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Reprogramming of somatic cells: possible methods to derive safe, clinical-grade human induced pluripotent stem cells

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Derivation of pluripotent stem cells from adult somatic tissues by reprogramming technology has opened new therapeutic possibilities. Current most efficient procedures for derivation of induced pluripotent stem (iPS) cells are based on the viral vectors, which represent the danger of insertional mutagenesis during incorporation of introduced genes into the host genome. To circumvent this problem, the new, safe, non-integrative and non-viral strategies of reprogramming have been developed. In this review we discuss novel DNA-free and viral-free methods of reprogramming to iPS cells including protein transduction, mRNA and microRNA delivery.

Key words: iPS cells, somatic cells reprogramming, protein transduction, mRNA and miRNA transfection

INTRODUCTION

Development of cloning technology in vertebrates clearly demonstrates that the nucleus of differentiated somatic cell may attain pluripotent stage in the cytoplasm of the oocyte (Gurdon et al. 1964). Phenotypic and molecular investigation of embryonic stem (ES) cells during the last three decades has enabled identification of genes which are responsible for the maintenance of cellular pluripotency in mammals (reviewed in Nichols and Smith 2012). However, *in vitro* conditions for successful derivation of pluripotent cells from differentiated somatic cells were not known. Yamanaka and his PhD student, Takahashi, undertook the challenge of finding the proper combination of transcription factors for genetic modification of mouse embryonic fibroblasts (MEF) into iPS cells (Takahashi and Yamanaka 2006). The procedure of reprogramming appeared to be the most efficient when a combination of 4 out of 24 tested genes encoding transcription factors Oct4, Sox2, Klf4, c-Myc (OSKM factors, also called Yamanaka factors) was applied. One year later Yamanaka's group successfully derived iPS cells also from human fibroblasts (Takahashi et al. 2007). The first method, employed for the introduction of reprogramming transcription factor genes to differentiated cells, was based on four monocistronic retroviral vectors. However, upon transduction, retroviral vectors are randomly integrated into the host genome, thus significantly increasing the risk of insertional mutagenesis and cancer (Schroder et al. 2002, Wu et al. 2003, Bushman et al. 2005, Okita et al. 2008). In order to lower the excessive amounts of random integration, polycistronic vectors containing the sequence for all reprogramming factors under a single promoter have been used (Carey et al. 2009, Zhang et al. 2011). However, this method involves integration of transgenes, and therefore it is unsuitable for the generation of clinical-grade iPS cells.

In addition to the stable integration approach, the transient transfection using episomal vector or minicircle DNA has been employed (Yu et al. 2009, Narsinh et al. 2011). This reprogramming method is non-integrating since the introduced genetic material persists in the nucleus as extrachromosomal DNA. However, potential spontaneous integration causing mutagenesis

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can occur, thus limiting the advantage of this technique for possible clinical applications (Okita et al. 2008). In that respect closer to the clinical application seems to be efficient, transgen-free induction of human pluripotent stem cells by the vectors derived from Sendai virus. However, this technique involves viral particles raising questions regarding the safety of generated iPS cells (Fusaki et al. 2009).

Safe methods for derivation of iPS cells have become the main goal of development in reprogramming technology. The most promising are DNA-free and viral-free protocols. They include introduction of reprogramming-inducing molecules into cells such as: (1) recombinant proteins (Zhou et al. 2009, Kim D. et al. 2009), (2) messenger RNA (mRNA) (Warren et al. 2010), and (3) mature microRNA (miRNA) (Miyoshi et al. 2011). The efficiency of non-integrating reprogramming methods is greatly enhanced by the use of low oxygen level conditions (Szablowska-Gadomska et al. 2011) and small molecules such as histone deacetylase inhibitors (Huangfu et al. 2008) and/or DNA methyltransferase inhibitors (Mikkelsen et al. 2008).

The strategies of somatic reprogramming such as using recombinant proteins, mRNA and miRNA are the safest and integration-free methods having the highest potential for therapeutic application of all known methods for reprogramming. These techniques are the main focus of this review and will be described below in detail.

RECOMBINANT PROTEIN TRANSDUCTION

The strategy to entirely replace gene delivery during the reprogramming process by recombinant protein transduction was reported for the first time by Kim D. and coauthors (2009) and Zhou and others (2009). Recombinant protein transduction is one of the DNA-free strategies of cellular reprogramming that rely on introduction of proteins into the target cells, bypassing the need to introduce exogenous genetic materials. However, some macromolecules including proteins have largely limited ability to cross cellular membrane, therefore, recombinant proteins have to be modified. The observation that some proteins are able to pass through the cell membrane barrier contributed to the identification of specific domains controlling this process. Cell penetrating peptides (CPP), also termed membrane translocating sequences (MTS) or protein transduction domains (PTDs), are peptides that have the ability to transport through the cell membrane large molecules in a process independent of classical endocytosis. These properties make CPP domains suitable for transfer of proteins and other molecules into living cells (Fig. 1), for spreading the protein from transfected to non-transfected cells (Beerens et al. 2003) and also for entering the nucleus (Matsui et al. 2003, Prochiantz 2000).

Naturally existing peptides having the ability to translocate through the cell membrane barrier are characterized by a high proportion of basic amino acids (e.g., arginine or lysine) (Ziegler et al. 2005, El-Sayed et al. 2009). Poly-arginine domains are the type of CPP which are frequently used in reprogramming protocols. They bind to the plasma membrane, facilitating chemical compounds or proteins to translocate through the cell membrane (Schwarze et al. 2000). Poly-arginine penetrating peptide is composed of six to twelve arginine residues and assigns recombinant proteins with high transduction capacity (Matsui et al. 2003). It has been reported that oligo-arginine residue (3R) was sufficient for delivery of functional transcription factors, but its delivery effectiveness is not as powerful as poly-arginine (11R) residue (Hitsuda et al. 2012). Thus, for cellular reprogramming pluripotency transcription factors, such as Klf4, Oct4, Sox2, Nanog and Lin28, have been produced as recombinant proteins containing nine or eleven "arginin tails" functioning as protein transduction domain (PTD).

The pioneers of the experimental procedure for reprogramming using recombinant proteins (Kim D. et al. 2009, Zhou et al. 2009) applied the method of serial transduction with proteins containing poly-arginine domain. Though the process of reprogramming somatic cells to pluripotency stage was completed, the efficiency of this process was low. However, using recombinant proteins for cellular reprogramming has some advantages, as it does not require complicated manipulation protocols and also does not incorporate any changes to the genome, thus representing a safe method for iPS cells derivation.

For the pioneering experiments, Zhou and colleagues used MEFs from OG2 transgenic mice (Oct4-GFP reporter). MEFs were cultured in media containing Oct4, Sox2, Klf4, c-Myc recombinant proteins associated with poly-arginine domain (11R) in the presence of valproic acid (VPA) – a histone deacetylase inhibitor (HDAC). After four rounds of protein supplementation and subsequent culture of $5x10^5$ MEF

cells for 23–28 days, three iPS cell clones positive for Oct4-GFP reporter gene were successfully generated. Resulting iPS cells formed compact small colonies, which were morphologically similar to mouse embryonic stem (mES) cell colonies. Global gene expression analysis revealed that derived iPS cells were similar to mES cells and subsequent genomic sequencing analyses showed that Oct4 and Nanog promoters were demethylated. Obtained iPS cells contributed to embryonic development of the three germ layers in mouse chimeras and they also possessed the ability to differentiate into neurons, cardiomyocytes, and pancreatic as well as hepatic cells (Zhou et al. 2009).

For the reprogramming of human newborn fibroblasts (HNF) Kim and colleagues (2009) used whole cell extracts from human embryonic kidney (HEK) 293 cells, which were transfected with plasmid encoding pluripotent factors, such as OCT4, SOX2, KLF4 and C-MYC. Proteins were fused to 9-arginine peptide tags to allow transport through the plasma membrane. Cell extracts were added to the fibroblast cultures six times within the first week. Efficiency of reprogramming was low (0.001%), but obtained cells possessed pluripotent stem cell properties including differentiation potency into three germ layers performed *in vitro* and *in vivo* (Kim D. et al. 2009).

Protein transduction method in reprogramming can be used routinely for developmentally immature newborn or fetal cells. However, it has been proven that it is generally more difficult for adult cells to undergo reprogramming procedures (Park et al. 2008). Szablowska-Gadomska and colleagues (2012) reported that human umbilical cord blood-derived neural stem cells (HUCB-NSC) can be reprogrammed using recombinant proteins fused with poly-arginine domains. HUCB-NSCs have been treated with HEK293 cell extracts containing KLF4-9R, OCT4-9R and SOX2-9R recombinant proteins. The induction of pluripotency in HUCB-NSC was successful when small molecules, such as histone deacetylase inhibitor Trichostatin A (TSA), DNA methyltransferase inhibitor RG-108, and 5% oxygen tension were applied in addition to the recombinant proteins. TSA and RG-108 in combination with low oxygen tension showed an important role in epigenetic stimulation and in the generation of induced pluripotent stem cells from HUCB-NSC (Szablowska-Gadomska et al. 2012).

Several studies have indicated that other peptide domains can also be functional as the potential protein transmembrane carriers (Wadia and Dowdy 2002). The cell penetrating TAT domain from HIV1 (HIV1 TAT) is one of these, and was used for reprogramming of human fibroblasts (Pan et al. 2010). However, the reprogramming to iPS cells was not fully successful, since TAT engineered proteins remained in the endosomes instead of being transported to the nucleus. To make TAT-protein-based reprogramming effective, the approach of the conjugation in complex with cationic liposomes (lipo-Tat) was applied resulting in higher transduction efficiency (by 1000 fold) (Li et al. 2012). iPS cell generation using TAT-conjugated reprogramming factors (Oct4, Sox2, c-Myc, Klf4, Nanog) was further supported by adding VPA to the culture medium (Zhang et al. 2012).

In summary, the application of recombinant proteins is considered to be a safe and non-integrative method of generation of iPS cells, although this technology has some limitations. One of them is the quality and quantity of recombinant proteins required for cellular reprogramming, since it is challenging to generate and purify sufficient quantities of desired proteins. The other limitation is linked to bacterial post-translational modification of proteins, which revealed essential disadvantages and low efficiency of iPS cell generation (Zhou et al. 2009). Since iPS cells obtained by recombinant proteins transduction have significant potential for clinical application, these technical difficulties need to be resolved (Yang et al. 2012). It is of note that the first clinical trial using iPS cells, which has already started in Japan in order to cure retinal disease age-related macular degeneration (AMD), is based on iPS cells obtained by the technology of reprogramming with recombinant proteins (Cyranoski 2013, Takahashi 2013).

TRANSFECTION WITH mRNA

Efficient reprogramming of somatic cells to pluripotency can be achieved by introducing mRNA molecules into living cells. mRNA can be obtained from purified lysed cells, synthesized from free nucleotides either chemically or enzymatically, and delivered to the cells by microinjection, electroporation or lipofection. Transfected cells translate the mRNA into the desired protein (Fig. 2), which can be transported to the nucleus for its functional outcome. The mRNA technique may offer several advantages over the classical reprogramming protocols. Technology based on

mRNA totally eliminates the risk of integration of genetic material into the genome and insertional mutagenesis inherent to all DNA-based methodologies, including those that are defined as non-integrating.

Transfection using mRNA encoding reprogramming factors was applied for the first time in 2010 (Warren et al. 2010, Yakubov et al. 2010). Warren and colleagues (2010) produced human iPS cells by repeated transfection of modified synthetic mRNAs designed to bypass innate antiviral responses. Fibroblasts and keratinocytes were transfected with four synthetic modified mRNAs encoding Oct4, Sox2, Klf4, and c-Myc in the presence of interferon inhibitor. Efficiency of synthetic mRNA transfection in cellular reprogramming was higher than using retroviral vectors (1.4% *versus* 0.04%, respectively). It was also shown that additional transfection of Lin28-encoding synthetic modified mRNAs, under hypoxic conditions, enhanced reprogramming of fibroblasts and keratinocytes to iPS cells.

iPS cells obtained using mRNA molecules show similar characteristics and morphology as derived by Yamanaka protocol using the retroviral transduction method (Takahashi et al. 2007). However, some distinctions in the molecular phenotype, differentiation capacity, and teratoma formation between viral iPS and the mRNA-iPS cells have been reported. The different capability of teratoma formation *in vivo* can be explained by the possibility of producing partially reprogrammed intermediates during mRNA-based reprogramming procedure (Chan et al. 2009).

The ability to maintain a high-level of expression of defined proteins in human cells for many days without introducing the cells to unsafe DNA-based transgenes makes the mRNA-based reprogramming procedure attractive for therapeutic applications. Today it is considered to be the optimal strategy for the fast generation of pluripotent cell lines with therapeutic potential as compared to other non-integrative methods using episomal DNA plasmids or highly-infective Sendai virus. The mRNA reprogramming method is considered to be the safest and a highly efficient method, which eliminates the need for screening the cells to confirm viral remnants (Warren et al. 2010). However, mRNA molecules delivered into the living cells usually induce a significant inflammatory response and may also cause a variety of nonspecific effects including translation block, cell cycle arrest and apoptosis (Warren et al. 2010). Frequently observed cell death after repetitive transfection of cells with even a small amount of mRNA was related to the cell immune response. The application of chemical compounds (Pepinh-TRIF, Pepinh-MYD, B18R, chloroquine, TSA) known for their ability to suppress such cellular responses did not evoke the desired effect (Drews et al. 2012). One of the possibilities to solve this problem is the use of RNA viruses to destroy or inhibit specific immune-related proteins which enable persistent infection (Bode et al. 2007). The other possibility to escape the response of the immune system during multiple transfections with mRNA is the induction of suppression of exogenous RNA-recognition receptors (PRRs). They include Toll-like receptors TLR3, TLR7, TLR8 (Alexopoulou et al. 2001, Diebold et al. 2004, Kariko et al. 2005), the RNA helicase RIG1 (RARRES3) (Yoneyama et al. 2004), protein kinase R (PKR, a.k.a. EIF2AK2) (Levin et al. 1981), and members of the oligoadenylate synthetase family of proteins (OAS1, OAS2, OAS3). These and other receptors trigger an inflammatory response upon detecting pathogen-associated molecular patterns (PAMPs) such as exogenous RNA. However, it is not yet clear how cells distinguish exogenous mRNA from the large amount of endogenous RNA (Hornung et al. 2006, Saito et al. 2008, Takahasi et al. 2008, Yoneyama and Fujita 2008, Schmidt et al. 2009).

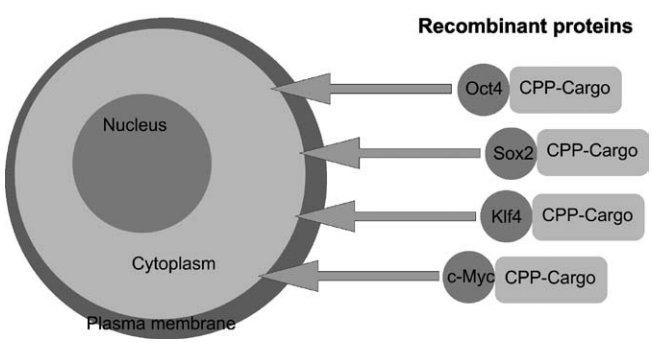

Fig. 1. Delivery of recombinant proteins to the somatic cells: (CPP) cell penetrating peptide.

The third possibility to increase the rate of recovery of cells transfected with mRNA is the knock-down of p53 (Angel and Yanik 2010). The same group has introduced the procedure of desensitizing cells to frequent transfection with mRNA. They applied small interfering RNA (siRNA) cocktail for the combined knock-down of interferon beta (IFNβ) and transcription factors Eif2ak2, Stat2 to allow sequential transfections with mRNA for the successful reprogram-

ming. Co-transfection of cells with siRNA cocktail designed to directly knock-down the expression of immune-related proteins allowed for repeated transfection with exogenous mRNA and increased viability of mRNA transfected cells (Angel and Yanik 2010). Further methodological advancement was the prolonged transfection with mRNA, which allowed for generation of iPS colonies without interferon-directed blocking (Arnold et al. 2012). Arnold and colleagues (2012) used this technology together with the combination of three transcription factors (OSK and ONT) for successful reprogramming of human Huntington's disease fibroblasts. However, the efficiency of reprogramming was low: 0.0005% of input cells as compared to 1.4% obtained by Warren and coworkers (2010) with the OSKN transcription factor combination.

Although the generation of iPS cells from adult patients is difficult, Heng and colleagues (2013) generated human iPS cells from adipose-derived mesenchymal stem cells (MSCs) from a 50-year old patient using synthetic modified mRNA encoding transcription factors in feeder-free defined conditions. Twelve karyotypically normal clonal iPS cell lines that were obtained revealed normal karyotype up to 10th passage, but after 24 passages displayed chromosomal mosaicism of normal and abnormal karyotypes. Reprogramming efficiency was at 0.005% level, and thus the procedure was considered just as the progress toward reaching clinical application (Heng et al. 2013).

Despite a few disadvantages which are recently being reduced by technological progress, the mRNA technique for iPS cell derivation is safer than viral-based classical reprogramming protocols, since it eliminates the risk of genomic integration and insertional mutagenesis. Application of the modified RNA strategy may serve in the future as the method for derivation of the clinical-grade human iPS cell lines.

TRANSFECTION WITH miRNA

miRNAs are 18–24 nucleotide-long single stranded RNA molecules usually generated from non-coding regions of gene transcripts, and function to suppress gene expression by repression of mRNA translation. miRNAs are associated with a protein complex called RNA-induced silencing complex (RISC) which inhibits the translation of targeted mRNA (Ambros 2004, Bartel 2004, Rana 2007, Kim VN et al. 2009)The reports showed that specific miRNAs can play a critical role in control of pluripotency-related genes. These conclusions were based on studies demonstrating that specific miRNAs are highly expressed in embryonic stem cells (Houbaviy et al. 2003, Suh et al. 2004, Marson et al. 2008). Several years earlier Leeand coworkers (1993) and Ruvkun (2001) confirmed the significant role of miRNAs in regulation of embryonic development and cell differentiation. Some important cellular processes that miRNAs have been implicated in include: expression of self-renewal genes in human embryonic stem (hES) cells (Xu et al. 2009), cell cycle control of ES cells (Wang et al. 2008), alternative splicing (Makeyev et al. 2007) and heart development (Latronico and Condorelli 2009).

Several miRNAs could mediate reprogramming of somatic cells to iPS cells, or they enhance iPS cell reprogramming when expressed with combinations of the OSKM factors (Judson et al. 2009). Specific miRNAs, such as miR290-295 in mouse or miR-302/367 in human, facilitate iPS cells to maintain the ES cell phe-

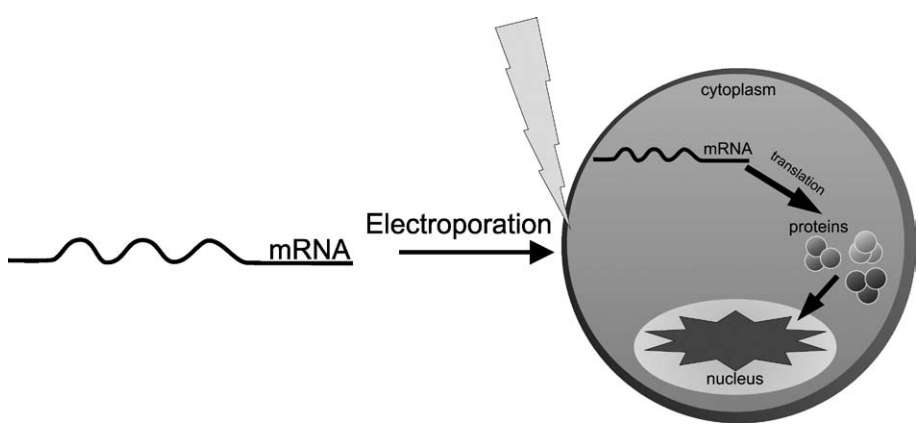

Fig. 2. mRNA delivery to the living cell and activation of the gene response.

notype by stimulation of expression of pluripotent genes (Wang et al. 2007, 2008, Babiarz et al. 2008, Wang and Blelloch 2009). These miRNAs have the ability to regulate the cell cycle which probably is connected with their capacity to enhance iPS cell reprogramming (Judson et al. 2009). Furthermore, cell-specific miRNAs can replace the function of c-Myc during reprogramming (Judson et al. 2009). Lin and others (2011) reported co-suppression of four epigenetic regulators: Lysine-specific histone demethylase 1A (also known as AOF2, KDM1 or LSD1), histone H3K4 demethylase (AOF1), histone deacetylase complex-repressor component (MECP1-p66) and Methyl-CpG-binding domain protein 2 (MECP2) by miR-302. The consequence of AOF2 silencing connected with DNA-methyltransferase-1 (DNMT1) deficiency resulted in global genomic DNA demethylation and H3K4 modification during somatic cell reprogramming (SCR), while supplementation of AOF2 changed pluripotent stage of iPS cells propagating their differentiation (Lin et al. 2011). Involvement of different miRNA clusters in the activation and inhibition of the specific cellular processes during reprogramming is presented on Figure 3.

The mir-302 cluster is located in the 4q25 locus of human chromosome 4 (Puca et al. 2001) and is predominantly expressed in hES and iPS cells (Suh et al. 2004, Wilson et al. 2009), while during early embryonic development and *in vitro* differentiation the expression of miR-302 is lost (Suh et al. 2004, Ren et al. 2009). The majority of miR-302-targeted genes are transcripts of developmental signals and oncogenes (Lin et al. 2008). The MiR-291/294/295 family presents a similar expression profile in mice (Judson et al. 2009).

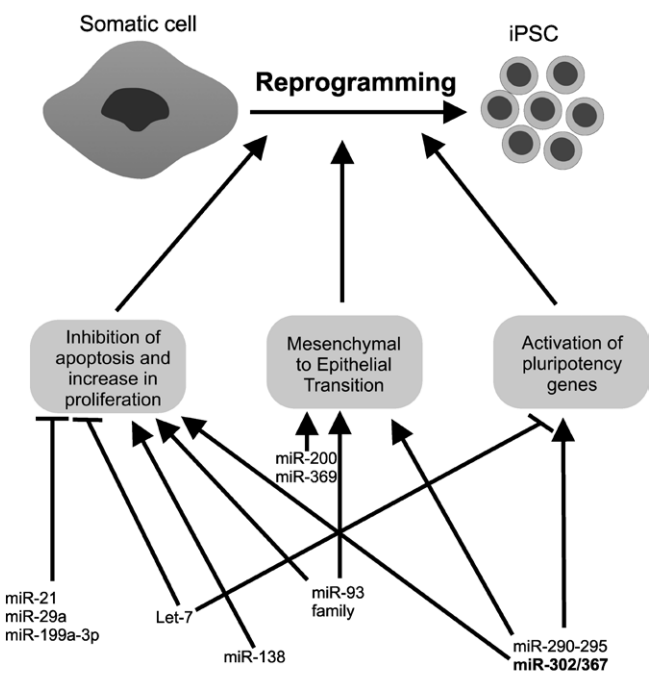

Fig. 3. The role of miRNA in the activation and inhibition of the specific cellular processes during reprogramming.

As demonstrated by Lin and coworkers (2008), miR-302 cluster not only improves the efficiency of SCR but also enhances the stemness and pluripotency of the reprogrammed cells. In addition, miR-302 may silence cyclin-dependent kinase inhibitor 1 (CDKI1, p21Cip1) thus promoting cell proliferation (Dolezalova et al. 2012). In human cells miR-302 cluster was also shown to be implicated in the inhibition of G1-S cell cycle transition by simultaneous suppression of cyclin E-CDK2 and cyclin D-CDK4/6 pathways (Lin et al. 2010).

Next to the miR-302s, the miR-367 expression is essential for iPS cell reprogramming by the miR-302/367 cluster (Betel et al. 2008, Zhang and Wu 2013). The miR-302/367 cluster has been shown to be a direct target of Oct4 and Sox2 transcription factors, since levels of miR-302/367 cluster correlate with Oct4 transcripts in ES cells during early embryonic development, indicating an important role of these miRNAs in ES cell homeostasis and maintenance of pluripotency (Card et al. 2008). Moreover, expression of the miR-302/367 cluster can directly reprogram mouse and human somatic cells to a pluripotent cell state without the presence of reprogramming transcription factors (Anokye-Danso et al. 2011). The efficiency of reprogramming obtained by Anokye-Danso and colleagues (2011) was higher when integrating viral vectors encoding miRNAs were used, compared to the method based on direct transfection of mouse and human cells with mature miRNAs (miR-200c, miR-302, miR369) (Miyoshi et al. 2011). The same combination of miRNA was investigated and found to successfully generate induced pluripotent stem cells from human somatic cells. The reprogramming was effective when transfections with mature miRNA were repeated 4 times in 48 h intervals. After 30 days post-transfection miRNA-derived iPS cells expressed genes typical for undifferentiated ES cells, including Nanog, Oct4, Sox2, Cripto, Dppa5 and Fbx15, Ssea-1, as well as E-cadherin which is the epithelial cell marker highly

expressed in ES cells (Miyoshi et al. 2011). However, the reprogramming efficiency using this method based on incorporation of mature miRNA molecules was about 0.01%. These experiments showed that miRNAs can reprogram somatic cells to pluripotency and miR-367 is required for miR-302/367 reprogramming. Moreover, the supplementation with histone deacetylase inhibitors, such as valproic acid or sodium butyrate in miR-302/367 reprogramming further enhanced this process (Anokye-Danso et al. 2011, Zhang and Wu 2013). The miR-302/367 expression along with HDAC2 suppression allows for highly efficient iPS cell reprogramming (10%) without the expression of the commonly used reprogramming factors. Moreover, the miRNA-based method was more efficient than previously described strategies, including transfection of synthetic mRNAs or OSKM factors (Warren et al. 2010).

Reprogramming methods using mature miRNAs do not require vector-based gene transfer, therefore they can be considered to be a potential solution for the personalized medical applications.

CONCLUSIONS

Reprogramming methods are constantly developing due to the amazing technological progress in the stem cell field. The performance of the applied methods is expected to be improved, while maintaining a high degree of safety. The present state of the art in the advancement of reprogramming procedures suggests that utilizing integration-free and virus-free methods under feeder-free conditions is the most promising step toward safe translation of iPS cells to future possible personalized regenerative medicine.

Improving both the efficiency and biological safety of reprogramming using recombinant proteins, mRNA and miRNAs is an opportunity for more rapid introduction of iPS cells to therapeutic application.

However, despite the overall methods of reprogramming, it is very crucial to extensively investigate the iPS cell-derived cell lines considered to be used in the clinic. The issues that must be evaluated in addition to the reprogramming technology raised in this review are the appropriate somatic origin of iPS cells and a proper differentiation of iPS in order to exclude the immunogenic potential of undifferentiated cells and elimination of the risk of tumorigenesis in the host tissue.

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