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Reprogramming methods for induced pluripotent stem cells generation

Ylenia Della Rocca[1,*], Antonella Mazzone[1], Jacopo Pizzicannella[2], Guya Dilletta Marconi[1], Oriana Trubiani[1], Francesca Diomede[1]

[1] *Department of Innovative Technologies in Medicine & Dentistry, University "G. d'Annunzio" Chieti-Pescara, via dei Vestini, 31, 66100 Chieti, Italy*
[2] *Department of Engineering and Geology, University "G. d' Annunzio" Chieti-Pescara, Viale Pindaro, 42, 65127 Pescara, Italy*
*Corresponding author. E-mail: ylenia.dellarocca@unich.it

Abstract. Regenerative medicine expects to replace the function of tissue or organs damaged by disease, trauma, or congenital issues. The tools used to realize these outcomes are tissue engineering and cellular therapies. Cellular therapy is considered a regenerative medicine strategy based on the use of stem cells. Pluripotent stem cells are the hotspots of cellular therapy due to their features that have been showed promising results. Embryonic stem cells (ESCs) are pluripotent, self-renewing cells that are derived from the inner cell mass (ICM) of the developing blastocyst. Pluripotency is the main feature that lead single cell to generate all cell lineages of the developing and adult organism. The use of human ESC (hESC) is ethically controversial, to overcome this problem the induced pluripotent stem cells (iPSCs) were developed. The aim of the present mini-review is to report a comprehensive summary of the different cellular reprogramming techniques from its initial conception to the present day.

Keywords: embryonic stem cells, pluripotency, human ESC, reprogramming.

INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent cells with the property to grow indefinitely maintaining pluripotency and to differente into cells of all three germ layers. ESCs derived from the inner cell mass of mammalian blastocysts [1] [2] [3]. Human ESCs (hESCs) could be a therapeutic perspective for the treatment of various pathologies but there are ethical problems with the use of human embryos, and clinical difficulties such as post-transplant tissue rejection. In recent years, the necessity of therapeutic purposes with greater regenerative potential, bypassing the ethical problems of the use of ESCs, led to the establishment of induced pluripotent stem cell lines (iPSCs). The iPSCs are cells with pluripotent properties obtained from differentiated cells by reprogramming. It results in a similar embryonic stem cell state [4] [5]. Yamanaka and Takahashi were the first to hypothesize the central factors to the induction of pluripotency in somatic cells and for the

maintenance of pluripotency in ESCs [6]. They selected 24 genes as candidate factors, starting from other previous studies in which it was demonstrated that several transcription factors, including Oct3/4, Sox2, Nanog are involved in the maintenance of pluripotency at the level of ESCs, as well as some genes demonstrated upregulated in ESCs. Tumors markers, such as Stat3, E-Ras, c-myc, Klf4 and β-catenin, contribute to the maintenance of ESCs phenotype in culture and their rapid proliferation. By combining four selected factors (OCT3/4, SOX2, c-Myc, and KLF4), it was possible to generate pluripotent cells directly from mouse embryonic or adult fibroblasts cultures, generating iPSCs [6]. Since the characteristics of iPSCs are similar to those of embryonic stem cells; they can be expanded indefinitely in vitro and differentiated into the three germ layers: endoderm, mesoderm and ectoderm. Takahashi and Yamanaka's revolutionary discovery has led to the extensive use of iPSCs and their differentiated cells in various research areas, especially in regenerative and personalized medicine. Among the reprogramming methods that allow to obtain iPS there are: nuclear transplantation, cell fusion, reprogramming by cell extracts, and direct reprogramming through gene manipulation [7]. Among the direct reprogramming methodologies, we can make a further distinction between integrating vector methods and genomic non-integrating methods. These two types of direct reprogramming are the most widely used methods today to generate iPSCs [8].

NUCLEAR TRANSPLANTATION

Nuclear transfer allows to obtain cells with embryonic characteristics through the reprogramming of differentiated somatic cells by transplanting cell nuclei into an enucleated oocyte [9] [10]. At this point, the cloned embryo reaches the blastocyst stage, from which ESCs can differentiate into pluripotent cells and thus into cells of the three embryonic layers and then in different tissues. However, this method has several limitations for clinical applications since it presupposes an unfertilized egg cell and, in addition, there is the risk of immunological rejection. In 2007, only 2 cells out of a total of 304 oocytes were successfully created as ES cells by nuclear transfer, demonstrating a low efficiency of the method [11].

CELL FUSION

Cell fusion is the reprogramming method which generate cells with pluripotency characteristics through the hybridization of an adult somatic cell with an embryonic stem cell. The resulting hybrid is determined by rearrangement in the DNA during cell division of the formed syncytium. ESCs therefore can induce somatic cell reprogramming, overwriting the somatic cell genome with ESC genetic information [11]. Hybrids thus formed have been seen to generate chimera embryos after blastocyst injection, demonstrating their pluripotency [12]. It is not clear whether the cytoplasmic elements of ESCs are sufficient to obtain pluripotent cells through this methodology or whether the nuclear elements are also necessary. Since it would be desirable to selectively remove only ESCs chromosomes from the melted nuclei, and it is practically difficult, this technique is still far from its possible use in clinical applications [13].

REPROGRAMMING BY CELL EXTRACTS

This method of reprogramming involves inserting cell extracts obtained from pluripotent stem cells into somatic cells. The cellular extract, chemically isolated from ESCs, consists of a set of reprogramming factors which, once inside somatic cells, induce their reprogramming. This technique has been shown to increase the expression levels of pluripotent markers such as Oct4 in host cells. Although these cells were able to differentiate into different cell lines, they were not able to give rise to the three germ layers [14], fundamental property for a pluripotent cell. For this reason, this method does not allow a complete reprogramming.

INTEGRATING VECTOR METHODS

Methods that use integrating vectors to induce pluripotency can first be distinguished based on whether they use viral or non-viral vectors.

As regards the viral delivery systems, the gene factors inducing pluripotency are inserted into the cells to be reprogrammed through a viral vector. The determining factor for efficient delivery is the infection system utilized by the virus, especially for entry into the host cell, into the nucleus, and for cytoplasmic trafficking. These processes change and vary according to different types of viruses. Persistent gene expression requires the use of integrating vectors such as lentiviruses or retroviruses. For transient expression, however, it may be sufficient to use an adenovirus since they are episomically maintained and do not integrate into the genome [15].

The original system for obtaining iPSCs is using retroviral vectors, which integrate the transgenes into the

host genome [16]. Adenovirus vectors are generally quite poor in cell gene transfer, probably due to the availability of primary receptors and/or co-receptors required for *in vitro* cell binding and internalization [17,18]. Stem cells gene delivery by these vectors is relatively weak, although there are capsid modification techniques that improve delivery efficiency [19]. Retroviruses are vectors that induce long-term expression through DNA integration. They have been used with exceptional effect in the reprogramming of mouse and human dermal fibroblasts into iPSCs [20] [6]. However, they have the important limitation related to their propensity for cellular transduction limited to actively dividing cells. Due to retrovirus limitations, the research has shifted to lentiviruses for several reasons. First, the lentivirus transduces both dividing and non-dividing cells. This distinguishes it from classic retroviruses [21]. Furthermore, unlike native adenoviruses which are selective for CD4 on T cells, recombinant lentiviruses can be pseudo typed with other envelope proteins to make them less selective and broaden the viral tropism [22]. Finally, producing lentiviruses in the laboratory is not overly complicated. The lentivirus allows a very efficient, stable, and reproducible gene delivery.

However, since viral integration methods lead to host genomic integration and bring safety risk associated with genetic manipulation, they are not ideal methods to obtain iPSCs for therapeutic purposes.

Therefore, a non-viral approach can be considered as an alternative to developing iPSCs.

The non-viral system usually employs plasmids, through which genes for pluripotency induction are transported. For delivery, the plasmid is encapsulated by lipid or cationic polymers which are needed to transfect the cells to be reprogrammed. Plasmids are episomically maintained and result in short-term gene expression [23].

In 2008 mouse iPSCs were generated with a plasmid vector, demonstrating that transient expression of reprogramming factors can be induced, identifying three essential reprogramming factors: Oct3/4, Sox2 and Klf4 [24]. These three factors were linked in a single plasmid with a constitutively active CAG promoter, allowing an high expression of multiple proteins from a single RNA transcript. The transfection trough plasmids was repeated several times to obtain the necessary expression for generating iPSCs. After four weeks, colonies of iPSCs were obtained, but with a low frequency: one third of the iPSCs clones did not integrate the transgene. The iPSCs clones lacking integration had the potential to differentiate into different cell types of the three germ layers. Furthermore, when transplanted into blastocysts, they were able to form chimeric mice, competent for germline transmission [25].

NON-INTEGRATING METHODS.

One of alternative reprogramming methods for obtaining iPSCs trough non-integrating methods is by episomal vectors derived from the Epstein-Barr virus without the viral packaging. It is seen that with this method the reprogramming efficiency was low although sufficient to have enough starting cells for culture. Furthermore, the addition of chemical compounds could increase the reprogramming efficiency of these episomal vectors [26]. Another method is the constitution of a minicircle vector. This is a small size double-stranded circular DNA. A mini-circle DNA vector was developed with a constituted single cassette of four reprogramming factors (Oct4, Sox2, Lin28 and Nanog) together with the GFP reporter gene, each separated by self-cleaving 2A peptide sequences [27]. This construct exhibits better transfection efficiency besides longer ectopic transcription factors expression, probably because it less activates exogenous silencing mechanisms. In the delivery of the minicircle vector electrotransfer gave the best results [28]. Furthermore, this method showed low cytotoxicity when electroporation was performed with minicircle DNA. Although these non-viral methods represent an important advance, they show poor reprogramming efficiency. Among the more recent non-integrating methods is synthetic transfection of mRNA, which has resulted in efficient and controlled gene expression in human cells, without genomic integration. Treatment with speific mRNA cocktail induces pluripotency in somatic cells [29]. Transfection with mammalian cell mRNA results in severe cytotoxicity which can be reduce by substituting cytidine and uridine with pseudo UTP and 5 methyl CTP respectively, still achieving stable transfection [30]. Furthermore, more recently iPSCs have been efficiently generated using synthetic self-replicating RNAs [31]. With this method it is avoided repeated mRNA transfection, so cytotoxicity is limited. More recently, these pluripotency-inducing nucleic acid have been placed inside state-of-the-art nanoparticles that interact with the membrane allowing target cell transfection [32].

CONCLUSION

Regenerative medicine has made great progress. Reprogramming tools represent a recent area of research with promising results. Although encouraging data have been obtained, further studies are needed, especially to establish the efficacy and safety of reprogramming methods. Cellular reprogramming is one of the most promising cell therapy approach for treatment of several diseases.

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