ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Review Acta Neurobiol Exp 2013, 73: 1–18

Genetic engineering of stem cells for enhanced therapy

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Stem cell therapy is a promising strategy for overcoming the limitations of current treatment methods. The modification of stem cell properties may be necessary to fully exploit their potential. Genetic engineering, with an abundance of methodology to induce gene expression in a precise and well-controllable manner, is particularly attractive for this purpose. There are virus-based and non-viral methods of genetic manipulation. Genome-integrating viral vectors are usually characterized by highly efficient and long-term transgene expression, at a cost of safety. Non-integrating viruses are also highly efficient in transduction, and, while safer, offer only a limited duration of transgene expression. There is a great diversity of transfectable forms of nucleic acids; however, for efficient shuttling across cell membranes, additional manipulation is required. Both physical and chemical methods have been employed for this purpose. Stem cell engineering for clinical applications is still in its infancy and requires further research. There are two main strategies for inducing transgene expression in therapeutic cells: transient and permanent expression. In many cases, including stem cell trafficking and using cell therapy for the treatment of rapid-onset disease with a short healing process, transient transgene expression may be a sufficient and optimal approach. For that purpose, mRNA-based methods seem ideally suited, as they are characterized by a rapid, highly efficient transfection, with outstanding safety. Permanent transgene expression is primarily based on the application of viral vectors, and, due to safety concerns, these methods are more challenging. There is active, ongoing research toward the development of non-viral methods that would induce permanent expression, such as transposons and mammalian artificial chromosomes.

Key words: stem cells, transfection, gene therapy, viral methods, physical- and chemical-based methods

INTRODUCTION

Stem cell therapy is a promising strategy for overcoming the limitations of current treatment methods. The efficacy of stem cell transplantation has been shown in multiple animal models of human disease, as well as by the clinical success in some fields, such as hematology (Hacein-Bey-Abina et al. 2010) or connective tissue restoration (Burt et al. 2009). However, in the case of non-regenerating organs, such as the heart or the brain, despite promising outcomes in small animals, clinical trials, to date, have failed to show a satisfactory effect. While the stem cells were proven to exert some positive effect due to cell replacement mechanisms, immunomodulation, or trophic effects, these effects may still require adjustment/modification to realize their full therapeutic potential (Bersenev and Levine 2012, Gage 2012).

Advances in stem cell and molecular biology have opened up new avenues for manipulating the fate and the functionality of stem cells. Genetic engineering, with an abundance of methodologies to induce gene expression in a precise and well-controllable manner, is particularly attractive.

There are several methods that enable gene delivery to eukaryotic cells. The first group includes typically the non-integrating techniques with transient expression, such as plasmid DNA and mRNA transfection,

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Received 12 February 2013, accepted 20 March 2013

minicircles and the Sendai virus based methods (Gracey Maniar et al. 2013). The second set of methods grouped typically the genome-integrating methods with stable expression, based on lentiviruses, retroviruses and site-specific recombinases. Both approaches have advantages and disadvantages and the selection of method should be determined by a careful evaluation of unique experimental needs and/or clinical conditions. Advanced genetic engineering techniques allow very specific genetic manipulations including targeted gene insertion, gene removal, or gene targeting. The introduction of a new gene followed, by its expression, proved highly practical, thus encouraging development in this field and a methodology for gene introduction that is impressively effective and straightforward, often with off-the-shelf, kit-based solutions.

With these methods, it is possible to engineer the cells and introduce completely new, specific, and well-defined features. It was recently shown that cells engineered by the insertion of genes encoding adhesion molecules dramatically increased the homing of glial progenitors to the brain after intracarotid transplantation (Gorelik et al. 2012). Insertion of gene-encoding growth factors is another example of a practical application for genetic engineering, potentiating the therapeutic effects of stem cells (Janowski and Date 2009). However, it should be emphasized that these studies were performed in animal models, and the clinical application of genetically engineered stem cells remains challenging and requires further investigation for better understanding and the control of genomic integration and transgene expression. Due to the risks associated with the viral vector-based methods (i.e. mutagenesis, aberrant transgene expression, and immunogenicity), integration-free solutions (or rather with a low probability of integration) have been solicited for clinical use.

Still, many of the integration-free methods require transfection agents, an additional means of enabling the shuttling of genetic material into the cell. This can be accomplished with various physical and chemical methods, but all are associated with some level of toxicity, which cannot be ignored. The expression time-course with the integration-free methods is usually short-lived, with natural silencing, but that silencing of the transgene may be considered an advantage in some applications. By using an appropriately selected method for cell engineering, ranging from stable viral transduction, DNA transfection, mRNA transfection, or protein introduction, it is now possible to select a technique that precisely suits the particular application, with an optimal expression time-course in stem cells and an acceptable balance of benefits and risks. In this review, we will characterize different techniques for expressing the transgene, with a focus on maximizing the therapeutic utility of stem cells. We will also discuss genetic engineering of stem cells from the perspective of clinical applications.

METHODS FOR GENETIC ENGINEERING BASED ON VIRAL VECTORS

The application of viral vectors for gene delivery is based on their natural ability to infect the cells. Their use as a relatively safe molecular biology tool was made possible by the modification of the viral genome by the deletion of some critical coding sequences to prevent spontaneous replication in target cells. Introducing a gene of interest as a component of the viral genome enables highly efficient shuttling of the payload into the cells and its long-term expression. Currently, viruses are commonly used as vehicles with both *in vitro* and *in vivo* delivery paradigms. Retroviruses, lentiviruses, and adeno-associated viruses are the three main integrating virus types that are used for a transduction of mammalian cells for long-term transgene expression. Adenoviruses and Sendai viruses are non-integrating types of virus that result in a transient expression of the transgene (Fig. 1).

Integrating viral vectors

Retroviral vectors

Retroviruses consist of a capsid with double-stranded RNA and reverse transcriptase and a lipid envelope with receptor binding proteins. While retroviral vectors infect proliferating cells with high efficiency, post-mitotic cells, such as neurons or myocytes, are not susceptible, which limits the spectrum of cells for targeting. The process of infection is initiated by the viral envelope glycoproteins binding to the cell's surface receptors. These receptors determine the infection specificity; for example, the human immunodeficiency virus type 1 (HIV1) binds only to lymphocytes and macrophages expressing the CD4 receptor (Dalgleish et al. 1984). After binding to the receptor, the external layer of the envelope integrates with the cellular mem-

brane and the virus is internalized, releasing the contents of the capsid into the cytoplasm. Reverse transcriptase is a hallmark of retroviruses, and it is necessary to rewrite viral RNA into DNA for its integration into the host genome. Following integration, viral genes, including the insert, are expressed.

Retroviral transduction of actively dividing cells is highly efficient, with a fairly large-capacity genetic payload of up to 8 kb. The integration of viral DNA into a host genome results in sustained expression of the gene of interest. However, long-lasting expression in some cell types, including hematopoietic (HSC) and mesenchymal (MSC) stem cells, has proved difficult (Zhang et al. 2002). It has been shown that the silencing of a viral transgene intensifies during cell differentiation (Laker et al. 1998). One of the suggested mechanisms for this is the methylation of a viral promoter sequence after its integration into the host genome (Challita and Kohn 1994). Another important feature of the retroviral vectors, with implications for the safety of genetic engineering, is the random integration of a transgene into the host genome. This can lead to the disruption of the host proto-oncogenes and tumorigenesis. An example of that scenario is a clinical trial performed between 1999 and 2002 in patients with X-linked severe combined immunodeficiency (SCID-X1) (Hacein-Bey-Abina et al. 2010). In that trial, CD34+ bone marrow cells (Huang and Terstappen 1994) were isolated and transduced *in vitro* with Moloney murine leukemia virus vectors carrying a cytokine receptor common γ chain (Hacein-Bey-Abina et al. 2010). Engineered, autologous cells were transplanted back into patients. The result of this gene therapy was positive, with the successful correction of an immunodeficiency in eight of nine patients. Thus overall, the study considered successful. Unfortunately, over the following nine years, four of the treated patients developed acute leukemia caused by the insertional oncogenesis (Hacein-Bey-Abina et al. 2010). Currently, retroviral vectors with some improved safety features are extensively used in clinical trials, accounting for 19.7% (370) of all vectors clinically used for gene therapy (http://www.wiley.com//legacy/wileychi/genmed/clinical/).

Lentiviral vectors

Lentiviruses are a subclass of retroviruses with a similar structure, including double-stranded RNA as their genetic material, reverse transcriptase, and vectors with a maximum payload of up to 9 kb. An impor-

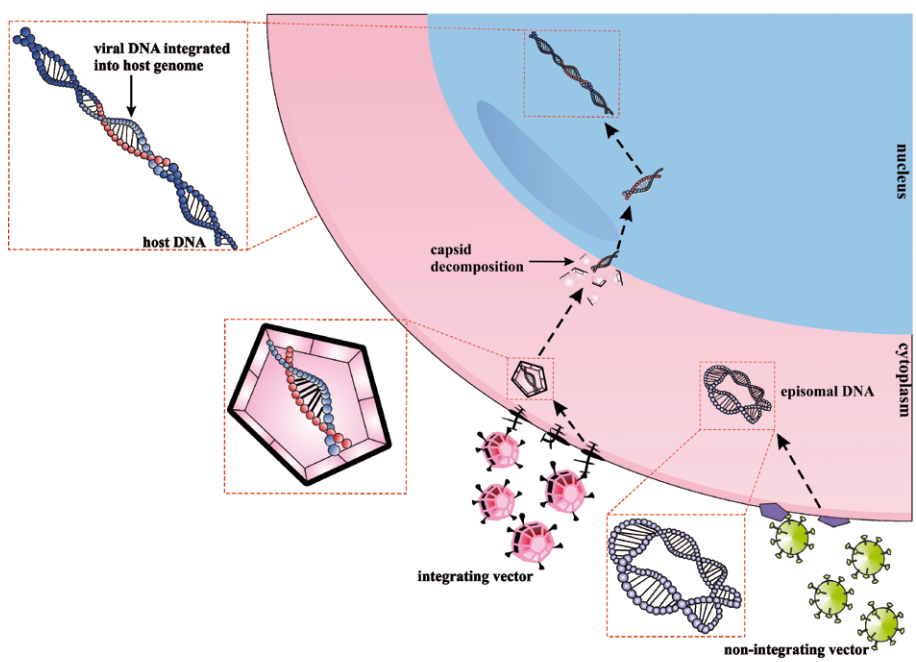

Fig. 1. Viral vectors can be divided into two types: integrating and non-integrating vectors. Most vectors require the presence of specific receptors on the cell surface. After binding to the receptors, the content of the viral capsid is released into the cytoplasm. In the case of integrating vectors, viral DNA translocates into the nucleus and integrates into the host genome. The genetic material of non-integrating vectors, in contrast, remains in the cytoplasm in an episomal form.

tant advantage of the lentiviral vectors is their ability to efficiently infect the non-dividing cells (Naldini et al. 1996). The integration of lentiviruses, similar to retroviruses, is random; however, it has been shown that, with targeted mutations of the integrase gene, it was possible to achieve episomal forms of the vector without the risk of a non-specific integration of the transgene (Apolonia et al. 2007).

The adaptation of lentiviruses as gene delivery vectors, with the minimized risk of viral replication in target cells, was achieved by the deletion of viral structural genes and by placing these genes on separate DNA-helper plasmids. In addition to the structural genes of the virus, helper plasmids may also include modified gene coding envelope proteins responsible for the interaction with cellular surface proteins, such as the vesicular stomatitis glycoprotein (VSV-G) or RD114 (Bell et al. 2010). This enables expanding the variety of potential host cells that can be infected. All helpers, as well as transgene-carrying viral backbone plasmid, are co-transfected into packing cells for the assembly of fully functional, yet replication-deficient virions. The safety of lentiviruses was further improved by the generation of self-inactivating vectors (SIN). SIN were created by Hiroyuki Miyoshi in 1998, based on HIV1 (Miyoshi et al. 1998). SIN were developed by the deletion of viral enhancer and promoter sequences in the U3 region of the 3' LTR sequence. This modulation prevents the replication-competent virus mobilization.

Lentiviral vectors, due to their transduction efficiency toward non-proliferating or slow proliferating cells, have proven to be a particularly useful tool for the engineering of stem cells. It has been shown that, at optimized conditions, a lentiviral transduction does not have a significant detrimental effect on stem cells with an example of rat MSC as their viability or differentiation potential was unaltered (McGinley et al. 2011). A comparative study showed that a single transduction of cynomolgus stem cells by an SIN, carrying the enhanced green fluorescent protein (eGFP) gene, resulted in higher efficacy compared to the retroviral method (Asano et al. 2002). The SIN eGFP expression persisted for months and was not altered by the embryoid body formation (Asano et al. 2002). Human embryonic stem cells (ESC) transfected by SIN carrying a transgene encoding GFP retain green fluorescence following co-culture with S17 mouse stromal feeders and the differentiation into CD34+ cells (Ma et al. 2003). The transduction of human CD34+ progenitors with SIN yielded 20–40% efficiency (Hanazono et al. 2003) (Table I), and was similar to that achieved in rat MSC transduced with GFP (35–40%) (McGinley et al. 2011). It has been shown that the transduction efficiency can be significantly improved by pseudotyping of the lentiviral vectors with the fusiogenic envelope G glycoprotein of the vesicular stomatitis virus (VSV-G), which results in 90–98% of positive cells and long-lasting expression (McGinley et al. 2011). An additional factor that affects the efficiency of lentiviral transduction is the number of passages. For rat MSC, the difference between p1 and p5 was about 8% (McGinley et al. 2011). Another important element with a direct effect on gene expression is the methylation of a transgene promoter. For some vectors, the supplementation with demethylation factors, such as 5-aza-2`-deoxycytidine or Trichostatin A, increased efficacy. The advantage of lentiviruses compared to retroviruses was highlighted in a study on the engineering of CD34+ progenitors. For the retroviral transduction, the standard culture protocol had to be modified with the addition of cytokines to boost proliferative activity. This led to the decline of proliferation capacity, probably due to the differentiation of CD34+ cells and the loss of multipotency (Dunbar et al. 2001). This problem does not exist in the case of lentiviral vectors since they can infect non-dividing cells (Schambach et al. 2013).

Adeno-associated Viruses (AAV)

AAV are small, non-pathogenic, single-stranded DNA viruses. Their nomenclature is due to their dependence on an adenovirus to replicate. AAV integrate specifically into the host genome on chromosome 19. The place of their integration is called AAVS1. After the deletion of the integration target gene Rep68 or Rep78, the DNA of AAV remains in an episomal form. AAV penetrate cells by endocytosis after binding to the integrin $\alpha_v\beta_5$ and FGF4 receptor. AAV can infect a broad spectrum of cells, although the particular serotype is very specific toward selected cell types. This feature makes these vectors very good candidates to use in gene therapy (Wright 2009). The transduction of human bone marrow or umbilical cord blood-derived MSC resulted in high efficiency and the

expression of the transgene was sustained during three months after transplantation into rat brain, without a detrimental effect on their differentiation (Kim et al. 2007). An important advantage of the AAV is their low immunogenicity (Zaiss et al. 2002).

Another subtype of viral vectors developed from adenoviral vectors is the helper-dependent adenoviral vectors. These vectors are characterized by low immunogenicity, and by a very high (up to 100%) transduction efficiency reported for human and mouse ESC, without a negative effect on their pluripotency. Moreover, these vectors enable an efficient (approximately 45%) gene targeting via homologous recombination in ESC (Suzuki et al. 2008).

Non-integrating viral vectors

Adenoviral-vectors (AV)

Adenoviruses are a non-envelope virus family with a double-stranded DNA as genetic material. Adenoviruses do not pose a serious danger for humans and induce only mild upper respiratory infection or food poisoning. The lack of pathogenicity is their great advantage. They are composed of icosahedral capsids and protein fibers situated on their surface. The function of the surface elements is to recognize and bind to membrane receptors of mammalian cells. These receptors belong to the Coxsackie family (CAR). The expression level of the CAR is crucial for the efficacy of cell infection. The integrins are also involved in this process. The viral particles undergo phagocytosis and the virus is internalized and routed to the endosome. The endosomes decompose, and a viral capsid disintegrates and releases adenoviral DNA into the cytoplasm. Adenovirus rarely integrates into the host genome, but rather, usually stays in the cytoplasm in an episomal form (Stadtfeld et al. 2008). This protects the host from the risk of an insertional mutagenesis, but the gene expression is transient. The maximum payload of adenovirus is much larger compared to the previously described types of viruses, and it can reach up to 36 kb (Alba et al. 2005). Human bone marrow-derived MSC and human HSC provide inherently low transduction efficiency for adenoviral vectors, due to the low level of the CAR receptors on their surface (Carson et al. 1999). One possible solution for this problem is to use adenoviral vectors with modified capsid fibers. This technique was developed by Gall and coworkers (1996). The transgene expression in MSC with an inhibited proliferation activity endured for up to 36 days. Similar experiments performed with dividing MSC resulted in a loss of the transgene expression after 21 days (Knaän-Shanzer et al. 2005). It demonstrates that the episomal DNA of an adenoviral vector is gradually lost during cell divisions, and that feature may be an advantage when only a transient expression of a transgene is desired. An improvement in the transduction efficiency of the AV has also been achieved with brief exposure to ultraviolet light (Ito et al. 2004). Reports about the effect on the differentiation capacity of stem cells engineered with AV are inconsistent. Some studies have reported no influence, including the preserved capacity of human MSC to differentiate into adipocytes or osteoblasts (Knaän-Shanzer et al. 2005), and some have reported detrimental effects (Zaldumbide et al. 2012).

A significant disadvantage of the AV is their high immunogenicity (Yang et al. 1994). It has been shown that the infection of host cells by the AV *in vivo* results in the activation of the antigen-presenting cells, CD4+ T-helper cells and CD8+ cytotoxic T cells. This effect is triggered by both the expression of the transgene protein, as well as the presence of a viral capsid protein (Yang et al. 1994). This leads to the degradation of the viral particle and the silencing of transgene expression. However, bone marrow-derived rat MSC transfected *in vitro* by an adenoviral vector, and then transplanted intravenously to the rat, did not cause an inflammatory response (Treacy et al. 2012). Another possible strategy to overcome the immunogenicity problem is to co-transduce adenoviral vectors with a second adenoviral vector carrying the gene for hem oxygenase-1. This enzyme prevents the development of an acute inflammation state through an unknown mechanism (McCarter et al. 2003).

Sendai Virus Vectors (SVV)

Sendai viruses belong to the paramyxviridae family with negative-strand RNA. They cause severe respiratory disease in mice, but are not pathogenic for humans (Lamb and Kolakofsky 2001). Similar to the adenoviruses, they replicate their genome in the cytoplasm without going through the DNA phase, thus precluding the integration and altering of cellular DNA. SVV are characterized by high transduction efficiency, and very rapid onset of expression, with the maximum

reached as early as 24 hours after infection (Hosoya et al. 2008). Due to these important advantages, SVV are being successfully used for cellular reprogramming and the generation of induced pluripotent stem cells (iPS) (Fusaki et al. 2009).

The viral transduction of stem cells is one of the most efficient gene delivery techniques; however, there are unfortunately still many problems associated with the use of viruses. The most important of these problems are high immunogenicity of the vectors and the risk of insertional mutagenesis. The last decade has brought significant progress in this field, with improvements in the safety of viral vectors to a level acceptable for clinical application (Candotti et al. 2012, Schambach et al. 2013). Nevertheless, further research effort is needed to maximize the safety and the therapeutic utility of these tools.

METHODS FOR GENETIC ENGINEERING BASED ON NON-VIRAL METHODS

Successful genetic engineering can be achieved with several non-viral methods. Recently, these methods have gained popularity due to their low immunogenicity (both intracellular and systemic) (Dewey et al. 1999, Gul-Uludag et al. 2012), a nearly unlimited size of the transgene (Raimondi 2011), and well-established, straightforward procedures. However, the disadvantage of these techniques is their relatively low efficiency compared to viral transduction. Based on transfection strategies, the non-viral based methods can be divided into two main groups: physical (Fig. 2) and chemical (Fig. 3). Physical methods include microinjection (Han et al. 2008), electroporation (Gehl 2003), microporation (Ziv et al. 2009), nucleofection (Gresch et al. 2004), magselectofection (Sanchez-Antequera et al. 2011), and sonotransfection (Otani et al. 2009). Chemical methods are based on the application of chemical compounds that facilitate the internalization of a genetic material and include calcium phosphate (Cao et al. 2011), cationic lipids (Cho et al. 2012), cationic polymers (Jeon et al. 2012), cationic peptides (Kim et al. 2010), cationic polysaccharides (Thakor et al. 2011), and inorganic nanomaterials (Kim et al. 2012).

Diversity of genetic material

Among DNA-based constructs, plasmids containing one or more therapeutic genes find widespread use. After transfection into the cell, exogenous plasmid DNA must reach the nucleus to be transcribed, and synthesized mRNA diffuses back to the cytoplasm for translation. The limitation of this approach is a maximum estimated insert size of 20 kb (Lodish et al. 2000).

Another group of DNA constructs are the artificial chromosome technologies. For stem cell transfection, YAC (yeast) and BAC (bacterial) systems have been used (Lamb and Gearhart 1995, Kang and Hebert 2012). The development of new generations of artificial chromosomes, such as MAC (mammalian) and HAC (human), where an artificial chromosome does not integrate into the host genome but is maintained in the nucleus as an extra chromosome, enables almost unlimited possibilities of introducing multiple genes (Lindenbaum et al. 2004, Kazuki and Oshimura 2011).

Another approach is based on the phiC31 integrase, which is a site-specific recombinase catalyzing genomic integration between two DNA recognition sequences: *att*B containing plasmids and a pseudo *att*P site within the host genome, leading to permanent transgene expression (Keravala et al. 2008). This technology was used in trials that explored the engineering of human cardiac stem cells (Lan et al. 2012).

An alternative vehicle for gene delivery is a "Sleeping Beauty" technology (SBTS). This approach is based on the synthetic transposase gene, encoding an enzyme that catalyzes genomic transposon insertion. The transposase gene was reactivated from an evolutionary sleep, hence the name "sleeping beauty," as accumulated mutations had made it inactive. Salmonid fish-derived genetic elements allowed the reactivation of the transcriptional activity of the transposase gene (Ivics et al. 1997). In practice, SBTS requires a vector containing a transposon sequence and a second vector with the transposase gene. The disadvantage of this method is a random genomic integration and the nuclear presence of an active form of the transposase enzyme, which also carries the risk of a native dormant transposon activation, dispersed throughout the host genome. Despite these shortcomings, SBTS has been used as an effective and stable transfection method for HSC (Hollis et al. 2006, Izsvak et al. 2009), and for iPS generation (Muenthaisong et al. 2012). The transposon-based technology advanced with the development of new transposase genes, including the insect-derived transposase piggy BAC, was utilized to gener-

ate iPS (Woltjen et al. 2011), and the medaka-derived Tol2 transposase was applied to the engineering of neural precursors (Yoshida et al. 2010).

In addition to the DNA-based methods, genetic engineering utilizing RNA is highly attractive. To achieve a transient presence of therapeutic proteins in the stem cells, the genes of interest can be transcribed *in vitro* into mRNA and introduced into the cytoplasm of target cells. The exogenous mRNA is already translationally active, and thus, nuclear transport is not required. Another advantage of this method is that exogenous mRNA cannot be integrated into the genome, so there is no risk of an insertional mutagenesis. In addition, due to the high transfection efficiency, it is possible to efficiently transfect cells that are resistant to transfection (Wiehe et al. 2007). However, it must be emphasized that the mRNA transfection is temporary and gradually declines over a period of two-to-three weeks, as mRNA is unstable and vulnerable to intracellular degradation. To circumvent this limitation, new strategies have been employed to stabilize exogenous mRNA molecules in the cytoplasm (Hayashi et al. 2010). However, a short-lived gene expression may actually be an advantage.

In addition to direct gene expression based on the induction of DNA or mRNA, it is possible to apply regulatory RNA sequences to regulate native gene expression (Liu et al. 2012, Snead and Rossi 2012). These short, non-protein-coding RNA sequences, miRNA and siRNA, interact with mRNA molecules, causing the inhibition of their translation and posterior cleavage, which results in silencing of gene expression. The use of this solution permits the silencing of selected genes, which causes some beneficial biological effects (Guzman-Villanueva et al. 2012).

Physical transfection methods

Electroporation

Electroporation is based on the application of an external electrical field to the cells suspended in an adequate buffer. In practice, cells are exposed to a series of short, high electric pulses, which results in a transient destabilization of the cell membrane and the formation of micropores. Thus, molecules present in the extracellular medium enter the cells through the micropores according to the concentration gradient. In the case of DNA delivery, negatively charged DNA molecules enter the cells on the cathode side of the cell and migrate inside the cell body toward the anode (Gehl 2003). Multiple data indicate the utility of this method for the enhancement of stem cell functionality, however, according to other reports electroporation is considered as a method with low transfection efficiency and high cell death (Nakashima et al. 2005, Cao et al. 2010, Lim et al. 2010). On the other hand, it has been shown that electroporation-mediated gene delivery was effective in improving the migration capacity of MSC (Ryser et al. 2008, Park et al. 2011) and in the induction of cellular differentiation into various tissues, such as bone (Ferreira et al. 2012) and carti-

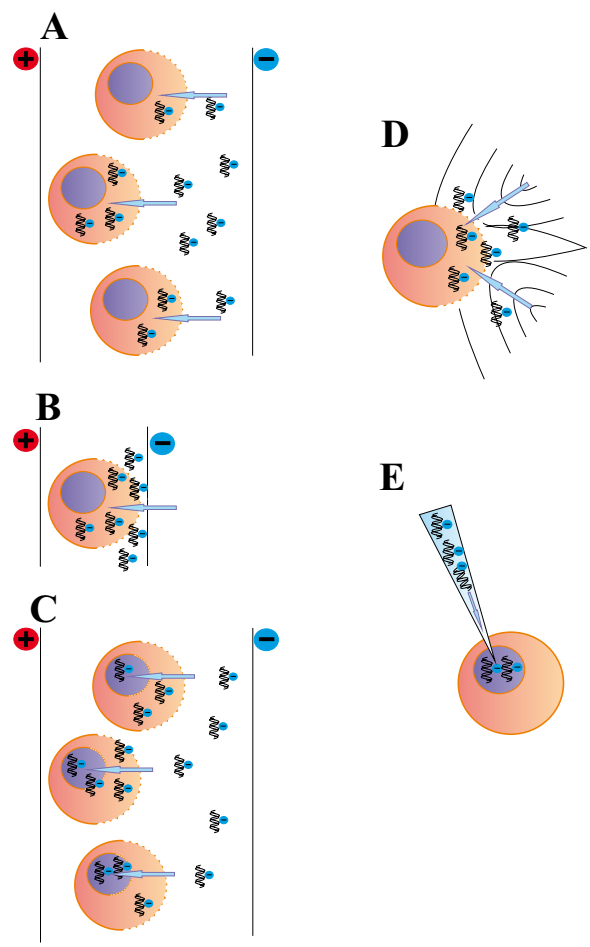

Fig. 2. Schematic representation of physical transfection methods (dark blue – nucleus; light orange – cytoplasm; black strings – genomic material; blue arrows indicate the direction of genetic material migration). (A) electroporation, (B) microporation, (C) nucleofection, (D) sonotransfection, and (E) microinjection.

lage (Kim and Im 2011). In one study, Park et al. used electroporation to introduce a DNA plasmid encoding the C-X-C chemokine receptor type 4 (CXCR4) gene to improve the homing of human MSC cells in glioma patients (Park et al. 2011). A similar strategy has been used to introduce mRNA for the CXCR4 protein into MSC (Ryser et al. 2008).

Microporation

Microporation, a variant of the classic electroporation, is a process that occurs in a small, confined space, allowing the controlled electroporation of single cells (Ziv et al. 2009). This approach was applied to the delivery of eGFP and brain-derived neurotrophic factor (BDNF) DNA constructs to human umbilical cord blood-derived MSC, and it was reported that microporation had the highest efficiency of all the compared transfection methods (Lim et al. 2010).

Nucleofection

Nucleofection is another modification of the electroporation method that is geared to deliver genetic material (DNA or mRNA) directly into the nucleus (Gresch et al. 2004, Flanagan et al. 2011). This method proved effective with many hard-to-transfect cell types, including MSC, resulting in higher transfection efficiency compared to conventional electroporation (Nakashima et al. 2005), and was shown not to alter the properties of MSC (Aluigi et al. 2006). In addition to the DNA-based delivery, nucleofection was also effective for mRNA transfection, thus opening new possibilities for a non-viral, safe, transient, and highly efficient expression of exogenous proteins (Wiehe et al. 2007, 2012). This approach satisfied good manufacturing practice conditions and is very attractive for application in future clinical trials.

Sonotransfection

Sonotransfection utilizes ultrasonic waves, and the principle of this technique is similar to that of electroporation, with a local sound wave introducing the transient cell membrane destabilization with local cavitation. When the cavitation bubbles collapse, the resulting forces lead to interruption of the local cell membranes and the internalization of DNA present in the extracellular environment (Nakashima et al. 2003, Rome et al. 2008). The same technique can be used for transfection with a small interfering RNA (siRNA), as exemplified by the silencing of the phosphatase and tensin homolog in MSC (Otani et al. 2009).

Microinjection

Microinjection is yet another transfection technique that enables the precise delivery of DNA into individual cells. Han et al. compared two techniques for the direct injection of DNA into cells: the classic microinjection method based on a needle of 1 μm diameter, and nanoinjection with a 200 nm diameter nanoneedle. Both techniques effectively delivered DNA into the cell nuclei of MSC (Han et al. 2008).

Chemical transfection methods

Calcium phosphate-based transfection

Calcium phosphate-based transfection uses the co-precipitation of positively charged Ca^{2+} cations and negatively charged DNA (Jordan and Wurm 2004). The modification of this technique with the encapsulation of DNA into calcium phosphate nanoparticles resulted in an improved transfection efficiency with the successful expression of the tumor growth factor beta-1 (TGF-β1) in MSC, leading to enhanced chondrogenesis (Cao et al. 2011).

Cationic lipid-based transfection

Cationic lipid-based transfection is, by far, the most popular chemical transfection technique. Cationic lipid-based transfection is called lipofection and is based on the use of nano-sized lipoplex particles. Lipoplexes can be created by the spontaneous self-assembly of cationic liposomes and DNA (Masotti et al. 2009). Generally, the liposomes contain an amphiphilic cationic lipid linked to a hydrophobic domain *via* a connector. Dioleoylphosphatidylethanolamine (DOPE) and cholesterol are employed as neutral lipids (Ciani et al. 2007, Pozzi et al. 2012). The DNA-lipoplex aggregates bind to the cells by the electrostatic interactions

between the positively charged complexes and the negatively charged cell membrane surface, followed by endocytosis (Ruponen et al. 2001, Rejman et al. 2006). It has been shown that DNA release into the cytoplasm occurs due to the endosome membrane destabilization elicited by the presence of cationic lipids (Simoes et al. 1999). The commercially available lipoplex compound, lipofectamine 2000, was used to introduce the forkhead box A2 (Foxa2) gene to MSC to enhance the regeneration of damaged liver tissue (Cho et al. 2012). Lipofection has also been applied for the transfection of siRNA and miRNA into stem cells, including transfection of miR-181a into MSC for the modulation of cell proliferation, immunosuppressive properties, and myogenic differentiation (Cai et al. 2012, Liu et al. 2012). The efficiency of lipofection-based methods is highly cell-type dependent, and ranges from about 20% for neural stem cells (NSC) (Tinsley et al. 2004) to over 90% for mouse ESC (McLenachan et al. 2007). The efficiency of RNA transfection into MSC is high and oscillates between 80–90% (Rejman et al. 2010, Yu et al. 2012).

Cationic polymers

Cationic polymers, in contrast to the cationic lipids, lack the hydrophobic groups, and thus, are incapable of endosomal membrane destabilization. However, the new generations of cationic polymers, such as polyethylenimine (PEI) and polyamidoamine dendrimers (PAMAM), provide the opportunity for endosomal escape, and result in the cytoplasmic targeting of delivered DNA (Wen et al. 2012). Cationic polymers consist of natural DNA-binding molecules, such as PEI, PAMAM, and Poly(L-lysine) (PLL). PLL and PEI are among the most widely tested compounds for gene delivery. Several reports claim the use of cationic polymers for transgene delivery to MSC. For instance, PAMAM-based transfection is reportedly a tremendous transfection method, with elevated capacity and low cytotoxicity (Santos et al. 2010). The examples of PEI-based transfection for MSC include Bcl-2 gene overexpression in a myocardial infarction model (Li et al. 2007), induced expression of type I interferon IFNα (Krause et al. 2011), or

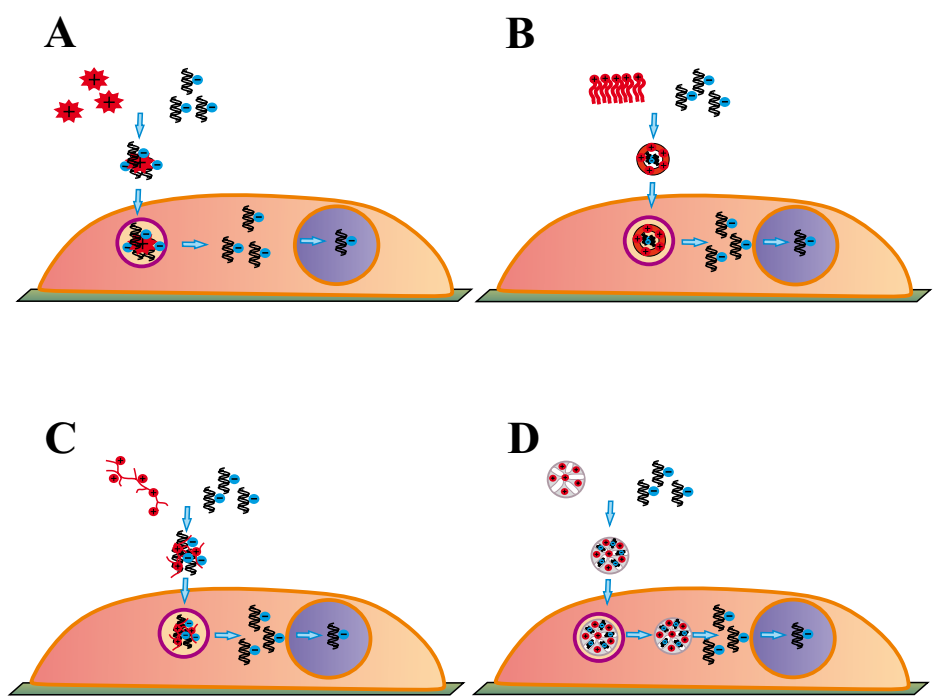

Fig. 3. Schematic representation of chemical transfection methods (dark blue – nucleus; light orange – cytoplasm; purple circles with bright inner – endosomes; black strings – genomic material; blue arrows indicate the direction of genetic material migration). (A) calcium phosphate, (B) cationic lipids, (C) cationic polymers, peptides, and polysaccharides, and (D) inorganic nanomaterials.

the Sox9 gene overexpression to enhance chondrogenesis (Jeon et al. 2012). To further increase the PEI-based transfection efficiency, a photosensitizer-induced gene delivery system was introduced by using the photosensitive compound, Pheophorbide-a. The authors claim that their approach ensures an effective gene delivery through the enhancement of cell membrane permeability, the facilitation of DNA internalization, and ultimately improves endosomal escape, leading to the subsequent enhanced transgene expression (Park and Na 2012). The examples provided by these investigators clearly indicate that cationic polymers are effective transfection factors and should be considered promising tools for the genetic engineering of stem cells.

Cationic peptides

It has been observed that application of basic, arginine-rich peptides results in an improved DNA delivery with low cytotoxicity (Kim et al. 2007). The method was based on the modification of a short arginine peptide by adding the hydrophobic group of a palmitic acid. The peptide incubated with DNA resulted in the formation of complexes, which were readily endocytosed when applied to the *in vitro* cultured cells (Kim et al. 2010).

Cationic polysaccharides

Dextran, a naturally occurring cationic polysaccharide, is one of the most frequently used transfection agents. Spermine-modified dextran is known to interact with DNA chains with high affinity (Bachrach 2005). The endocytosis of spermine-dextran-DNA complexes is thought to occur through the sugar-chain-recognizable cell surface receptors (Jo et al. 2007). This transfection method also proved successful for the internalization of siRNA (Nagane et al. 2010). Another polysaccharide, pullulan, complexed with spermine DNA, efficiently improved MSC transfection, with a toxicity significantly lower than that of lipofection (Thakor et al. 2011).

Inorganic nanomaterials

Recently, it has been shown that inorganic nanomaterials can be used as effective DNA carriers. Mesoporous silica nanoparticles are porous nanostructures, and, due to their opened-channel arrangement, they can be loaded by small molecules (Slowing et al. 2008). Particularly for DNA delivery, the inner tube structure was modified to generate a positively charged amine surface, electrostatically retaining negatively charged DNA molecules. The application of such prepared particles to cell suspensions results in their efficient endocytosis. It has been shown that mesoporous silica effectively shuttled the plasmid-containing bone morphogenetic protein-2 (BMP-2) gene into MSC (Kim et al. 2012).

CLINICAL ASPECTS

Various methods of the gene delivery have been employed in preclinical studies, with DNA being a predominant genetic material. The efficiency of non-viral methods is highly cell-type-dependent, with the best results achieved in immortalized cells, which, due to a loss of therapeutic activity or to the danger of tumor development are not clinically relevant. Primary stem/progenitor cells demonstrate high therapeutic potential; however, they are often resistant to DNA-based genetic engineering.

An important implication for gene therapy is the duration of transgene expression, and, in that respect, there are two strategies for the introduction of transgenes into therapeutic cells for either transient or long-term expression.

The transient expression of a transgene is, in many cases, sufficient to achieve the desired therapeutic goal, and prolonged expression may actually even be undesirable. Traditional methods, such as plasmid DNA transfection, are characterized by low transfection efficiency, prohibiting wide application for primary, highly therapeutic stem/precursor cells. A high efficiency of gene delivery for the primary cells is essential, as, in most cases, the cell engineering directly precedes the stem cell transplantation, and sorting to enrich for positive cells may compromise the cell viability or function. In addition, the genetic engineering methods utilizing DNA are always associated with the risk of an insertional mutagenesis, including aberrant cell function, and, the most feared, latent oncogenesis. Recent advances in mRNA technology, including improved stability and translation efficiency, confirmed that mRNA is a versatile tool for transient gene expression (Kuhn et al. 2012). The mRNA results in a

Table I

The comparison of transfection methods efficiency depending on genetic material and cell type used

Transfection Method	Genetic Material	Efficiency (%)	Cell Type	Reference
Viral vectors				
Lentiviral vectors	RNA	25	ESC	Cao et al. 2010
		20–40	CD 34 + progenitor cells	Hanazono et al. 2003
		35–40	MSC	McGinley et al. 2011
Adeno-associated viruses	DNA	65	MSC	Kim et al. 2007
Helper-dependent adenoviral vectors	DNA	~100	ESC	Suzuki et al. 2008
Adenoviruses	DNA	~100	MSC	Knaän-Shanzer et al. 2005
Non-Viral methods				
Electroporation	DNA	80	MSC	Park et al. 2011
		2	ESC	Cao et al. 2010
	RNA	>90	MSC	Ryser et al. 2008
Microporation	DNA	83	MSC	Lim et al. 2010
Nucleofection	DNA	41	MSC	Nakashima et al. 2005
	RNA	>90	MSC	Wiehe et al. 2012
Sonotransfection	siRNA	47	MSC	Otani et al. 2009
Microinjection	DNA	10	MSC	Han et al. 2008
Nanoinjection	DNA	70	MSC	Han et al. 2008
Lipofection	DNA	20	NSC	Tinsley et al. 2004
		>90	ESC	McLenachan et al. 2007
	RNA	80–90	MSC	Rejman et al. 2010
Cationic polymers	DNA	24	MSC	Krause et al. 2011
Cationic peptides	DNA	12	MSC	Kim et al. 2010
Cationic polysaccharides	DNA	100	MSC	Thakor et al. 2011
Inorganic nanomaterials	DNA	66	MSC	Kim et al. 2012

(ESC) embryonic stem cells; (MSC) mesenchymal stem cells; (NSC) neural stem cells

rapid onset, high gene expression that is relatively uniform across the treated population of cells, with gradual silencing within three weeks. This characteristic seems ideally suited for many clinical applications. Using this approach, therapeutic cells can be thawed, transfected overnight, with a resulting high transgene expression the following day, and, then, are ready for transplantation. This strategy could be used, for example, for the "biological navigation" of stem cell trafficking. Transplanted cells usually need to extravasate or migrate (disperse) throughout the tissue until they reach the desired destination. Thus, such temporary mRNA-based transgene expression could be useful for cell guidance, with the subsequent cessation of expression limiting the risk of interference with cell differentiation. This method would also be useful for rapid-onset diseases, such as stroke or trauma, where the pathological processes are initially very active, with a gradual decrease. In such cases, stem cells could be transiently transfected for the expression of immunomodulatory trophic factors to facilitate tissue healing. Indeed, the mRNA method has been used already in a preclinical study to increase cell migration by the overexpression of the CXCR4 gene (Ryser et al. 2008).

Permanent transgene expression is more difficult to achieve and is potentially less safe; however, in some circumstances, it is still superior to the transient methods. The engineering of stem cells for the stable expression of the transgene is preferable for the slow, progressive degenerative diseases, or in oncology, where the time-frame of cell activity is expected to be very long. Because the safety of these methods is a concern, they are usually considered for diseases with a rather poor prognosis where greater therapy-associated risks are acceptable. For permanent expression, it is feasible and advisable to perform all cell-engineering steps during the process of cell production at the manufacturing site. Following engineering, cells should be carefully evaluated *in vitro* for the stability of transgene expression and for functionality and proliferative properties. Permanent transgene expression is typically accomplished by the integration of a genetic material into the genome using viral transduction. The MMLV-type retrovirus encoding the mycER^{TAM} fusion gene was employed for the introduction of conditional immortalization of neural stem cells (Pollock et al. 2006). This strategy has been applied in the PISCES clinical trial (NCT01151124) to enhance the recovery from stroke (Stroemer et al. 2008, Sinden et al. 2012).

Although retroviruses used for HSC gene therapy in patients has led to leukemia in some patients (Wu et al. 2011), these viruses continue to be used clinically (Candotti et al. 2012). The random integration of genetic material into the genome, including proto-oncogenic sites, is one of the highest risk factors. Thus, significant efforts have been directed to develop new, safer techniques. Recombinant lentiviruses are characterized by several more advantageous features than retroviruses, including a better safety profile, additional built-in safety features (Scaife et al. 2009), and their safe use in several clinical trials (D'Costa et al. 2009). There have also been attempts to use MAC, which have several positive features, including stable episomal maintenance that avoids insertional mutations (Katona et al. 2011), which makes them potentially safer, and MAC have the ability to carry large gene inserts. However, the frequency of a successful MAC introduction is relatively low; thus, multiple cell divisions with sorting is necessary to reach a therapeutic number of cells. Consequently, this technology is of a low value for primary stem cells with limited proliferation capacity, but was shown to be applicable for iPS. This method enabled a complete genetic correction of iPS cells from Duchenne's muscular dystrophy (Kazuki et al. 2010). Another potentially clinically applicable non-viral method is to use transposons, which combines the advantages of viruses and naked DNA. Specifically, the Sleeping Beauty and Pigg Bac transposon systems were developed over the last several years for clinical application (Aronovich et al. 2011, Di Matteo et al. 2012). Recently, the site-specific recombinases, such as Zinc Finger (Ramalingam et al. 2013), TALEN (Sakuma et al. 2013), or phiC31, were introduced as highly efficient methods for the targeted integration of genes into a known chromosomal context, avoiding the risk of proto-oncogene activation (Lan et al. 2012).

CONCLUSION

The present work provides an overview of the various transfection methods applicable to genetic modification of stem cells. Exogenous DNA, mRNA, and/or short regulatory RNA can be delivered to stem cells, resulting in modification that is important from the cell therapy point of view. This cell engineering technology opens up new possibilities for the modulation of stem cells to achieve beneficial clinical effects in the near future.

ACKNOWLEDGMENTS

The study was supported by a National Centre for Research and Development grant No 101 in ERA-NET NEURON project: "MEMS-IRBI".

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