UDC 616-085:577.218+577.218

Claudius Conrad[1], Rashmi Gupta[2], Hanno Niess[1], Karl-Walter Jauch[1], Ralf Huss[3]

GENETIC ENGINEERING AND GENETIC THERAPY

[1]Department of Surgery, Clinic Grosshadern, University of Munich, Munich, Germany;
[2]Max-Planck-Institute for Biochemistry, Martinsried, Germany;
[3]Roche Diagnostics GmbH Pathologic Institute, University of Munich, Munich, Germany

УДК 616-085:577.218+577.218
Клаудіус Конрад[1], Рашмі Гупта[2], Ханно Ніcс[1], Карл-Вальтер Джох[1], Ральф Хус[3]
ГЕННА ІНЖЕНЕРІЯ І ГЕННА ТЕРАПІЯ
[1]Клініка Гроссхадерн, Університет, м. Мюнхен;
[2]Макс-Планк-Інститут біохімії, м. Мартинсрид;
[3]Роше Діагностикс, Інститут патології, Університет, м. Мюнхен

У статті подано огляд різних методів зміни експресії генів і їхньої доставки в дорослі стовбурові клітини. Описано сучасні технології управління генами. Авторами запропоновано можливі шляхи застосування в медицині генетично модифікованих стовбурових клітин як різновиду комбінації клітинної і генної терапії — перспективний напрямок лікування деяких захворювань у майбутньому.
Ключові слова: гени, стовбурові клітини, генна інженерія, генна терапія.

UDC 616-085:577.218+577.218
Claudius Conrad[1], Rashmi Gupta[2], Hanno Niess[1], Karl-Walter Jauch[1], Ralf Huss[3]
GENETIC ENGINEERING AND GENETIC THERAPY
[1]Department of Surgery, Clinic Grosshadern, University of Munich, Munich, Germany;
[2]Max-Planck-Institute for Biochemistry, Martinsried, Germany;
[3]Roche Diagnostics GmbH Pathology Institute, University of Munich, Munich, Germany

The different methods of gene expression modulation and gene delivery in adult stem cells are reviewed in the article. All the latest techniques for genetic manipulation are also described. Possible implications of genetically engineered stem cells in medicine as a form of combined stem cell and gene therapy are proposed by authors to be a promising field for the relieve of the diseases in the future.
Key words: gene, stem cells, genetic engineering, genetic therapy.

INTRODUCTION

Gene therapy is still a novel form of molecular medicine that will have a major impact on human health in the future. The emerging and sophisticated DNA technology has reached new heights since the completion of the Human Genome Project (HGP). The comprehensive knowledge of the entire set of human genes and their chromosomal organisation has particularly entered the diagnostic laboratory, which enables scientists and clinicians to screen for inborn genetic errors and allow genetic counseling of the affected families. The scope and definition of gene therapy has changed and expanded in the past few years. In addition to the possibility of correcting inherited genetic disorders like cystic fibrosis, hemophilia and several others entities, gene therapy approaches are being used also to combat acquired diseases like cancer, AIDS, chronic vascular ischemia, osteoarthritis, diabetes, Parkinson's and Alzheimer's disease.

Nevertheless, gene therapy has entered a very painful adolescence. Although the possible success of this therapy was demonstrated in 22 children suffering from different types' of severe combined immunodeficiency (SCID), three of the children in a French trial developed leukemia and one child died thereafter. It was discussed later that the application of retroviruses to fix broken or incomplete genes may increase the risk of cancer. This might be due to the fact that retroviruses tend to

insert in active genes, perhaps because the condensed chromatin opens up in these chromosomal regions. This is also true for the genetic engineering of stem cells, because the genetic material inserts closely to those genes which are involved in cell proliferation. On the other hand, there are also stories of complete success like in those patients with chronic granulomatous disease (CGD), where the NADPH oxidase activity was almost completely restored after the infusion of genetically altered blood stem cells. But at present, germ line gene therapy is not being contemplated due to the complex technical and ethical issues. Instead, the scientific community is rather interested in pursuing somatic cell gene therapy, which is exclusively for the benefit of one individual and cannot be passed on to the succeeding generation. The minimal requirement for gene therapy is sustained production of the therapeutic gene product without any harmful side effect.

However, efficient use of stem cells in genetic therapy still require intelligent strategies for modulating gene expression and efficient protocols for delivering foreign genes in stem cells.

Modulation of gene expression in stem cells is necessary to precisely control their differentiation into various lineages, maintenance of their undifferentiated state for later transplantation, controlling their proliferation and regulating the secretion of various cytokines and growth factors. Traditionally gene expression modulation has been done through the use of DNA technology which ultimately causes permanent alteration in the genetic makeup of the cells.

Here we review the different methods of gene expression modulation and gene delivery in adult stem cells (Figure). Even with all the latest techniques and means of genetic manipulation, it is still difficult to transfect them at high efficiency and maintain their undifferentiated phenotype at the same time. One more important consideration during gene delivery in stem cells is the safety aspect of the delivery system.

GENE EXPRESSION MODULATION

Conventionally, the gene expression pattern in stem cells is modulated by using recombinant

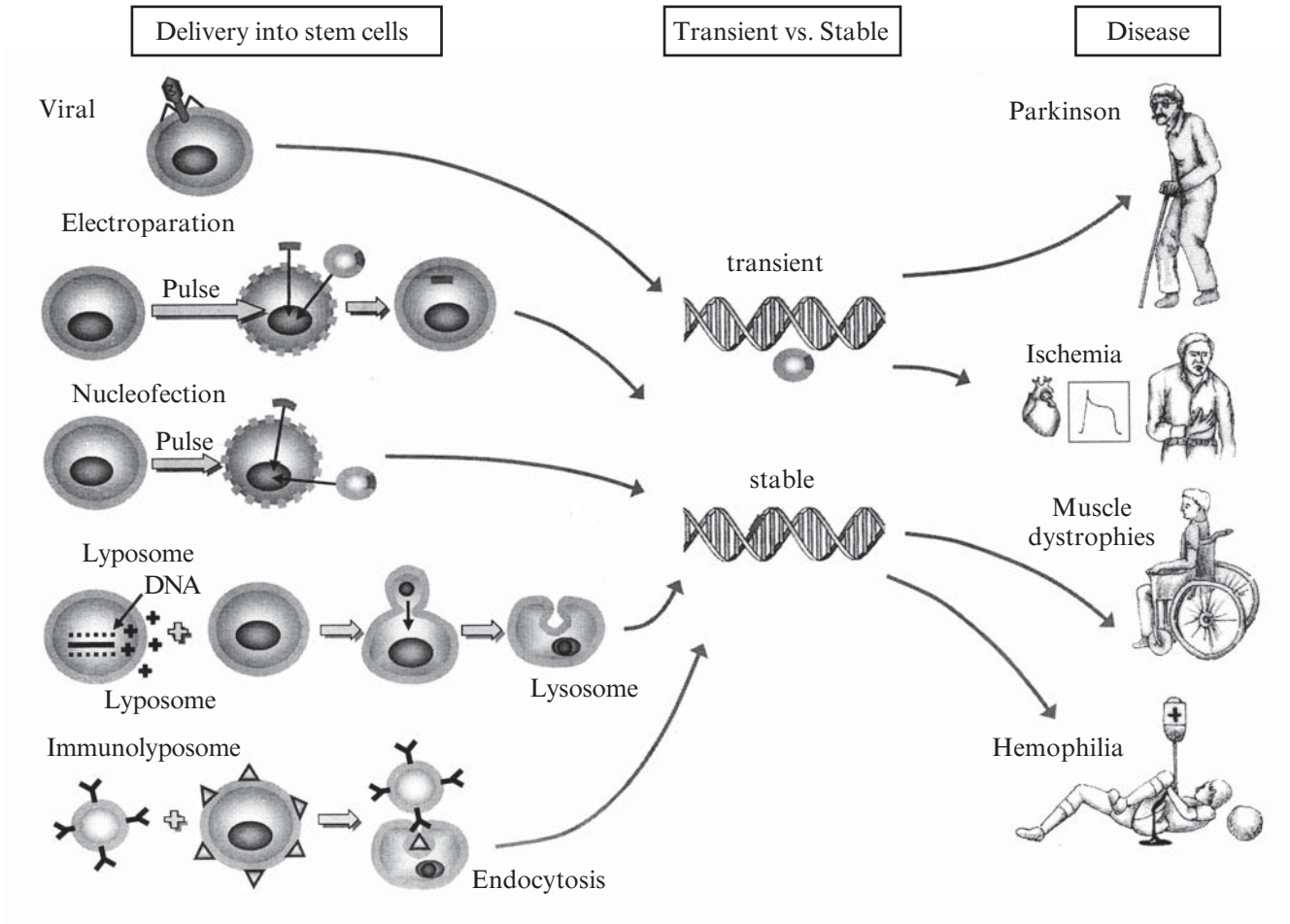

Figure. Different methods of producing genetically engineered stem cells. The stem cells are ideally obtained from the patients to be treated. They include viral gene transfer, electroporation, nucleofection, and gene transfer using liposomes and nucleosomes. The means of transfection are either stable or transient. After returning the modified stem cells to the patient systemically, they home to the relevant niche, where they produce the missing protein or substitute the defect cell type

DNA. But due to overwhelming safety concerns about the use of such genetically modified stem cells in human gene therapy it is essential to look for safer options for controlling expression patterns in stem cells. Apart from the risk of becoming malignant these stem cells also have a tendency of behaving in unpredictable manner upon transplantation in host system.

Some obvious alternatives for recombinant DNA technology would be direct delivery of proteins, RNA and synthetic molecules like peptide nucleic acid (PNA) into the cell. These molecules have the advantage of being short lived in the cytosol and since they are not able to integrate in the host genome they don't alter the genetic makeup of the host.

The most interesting candidates among intracellular proteins that can modify gene expression patterns would be transcription factors. These molecules have a profound affect on the transcription machinery of a cell and hence incorporation of these in stem cells would stimulate the cells to express a defined set of genes (Urnov and Rebar, 2002).

Use of antisense KNA could also block expression of certain genes in stem cells. These small RNA molecules are complimentary to the sequence of a particular mRNA and could therefore form double stranded RNA complex with the mRNA to inhibit its translation by the ribosome.

Other promising RNA candidate in modulation of gene expression is siRNA. These small 21–25 bp long molecules are generated by the cleavage of long double stranded RNA by the dicer enzyme. They are then incorporated into a RNA-induced silencing complex which degrades homologous mRNA molecules in the cell.

Both antisense RNA and siRNA have short half life inside the cytosol. In this respect the synthetic RNA analogs are more effective in modulating gene expression. For example peptide nucleic acids (PNA) are synthetic molecules which have a flexible pseudo-peptide polymer backbone in place of the natural phosphate-sugar backbone. This chimeric structure confers them resistance from intracellular RNAses and proteinases. Since they have nucleobases attached to the pseudo-peptide backbone, they can hybridize with high affinity and specificity to complementary sequences of RNA. This backbone also enables the PNA to be covalently linked to the protein transduction domains facilitating their direct entry into the cells (Mann et al., 2004).

Delivery of proteins, RNA and PNA is a difficult task and requires efficient means of delivery into stem cells.

Gene delivery methods in stem cells can be characterized in many ways like stable versus transient, viral versus non viral and biological means versus physical and chemical means.

VIRAL GENE DELIVERY SYSTEMS

Genetically modified viruses have always been the favorites for delivering genes in stem cells because of their natural ability to insert their genetic material into the host genetic material and also to take over the replication, transcription and translation processes of the host for its own survival.

Lentiviruses

These viruses have been derived from human immunodeficiency vims (HIV-I) by removing the genes necessary for viral replication. This ensures that virus doesn't cause any disease when transfected. Although they are devoid of these genes, they can still integrate with the host chromosomes highly efficiently and lead to stable expression of the delivered gene. Using these viruses is really advantageous because of their ability to infect many different cell types and low cytotoxicity.

Under various promoters reporter genes like GFP have been expressed for long periods in both human stem cells and mouse stem cells without considerable loss in activity (Kosaka et al., 2004).

Adenoviruses

These viruses have also been routinely used for gene delivery in adult stem cells. They have many advantages and disadvantages too. The most important advantage which makes this highly used is that these viruses can be amplified at high titers. They are easy to handle due to their stability in various storing conditions. Like lentiviruses they can also infect wide range of cells in dividing and nondividing state (Benihoud et al., 1999). Adenovirus type 5 (Ad5) has been successfully used in delivering genes in human and mouse stem cells (Smith-Arica et al,, 2003). The disadvantage about adenoviruses is that they are not able to integrate into host cell genetic material and hence the expression of transfected gene is transient not prolonged. Many therapies or experiments do not need stable gene expression, in these cases use of adenoviruses is a good option since there gene expression gets reduced after some time. They are also known to elicit strong immune response upon transfer in host (Bradfute and Goodell, 2003).

Adeno-associated viruses

They are similar to adenoviruses in being able to infect wide range of dividing and nondividing cells but they also have the ability to integrate into the host genome. An important feature of these viruses is that they only integrate at a specific site in human chromosome 19; hence they are not preferred in cases where there is a need of wide spread mutations. Also they have a very limited capacity for foreign inserts due to their bulky genome (Wu and Ataai, 2000).

NON VIRAL GENE DELIVERY SYSTEMS

Physical methods

Electroporation

The cell membrane can be permeabilized by brief, high voltage electric pulses. These pulses create transient pores in the membrane by overcoming its capacitance. Once the membrane is permeabilized various molecules can be delivered into the cell with high efficiency. Most of the small uncharged molecules enter such a cell by simple diffusion process but, large charged molecules like DNA and RNA move into the cell through a process driven by their electrophoretic gradient. The amplitude of the pulse governs the total area that would be permeabilized on the cell surface and the duration of the pulse determines the extent of permeabilization (Gabriel and Teissie, 1997). After entry into the cell, DNA is further processed by the cellular machinery in an ATP dependent manner.

The permeabilized state of the cell depends on the strength of the pulses. Strong pulses can lead to irreversible permeabilization, hence causing irreparable damage to the cell and ultimately cell death. For this reason electroporation is probably the harshest of gene delivery methods. There is also strong evidence that electroporation induced damage is more serious *in vitro* than *in vivo* due to the positive cell-cell interactions and smaller extracellular volume in tissues.

The effectiveness of this method depends on many crucial factors like the size of the cell and temperature during the application of pulse (Rols and Teissie, 1990).

Nucleofection

This is one of the most highly efficient non viral methods of gene transfer. It is based on the previously knovm method of electroporation but it is a lot more efficient than that. The most advantageous feature of this technique is that DNA can be transferred directly into the nucleus increasing its likelihood of getting integrated in the host genome and hence even cells that are difficult to transfect can be stably transfected (Aluigi et al., 2005; Zemecke et al, 2003).

Biomolecules based methods

Protein transduction domains (PTD)

These are short peptides that are capable of entering into the cell without the requirement of endocytotic pathway and protein channels. The actual mechanism of their entry into the cell is not well understood but it occurs even at low temperature (Heng and Tong, 2005; Derossi et al., 1996.)

The two most commonly use naturally occurring PTDs are the TAT transactivator domain of human immunodeficiency virus and the homeodomain of *Antennapedia* transcription factor. Apart from these naturally occurring PTDs there are a number of artificial peptides that have the ability spontaneously crossing the cell memebrane (Joliot and Prochiantz, 2004).

Since these are peptides they can be covalently linked to the pseudo-peptide backbone ofPNA and deliver them into the cell.

Liposomes

Liposomes have been used in delivering genes, drugs, reporter proteins and other molecules into the cell since a long time. Liposomes resemble the cell membrane in the fact that they are also double membrane compartments surrounding aqueous environment but they are much simpler than the cell. When lipid molecules are agitated with water they spontaneously form spherical structures called liposomes surrounding an aqueous centre. Many hydrophilic molecules can beincorporated in them during their formation. For example when lipids with positively charged head group are mixed with a solution of recombinant DNA they can form lipoplexes in which negatively charged DNA is complexed with the positive head groups of lipid molecules. These complexes can then enter the cell through the endocytotic pathway and deliver the DNA into lysosomal compartment. The DNA molecules then escape this compartment with the help of dioleoylethanolamine (DOPE) and gain entry into the nucleus where they can be transcribed (Tranchant et al, 2004).

Despite their simplicity liposomes suffer from low efficiency of transfection because they are not always able to enter the cells by the endocytotic pathway. And if they are able to enter the cell then later in the pathway the DNA may not be able to escape from the endosome and hence be degraded by the efficient nucleases there.

Immunoliposomes

These are liposomes with antibodies inserted in their membrane. These antibodies are against the surface molecules of the target cell. Apart from providing specificity to gene delivery, these antibodies also provide a protective covering to the liposomes and hence prevent degradation by the RNAses and proteinases (Bendas, 2001).

STEM CELLS AS GENE VEHICLES

The *in-vivo* application of viral particles or nonviral nucleic acids still implies the risk of an uncontrolled integration of genetic information into unselected tissues, which bears an unpredictable risk of the disruption of genes, promoter or enhancer

organization. There are currently two ways to control this high-potential risks: (a) the use of the phage integrase system and (b) the transfection of stem cells as vehicles for genes (Ginsburg and Calos, 2005). Such an approach can be used for the treatment of a variety of degenerative disorders, but also as an anti-cancer therapy.

IMPLICATIONS IN MEDICINE

Stem cells have the ability to differentiate into cells, tissues and organs and due to their potent homing mechanisms they have the ability to deliver genes or proteins to sites of interest. Transfected stem cells can be used as a mechanism to transport selectively genes to areas of defects, thereby releasing the product of the transfection only where it is needed. Diseases, where genetically engineered stem cells might play a role in future are diseases where a protein or an entire enzyme is missing or not functioning or where certain factors are needed for improved function in that area. There have been phase I and II studies been conducted already in order to treat cancer, neurodegenerative disorders like Parkinson's disease or Alzheimer's disease, ischemic disease of the brain and the heart and infectious diseases. Especially Alzheimer's disease and spinal cord injury have been studied for the used of genetically engineered stem cells. The Aizheimer's is a disease where neurons in the central nervous system undergo widespread degeneration. Especially cholinergic neurons in the basal forebrain degenerate. Although the direct injection of nerve growth factor in the ventricles led to disappointing results the delivery of nerve growth factor to the brain using stem cells seems a more promising approach. There has been data showing a beneficial effect of transfected stem cells in injured axons in experimental spinal cord models. In some instances partial function was recovered through this combined stem cell gene therapy (Grill etal., 1997).

These data indicate that genetically engineered stem cells as a form of combined stem cell and gene therapy are a promising field for the relieve of diseases in the future.

REFERENCES

1. *Urnov F. D., Rebar E. J.* Designed transcription factors as tools for therapeutics and functional genomics // Biochem Pharmacol. — 2002. — Sep. — Vol. 64 (5-6). — P. 919-923.

2. *Marin V. L., Roy S., Armitage B. A.* Recent advances in the development of peptide nucleic acid as a gene-targeted drug // Expert Opin Biol Ther. — 2004. — Mar. — Vol. 4 (3). — P. 337-348.

3. *Lentivirus-based* gene delivery in mouse embryonic stem cells / Y. Kosaka, N. Kobayashi, T. Fukazawa et al. // Artif. Organs — 2004. — Mar. — Vol. 28 (3). — P. 271-277.

4. *Bradfute S. B., Goodell M. A.* Adenoviral transduction of mouse hematopoietic stem cells // Mol. Ther. — 2003. — Mar. — Vol. 7 (3). — P. 334-340.

5. *Wu N., Ataai M. M.* Production of viral vectors for gene therapy applications // Curr Opin Biotechnol. — 2000. — Apr. — Vol. 11 (2). — P. 205-208.

6. *Electropermeabilization* of mammalian cells to macromolecules: control by pulse duration // Biophys J. — 1998. — Sep. — Vol. 75 (3). — P. 1415-1423.

7. *Nucleofection* is an efficient non-viral transfection technique for human bone marrow-derived mesenchymal stem cells / M. P. Rols, J. Teissie, M. Aluigi et al. // Stem Cells. — 2005. — Aug. — Vol. 11 [Epub ahead of print].

8. *Suppression* of endothelial adhesion molecule up-regulation with cyclopentenone prostaglandins is dissociated from IkappaB-alpha kinase inhibition and cell death induction / A. Zernecke, W. Erl, L. Fraemohs et al. // FASEB J. — 2003. — Jun. — Vol. 17 (9). — P. 1099-1101 [Epub 2003 Apr 8].

9. *Potential* utility of cell-permeable transcription factors to direct stem cell differentiation / B. C. Heng, T. Cao, G. Q. Tong, S. C. Ng // Stem Cells Dev. — 2004. — Oct. — Vol. 13 (5). — P. 460-462.

10. *Cell* intemalization of the third helix of the Antennapedia homeodomain is receptor-independent / D. Derossi, S. Calvet, A. J. Trembleau et al. // Biol Chem. — 1996. — Jul. 26. — Vol. 271 (30). — P. 18188-18193.

11. *Joliot A., Prochiantz A.* Transduction peptides: from technology to physiology // Nat Cell Biol. — 2004. — Mar. — Vol. 6 (3). — P. 189-196.

12. *DNA* complexing lipopolythiourea / I. Tranchant, N. Mignet, E. Crozatet et al. // Bioconjug Chem. — 2004. — Nov-Dec. — Vol. 15 (6). — P. 1342-1348.

13. *Bendas G.* Immunoliposomes: a promising approach to targeting cancer therapy // BioDrugs. — 2001. — Vol. 15 (4). — P. 215-224.

14. *Grill R. J., Blesch A., Tuszynski M. H.* Robust growth of chronically injured spinal cord axons induced by grafts of genetically modified NGF-secreting cells // Exp Neurol. — 1997. — Dec. — Vol. 148 (2). — P. 444-452.

15. *Ginsburg D. S., Calos M. P.* Site-specific integration with phiC31 integrase for prolonged expression of therapeutic genes // Adv Genet. — 2005. — Vol. 54. — P. 179-187.