

Gene Therapy

General overview

Rapid development of molecular medicine has led to molecular diagnosis of human diseases, like foreign nucleic acids presented in the cell (infections), somatic mutations (carcinogenesis) and germline mutations (inherited diseases). The extensively growing theoretical knowledge (e.g. the knowledge produced through the Human Genome Project) made characterization and treatment of the diseases possible at molecular level.

During the past few decades these therapeutic approaches that we call molecular therapy or gene therapy have gone through massive development. However, it should be borne in mind that gene therapy is still experimental. The first promises were made twenty years ago (in comparison, bone marrow transplantation began in 1958 and by the early 1970s, the survival rate was only around 1 percent). The trials are mainly carried out on cases in which the severity of the disease exceeds the risk of therapy.

The website at <http://www.clinicaltrials.gov/> provides information about current ongoing clinical research studies.

For ethical reasons, patients treated with gene therapy received also conventional treatment. This made and makes it difficult to determine the effectiveness of gene therapy on its own.

The aim of gene therapy is to replace, modify, or knock out the defective, disease-causing gene by introducing therapeutic *nucleic acids* into the target cells. The nucleic acids are mostly inserted into a *vector* that helps the molecules enter the cells. The vector is transferred either *in vivo* or *ex vivo*. In the *ex vivo* approach the patients cells are modified in the laboratory then delivered to the target tissue (autologous transplant.) Vectors can be divided into two main categories: viral and non-viral. At the moment, 90% of clinical trials use viral vectors. Generally, integration of the DNA into the genome is called insertion while virus-mediated DNA transfer is called transduction. Depending on the vector of our choice the therapeutic DNA molecule is either integrated into the genome (and inherited by the progeny cells) or stays in extrachromosomal form. A *human artificial chromosome* (HAC) also can be created that operates as a new chromosome providing safety and long-term expression. However, these micro-chromosomes are technically challenging to construct and difficult to deliver to target cells.

Gene addition is the most commonly accepted clinical approach; the missing/mutant protein is supplied by the expression of the normal gene. Initial targets for gene therapy were recessive monogenic loss-of-function diseases. These diseases are fatal unless the missing protein is supplied.

Gene modification and *knock out* of defective genes are technically much more challenging. Much progress has been made in this area by the discovery of the zinc finger endonucleases (ZFNs). ZFNs are modified transcription factors designed to cut at specific DNA sequences followed by recombination.

Their development is continuous (<http://www.zincfingers.org/>).

By introducing exogenous nucleic acids into eukaryotic cells regulation of gene expression and modification of protein function is also possible. In these therapies the genetic material of the cell remains unchanged. The 2006 Nobel Prize in Medicine

was awarded for the “discovery that double-stranded RNA triggers suppression of gene activity in a homology-dependent manner, a process named *RNA interference* (RNAi).” RNA interference is mediated by micro-RNA. These short (19-23 nucleotide) RNA molecules mediate numerous physiological processes (ontogenesis, proliferation, apoptosis, differentiation) of sequence-specific degradation of mRNA molecules in the cell.

Micro-RNA expression or function is significantly altered in many diseases including cancer. RNA interference is also an important experimental tool that makes possible the silencing of any gene. The human diagnostic and therapeutic applications range is growing as well. Small interfering RNA transfected into the cell or expressed inside the cell target complementary mRNAs leading to gene silencing by sequence-specific endonucleolytic RNA degradation. *RNA aptamers* have specific and stable three-dimensional structure that allows them to bind to specific targets. Aptamers that target cell surface receptors transfer aptamer-bound molecules into the cells. Cargo molecules may include anti-tumor drug, enzyme, or siRNA. *Antisense oligonucleotides* are complementary to the sense mRNA chain. Their binding prevents the translation of the mutant mRNA. Antisense drugs (antiviral, cholesterol-lowering medications) have been approved for clinical application. Similarly to antisense drugs *ribozymes* specifically bind and eventually degrade target mRNA molecules. Dominant negative mutations have a gene product that acts antagonistically to the wild-type protein. The mutant protein coding RNA molecules can be degraded by the above described methods.

Technical difficulties

Over the past 10 years, vector systems were refined, production methods were improved and greater safety was achieved. Ideally the locally or systematically administered vectors reach exclusively the target cells (cell-specific delivery) during in vivo therapy. In order to achieve this, non-viral vectors with specific tags and viral vectors with re-engineered capsids or envelope proteins were designed. The vectors might have to cross the endothelial barrier and have to enter the nucleus. In the nucleus vectors integrate into the host chromosome or stay extrachromosomal. In dividing cells, extrachromosomal vector becomes rapidly diluted while the integrated vectors are inherited by the progeny. Their disadvantage is that the generally random integration might lead to insertional mutagenesis. The optional duration of the therapeutic gene expression depends on the type of disease: temporary for acquired diseases (infection, tumor) or life-long for genetic disorders.

One of the most significant barriers to successful gene therapy is that virus-based vectors evoke an immune response especially those serotypes that most adults have already met during their life.

Vectors used in gene therapy and related clinical trials

1. Viral vectors

1.1 Retroviral vectors

The advantage of retroviral vector is that the coding region of the provirus is easily replaceable by the therapeutic gene. They infect cells at high efficiency and they integrate a copy of their genome (provirus) into the host cell's genome.

The maximum size of the insert is 8-10 kilobase (kb).

Their disadvantage is that their insertion results in an alteration of the chromosomal DNA and might lead to insertional mutagenesis and tumor formation. Their infectivity is mainly limited to dividing cells. Retroviral vectors were the first choice in human gene therapy trials (*Adenosine deaminase deficiency (ADA)*) and in the first successful gene therapy treatment (*Severe combined immunodeficiency (SCID)*). Leukocytes as part of the immune system undergo differentiation that can be disturbed on several levels resulting in SCID. One form of SCID is caused by mutation in the adenosine deaminase gene, the protein product of which is involved in nucleotide metabolism. If left untreated, the disease causes death within two years. In 1990 a 4-year-old girl was not eligible for bone marrow transplantation because of the lack of suitable donor. After informed consent *ex vivo* gene therapy was performed. The patient's bone marrow cells were transduced with functional ADA gene carrying retrovirus and returned to the patient (*autologous bone marrow transplantation*). The patient recovered well and is living a normal life until the present day. However the success of gene therapy was not unequivocal. A new enzyme replacement therapy has been developed (ADA-PEG) which was given in parallel for ethical reasons. This made both the judgment of efficacy of treatment and the treatment itself difficult since the cells containing the functional genes had no growth advantage.

Mutations in a cell-surface receptor gene that mediates the differentiation effects of the cytokines result in other form of SCID. *Ex vivo* retroviral gene transfer was successful in correcting a form of SCID caused by defective cytokine receptors (in particular a subunit called γc), as reported in 2000. Immune system function was restored and the quality of life of the treated children was normal providing confirmation that human gene therapy could cure a serious genetic disease. However, later one-fifth of patients developed leukemia as a result of insertional mutagenesis by the retroviral vector. This setback put forward the safety reconsideration of this therapeutic modality and necessitated further development of gene therapy vectors.

Human gene therapy is not limited to single gene disorders (like cystic fibrosis or Duchenne muscular dystrophy), but carries the promise of cures for complex genetic diseases; heart disease, AIDS and cancer. As an example, autologous cytotoxic T lymphocytes transduced with tumor-specific antigens caused tumor remission in clinical trials.

1.2 Lentiviral vectors

The Lentivirus subgroup of retroviruses (e.g. HIV) is able to transduce non-dividing cells as well. In order to achieve better safety self-inactivating lentiviruses were constructed where an enhancer element was deleted. Hematopoietic cells from thalassemia patients were *ex vivo* transduced by β -globin expressing lentivirus. As a result, the patients became transfusion independent.

In clinical trials with HIV-positive patients, researchers found that HIV-based lentiviral vector was a safe and potentially effective choice. Non-coding RNA molecules expression vector inhibited HIV receptor expression and HIV replication.

1.3 Adenoviral vectors

Adenoviral vectors can accommodate very large inserts of DNA (30 kb) and infect a broad range of mammalian cells both replicative and non-replicative. The vector remains episomal (extrachromosomal) therefore, no risks of insertional mutagenesis but their effect is transient in rapidly dividing cells. In quiescent cells therapeutic levels of plasma proteins have been achieved (e.g. coagulation factors, α_1 -antitrypsin, erythropoietin and metabolic enzymes). Clinical trials on diabetes

treatment efficiently reprogrammed β cells and eliminated hyperglycemia by transcription factors. Oncolytic adenoviruses, which replicate preferentially in tumor cells were designed, but these trials are controversial so far.

Systemic adenovirus treatment might evoke the immune system's response which causes serious toxicity. Attempts are underway to modify the serotype of adenoviruses which can reduce the immune response, but the case of adenovirus is technically more difficult compared to retroviruses.

Massive immune response triggered by the therapeutic adenovirus led to the first fatal complication in gene therapy in 1999. The patient suffered in ornithine transcarbamoylase (OTC, a key enzymes in the urea cycle) deficiency.

1.4 Adeno-associated viruses (AAV)

Among the numerous advantages of AAV they are easily produced at high titers, they transduce both dividing and non-dividing cells, but don't trigger immune response and integrate into the genome. Their integration is somewhat more predictable than retroviruses; they insert their genes into a specific site on the 19th chromosome. However, the vector packaging capacity is limited (5 kb). The virus was used in several experiments that affected gene regulation where small insert is not a disadvantage. Virus encoded RNA molecules successfully induced exon skipping to restore the correct reading frame in Duchenne muscular dystrophy (DMD). DMD is caused by mutation of dystrophin, the largest known human gene. In tumor therapies, oncogene mRNAs were degraded by AAV expressed siRNA molecules.

Gene therapy treatment has successfully halted an inherited form of blindness; in Leber's congenital amaurosis subretinal infusion of the normal gene proved to be a promising and safe treatment. All patients involved in the clinical trial had improved vision. The effect was age-dependent, younger eyes showed a more marked improvement.

Current trials use skeletal muscle as „protein factory” that produce lipoprotein lipase (LPL), α_1 -antitrypsin and blood coagulation factors.

In 2010 the manufacturer sought permission to market Glybera an AAV-derived LPL expression vector. (Note of 2012: Following the meeting of the EU Member States Standing Committee on human medicinal products (CHMP) on 23 January 2012, the European Commission asked the Agency to re-evaluate the application for Glybera in a restricted group of patients with severe or multiple pancreatitis attacks. This was first considered in April 2012. Following detailed scientific discussions in both committees, the Committee for Advanced Therapies adopted a positive draft opinion in June 2012, which has now been endorsed by the CHMP. The CHMP's opinion on Glybera will now be sent to the European Commission for the adoption of a marketing authorisation.) Clinical trials have proved positive effects of Glybera including prevention of life-threatening complications as pancreatitis even after single dose administration.

In neurological diseases as Parkinson disease transduction of AAVs into neurons has changed the production of neurotransmitter (dopamine) synthesizing enzymes; whereas growth factor expression inhibited neurodegeneration.

The development of advanced imaging techniques like Real-time Magnetic resonance imaging made possible directed, even cell type-dependent transduction of the therapeutic gene.

2. Non-viral vectors

Non-viral vectors offer several advantages like almost no immunogenicity and flexibility towards the size of the therapeutic DNA that they can deliver. Once the DNA enters the cytoplasm (mostly through endocytosis), it forms complexes with proteins that influence and regulate its intracellular transport. The main barrier that must be overcome by the non-viral vectors is the nuclear membrane. Investigations to improve the efficiency of the transfer into the nucleus through peptides, native or artificial proteins or transcription factor binding sites are ongoing. Large numbers of biodegradable polymers have been created that form nanoparticles when mixed with DNA. As a result of developments the disadvantage of the non-viral vector that the efficiency is lower than the efficiency of viral vectors was overcome in certain cases. Although vectors are mainly episomal, experiments have been carried out where directed gene insertion is possible by modified transposons (e.g. Sleeping Beauty transposon).

The future of gene therapy

We are in the era of personalized medicine where the cost of whole genome sequencing is decreasing rapidly and it will become a routine medical test. Genetic research is progressing in parallel with stem cell research. Gene therapy combined with stem cell therapy is expected to be among future therapies. For instance in chronic form of viral hepatitis (HBV, HCV) that require liver transplant hepatocytes might be derived from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Unfortunately the risk of transplanted organ reinfection is an issue. This can be addressed through gene transfer of a vector encoding siRNA directed against the virus.

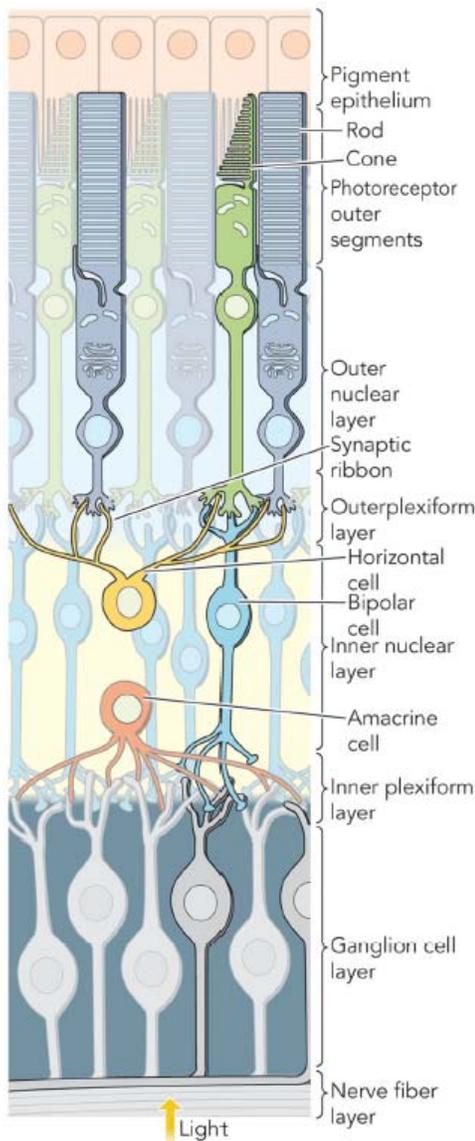
Appendix: Case Report

Leber Congenital Amaurosis 2 (<http://omim.org/entry/204100>)

Background information

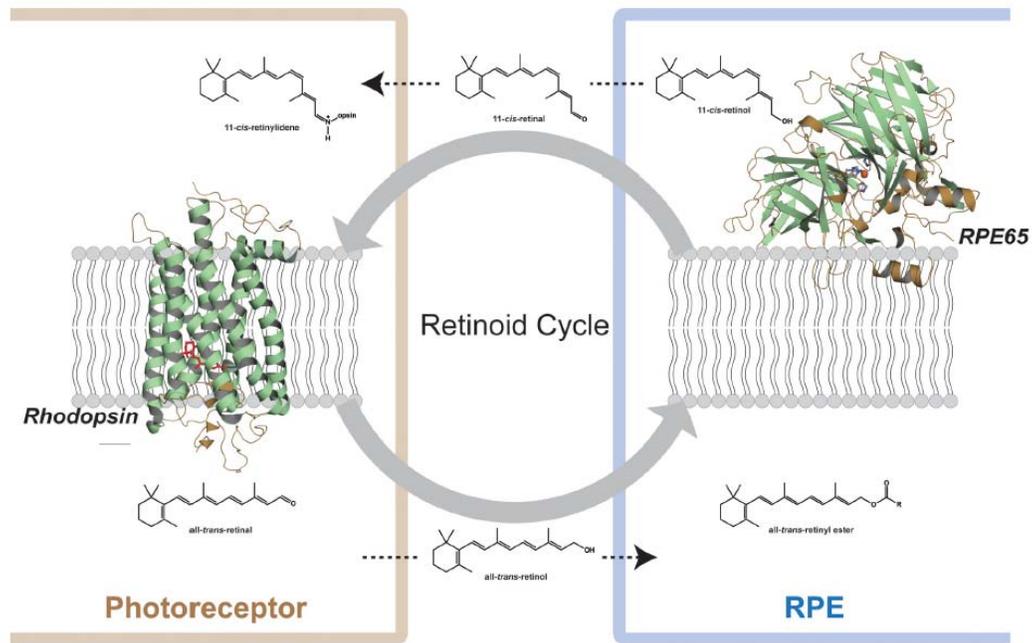
The Chemistry of vision and Retinal Pigment Epithelium Specific Protein, 65 KD, RPE65 (<http://omim.org/entry/180069>)

The RPE65 protein is the source of isomerohydrolase activity (conversion of all-trans retinyl ester to 11-cis retinol) in the retinal pigment epithelium (RPE). The RPE is a monolayer simple epithelium apposed to the outer surface of the retinal photoreceptor cells (see figure below). It is involved in many aspects of outer retinal metabolism that are essential to the continued maintenance of the photoreceptor cells, including many RPE-specific functions such as the retinoid visual cycle and photoreceptor outer segment disc phagocytosis and recycling.



Cellular morphology of the retina (K. Palczewski, J Biol Chem 2012; 287: 1616-)

In photoreceptor cells, 11-*cis*-retinal is bound to opsin, forming rhodopsin. Absorption of a photon of light by rhodopsin causes photoisomerization of 11-*cis*-retinal to all-*trans*-retinal and productive signaling, eventually leading to release of all-*trans*-retinal from the chromophore-binding pocket of this opsin. All-*trans*-retinal is reduced to all-*trans*-retinol in a reaction catalyzed by NADPH-dependent all-*trans*-retinol dehydrogenases. Then, all-*trans*-retinol must diffuse into the adjacent RPE cell layer. This process is enabled by esterification of retinol with fatty acids in a reaction catalyzed by lecithin:retinol acyltransferase. In the RPE, these all-*trans*-retinyl esters tend to form intracellular storage structures called retinosomes. These esters serve as substrates for the RPE65 retinoid isomerase, which converts them to 11-*cis*-retinol, which is further oxidized back to 11-*cis*-retinal by retinol dehydrogenases. 11-*cis*-Retinal formed in the RPE diffuses back into the photoreceptor, because this reaction is virtually irreversible. This last step also completes the cycle by recombining 11-*cis*-retinal with opsin to form rhodopsin. The chemical cycle of visual pigment is summarized in the figure below.



The chemical cycle of visual pigment (K. Palczewski, J Biol Chem 2012; 287: 1616-)

Autosomal recessive childhood-onset severe retinal dystrophy is a heterogeneous group of disorders affecting rod and cone photoreceptors simultaneously. The most severe cases are termed Leber congenital amaurosis, whereas the less aggressive forms are usually considered juvenile retinitis pigmentosa. Leber congenital amaurosis comprises a group of early-onset childhood retinal dystrophies characterized by vision loss, nystagmus, and severe retinal dysfunction. LCA2 is distinguished by moderate visual impairment at infancy that progresses to total blindness by mid to late adulthood. One of the unique qualities of LCA2 is that, even with profound early visual impairment, retinal cells are relatively preserved in the early stages of the disease. The molecular background of the disease is RPE65 deficiency.

Gene Therapy of LCA2

The gene therapy of LCA2 is the success story of this therapeutic approach and the only one that has already entered its Phase III clinical trial (<http://clinicaltrials.gov/ct2/show/NCT00999609>, start day August 2012, end day December 2014, completion December 2028). The successful approach was developed by Katherine High's és Jean Bennett's team (<http://www.ncbi.nlm.nih.gov/pubmed/19854499>). They assessed the retinal and visual function in 12 patients aged 8 to 44 years with RPE65-associated Leber congenital amaurosis who had received 1 subretinal injection of AAV containing the RPE65 gene in the worse eye at low (1.5×10^{10} vector genomes), medium (4.8×10^{10} vector genomes), or high dose (1.5×10^{11} vector genomes) for up to 2 years. Patients had at least 2 log unit increase in pupillary light responses, and an 8-year-old child had nearly the same level of light sensitivity as that in age-matched normal-sighted individuals. The greatest improvement was noted in children, all of whom gained ambulatory vision (http://www.hhmi.org/bulletin/aug2011/features/gene_therapy_high2.html). Gene

therapy was well tolerated and all patients showed sustained improvement in subjective and objective measurements of vision.