Chapter

Non-integrating Methods to Produce Induced Pluripotent Stem Cells for Regenerative Medicine: An Overview

Immacolata Belviso, Veronica Romano, Daria Nurzynska, Clotilde Castaldo and Franca Di Meglio

Abstract

Induced Pluripotent Stem cells (iPSC) are adult somatic cells genetically reprogrammed to an embryonic stem cell-like state. Due to their autologous origin from adult somatic cells, iPSCs are considered a tremendously valuable tool for regenerative medicine, disease modeling, drug discovery and testing. iPSCs were first obtained by introducing specific transcription factors through retroviral transfection. However, cell reprogramming obtained by integrating methods prevent clinical application of iPSC because of potential risk for infection, teratomas and genomic instability. Therefore, several integration-free alternate methods have been developed and tested thus far to overcome safety issues. The present chapter provides an overview and a critical analysis of advantages and disadvantages of non-integrating methods used to generate iPSCs.

Keywords: induced pluripotent stem cells (iPSCs), cell reprogramming, dermal fibroblasts, integration-free methods, regenerative medicine

1. Introduction

The entrance of induced Pluripotent Stem Cells (iPSCs) in the stem cell scene represents a novel approach for studying human diseases and a promising tool for regenerative medicine [1].

The compelling need to overcome ethical and technical issues related to the production and utilization of Embryonic Stem Cells (ESCs) has prompted to search for a method to induce the pluripotency in terminally differentiated cells pushing them to an embryonic-like state.

Several studies, therefore, have been focused on characterizing and isolating unique transcriptional factors expressed by ESCs, presuming that their expression was sufficient to confer to adult cells the peculiar features of pluripotent cells [2]. The hypothesis that genome is not irreversibly modified during the differentiation and that some factors residing in ESCs can confer pluripotency to terminally differentiated nuclei has given a boost to bypass both the practical and ethical concerns

related to the use of ESCs and has paved the way for the development of cutting-edge approaches for tissue regeneration, like cellular reprogramming by artificially inducing the pluripotency [3].

Cell reprogramming consists in converting adult somatic cells in undifferentiated cells defined by an acquired pluripotency, typically showed by ESCs. Many techniques have been developed to achieve the goal since in 2006 Yamanaka and colleagues succeeded in the undertaking challenge of identifying four specific transcription factors (Oct4, SOX2, c-Myc, and KLF4) capable of reprogramming murine or human fibroblasts to embryonic-like cells, and termed them "induced pluripotent stem cells" [4]. The four factors recognized by Yamanaka are involved in multiple mechanisms and are pivotal for the pluripotency of embryonic stem cells, for embryonic development and to determine cell fate [5].

The great potential residing in iPSCs was soon noticeable, primarily for the possibility to obtain stem cell lineages customized for each patient, able to give rise to the needed cell type, then, for the chance to overcome organ shortage difficulties and to avoid invasive medical procedures to treat degenerative diseases [6].

Additionally, iPSCs share several features with ESCs showing similarities for morphology and culturing conditions: they both grow arranging in dome-shaped colonies (**Figure 1**) and need to be cultured in presence of a layer of feeder cells and/or specific cytokines [7]. Furthermore, iPSCs express equal stemness markers showed by ESCs, a common proliferation potential, the capability to self-renew and differentiate into the three fundamental germ layers [8].

It is considerably relevant that iPSCs can also provide effective disease models to investigate cellular and molecular mechanisms involved in the development of pathologies and a platform for toxicological and pharmacological screening [9].

Until the sprawl of cell reprogramming, ESCs were considered the most promising and innovative tool for the research and clinical application in the field of regenerative medicine. Due to their ability to grow indefinitely and to differentiate into cells of the three germ layers while maintaining the pluripotency, ESCs rapidly gained the attention of the scientific community [10]. Despite the tremendous potential they hold for tissue and organ regeneration, at now, the clinical application of ESCs is limited and still faces many obstacles. The use of ESCs, in fact, raises several controversies and the studies focused on the understanding of their biology are strictly regulated or even forbidden in many countries. The reason of such restraint primarily resides in their origin and isolation techniques, as ESCs derive from the inner cell mass of mammalian blastula, the early stage of embryonic development, and common methods for their isolation require the destruction of the embryo, triggering ethical concerns [11].

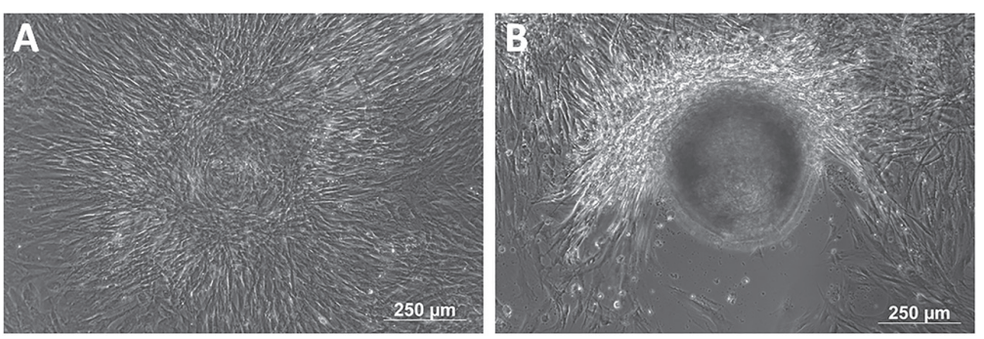

Figure 1.
Skin fibroblast reprogrammed by mRNAs codifying for Oct4, SOX2, c-Myc, KLF4, Nanog, LIN28. Representative images of iPSCs arranging after 24 hours to form a colony (A) that appeared clearly visible ten days later (B). Scale bar is 250 μm.

Beside the ethical issue, several other hurdles limit the concrete employment of ESCs, such as the risk of rejection related to their immunogenicity, the challenging conditions to culture and expand ESC lines and then to maintain the undifferentiated state ensuring their stability [12–14]. Additionally, there is a risk for tumor formation if ESCs are not fully addressed into a specific differentiated cell type prior to implantation [15]. Finally, about the therapeutic application of ESCs in human studies, another major concern is the use of non-human and xenogenic materials such as fetal bovine serum for cell culture [16].

However, the pluripotency makes ESCs unique, and this astonishing differentiation capability renders them very attractive to research studies, to the extent that a big effort has been put recently into the search for methods to artificially reproduce their pluripotency (**Figure 2**).

Nonetheless, the efficiency of cell reprogramming remains low, hence, the reprogramming techniques are under intense investigation so as to generate induced pluripotent stem cells ameliorating the efficiency of the process, the quality and safety of the derived cells [17]. The improvement generally targets several aspects of the reprogramming methods: primarily the source of somatic cells, as many studies suggest that some cells are more prone than others to be reprogrammed into certain cell types; [18] reprogramming factor cocktail; [19] the conditions to culture and maintain the iPSCs and, above all, the technique to introduce the reprogramming factors [20].

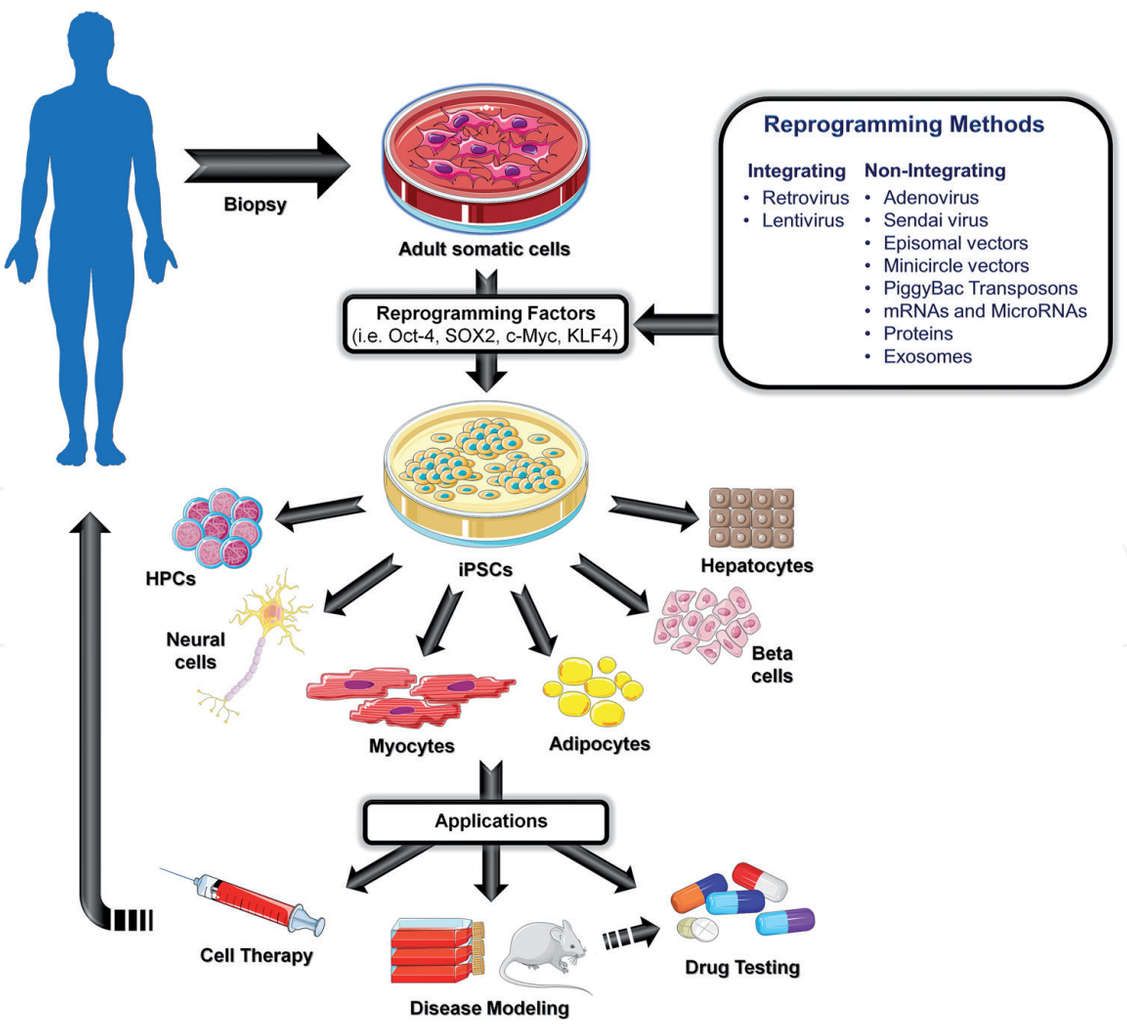

Figure 2.
Human iPSC technology allows, through the introduction of reprogramming factors into adult somatic cells, to obtain pluripotent cells capable of differentiating towards several mature cells which can be used for providing cells for regenerative medicine, for in vitro or in vivo disease modeling, and for screening and developing new drugs. (The figure was prepared with the support of Servier Smart Medical Art, https://smart.servier.com/).

A major issue related to the production of iPSCs, in fact, is the use of retroviruses to obtain a permanent integration of the reprogramming factors in the host cell genome, leading to teratoma formation due to the residual expression of oncogenes like c-Myc and KLF4 [21].

Different methods are known, at the present, to induce the expression of the reprogramming factors, classified in two major categories: non-viral and viral vector-based methods [22]. Viral-based methods include integrating viruses like Retrovirus and Lentivirus and non-integrating viruses, such as Adenovirus, Sendai virus [23].

According to several studies, all these methods provide good results in terms of effectiveness of cell reprogramming, hence, the choice of the suitable method strictly depends on the cell type used and on the subsequent applications of the iPSCs obtained [24].

Since from the first studies on reprogramming programs the most common method to generate iPSCs included the employment of retroviruses or lentiviruses to deliver Yamanaka factors [25–27]. Retroviruses integrate into host's genome allowing a satisfying expression of reprogramming factors. The first retrovirus used to deliver specific transcription factors into mouse and human fibroblasts was the Moloney Murine Leukemia Virus (MMLV), capable to infect only actively dividing cells and silent in immature cells such as ESCs [22, 28].

Conversely, the most common lentivirus used as a delivery vector derives from HIV. Usually lentiviruses have higher cloning capacities and infection efficiency than retroviruses. Unlike MMLV based retroviruses, lentiviruses could replicate both in dividing and non-dividing cells. Lentiviruses, with the respect to retroviruses have two safety advantages, the lack of integration near the transcription site of start and the capacity to deliver simultaneously different reprogramming factors in a single construct [29].

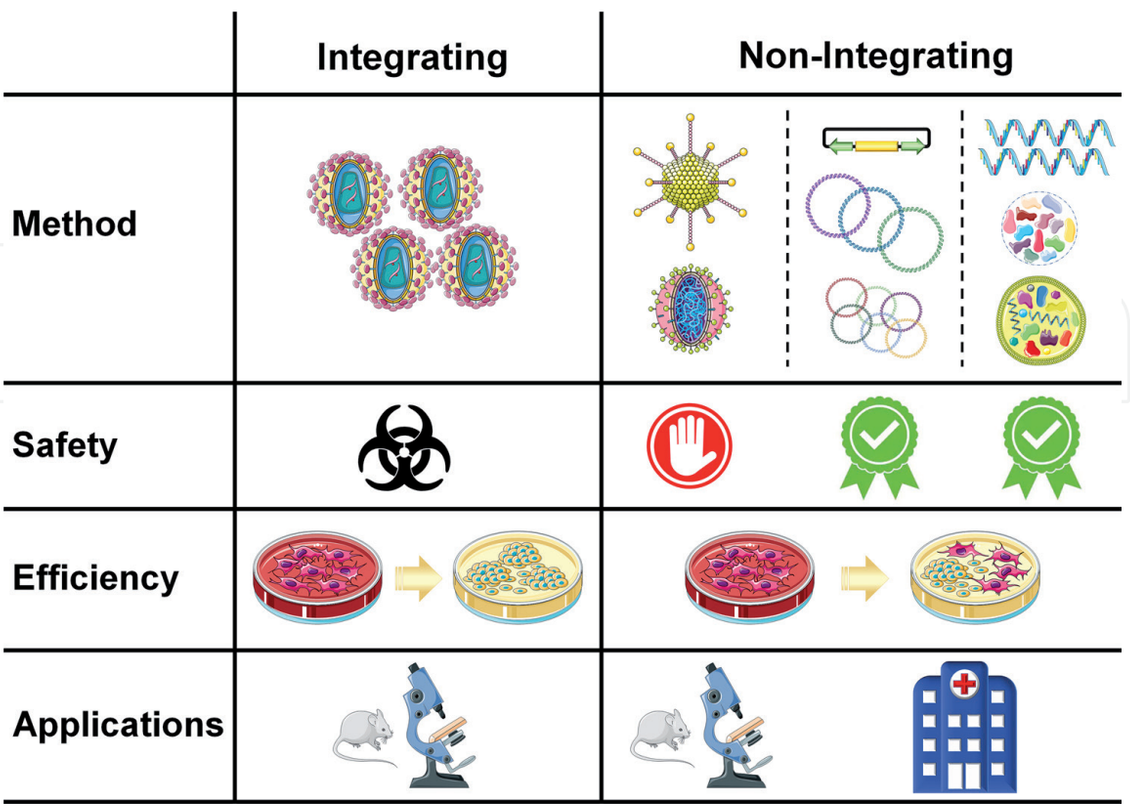

Figure 3.
The scheme summarizes the major advantages and disadvantages of integrating vs non-integrating methods currently employed for adult somatic cells reprogramming. (The figure was prepared with the support of Servier Smart Medical Art, https://smart.servier.com/).

However both vectors made of retrovirus and lentivirus carry significant risk of insertion mutagenesis during transfection related to their genomic integration; [26] therefore, even if they are properly silenced, viral transgenes can eventually be reactivated during differentiation or during the maturation of iPSCs, with high risk for tumorigenicity [6].

Therefore, while they represent a valuable research tools, they cannot be safely employed in the clinical application (**Figure 3**).

2. Introducing non-integrating methods

The efficiency and safety of generating and using iPSCs show a negative balance, and thus clinical employment of iPSC technology is still waiting for an effective protocol better poising these two fundamental features [30].

Even though the integration of reprogramming factors in iPSCs generated using viral methods offers high efficiency and a good yield, it is risky and represents a strong limitation for further clinical applications. Indeed, the residual expression of Yamanaka factors in the derived cells, such as the oncogene c-Myc, causes several genetic and epigenetic mutations, along with transcriptional abnormalities, despite the silencing of these genes during reprogramming. The integration of the reprogramming factors, in fact, is responsible for disruption of coding regions, promoters, and enhancers/repressors causing the instability of the gene network of the iPSCs obtained. Genetic aberrations are strongly related to cancer onset, hence, the maintenance of genomic stability of iPSCs without dragging integrating viral vectors sequences is highly desirable (**Figure 3**). To overcome all these relevant safety issues newer protocols are currently under development for deriving iPSCs without any integration while addressing the low efficiency showed by earlier reprogramming methods [31].

3. Non-integrating viral-based methods: advantages and disadvantages

In order to fulfill the above-mentioned requirements several integration-free methods have been developed, many of them employing viruses. It is important to underline that, even if all these methods are classified as "non-integrating", avoiding even a partial and negligible integration of viral genome into the host cells is not possible (**Figures 2** and **3**).

3.1 Adenovirus

Adenovirus is DNA virus that can reprogram cellular metabolism in a variety of ways, like increasing glucose uptake in cells and stimulating the synthesis of lactate, and produce many other metabolic changes related to cancer. Several studies have shown the effectiveness of the adenovirus as a vector to deliver specific differentiation factors to generate iPSCs without integration into host's genome.

The use of Adenovirus as a delivering method for reprogramming factors shows a certain effectiveness, allowing, at the same time, the production of quite safe and integration-free iPSCs. As a matter of fact, Stadtfeld and colleagues have demonstrated that human iPSCs generated from adenovirus are pluripotent and can be differentiated into all three germ layers in vitro and in vivo [32]. Zhou and Freed produced iPSCs from human embryonic fibroblasts using adenoviral vectors expressing c-Myc, KLF4, Oct4, and SOX2, and the iPSCs obtained expressed ESCs specific markers, showed a great differentiation potential and were free of any viral

or transgene integration [33]. However, despite the employment of the adenovirus method eliminate the risk for malignant transformation associated with retrovirus or lentivirus, it shows disadvantages, such as the lower efficiency and the shorter expression kinetics requiring repeated transductions to maintain an adequate level of transgene expression [33].

Additionally, not all the cell types are capable to generate iPSCs with this method alone as shown by Okita and colleagues in their studies they were unable to obtain murine hepatocyte iPSCs clones introducing the four reprogramming factors in the adenoviral vector alone, but the entire process required additional transfections of Oct3/4 and KLF4 or Oct3/4 and SOX2 by retrovirus [34].

3.2 Sendai virus

Sendai virus is an RNA virus that can infect a wide range of cell types either proliferating or quiescent and does not enter the nucleus of host cells. RNA virus-derived vectors are considered an attractive tool to vehicle Yamanaka factors, as they show a low risk of genomic insertion and are commonly used to reprogram neonatal and adult fibroblasts, and blood cells. The virus, while replicating, remains in the cytoplasm after infection and can be washed out of the host cells by several passages. Sendai virus shows a high transduction efficiency as confirmed by the expression of transgenes delivered yet detectable within a few hours after transduction, with a maximum expression after 24 hours after transduction. Sendai virus vectors have been largely studied and have emerged for their capability of successfully produce iPSCs with a non- detectable presence of viral RNA reprogramming adult human fibroblasts and circulating T cells [35].

As Sendai virus is an RNA virus it holds the great advantage that does not enter the nucleus of the host cells and allows a highly efficient reprogramming [36]. Further, Sendai virus-based vectors are replication deficient, and their copies became diluted during cell divisions, and eventually virus-free iPSCs are available after about 10 passages. Thus, this reprogramming technique works to obtain iPSCs without introducing changes to genome. However, their relatively short expression strongly limits their use in biological and research application that require long-term manipulation for somatic cell reprogramming.

4. Non-viral-based reprogramming methods

Due to the highlighted safety issues, it is necessary to develop efficient non-viral reprogramming methods. To generate iPSCs completely free from any viral contamination, researchers have modified and used DNA-based vectors, such as plasmids, episomal vectors, minicircle vectors, piggyBac transposons and non-DNA-based methods to deliver Yamanaka factors as mRNAs, microRNAs and proteins, as well as the direct reprogramming by exosomes (**Figure 2**) [37].

The common denominator of these methods is a much lower reprogramming efficiency as compared to lentiviral vectors-based reprogramming (**Figure 3**).

4.1 DNA-based methods

They require the use of elements composed of DNA to induce the expression of the reprogramming factors into the target cells. The most used elements include circular DNA vectors of different sizes (episomal vectors and minicircles) and mobile DNA sequences able to move and integrate to different locations within the genome by a cut and paste mechanism (PiggyBac).

4.1.1 Episomal vectors

One of the first integration-free techniques used for cell reprogramming includes the employment of non-replicating or replicating episomal vectors. This method is a quite simple technique not requiring special skills by the operators performing the experiments. Beside these advantage, reprogramming by the means of episomal vectors produces iPSCs still containing fragments of plasmidic DNA, due to the low transfection power that requires multiple transfections to obtain an appropriate expression level of the desired genes in the derived cells. Due to such a low transfection efficacy, the possibility of DNA fragments integration is highly increased, and it is crucial to improve the technique focusing on the reduction of transfection frequency and genetic fragments integration. Reprogramming by episomes is an excellent choice if employing blood cells but needs modification of standard culture conditions to reprogram fibroblasts into iPSCs [34].

4.1.2 Minicircle vectors

Minicircle vectors were first developed as smaller alternates to episomal vectors with a higher efficiency of transfection. They are circular, non-viral DNA elements that have been freed from a prokaryotic vector containing sequences of interest i.e. Oct3/4, SOX2, Nanog, LIN28, Green Fluorescent Protein (GFP). Expression of minicircle-coded genes occurs in both dividing and non-dividing cells with high efficiency, and typically yield higher expression levels of desired proteins, as they are less likely to be inactivated and silenced by cellular mechanisms targeting foreign nucleic acids [38, 39].

4.1.3 PiggyBac transposons

In PiggyBac transposon reprogramming, transgene sequence can be removed from integration site without changing host's DNA. It requires only the inverted terminal repeats, flanking a transgene and transient expression of the transposase to catalyze insertion or excision events [40]. All the mentioned methods show disadvantages, for example, the Sendai virus is effective on all cell types, but requires a lot of passages to obtain iPSCs. The PiggyBac method could represent an attractive alternate but studies in human cells are still limited and weak [40].

4.2 Non-DNA-based methods

Most common reprogramming strategies are based on the use of DNA. All these techniques are effective in achieving a successful reprogramming of the somatic cell, but they are considered less safe as some fragments of the DNA employed can integrate into the host cells genome due to the repeated treatments required to obtain the appropriate expression level of the desired genes. To avoid this safety issues, several groups focused on the development of protocols including non-DNA based methods for reprogramming, such as the use of mRNAs, microRNAs, or recombinant proteins (**Figures 2** and **3**).

Beside reprogramming, and subsequent differentiation into desired cell type, several authors have recently reported the possibility of trans-differentiation, or conversion of one cell type to another one, while bypassing the iPSC-state. An example is the direct conversion of myocardial scar fibroblasts (MSFs) to cardiomyocytes by infection of human MSFs with a lentivirus vector encoding the potent cardiogenic transcription factor myocardin [41].

The direct reprogramming, in fact, is a procedure by which a mature, fully differentiated cell is converted into another cell type, completely or partially bypassing

an intermediate pluripotent state. The direct reprogramming is an interesting new approach of regenerative medicine allowing to overcome the numerous problems related to the use of stem cells. Additionally, it has a low risk for genetic alterations and tumor development, as the reprogramming by this technique avoids risky genetic manipulation and the use of viruses or other strategies causing the residual integration of exogenous genetic material.

4.2.1 mRNAs

High-efficiency, synthetic mRNA-based reprogramming was recently described [42]. Synthetic mRNAs codifying for Yamanaka factors are modified to overcome innate antiviral responses. Since mRNA is translated to protein in the cytoplasm, it does not enter the nucleus, minimizing chance of unwanted modifications of hosts DNA. This method appears to work fast and efficiently, but the major disadvantage is that mRNA is degraded in few days. As such, repeated transfection is required for successful reprogramming [42, 43].

The direct delivery of synthetic mRNAs for the conversion of adult mature cells into iPSCs is an example of direct reprogramming. An effective protocol including the employment of this method was proposed by Warren et al. The idea of delivering mRNA directly raised from the possibility of random DNA fragment integration when DNA is used to derive iPSCs. This procedure is based on the in vitro transcription by the means of templates previously amplified by molecular biology techniques to encode the four Yamanaka reprogramming factors. A strong limitation related to the employment of this procedure is due to the multiple administrations required to gain an adequate protein expression levels, therefore the entire reprogramming process consists in a daily mRNA transfection and the derivation of iPSCs can take up to 18 days. Nonetheless, the transfection of human dermal fibroblast with Yamanaka's reprogramming factors combined with Nanog and LIN28, from Thomson's approach, have been reported as inducing the arrangement of cells in colonies as early as 24 h after the first transfection (**Figure 1**) [18, 19]. To increase the efficiency of the technique, the delivery of mRNAs is combined with hypoxic culture conditions that seem to double the efficiency of reprogramming. However, direct cell reprogramming mediated by mRNA is risky, as the numerous and repeated administrations of them to ensure a high expression level of proteins of interest can eventually trigger the activation of c-Myc, with a high risk for tumor development. A pivotal improvement for this procedure could target the frequency of mRNAs administration and the activation of the oncogene c-Myc.

4.2.2 MicroRNAs

MicroRNAs are small molecules of non-coding RNA primarily involved in gene expression regulation at both transcriptional and post-transcriptional level; in particular, they are responsible for gene silencing. Several studies have reported that including microRNAs in the traditional procedures employed for reprogramming can positively impact the efficiency of the process. Equally to other procedures not requiring DNA, reprogramming by microRNAs produces iPSCs free from exogenous DNA integration, but the needing of multiple administrations makes the procedure complicated and time consuming [44].

4.2.3 Recombinant proteins

To overcome the issue related to the introduction of exogenous DNA into derived iPSCs, another approach consists in the employment of recombinant proteins as

reprogramming factors. Protein-based reprogramming carries the advantage that it does not cause any genetic changes. As already mentioned, current methods of protein-based reprogramming are less efficient that lentiviral delivery of Yamanaka factors [42, 45]. Typically, synthesized in bacteria, Yamanaka factors are modified so that they express basic amino acids or other transport peptides enabling to cross the cell membrane [4].

Some studies have led to the development of different methods to isolate, purify, and then deliver reprogramming factors in form of recombinant proteins [33, 45].

The reprogramming mediated by recombinant proteins is challenging and need several improvements. The synthesis of a consistent amount of proteins is quite hard and requires specific skills that make the technique ineffective for a number of laboratories.

4.2.4 Exosomes

The modern trend for cell reprogramming consists in the direct conversion of a cell into another by the means of exosomes containing a cocktail of reprogramming factors for a specific purpose, named reprosomes. With the respect to the iPSCs, reprogramming cells by exosomes seems to be more likely for clinical applications, as it requires easier procedures and the risk for tumor formation and mutations is low [46].

Exosomes are nanovesicles with a size ranging between 30 and 200 nm. They are secreted by all cell types and circulate in many body fluids, from where they can be easily isolated. After the discovery that exosomes are able to transfer molecules of biological relevance, like mRNA, miRNA and proteins to one cell to another eliciting phenotypical changes, several studies are ongoing to define their potential as an integration-free method for cellular reprogramming. Despite several advantages offered by the use of exosomes, like the easy extraction method, the reduction of immunological host response and the possibility to reprogram cells without genetical manipulation, their effective employment is still under investigation and the procedures for their isolation and characterization are still limited by a low efficiency and a poor specificity [47, 48].

5. Conclusions

The improvement of non-integrating methods is now the target for cell reprogramming to derive iPSCs. In fact, these methods do not require the incorporation of viral genome into the host cells, avoiding the risk of tumor development. The safety of these methods, that makes the derived cells more appealing for clinical applications, is a common strong point, although beside the above-mentioned specific issues related to each method, the common major weakness is represented by a general low efficiency respect to the traditional integrating approaches. The original protocol proposed by Yamanaka for generating iPSCs from adult somatic cells was based on the insertion of only four factors: octamer-binding transcription factor-3/4 (OCT3/4), SRY-related high-mobility group (HMG)-box protein-2 (SOX2), Myc, and Kruppel-like factor-4 (KLF4), or Nanog and LIN28 instead of Myc and KLF4 [4]. A major obstacle in cellular reprogramming, beside the risk of tumor formation due to the integrating methods, is the very low efficiency of the reprogramming procedure, strictly related to several factors, such as the type of cell to be reprogrammed, the method of delivering the reprogramming factors and culture conditions. Although the non-integrating methods offer a safer way to produce iPSCs for further clinical application, it is crucial to focus on the enhancement

of the efficiency of the existing and ongoing protocols. In this respect, several strategies have been developed, such as the employment of promoters or enhancers boosting the reprogramming of somatic cells have being developed. Regulatory genes involved in proliferation and cell cycle modulators represent a valid example among the approaches proposed, although if on one side they allow a better yield, on the other they have the disadvantage of being potentially tumorigenic [49, 50].

Additional candidates investigated for their ability to increase up to 100 folds the efficiency of reprogramming, due to their capability of remodeling chromatin, are small molecules and inhibitor factors, such as valproic acid (VPA) and histone deacetylase (HDAC) inhibitor. Further, the use of VPA together with hypoxic conditions greatly boosts the efficiency of reprogramming [51–53]. The remodeling of chromatin induces a dynamic modification of chromatin architecture that allows the access to the condensed DNA by proteins involved in transcriptional regulation mechanism and responsible for the modulation of the gene expression in the cells [54].

Other factors heavily impacting on the efficiency of reprogramming are culture conditions, the possible employment of supporting feeder cells, and the composition of culture medium [55, 56]. It is well documented that reprogramming under hypoxic conditions of 5% O2 instead of the atmospheric 21% O2 increases the reprogramming efficiency of mouse embryonic fibroblasts (MEFs) and human dermal fibroblasts. The presence of a layer of feeder cells is extremely important to support cells during the reprogramming procedures, as feeder cells are responsible for the secretion of growth factors essential for cell survival. Usually, mouse feeder cells are used to support the growth and culture of iPSCs, but they must be removed before the use in clinical applications. Basically, feeder cells consist in a layer of growth-arrested cells unable to divide, which provides extracellular secretions to help other cells to proliferate. However, the use of animal derived feeder cells rises safety issues for the clinical applications due to the contamination of pathogens cross-transfer. To overcome this limitation, the use of Matrigel, a mixture of extracellular matrix proteins such as laminin, collagen and fibronectin, and supplemented with a medium conditioned by feeder cells, as substitute supporting layer is widely popular to produce and support iPSCs [19, 57–58].

A successful reprogramming also depends on the choice of the proper cell type to reprogram. The original protocol proposed by Yamanaka included the use of fibroblasts, first from mouse, then from humans, and these cells still remain the favorite cell type, primarily for the easiness of harvesting by skin biopsy. However, even among the different types of fibroblasts several studies highlighted that they are not reprogrammable with the same efficiency [18]; hence, other cell sources need to be found. In fact, the specific promptness of cell to be reprogrammed is strictly related to the endogenous expression of some reprogramming factors and from the starting differentiation state. Currently, there are different strategies, which allow choosing the appropriate cell source, the delivery method, and the system to boost the efficiency of cell reprogramming to derive iPSCs in the safer manner. Nevertheless, all these techniques need to be strongly boosted in order to be considered useful for a clinical application of the derived iPSCs.:

Conflict of interest

The authors declare no conflict of interest.

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