$^+$ muscle precursors can convert into brown fat cells *in vivo* and *in vitro* [124], while studying the role of PRDM16 during development. Overexpression of PRDM16 differentiated primary mouse myoblasts with nearly 100% efficiency to brown fat. *Vice versa,* downregulation of PRDM16 in primary brown fat cells resulted in the expression of MyoD and Myogenin and in a myotube-like morphology. Interestingly, forced expression PRDM16 was not able to induce the same conversion in non-myogenic cell lines, like fibroblasts. Performing proteomic studies, they identified C/EBPα as a partner of PRDM16 in brown fat. Subsequently, they used combined overexpression of C/EBPα and PRDM16 [125] and demonstrated that mouse and human dermal fibroblasts could differentiate into brown-fat cells, which functional features (fat pad formation and glucose uptake after transplantation into mice).

Human dermal fibroblasts were converted into multipotent blood progenitors by just Oct4 overexpression [126] in combination with treatment with a hematopoietic permissive medium, containing growth factors and cytokines. Oct4 is a key TF for pluripotency but it is not expressed in the hematopoieic system [127]; probably, Oct4, in this case of lineage reprogramming, is mimicking the effect of Oct1 and Oct2, two other members of Pou family of TFs expressed in lymphoid development [128]. The induced progenitors express CD45 and express adult globin protein (unlike hematopoietic cells derived from human ESCs and iPSCs). Multipotent blood progenitors have myeloid, erythroid and megakaryocytic but not lymphoid potential, as shown by transplantation experiments.

The forced overexpression of TFs involved in cardiac development (Tbx5, Mef2c and Gata4) converts mouse cardiac and dermal fibroblasts into cardiomyocyte-like cells, termed induced cardiomyocyte (iCMs) [129]. Around 20% of the cells appear to be 'converted' in three days as measured by the expression of alpha-myosin heavy chain (αMHC), although one month is required for their complete maturation, which resulted in spontaneous beating capacity. Transplantation of iCMs, the day after the viral transduction, in injured hearts results in their engraftment and differentiation *in vivo*. Interestingly, the same strategy is able to convert cardiac fibroblasts *in vivo* [130]. When retroviral vectors, carrying the above factors, are injected after myocardial infarction, this results in the efficient conversion (>50%) of cardiac fibroblasts into functionally beating cardiomyocytes. Efficiency of cardiac-conversion is increased when Hand2 is added to the above cocktail of genes [131].

3.2. Conversion into endodermal types

The lineage reprogramming into β-cells is of particular interest, considering the potential for the treatment of diabetes. Zhou et al., [132] were able to *in vivo* convert exocrine acinar cells into functional β-like cells, combining three genes essential for pancreatic development (Ngn3, Pdx1 and MafA). Adenoviral vectors, carrying the pancreatic cocktail, were injected; again, conversion occurred in three day and efficiency of conversion was relatively high (20%). Analysis, one month later, showed that induced β-like cells produced insulin and rescued the hyperglycemic level after streptozotocin-treatment. However, the same combination of factors

failed to reprogram myocytes *in vivo* and mouse embryonic fibroblast *in vitro*, indicating that additional factors will be needed to achieve this conversion from unrelated cell types.

More recently, mouse fibroblasts were converted into hepatocyte-like cells by overexpressing Hnf4α, FoxA1, FoxA2 and FoxA3 [133] or by Gata4, Hnf1α, Foxa3 together with $p19^{Arf}$ inactivation [134]. The reprogrammed cells were termed induced hepatocytes (iHeps) and had a gene expression profile and features (albumin production and cytochrome P450 activity), which closely resemble mature hepatocyte. iHeps *in vivo* were able to reconstitute hepatic tissues and to support hepatic function in the fumoaryl-acetate hydrolase ($FAH^{-/-}$) deficient mice.

3.3. Conversion into neuronal types

The conversion into neuronal types is, probably, the one that received more attention in the field of lineage reprogramming. The increasing attention is due to their possible application for the treatment of diseases involving the nervous system.

In 2010, Vierbuchen et al. [135] were the first to describe how overexpression of Ascl1, Mytl1 and Bm2 (also known as Pou3f2, again a member of Pou family) can convert embryonic and tail-tip fibroblasts into a mixed populations of induced neurons (iNs). iNs generate functional synapses with mouse cortical neurons and have action potentials; the detailed electrophysiological analysis showed that iNs contains mainly cells with features of glutamatergic neurons (with just a small percentage of GABAergic neurons). Remarkably, the addition of NeuroD1 to the above set of genes was necessary to achieve the same conversion in human cells [136]. The enriched cocktail of factors was able not only convert fibroblasts but also mouse hepatocyte into iNs [137].

Several groups, differently, converted fibroblasts into induced neural stem cells (iNSCs), that differently from the previous examples, can still self-renew and differentiate into different neuronal subtypes (multipotent). Different cocktail of factors and inductive media have been used to obtain multipotent neuronal stem cells from human and mouse fibroblasts: the group of Scholer [138] used Sox2, Klf4, c-Myc, together with Tcf3 and Brn4 (also known as Pou3f4); our group [139] by adding Zic3 to Oct4, Sox2 and Klf4; Ring et al. [140], by just overexpressing Sox2.

Different laboratories focused on a more direct conversion into specific neuronal subtypes, with a particular interest on neuronal cell types affected in neurodegenerative diseases. Two groups have been able to convert mouse and human fibroblasts into induced Dopaminergic Neurons (iDAs), the subtype affected in Parkinson's disease. The first laboratory [141] achieved this by adding FoxA2 and Lmx1a to Ascl1, Mytl1 and Bm2; the second [142] by overexpressing Ascl1, Lmx1a and Nurr1 (also known as Nr4a2). iDA cells, upon transplantation in mice, were capable to integrate into the host neuronal circuitry and express markers typical for mature dopaminergic neurons.

Lineage conversion was also achieved into spinal motor neurons, the subtypes involved in amyotrophic lateral sclerosis and spinal muscular atrophy. Conversion into induced Motor Neurons (iMNs) was achieved for both mouse and human fibroblasts; mouse embryonic

fibroblasts were converted with Ascl1, Brn2, Mytl1, Lhx3, Ngn2, Isl1 and Hb9 whereas human cells also required NeuroD1 [143]. iMNs displayed markers, electrophysiological features and gene expression profile, which strongly resemble motor neurons. Moreover, iMNs engrafted into the developing chick spinal cord, forming axonal and dentritic projections toward the adjacent musculature.

Of note, Qiang et al [144] demonstrated that lineage reprogramming is also useful for drug screening and disease modeling. iNs, again with glutamatergic features, were induced by overexpressing Ascl1, Bm2, Mytl1 together with Zic1 and Olig2. iNs were produced from both healthy donors and Alzheimer's patients. iNs produced from patients displayed the typical accumulation of beta amyloid peptides (Aβ40 and Aβ42). Combining lineage reprogramming with gene-targeting technology, similar cells could also be used for autologous transplantation.

Starting cell type	Conversion into	Factors
(m) B-Cells	Macrophage-like cells	C/EBPα or β, PU.1 (121)
(m/h) dermal fibroblasts, myoblasts	Brown-fat cells	C/EBPα and PRDM16 (125)
(m) embryonic fibroblasts	Myoblasts	MyoD (114, 115)
(h) dermal fibroblasts	Multipotent blood progenitors	Oct4 (126)
(m) cardiac and tail tip fibroblasts	Cardiomyocytes	Tbx5, Mef2c, Gata4 ± Hand2 (129) (131)
(m) embryonic fibroblasts	Cardiomyocytes	Oct4, Sox2, Klf4 and cMyc (145)
(m) exocrine cells	β-like cells	Ngn3, Pdx1 and MafA (132)
(m) embryonic and dermal fibroblasts	Hepatocyte-like cells	Hnf4α, FoxA1, FoxA2 and FoxA3 (133)
(m) embryonic and tail tip fibroblasts	Hepatocyte-like cells	Gata4, Hnf1α, Foxa3 and $p19^{Arf}$ KD (134)
(m) embryonic fibroblasts	Neural progenitor cells	Oct4, Sox2, Klf4 and cMyc (146)
(m/h) fibroblasts	Neural progenitor cells	Sox2, Klf4, c-Myc,Tcf3 and Brn4 (138)
(h) fibroblasts	Neural progenitor cells	Oct4, Sox2, Klf4 and Zic3 (139)
(m) embryonic and (h) fetal fibroblasts	Neural progenitor cells	Sox2 (140)
(m) embryonic and (h) fetal, postnatal and dermal fibroblasts	Neurons	Ascl1, Mytl1, Bm2 and NeuroD1 (135) (136)
(m) tail and (h) embryonic fibroblasts	Dopaminergic neurons	Ascl1, Lmx1a and Nurr1 (142)
(h) embryonic and fetal lung fibroblasts	Dopaminergic neurons	Ascl1, Mytl1, Bm2, Lmx1a and FoxA2 (141)
(h) skin fibroblasts	Glutamatergic neurons	Ascl1, Bm2, Mytl1, Zic1 and Olig2 (144)
(m/h) embryonic fibroblasts	Motor neurons	Ascl1, Brn2, Mytl1, Lhx3, Ngn2, Isl1, Hb9 and NeuroD1 (143)

Table 2. Examples of lineage conversion. (m)= mouse (h) = human

3.4. Direct versus indirect strategy

Most of the examples, given in the previous section, describe the direct conversion from one cell type to another, in which the reprogramming is achieved without any intermediate state. However, other reports clearly demonstrated the possibility to achieve similar results, by using an alternative strategy, in which lineage conversion is indirect. Indirect conversion is achieved passing through a limited de-differentiation state by overexpressing Yamanaka factors for a shorter time. Like for the direct conversion, the indirect conversion is strongly dependent on the specific culture medium (growth factors and cytokines) given during the reprogramming phase.

The laboratory of Sheng Ding, at the Gladstone Institute of San Francisco, was the first to describe the possibility of lineage reprogramming through an indirect strategy. Short temporal overexpression of the Yamanaka factors induced a partial dedifferentiatied state, that allowed the subsequent conversion into cardiomyocytes-like cells by applying extracellular factors [145]. OSKM factors were overexpressed for six days in a medium free of signals necessary for pluripotency (i.e. leukemia inhibitory factor). After this short priming phase, cells were then cultured in media promoting cardiogenesis, i.e. cointaining BMP4. Three day after the cardiac induction, the expression of Nkx2.5, Gata4 and Flk1 (mid-stage markers of cardiac developments) could be detected. The further development into more mature cardiomyocytes, showing sarcomeric structures and cardiac features (expression of cardiac markers and cell-cell interaction) required at least two more days.

Interestingly, the authors also demonstrated that this indirect lineage conversion does not pass through a pluripotency-state, i.e. ESC/iPSC culture media in the induction phase drastically decrease the efficiency of conversion; *vice versa*, the addiction of a Jak inhibitor (which blocks the most important pluripotency-pathway) increased the efficiency of the process. The same group also demonstrated that a similar strategy induced expandable Neural Progenitor Cells (NPCs), having multipotent potential [146].

Both direct and indirect lineage conversions have pros and cons. The direct conversion, as in case of SCNT or cell fusions, occurs in hours-days. Induced cells are unipotent, are produced with a high efficiency, without the requirement of cell proliferation and with a lower risk for teratoma. The indirect strategy requires days-weeks and produces cells, which can be unipotent or multipotent. Cells induced by this strategy can be expanded but have a moderate risk for teratoma.

3.5. Mechanisms, differences with iPSC technology and unsolved questions

Reprogramming to the pluripotency-state occurs via a gradual and genome-wide de-differentiation, involving a first phase where epigenetic marks of differentiation are erased and a second phase in which the epigenetic marks of pluripotency are established to initiate the endogenous pluripotency-network. In lineage conversion, specific TFs are able to modulate cell fate in two different ways (direct or indirect), which does not involve a pluripotent-state and is associated with a lower tumor risk, still a major obstacle to achieve clinical applications with ESCs and iPSCs. In the direct conversion, ectopic TFs, involved in cell fate determination

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or maintenance during embryonic development, overcome the pre-existing epigenetic marks and generate a new state. In the indirect conversion, the TFs, which allow the reprogramming to the pluripotency-state, are temporally overerexpressed together with fate-specific signals to convert original cell type into a new state. Differently than iPSC technology, the efficiencies are much higher (even 20 % in some cases) and the kinetics of conversion are rapid (a few day to a week maximum, and not two weeks to a month, like for iPSC, see Table 3).

Strategy	Efficiency	Kinetic	Potentiality	Expandibility	Tumorigenicity	Cell Proliferation
iPSC	Low	Weeks-Months	Pluripotent	Yes	High	Required
Direct conversion	High	Hours-Days	Unipotent	No	Low	Not required
Indirect conversion	High	Days-Weeks	Multi/Unipotent	Yes	Moderate	Required

Table 3. Comparison of different strategy of TF-based Reprogramming

As for iPSCs, many questions still remain unsolved in lineage conversion. It is not clear whether the new cell type, generated upon conversion, is a hybrid between the original and the new cell. It is intriguing that, in direct conversion, TFs erase partially or completely the previous epigenetic marks, without cell divisions (in which chromatin marks are lost) but it is totally unknown how this is possible. Remarkably, in both iPSCs and lineage conversion, efficiencies are lower with human cells, if compared with mouse. It is unknown whether this is due to the intrinsic karyotypic instability of mouse cells in culture or to molecular mechanisms.

4. Culture mediated reprogramming

Reprogramming to the pluripotency-state and lineage conversion are achieved through the forced expression of TFs. However, in the last decade, several reports have highlighted how culture medium per se can be responsible for (partial) reprogramming. Moreover, there is an increasing amout of evidences showing that small molecules, including epigenetic modifiers and signaling pathway inhibitors, enhance the efficiency and kinetics of reprogramming.

Epiblast stem cells (EpiSCs) are isolated from post-implantation embryos between E5.5-E7.5. EpiSCs are the post-implantation equivalent of ESCs; they still express Oct4, Nanog and Sox2 but express lower levels of Stella and Rex1 [147]. ESCs and EpiSCs have also different culture requirements and features. While ESC selfrenewal is LIF dependent, EpiSC proliferation requires bFGF and Activin signaling. EpiSC female lines, but not ESC lines, have one of the X chromosome inactive. Importantly, EpiSCs, differently from ESCs, do not have the ability to contribute to chimeras *in vivo*, when aggregated into recipient morula/blastocysts.

In 2009, Bao et al. [148] demonstrated that established EpiSC lines could de-differentiate/revert into an ESC-like state by culturing EpiSCs in ESC medium (cointaining LIF) for 2-5 weeks. Once 'reverted' cells lost all the features of the original EpiSCs and acquired all ESC-characteristics (X was reactivated, growth was LIF-dependent and cells were capable to

contribute to chimeras). This report showed that the simple manipulation of culture medium can dedifferentiate EpiSCs to a more primitive ESC-state but this is not the only case reported in literature.

In 2004, Kanatsu-Shinohara et al. [149] descibed that mouse germline stem cells (GSCs) isolated from neonatal testis reverted occasionally into cells with ESC-like colonies morphology within 4-7 weeks if cultured in LIF, epidermal growth factor (EGF), glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2). The reverted cells were named multipotent germ stem cells (mGSs); they expressed not only Oct4 (already present in GSCs) but also Nanog and Sox2 at ESC-level. Analysis on mGSs showed the loss of spermatogonial properties (although the erasure of the androgenic imprinting was not complete) and the gain of ESC features (teratoma formation and contribution to chimeras with germline transmission). However, despite their siimilarity to ESCs, mGSs were not able to form offspring, after tetraploid complementation. Unipotent germline stem cells, but this time from adult testis, were converted into germline-derived pluripotent stem cells (gPSs) by Ko and colleagues [150]. Reprogrammed cells, like in the above case, were higly similar to ESCs but again, they could not form live animals in tetraploid complementation assay. The reason for this is most likely the residual persistence of androgenetic imprinting. The possibility to reprogram a germline stem cell into a cell with pluripotent features, even without the capacity of forming chimeric animal, is interesting because it might allow autologous cell therapy without embryo-manipulation. Similar conversions with mouse cells were also described by other laboratories [151, 152].

In 2008, Conrad and colleagues [153], showed that cells derived from human testis can be converted into cells with human ESC-like features. Cells isolated from human testis were cultured in GDNF-containing medium for 4 days and then selected based on the expression of CD49f and further selection on laminin matrix in medium cointaining LIF. 3-4 weeks later colonies with ESC-morphology appeared; human adult GSCs (haGSCs), like human ESCs, expressed SSEA4, TRA 1-60, TRA 1-81 and generated EBs and teratomas. However, a later report [154] questioned the previous finding of Conrad, arguing whether haGSCs really expressed Oct4, Nanog and Sox2; moreover, microarray data comparison further showed that haGSCs are similar to fibroblasts-derived from human testic biopsies but not to hESCs.

These studies strongly suggest that stem/progenitor cells derived from testis can to some extent be converted, by long-term culture, to cells with ESC-like properties, without any reprogramming factors; however, converted cells differ significantly from ESCs. The propensity of GSCs to be converted to ESC-like cells may depend on their Oct4 expression. Although gonads are the only place where Oct4 is functionally expressed in adult healthy-rodents [127], many reports described the isolation of $Oct4^+$ cells from rodents [155-169]. It remains to be determined whether culture mediated reprogramming is responsible for the Oct4 re-activation in such cell lines.

In 2002, our group [170] isolated multipotent adult progenitor cells (MAPCs) from rodent bone marrow (BM), upon prolonged culture at low density in a medium cointaining LIF, PDGF and EGF. Murine MAPCs differentiated *in vitro* into cells of the three germ layers and one murine line was also able to contribute to chimeric mice, although at low efficiency and without

germline trasmission. Subsequently analysis on rodent MAPC lines [171, 172] showed a lineage marker profile (Oct4, Gata4, Gata6, Sox7 and SSEA1) found also in the nascent hypoblast of the blastocyst and in rat blastocyst-derived Extraembryonic Endoderm Precursor cells (XEN-Ps) [173]. Recently, we demonstrated that similar cells are not present in fresh BM but appear after prolonged *in vitro* culture. To ascertain whether the MAPC culture system reprograms BM cells to the equivalent of XEN-P, we, first, showed that rMAPC and XEN-P cells exhibit similar features under reciprocal culture conditions. Second, we reported, using the same MAPC medium, the quick and efficient isolation of new cell lines directly from blastocyst, which we termed Hypoblast Stem Cells (HypoSCs) and which strongly resemble XEN-P in features and developmental potential [174].

Moreover, specific culture media may also be responsible for the broader differentiation potential described for some adult stem cell types [175] and this should be more considered in stem cell research, especially when reaching clinical trials phases [176].

4.1. Small molecules in stem cells and reprogramming

Small molecules are acquiring, on a daily basis, more relevance in the stem cell field because they can control protein functions selectively, reversibly and in a tunable way. Strikingly many reports have also shown how pathway inhibitors and epigenetic modifiers play a crucial role in the reprogramming process [177]. In 2010, the group of Ding reported that human primary somatic cells can be reprogrammed into human iPSCs with only Oct4 and a cocktail of small molecules [178].

4.1.1. Signaling modulators

Mouse (m)ESCs were first isolated more then three decades ago [179, 180]. mESCs have been derived and cultured in LIF and bone morphogenetic protein (BMP, contained in the serum) to inhibit their differentiation [181]. However, the efficiency of mESCs derivation was low in general and almost not possible from some mouse strains (like C57BL/6). More recently, several reports have now demonstrated that mESC culture in MEF or feeder-free are heterogeneous and fluctuates between a pre-implantation ESC and a post-implantation EpiSC-state [182, 183].

Ying and colleagues [184] demonstrated that mESCs can be maintained in an homogenous ground-state without the requirements of external stimuli, provided by growth factors and/or feeders. This achievement was possible by using two signaling modulators that regulate pathways involved in mESC differentiation: *PD0325901*, which blocks the differentiation-inducing signalling from mitogen-activated protein kinase (MEK), inhibiting the phosphorylation of ERK1/2; and *CHIR99021*, which inhibits the glycogen synthase kinase 3 (GSK3) and decreases the phosphorylation of β-Catenin, supporting their growth and further suppressing residual differentiation. The isolation of mESCs, with the two inhibitors (2i), together with LIF, allows now the efficient derivation of ESCs regardless of the mouse strain as well as from rat for the first time [185]. ESC lines cultured in 2i and LIF can be clonally propagated without feeders and support superior chimerism and germline transmission. The two inhibitors have also been used to increase the efficiency of iPSCs generation [178].

Mesenchymal-to-epithelial transition (MET) is a reversible process which drives cells from a multipolar, spindle and motile mesenchymal shape to a planar and polarized epithelial shape. MET is an important process during embryo development but also in reprogramming; i.e. fibroblasts change shape towards an epithelial morphology at the early stage of iPSC generation. TGFβ pathway negatively regulates an epithelial phenotype. The block of TGFβ1-2-3 receptors, using *SB431542*, in combination with PD0325901, enhances both the kinetics and the efficiency of reprogramming, during iPSC generation [186].

Cellular senescence is a pathway that negatively interferes with reprogramming. Expression of OSKM increases oxidative stress and DNA damage, inducing senescence. *Vitamin C* (or ascorbic acid), is an important cofactor for metabolic processes but also has a strong antioxidant effect; i.e. Vitamin C reduces reactive oxidandant species (ROS). In iPSC reprogramming, Vitamin C enhances the conversion from a partial reprogrammed to a fully reprogrammed-state [187], capable of forming completely iPSC-derived mice in tetraploid complementation assay [188].

Stem cells have a different metabolism if compared to differentiated cells [189]. Stem cells have a strong energetic and metabolic demand to meet their self-renewal and to do this, they mainly rely on glycolysis followed by fermentation of lactic acid in the cytosol. Differently, differentiated cells mainly rely on a low rate of glycolysis followed by oxidation of pyruvate in the mitochondria, which results in the production of ROS. Consistent with this, *PS48*, an activator of 3-phosphoinositide dependent protein kinase-1 (PDK1) that activates the PI3/Akt pathway, results in the upregulation of glycolytic genes and strongly facilitates iPSC reprogramming [178].

4.1.2. Epigenetic modifiers

The structure of eukaryotic genome is higly organized; genomic DNA is wrapped around structural proteines, called histones. DNA and histones, together, form the chromatin. Protein complexes are responsible for chromatin modifications. Histones then determine the transcriptional status; i.e. in an open and closed form. In somatic cells, chromatin is mainly in a closed conformation, while in pluripotent cells, chromatin is in an open conformation and it is dynamically associated with chromatin proteins. Obviously, during iPSC generation, the chromatin must change from a somatic to a pluripotent state. Therefore, many small molecules, which modulates chromatin have been described to enhance the efficiency of reprogramming and even to substitute for some of the reprogramming factors.

Pluripotent stem cells have, in general, a more demethylated DNA, in comparison with somatic cells; in fact, *5-azacytidine* [5-aza) and *RG108*, two inhibitors of DNA methyltransferases (DNMTs), are responsible of DNA methylation and methylation maintanance, increases efficiency of reprogramming [190, 191].

G9a is an histone methyltransferase (HMTase), which induce silencing of Oct4, through methylation of H3K9. *BIX-01294*, an inhibitor of G9a, enhances reprogramming [192]. Recently, *parnate*, an inhibitor of LSD1, a H3K4 demethylase, was used to reprogram human somatic cells with only Oct4 and Klf4 [193].

Similarly, *Trichostatin A* [137], suberoylanilide hydroxamic acid [71] and valproic acid (VPA), inhibitors of histone deacetylases (HDACs) also increases efficiencies of reprogramming, even with only Oct4 and Sox2 overexpression. [190, 194].

Small molecules	Category	Effect on
PD0325901	Signaling modulators	MEK inhibitor
CHIR99021	Signaling modulators	WNT/β-Catenin
SB431542	Signaling modulators	MET
Vitamin C	Signaling modulators	Cellular Senescence
PS48	Signaling modulators	Glycolysis
5-aza, RG108	Epigenetic modifiers	DNMT inhibitor
BIX-01294	Epigenetic modifiers	HMTase inhibitor
Parnate	Epigenetic modifiers	LSD1 inhibitor
TSA, SAHA, VPA	Epigenetic modifiers	HDAC inhibitor

Table 4. Small molecules in reprogramming

5. Conclusions

The importance and the impact on society of reprogramming has been recently recognised by the Nobel Assembly at the Karolinka Institute of Stockholm, which co-awarded John Gurdon and Shiniya Yamanaka with the Nobel Price in Medicine 2012. Their outstanding reports demonstrated that cellular fate is plastic and that differentiation is a reversible process. Epigenetic markers imposed by development can be erased through the multiple pathways to reprogramming. This means the epigenetic landscape as described by Waddington should be revised, as balls are capable of rolling back up and over the hill. The SCNT and the forced expression of TFs show that somatic cells can re-acquire all the features, lost upon their differentiation. Adult somatic cells can be redirected to the pluripotency-state or can be converted into cells of another lineage.

Although the precise mechanism via which the phenotype of all these cells can be changed remains to be fully elucidated, the iPSC technology is drastically changing and boosting the stem cell field; it allows one to obtain pluripotent stem cells for autologous therapy, avoiding the problems of immune rejection as well as the ethical issues related to the use of human embryo for scientific purposes. The possibility to also obtain precursors, with restricted differentiation potential, may be another alternative to reach the bedside, as it is likely associated with lower tumorigenicity. It is also clear that culture conditions have such a significant effect on cell fate, not only during reprogramming but also in establishing the potential of different adult stem cells, that this should be kept in mind when comparing studies across laboratories, and definitely when contemplating clinical trials with cultured stem cells.

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Author details

Antonio Lo Nigro* and Catherine M. Verfaillie

*Address all correspondence to: antonio.lonigro@med.kuleuven.be

*Address all correspondence to: catherine.verfaillie@med.kuleuven.be

Interdepartmental Stem Cell Institute Leuven (SCIL) & Department of Development and Regeneration, KU Leuven, Leuven, Belgium

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