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Kidney regeneration in vivo

Gene based therapies for kidney regeneration

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ABSTRACT

In this review we provide an overview of the expanding molecular toolbox that is available for gene based therapies and how these therapies can be used for a large variety of kidney diseases. Gene based therapies range from restoring gene function in genetic kidney diseases to steering complex molecular pathways in chronic kidney disorders, and can provide a treatment or cure for diseases that otherwise may not be targeted. This approach involves the delivery of recombinant DNA sequences harboring therapeutic genes to improve cell function and thereby promote kidney regeneration. Depending on the therapy, the recombinant DNA will express a gene that directly plays a role in the function of the cell (gene addition), that regulates the expression of an endogenous gene (gene regulation), or that even changes the DNA sequence of endogenous genes (gene editing). Some interventions involve permanent changes in the genome whereas others are only temporary and leave no trace. Efficient and safe delivery are important steps for all gene based therapies and also depend on the mode of action of the therapeutic gene. Here we provide examples on how the different methods can be used to treat various diseases, which technologies are now emerging (such as gene repair through CRISPR/Cas9) and what the opportunities, perspectives, potential and the limitations of these therapies are for the treatment of kidney diseases.

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1. Introduction

All the cells in our body fulfill a particular function, express numerous genes and respond in a different way to their environment. In a healthy situation, cells will adequately react to changes in oxygen, temperature, pH, metabolites, hormones, cytokines, pressure and more. However, in case of a genetic defect or in a chronic kidney disease, some of these pathways will be affected and can result in loss of cell function or cell death. When the genes or molecular pathways in these processes are known, gene based therapies can be used to target the defective pathway on a RNA, DNA or epigenetic level to restore cell function. With gene based therapies we refer to all therapies in which recombinant DNA is delivered to a cell to produce a therapeutic protein or RNA sequence. In this way, gene based therapies can be used to supplement a missing gene, inhibit a gene from being translated into a protein, change splicing of a specific gene, permanently repair or even delete a genetic sequence.

The hallmark of genetic therapies is that it requires knowledge of the mechanism underlying the disease. For genetic kidney diseases the most important step is identification of the affected gene, which is greatly facilitated by the availability of fast and cost effective whole genome sequencing techniques. The greater our knowledge on disease mechanisms, the more pathologies will become realistic targets for gene therapy. Currently, clinical trials are being conducted using gene based therapies in a wide variety of diseases which can be categorized in four main groups: monogenetic diseases, infectious diseases, cardiovascular diseases and cancer www.abedia.com/wiley and (Ginn et al., 2013). Anti-cancer therapies (Sterman et al., 2016) represent the biggest group and here gene therapy is used to either directly damage the cancer cells, empower the immune system to induce a specific immune response against the tumor, or to protect sensitive tissues from high doses of chemotherapy (Salem et al., 2015). Monogenetic diseases are the second biggest disease category targeted by gene therapy. Here, cDNA of the affected gene is transiently or stably introduced into cells to restore cell function and halt disease progression. A new tool that has recently become available and may prove very valuable for the treatment of both dominant and recessive genetic diseases is nuclease induced gene repair. To battle infectious diseases and to reduce chronic inflammation in cardiovascular diseases, recombinant therapeutic proteins are

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produced by liver or muscle cells and released in the bloodstream of patients.

In nephrology, therapies may be directed to a defect that directly affects the kidney cells, to target the production of toxic metabolites produced by other cells, or to ameliorate a defect in the immune system leading to chronic kidney inflammation. Some of these approaches have already shown to be effective in pre-clinical studies of kidney diseases. For other applications a proof-of-principle study in another disease will pave the way to new therapies that could also be applied to the kidneys. Here we will discuss the various different ways in which gene therapy can be used to target a disease and how delivery methods play an important role in the effectivity and specificity of a treatment.

2. The molecular toolbox of gene therapy

The proteins or RNA sequences required for gene therapy are delivered to the cell through recombinant DNA sequences that represent a functional gene-expressing unit, including a promotor and the gene that should be expressed (Fig. 1). When introduced into a cell and transported to the nucleus this DNA sequence is recognized as a gene and, depending on the promotor incorporated in the recombinant DNA, it is transcribed. The promotor sequence functions as an on/off switch and if the application requires the recombinant gene to be activated in all cells, a constitutively active promotor can be used. However, in some cases the recombinant DNA should only be transcribed in a subset of cells or only under a specific condition. This principle is also

commonly used in animal models in which a transgene only becomes active in one particular tissue or cell type after a specific signal, such as tamoxifen induced Cre expression (Ly et al., 2011). Specific promotor sequences exist for the proximal tubules, cortical tubules, and podocytes, which makes it possible to express the recombinant gene only in a specific part of the kidney. The recombinant DNA can be delivered to the cell for temporary gene expression, or stably integrated in the genome of the cell. This depends on the therapy and the delivery method (see also Section 3). The routes of gene therapy delivery are normally intravenously, intramuscularly, intra-ocular or *ex-vivo*. For *ex-vivo* therapy blood cells or stem cells from the patient are manipulated outside of the body and transplanted back into the patients. In this way only a specific cell type is targeted and quality checks can be performed before cells are placed back. Here we provide an overview on how gene based therapies can be used to express a recombinant protein in the cell (Section 2.1), to permanently modify the genome of a cell (Section 2.2), or to regulate gene expression (Section 2.3) (Fig. 2).

2.1. Gene addition

Inducing the expression of a recombinant protein can be done to compensate for a genetic defect (Fig. 2E) or to trigger a pathway that will ameliorate disease development (Fig. 2F). Some therapies, like enzyme replacement therapies, insulin injections and immunoglobulin therapy, depend on regular intravenous or subcutaneous injection of recombinant proteins produced by pharmaceutical companies. These diseases are currently candidates for
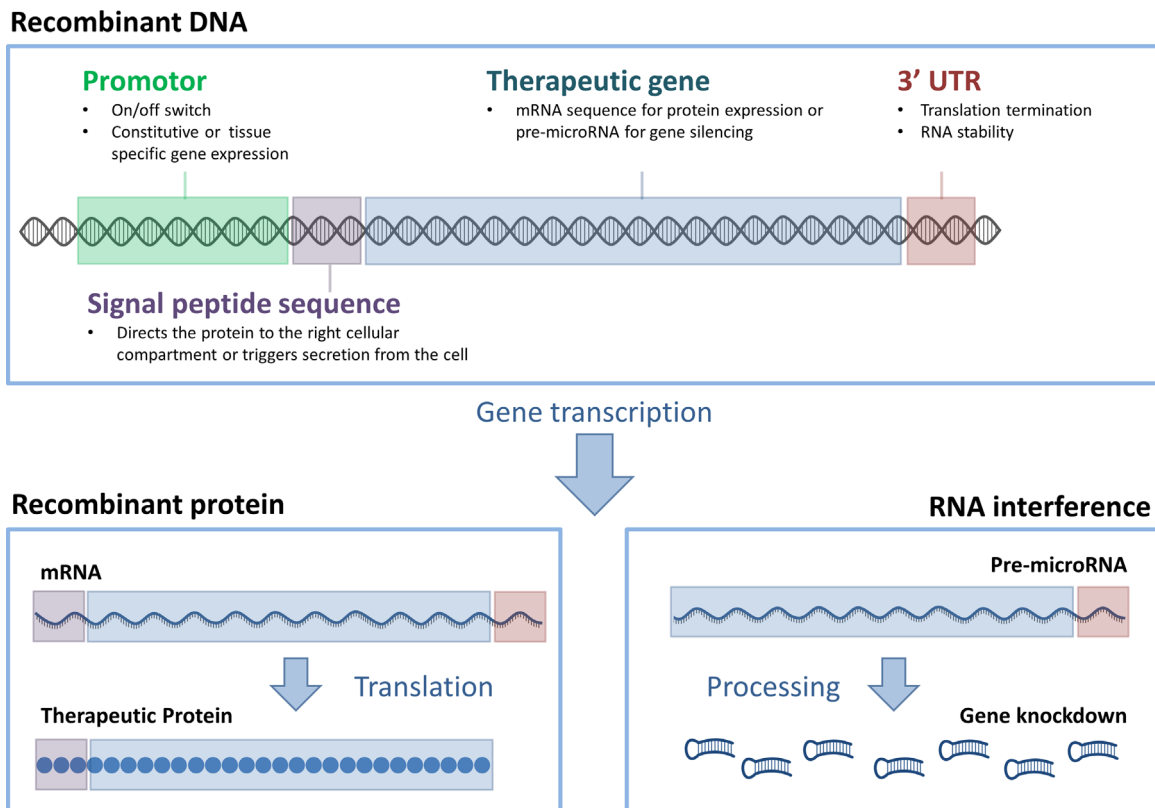

Fig. 1. Expression of recombinant DNA. Recombinant DNA resembles an endogenous gene and will be recognized as such by the cell resulting in the expression of a recombinant protein or the production of pre-microRNA. The promotor sequence will be bound by available transcription factors in the cell and the transcription machinery will produce the mRNA or pre-microRNA. After transcription termination the RNA sequences will be capped with a poly-A tail. The mRNA will be transported from the nucleus to the cytoplasm for translation. Depending on the presence of a signal peptide the protein will stay in the cytoplasm or be directed to the Golgi where the protein will be either transported to specific cell organelles or be secreted from the cell. The pre-microRNA will be processed in the nucleus into short hairpins which are transported to the cytoplasm where they will be substrate for the RNA interference machinery. The sequence of the RNA hairpins will be used as a template for the destruction of specific mRNA molecules (target genes) and in this way prevent their translation into protein (gene knockdown).
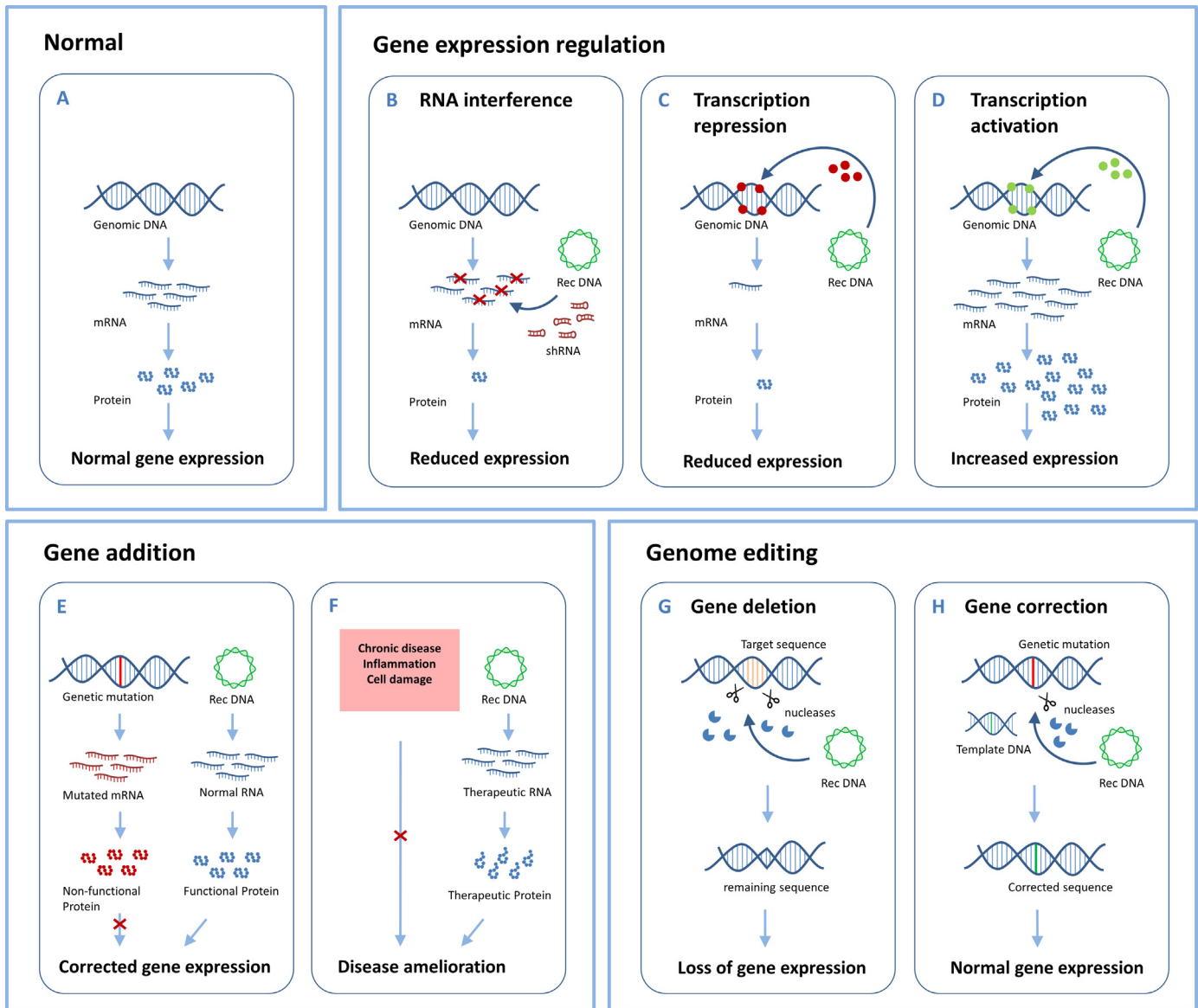
Fig. 2. Mechanisms underlying gene based therapies. In every cell the information on genomic DNA is transcribed into RNA which can be translated to protein (A). For all gene based therapies the recombinant DNA (rec DNA) can either be stably integrated in the genome or be present as a separate (circular) DNA sequence. The expression of endogenous genes can be modulated by degrading the mRNA from a specific gene (through micro RNA or short hairpin RNA) (B), or binding gene regulatory elements in the genome to repress (C) or activate (D) gene transcription. In the case of gene addition a recombinant protein is used to restore cell function by adding the missing gene in a genetic disease (E) or to improve disease outcome in a chronic or acute disease setting (F). For genome editing nucleases are targeted to a specific place in the genomic DNA to introduce a permanent change. An exon or a pathogenic gene can be excised (G) or a specific genomic region can be replaced with a new sequence by providing a homologous DNA template (H).

gene therapy. Muscle cells or liver cells can be targeted to become a "biofactory" and provide a short or lifelong supply of a certain recombinant protein into the bloodstream. Gene therapy has several important advantages over recombinant protein therapies. It does not require repeated intravascular injections to administer the protein and the sustained production and secretion of the protein by muscle cells may lead to higher tissue concentrations and a greater therapeutic effect. Expression of proteins by the liver has been reported to reduce the chance of an immune response to a recombinant protein, and can even induce tolerance in case of an autoimmune disorder (Sack et al., 2014).

2.1.1. First gene therapy treatments approved for market

The first gene therapy that was approved and that became commercially available was the anti-cancer drug Gendicine. Gendicine is a recombinantly engineered adenovirus carrying the tumor suppressor gene p53 (rAd-p53) and has been approved in China to treat head and neck cancers (Li et al., 2014). p53 is well known for detecting DNA damage and facilitating DNA repair, and also for initiating cell cycle arrest and apoptosis after extensive DNA damage. Most tumors have already accumulated many genetic mutations and frequently p53 has been mutated or reduced in cancer cells to evade cell death. Re-introduction of p53 expression in tumors can make these cells susceptible again for cell cycle arrest and apoptosis whereas most healthy cells will not be affected. In the phase III clinical study rAd-p53 alone had a similar effect on tumor mass and 5 year survival compared to chemotherapy alone, but with less side effects. Combination therapy with both rAd-p53 and chemotherapy resulted in complete disappearance of all tumor masses for more than 1 month in 50% of patients, compared to 17% of patients receiving only chemotherapy or rAd-p53 (Li et al., 2014). Unresectable head and neck cancers

are now often treated with chemotherapy containing cisplatin, which has a cumulative and dose-dependent nephrotoxic effect and often requires dose reduction or withdrawal due to acute kidney injury (Ozkok and Edelstein, 2014; Vermorken et al., 2007). Combining this treatment with rAd-p53 (like Gendicine) could therefore overcome the problems with kidney toxicity.

Glybera is a drug used for patients with lipoprotein lipase deficiency and was the first gene therapy to receive marketing authorization in Europe (2012). Lipoprotein lipase deficiency is a rare autosomal recessive disorder caused by a mutation in the gene coding for lipoprotein lipase. Intramuscular injection of the human lipoprotein lipase gene packaged in an adeno-associated viral (AAV) vector was shown to transduce muscle cells and result in the long-term production and secretion of lipoprotein lipase into blood of these patients (Gaudet et al., 2013).

Another gene therapy, called Strimvelis, was recently endorsed by the European Medicines Agency and will be used to treat children with severe combined immune deficiency (ADA-SCID). Children with ADA-SCID lack the enzyme adenosine deaminase, which affects the development and maintenance of the immune system. The *ex-vivo* lentiviral integration of adenosine deaminase in autologous hematopoietic stem cells seems to be safe and effective (Carbonaro et al., 2014). The production of both recombinant proteins and viral vectors are expensive and subjected to many regulations. However, because gene therapy aims for a long lasting cure, the lifelong therapy costs are expected to be lower than those for the repeated administrations of recombinant protein.

2.1.2. Gene therapy for the treatment of severe genetic kidney diseases

Approximately 3% of people ≥ 25 years suffer from a genetic renal disease (Joosten et al., 2010; Mallett et al., 2014), including severe recessive kidney diseases like Fabry disease, cystinosis, nephronophthisis and Alport's syndrome (Hildebrandt, 2010).

Fabry disease is a lysosomal storage disease caused by deficient lysosomal alpha-galactosidase A (α-gal A) activity, resulting in globotriaosylceramide accumulation and a progressive decline in renal function (Parenti et al., 2015). Regular intravenous injections with recombinant α-gal A (enzyme replacement therapy) have shown to be effective against loss of kidney function in these patients. In a mouse model of Fabry disease, a single intravenous injection with an adeno-associated virus (AAV) vector resulted in stable α-gal A expression and normalization of globotriaosylceramide levels for more than 6 months (Park et al., 2003).

Alport syndrome can be caused by mutations in the α5 chain of collagen type IV, affecting the glomerular basement membrane and resulting in end stage renal disease. In contrast to Fabry disease, supplementing a recombinant collagen to the circulation is not able to restore the defect as the protein must be produced and integrated in the basal membrane that is deposited by glomerular cells. With recombinant adenoviral delivery it was possible to induce expression of the type IV collagen α5 chain in pig kidney glomeruli, but the effect on disease progression still has to be determined (Heikkila et al., 2001). Another approach in Alport syndrome focusses on inhibiting microRNA-21 using antisense oligonucleotides (see also gene knockdown). Cystinosis is caused by mutations in the *CTNS* gene resulting in lysosomal accumulation of cystine. Patients develop renal Fanconi syndrome around the age of 6 months and progress to renal failure in the first decade of life. Treatment with cysteamine can reduce cystine accumulation and delay disease progression but does not prevent end stage renal disease. *Ex-vivo* gene therapy in a cystinosis mouse model showed that overexpression of the *CTNS* gene product in hematopoietic stem cells was able to prevent kidney function loss (Harrison et al., 2013).

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease (prevalence 1:1000). Patients have only one functional copy of the PKD1 or PKD2 gene, and low gene expression levels or somatic second hit mutations are thought to trigger cyst formation in a small subset of kidney cells. The subsequent expansion of these affected cells into cysts disrupts the normal structure of the kidney, triggers inflammation and leads to kidney failure. So far, the only curative treatment is kidney transplantation. Gene addition may be challenging for this disease as the cyst epithelial cells must be specifically targeted and most mutations are found in PKD1, which is an extremely large gene. Therefore, gene repair (rather than gene addition) may be used to restore PKD1 or PKD2 function in these cells. Recently, it was also found that in a mouse model of polycystic kidney disease macrophage recruitment and cyst growth could be halted by systemic inhibition of macrophage migration inhibitory factor or by reducing activin signaling (Chen et al., 2015; Leonhard et al., 2016). This indicates that targeting the immune system or downstream effects of the genetic mutation can also be very effective in treating a genetic disease.

2.1.3. Gene therapy approaches to ameliorate kidney disease

Fibrosis and inflammation are the common pathways to progressive chronic kidney disease and result in the deposition of extracellular matrix, microvascular compression, interstitial ischemia, deterioration of the functional tubular epithelium and eventually destruction of organ architecture and function. There are indications that this process can be halted or reversed by targeting various components of the fibrotic pathway (B. Tampe and Zeisberg, 2014; D. Tampe and Zeisberg, 2014). Overproduction of cytokines, like TGF-β1 and IL-6, can induce fibrosis in various organs and is directly linked to glomerulonephritis and diabetic nephropathy. Consequently, gene therapy through hepatic secretion of a IL-6 receptor fusion protein was able to block IL-6 signaling and improve kidney function in an ischemia and reperfusion injury mouse model (Gortz et al., 2015). Another regenerative approach involves the expression of exogenous recombinant BMP7, which could not only inhibit progression of experimental kidney fibrosis but also facilitated the reversal of established fibrotic lesions in mice (Zeisberg et al., 2005). In a rat transplantation model the expression of recombinant hepatocyte growth factor was achieved by intramuscular delivery of recombinant DNA through electroporation, and here the hepatocyte growth factor was able to reduce allograft scarring and improve renal function (Herrero-Fresneda et al., 2006). Furthermore, patients with chronic renal failure often receive recombinant human erythropoietin for the correction of anemia, which was effectively replaced by erythropoietin gene therapy in uremic rats (Ataka et al., 2003).

The expression of recombinant proteins can also be used to treat the underlying causes or contributing factors associated with chronic kidney disease, like diabetes and cardiovascular disease. In the previous examples a constitutively active promotor was used to drive expression of the recombinant gene, but recombinant DNA can also utilize the biological gene regulation and feedback mechanisms of the host cell. This mechanism may be used for dynamic insulin production in type 1 diabetes mellitus patients. Studies in a rat model for diabetes have shown that insulin production can be induced in liver cells (using the albumin promotor) and in the presence of high glucose levels (due to glucose inducible responsive elements) (Alam et al., 2013; Handorf et al., 2015). This shows that the specificity of a treatment does not only depend on the delivery of the recombinant DNA to the right tissues, but that the recombinant DNA can be designed to become active only in the designated cells. Recombinant DNA technology also allows the design and production of tailor made proteins that

are normally not found in nature. One example of this can be found in an human immunodeficiency virus (HIV) treatment study in which a recombinant fusion protein (consisting of CD4-Ig with a small CCR5-mimetic sulfopeptide) could bind with high affinity to conserved regions within the HIV envelope and that expression of this protein from muscle cells could provide durable protection from multiple HIV challenges in Rhesus macaques (Gardner et al., 2015). Furthermore, in the anti-cancer field many different gene therapy approaches are being developed that either directly target the tumor cells (knocking down oncogenes; re- or overexpressing tumor suppressor genes; expressing cytokines to induce immune response; introducing a suicide gene followed by (pro)-drug administration) or use other cells for tumor eradication (expression of tumor specific antibodies; enhance the anti-tumor immune response by the immune system; inhibiting angiogenesis) (Salem et al., 2015). Some of these methods may also be used in the future to tune the immune system during chronic kidney disease or to eradicate diseased cells in, for instance, polycystic kidney disease.

2.2. Genome-editing

In nuclease based gene editing a DNA-cutting enzyme is directed to a specific location in the genome. Initially this method was only used to repeatedly cut the genomic sequence of a gene of choice to introduce mutations and generate a knockout cell line or mouse strain. Later it was found that by supplying a homologous DNA sequence while introducing the cut, the chance of repair through homologous recombination was highly increased. This made it possible to introduce precise changes in the genome and repair a mutated gene, ideally leaving no additional marks on the DNA (Fig. 2H). So far several nucleases have been described, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Cas9 (CRISPR-associated 9) (Maggio and Goncalves, 2015). TALEN and ZNF are proteins that can bind a specific region in the DNA as well as cut the DNA. In the case of CRISPR/Cas9 the protein Cas9 has the enzymatic activity to cut DNA, but only after forming a complex with a site-specific guide RNA that can bind the DNA. This means that only the guide RNA needs to be specific for the DNA sequence and, because the binding occurs through base-paring with the DNA, it is easy to design. Therefore, CRISPR/Cas9 is now widely applied in basic research and (pre-)clinical trials. Thus far, CRISPR/Cas9 has been used to create knockout mouse models, repair human gene mutations in cell lines, modify genes *in vivo* in animal models, target viral infections or kill cancer cells (Hsu et al., 2014; Sander and Joung, 2014).

For genetic kidney diseases, gene editing may be used as a tool to permanently repair a genetic mutation and cure the affected cells. This approach may prevent kidney function loss in severe recessive diseases like Fabry disease, cystinosis, nephronophthisis or Alport's syndrome, but may also benefit autosomal dominant polycystic kidney disease (Miyagi et al., 2016). The following examples show how gene editing can be used in a large variety of diseases.

Duchenne muscular dystrophy is a life threatening muscle degenerative disease, due to a mutation in the gene coding for dystrophin. Most mutations lead to a frame shift and premature stop codon in the gene transcript, thereby preventing its translation into a protein. Several approaches have aimed at skipping a specific exon in the gene so that the resulting transcript can be translated into a (partially) functional protein. One approach consisted of directing Cas9 to the dystrophin gene in muscle cells of a Duchenne mouse model, with the hope that small insertions or deletions would restore the reading frame of dystrophin in part of the cells. Indeed this was the case, as 6 weeks after in the intramuscular administration of Cas9 using an AAV vector, 25% of the myofibers expressed dystrophin (Long et al., 2016). Another study used Cas9 to cut at both sites of the exon, resulting in excision of this exon from the genome (Fig. 2G), thereby restoring the reading frame in 67% of the myofibers (Nelson et al., 2016). Introducing a mutation or deletion seems feasible and so far no side effects have been reported. For many of these diseases only a subset of the cells needs to be correctly targeted to restore the function of the organ. Repairing a mutated gene into the correct sequence is less efficient and requires the delivery of a homologous DNA template (Fig. 2H). In patients with hereditary tyrosinemia type I an essential metabolic enzyme is mutated which leads to the accumulation of toxic metabolites and liver damage. In a mouse model for this disease the delivery of Cas9 together with a homologous DNA template was able to restore gene function in 6% of the liver cells. The repaired cells are permanently cured and this number of cells was sufficient to correct this disease and regenerate the liver (Yin et al., 2016).

Hepatitis B is replicated via covalently closed circular DNA (cccDNA) that is present in the nucleus of the infected cell. If cccDNA is destroyed, no further replication of the virus can take place. Hydrodynamic tail injection of Cas9 targeting several conserved regions in the hepatitis B cccDNA in a mouse model of hepatitis B resulted in a 93% drop in hepatitis B serum antigen levels which remained low for at least ten days (Zhen et al., 2015). HIV-1 can stably integrate itself into the genome of the host cell after infection. Recently Cas9 was used to specifically excise integrated copies of HIV-1 from human CD4+ T-cells. Continuous expression of Cas9 targeting HIV could also suppress viral replication and inhibit HIV-1 infection in primary cultured human CD4+ T-cells. Importantly, this procedure did not result in any off target mutations and did not affect cell viability suggesting that this approach is also safe (Kaminski et al., 2016).

2.3. Gene expression regulation

In the case of gene addition (see Section 2.1) the full coding sequence of a gene is delivered to a cell in order to express the recombinant protein. In the case of gene expression regulation, a (small) recombinant protein or RNA sequence is produced by the recombinant DNA to regulate the endogenously expressed genes (Fig. 2B–D).

Genes can be silenced by the exogenous expression of micro-RNA, small interference RNA (siRNA) or short hairpin RNA (shRNA) to degrade a specific mRNA (Fig. 2B). The RNA interference sequences are very small and specific for a target gene. In addition to gene knockdown, gene expression levels can also be modulated on a gene transcription level. DNA binding proteins or triplex-forming oligonucleotides that bind a specific DNA sequence may block the transcription of that gene. Furthermore, directing epigenetic modulators to the regulatory sequences of that gene may lead to long-term gene expression or repression (Tampe et al., 2014; Smyth et al., 2014).

Several proteins, like TGF-β or CTGF, are known to play a central role in the development of chronic kidney disease due to their pro-fibrotic or pro-inflammatory effects (see also Section 2.1). Reducing their expression may therefore halt the disease and improve renal function, even if the cause of the kidney disease cannot be cured. The expression of microRNA-21 is tightly linked to TGF-β and also has a strong pro-inflammatory and fibrotic effect on the kidney. In a mouse model of Alport nephropathy, the administration of anti–microRNA-21 oligonucleotides significantly inhibited disease progression and delayed the onset of renal failure, resulting in increased life span of almost 50% (Gomez et al., 2015). In a mouse model of type 2 diabetes, knockdown of miR-21 using shRNA prevented renal injury by halting the progression of renal fibrosis and reducing microalbuminuria and inflammatory

markers. The shRNA plasmid was delivered using ultrasound-microbubble-mediated gene transfer together with a regulatory plasmid which allowed controlled expression of the shRNA through the administration of doxycycline in the drinking water of the mice (Zhong et al., 2013). Knockdown of multiple target genes at the same time was shown to be very effective after cisplatin induced kidney damage, siRNAs targeting both Mep1b and Trp53 resulted in injury-free survival in 88% percent of the mice in contrast to none of the untreated mice and less than 40% of the mice treated with only one siRNA (Alidori et al., 2016). Another application of gene therapy may be found during kidney transplantation, where the donor kidney can be perfused before transplantation with factors to reduce the reperfusion injury (Hosgood et al., 2015). Also for dominantly inherited diseases in which the mutant allele disrupts cell function (like alpha 1 antitrypsin deficiency and Huntington's disease) an allele specific siRNA may be developed (Pfister and Zamore, 2009).

Gene regulation seems an elegant way to modulate the immune response to prevent kidney damage in different disease models and can be used to target several genes at the same time.

3. Delivery systems

For all applications of gene therapy it is important that the recombinant DNA can pass the lipophilic cell membrane to fulfill its function in the target cells. DNA without any packaging (naked DNA) is in general not very efficient in entering cells. Therefore, depending on the application and the target cells, different delivery vehicles and routes of administration are being used. Important criteria for delivery vectors are: 1) their transduction efficiency (percentage of target cells receiving the DNA), 2) the duration of transgene expression (ranging from a few days to lifelong expression), 3) toxicity or immune response caused by the delivery and 4) size of the DNA that can be packaged and transduced into the cells (Salem et al., 2015). Viruses hold the machinery to pack DNA into

compact particles and transport it across the plasma membrane with high efficiency and approximately two-thirds of the trials performed to date use viral vectors (Ginn et al., 2013). Years of evolution have made viruses superior in finding different ways to interact with receptors on the cells and deliver their DNA or RNA into the cell. For gene therapy, this viral machinery can therefore be modified to, instead of the viral DNA, package and deliver a recombinant DNA sequence. On the other hand, our immune system is also specialized in detecting and fighting viral particles, which can interfere with this route of administration (Miest and Cattaneo, 2014). In many cases non-viral delivery is preferred as a safe administration route and a lot of research has been done to improve its efficiency. Non-viral delivery of DNA is achieved using cationic lipids or polymers that form condensed complexes with the DNA and facilitate transport through the plasma membrane (Al-Dosari and Gao, 2009). In addition, gene and miRNA transfer has been shown to occur via extracellular vesicles or exosomes and tunneling nanotubes, which represent an alternative and safe route to deliver genetic material to cells. The most common delivery systems are summarized in Table 1.

3.1. Naked DNA

In general, DNA with no packaging (naked DNA) is not very efficient in entering cells due to its hydrophilic polyanionic nature and its large size. Furthermore, after intravenous injection the half-life of naked DNA is very short due to serum nucleases and degradation by the liver (Liu et al., 2007). However, several methods have been described to temporarily increase cell permeability, including hydrodynamic injection, the use of a gene gun, magnetofection, electroporation and sonoporation (Yin et al., 2014).

Hydrodynamic injections are based on the (systemic) injection of a large volume in a short time, which induces mechanical stress on the capillary endothelium. Stretching of the cells is thought to introduce small pores that allow macromolecules to enter. In

Table 1
Comparison of gene delivery systems.

Delivery system	Advantages	Limitations
Naked DNA) Injection of purified double-stranded DNA, sometimes in combination with electrical or mechanical stimulation (Herweijer and Wolff, 2003)	• Low immunogenicity and therefore can be administered repeatedly • The simplest vector system to manufacture in large qualities, strict quality control • Can be stored for long periods of time in a lyophilized form	• Inefficient gene delivery • DNA is not protected from degradation by nucleases
Viral vectors Uses the machinery of known viruses to deliver DNA to target cells (Nayerossadat et al., 2012)	• Good transfection efficiency in a large variety of cells • Properties can be adjusted to the target cells and desired expression • Can also lead to stable integration in non-dividing cells	• Maximum size of recombinant DNA can be a limitation • Can induce an immune response in patients resulting in adverse events or reduce treatment efficacy due to fast clearance of the virus
Non-viral vectors Combines naked DNA with synthetic or natural compounds to improve DNA stability and delivery (Al-Dosari and Gao, 2009)	• No size limit for recombinant DNA • Low immunogenicity and potential for repeated administration • The complexes protect DNA from degradation and facilitate cell uptake	• Less efficient gene delivery compared to viruses • Potential toxicities such as inflammatory response and formation of aggregates in blood
Extracellular vesicles Uses the intracellular signaling machinery from our body to deliver complex biological molecules to a specific tissue (Andaloussi et al., 2013)	• Biocompatible and immunologically inert if derived from appropriate cells • Innate ability to cross major biological barriers including the blood–brain barrier • Intrinsic homing ability to their target tissue	• Current limitations lay in the manipulation and production of these vesicles • No efficacy established for human applications

rodents hydrodynamic tail injection works well in targeting the liver, but systemic injection may not be feasible in humans because the heavy overload of fluid can lead to transient heart failure (Suda and Liu, 2007). However, the same principle may also be used to target a specific organ or tissue, and kidney specific targeting has been achieved by hydrodynamic injection of naked plasmid DNA into the vena cava inferior (Wu et al., 2005). With electroporation an electrical field is applied to the tissue to improve the uptake of large macromolecules. This technique requires the application of electrodes to the target organ which can only be obtained through surgery or may be applied on a donor kidney in transplantation settings (Sandovici et al., 2010). Magnetofection uses a magnetic field to attract and concentrate DNA coated nanoparticles to the target tissue. Sonoporation uses ultrasound to improve the permeabilization and uptake of DNA by tissues. Although less effective than hydrodynamic gene delivery, there are no adverse effects associated with ultrasound and it is therefore considered to be easy and safe. This method has also been used to activate the release of oligonucleotides from microbubbles in the kidneys (Deelman et al., 2010; Zhong et al., 2013).

3.2. Viral delivery

A natural occurring virus consists of a viral genome (DNA or RNA based) encapsulated by a protein coat (capsid) and is in some cases covered with a lipid bilayer derived from the host cells (viral envelope). The protein coat or lipid layer will be tailored to enter specific cell types and the information on viral genes will turn the host cell into a factory that can produce more infectious viral particles. Knowledge of the viral genome allows researchers to remove the genes required for viral replication and to use the properties of the viral particles to package and deliver a therapeutic recombinant DNA sequence instead. The size of DNA sequence that can be packaged varies depending on the viral vector, ranging from 5 kb up to 150 kb (Giacca and Zacchigna, 2012). This is especially relevant for gene delivery when large cDNA sequences must be delivered (gene addition) or for gene repair when a DNA template is required (gene editing). Some viruses also express proteins that help them to stably insert their genome into the host cell. This facilitates long-term transgene expression but can also lead to insertional mutagens.

The early viral vectors were based on gamma retroviral vectors which could efficiently transduce dividing cells but also exerted a high risk of tumorgenesis due to their integration sites and strong viral promoter elements activating oncogenes (Nayerossadat et al., 2012). Lentiviral vectors based on the HIV-1 long terminal repeats also integrate into the host genome but have not been associated with insertional mutagenesis or cancer in patients. In addition, lentiviral vectors efficiently transduce non-dividing cells and have low anti-vector immunity *in vivo*, which makes them a valuable tool for gene therapy. Adenoviral vectors can also transduce both dividing and non-dividing cells in a large range of tissues. Inserted transgenes can be maintained through successive rounds of replication without integration into the host genome and without the risk of insertional mutagenesis (Giacca and Zacchigna, 2012). Because the expression of viral genes in the host cell can trigger an immune response that kills the transduced cells, the next generation adenoviral vectors have been designed with very few viral sequences. In this case, all the genes required for the formation of viral particles have been moved to helper cells and are no longer transferred to the target cells (gutless adenoviral vector). These gutless vectors can package large (up to 36 kb) DNA sequences and are very suitable for the delivery of template DNA for gene repair (Holkers et al., 2014). Adeno-associated virus is not associated with any disease and does not trigger a strong immune response in humans. It can enter both dividing and non-dividing cells and

integrate itself in the DNA at one specific location, which highly reduces the chance of insertional mutagenesis. In most AAV vectors the integration capacity is removed and here the recombinant DNA can stay in the host cell as an episomal concatemer, providing long-term gene expression. Many clinical trials are using AAV delivery of a transgene to restore gene function in genetic diseases, including hereditary blindness, cystic fibrosis and severe bleeding disorders. The injection site and use of different AAV serotypes can have a big impact on the *in vivo* transduction efficiency and transgene expression levels. When optimizing transgene expression in mice, renal vein injection of rAAV9 resulted in high transgene expression in the kidneys (Rocca et al., 2014).

Previous exposure of people to viral infections from a related strain, or repeated injections with the same viral vector, may induce a fast immune response that clears the viral vector and renders the therapy ineffective. By combining properties from different viral vectors new hybrid viral vectors can be formed and epitopes present on the surface of the virus may be altered to evade the immune system or improve targeting to specific cells (Huang and Kamihira, 2013).

3.3. Non-viral delivery vectors

3.3.1. Synthetic delivery systems

Synthetic delivery systems have gained increasing popularity for nucleic acid delivery because of their excellent safety profile and (often) lack of immunogenicity. The basis of such synthetic carriers is a well-defined synthetic molecule (e.g. lipid, polymer, sugar or peptide) that, upon electrostatic interaction with the nucleic acid or by molecular self-assembly, forms nano-sized structures in which the nucleic acid is entrapped and protected from enzymatic degradation. Cationic lipids as well as polymers have been widely used for this purpose as these molecules enable entrapment of relatively large amounts of nucleic acids by electrostatic complexation (for review see (Allen and Cullis, 2013; He and Wagner, 2015; Pezzoli et al., 2013)). Although such cationic polyplexes and lipoplexes provide good protection against enzymatic degradation and enable efficient uptake by cells via adsorptive endocytosis, the subsequent intracytosolic release of nucleic acids is not well understood and leaves much room for improvement (Rehman et al., 2013). Moreover, exposed cationic charges may cause problems upon systemic administration as it may lead to blood cell aggregation within the bloodstream. To prevent this, the cationic charges are often temporarily shielded using polymers such as poly(ethylene glycol) or neutral or negatively charged lipids or by using ionizable lipids or polymers that are neutral at physiological pH. However, this charge shielding also has a negative effect on the uptake in target cells (Zhang et al., 2012). To improve intracellular delivery of nucleic acids with synthetic nanocarriers, several biomimetic approaches have been followed in which fusogenic peptides derived from viruses were used to enhance endosomal escape and peptides containing a nuclear localization signal to subsequently facilitate the transport of therapeutic gene constructs into the nucleus of target cells (Moore et al., 2009; Raad et al., 2014).

Systemic administration of synthetic or biomimetic nanocarriers often leads to accumulation of these particles in cells of the reticuloendothelial system. As a consequence, the majority of nanocarriers end up in liver and spleen. A typical biodistribution profile shows > 50–70% of injected dose ending up in these organs. Targeting beyond liver and spleen is limited to inflamed tissues and organs characterized by fenestrated vasculature, enabling passive extravasation and accumulation of the nanocarriers in the tissue interstitium. Via this mechanism nanocarriers have been targeted to solid tumors, inflamed joints, intestines and to atherosclerotic plaques (Ozbakir et al., 2014; van der Valk et al., 2015).

When designing a strategy to target gene-based nanocarriers to the kidneys, the location of the target cell population within the kidney is important. Most nanoparticles are bigger than the cut off size for renal filtration. As such, gene-based nanocarriers have difficulties reaching the renal tubular cells. In contrast, mesangial cells represent a particularly suitable target for nanoparticular gene delivery, because the glomerular endothelium is fenestrated and a basement membrane to separate glomerular capillaries from the mesangium is absent (Tuffin et al., 2005). Also here, nanoparticle size plays a crucial role in kidney mesangium accumulation. Davis and co-workers tested a range of gold nanoparticles with defined particle sizes and showed that gold nanoparticles in the range of 75 ± 25 nm accumulated in kidney mesangial cells (Choi et al., 2011). Renal accumulation of systemically administered nanocarriers can be further increased by the use of specific targeting ligands attached to the gene carriers (Wischnjow et al., 2016; Yuan et al., 2014).

Synthetic vectors have been used for the delivery of a variety of nucleic acids, including plasmid DNA, mRNA, siRNA and miRNA. Various lipid-based delivery systems for siRNA are being tested in clinical trials showing promising results in targeting liver diseases (Kanasty et al., 2013). mRNA delivery has been used as a genetic vaccine and miRNA has been delivered into tumors. An often-mentioned limitation of synthetic vectors is the transient nature of gene expression or silencing. As opposed to retroviral vectors, synthetic vectors do not have the auxiliary elements required for DNA integration into the genome of transduced cells. The exogenous DNA or RNA is therefore lost depending on time of the metabolic activity of the target cells and on how often cells divide. Episomal replication of the exogenous nucleic acids has been used as a strategy to prolong expression (Einav et al., 2003; Mizuguchi et al., 2000). By using transposable elements such as *Sleeping Beauty* and *Piggy Bag* genome integration with non-viral vectors has been demonstrated. Liu et al. used a lipid nanocarrier to systemically deliver the *Sleeping Beauty* transposon into lung endothelial cells and demonstrated transgene expression of over 2 months after transfection (Liu et al., 2004). This demonstrates that durable gene modulation with fully synthetic vectors is possible.

3.3.2. Cell-based gene therapy

Extracellular vesicles form a novel approach for nucleic acid delivery purposes. These vesicles are small particles released from any type of cell and represent an integral part of the cell-to-cell communication in a bidirectional manner and have physiological functions including transport of genetic material and modulation of the immune system, as discussed in detail in the review by Bruno et al. in this issue of European Journal of Pharmacology. They can be categorized into exosomes and microvesicles (Biancone et al., 2012) and contain a cargo that includes miRNA, mRNA, lipids, proteins and occasionally DNA from their cells of origin (Biancone et al., 2012; Rani et al., 2015). Exosomes are particles released from the endocytic pathway and have a size of about 30–100 nm, while microvesicles are formed by budding of plasma membrane of about 100–1000 nm sized vesicles (Biancone et al., 2012). The delivery of proteins, mRNA and miRNA to injured cells may induce cell reprogramming and *de novo* expression of factors involved in tissue proliferation and repair (Bruno and Bussolati, 2013).

Horizontal transfer of RNA and proteins between cells by extracellular vesicles has been first shown by Ratajczak and collaborators. They demonstrated that microvesicles produced by murine embryonic stem cells reprogrammed hematopoietic progenitor cells by delivering RNA of several pluripotent transcription factors that could be subsequently translated into proteins (Ratajczak et al., 2006).

Later, animal model-based studies suggested that extracellular vesicles have significant potential as a novel alternative to whole cell and gene therapies, presenting a superior safety profile. The release of extracellular vesicles by stem cells and the horizontal exchange of genetic information may partially explain mechanisms involved in the stem cell-mediated tissue repair after injury (Quesenberry et al., 2015). Microvesicles released by embryonic stem cells contain a significant amount of miRNA and part of this cargo could be transferred to mouse embryonic fibroblasts *in vitro* (Yuan et al., 2009). Bruno et al. (Bruno et al., 2009) have shown that extracellular vesicles isolated from mesenchymal stem cells (MSC) could protect against acute kidney injury in mice and that the uptake of vesicles into renal epithelial cells is mediated by cell surface receptors on the vesicle and it seems that the expression of CD44 and CD29 are important in this context. In a glycerol induced acute kidney injury in SCID mice, the intravenous injection of human MSC or MSCs-derived microvesicles presented similar outcomes in the recovery of the renal tissue and these microvesicles induced proliferation of resident tubular cells. Pre-treatment of microvesicles using RNase abolished the beneficial regenerative effects suggesting that the mechanism of action might be due to mRNA transfer (Bruno et al., 2009).

Furthermore, congenital genetic kidney diseases may also benefit from horizontal transfer of extracellular vesicles from cells expressing the missing gene. For the genetic kidney disease cystinosis it was shown that co-culture with human MSCs could reduce the pathologic cystine accumulation in patient's cells. Furthermore, a tagged version of the *CTNS* gene expressed by MSCs was later also expressed by cystinotic fibroblasts (Iglesias et al., 2012). This effect was seen in a mouse cystinosis culture model where the cells could not directly touch each other (transport through microvesicles), but was much more pronounced when cells could interact directly (Naphade et al., 2015). Direct interaction resulted in the formation of tunneling nanotubes through which cystinosin-bearing lysosomes were transferred into *Ctns*-deficient cells. These transfer mechanisms also explain how a hematopoietic stem cell transplantation can reduce cystine accumulation and is able to prevent kidney function loss in mice with cystinosis (Harrison et al., 2013).

Genetic exchange between diseased resident cells and administered stem cells or simply the vesicles released from them, appears to be a stable and safe tool for gene therapy leading to functional effects. Although promising, the biological nature of these carrier systems and their poor characterization may hamper widespread use as gene-based carriers for therapy.

4. Future perspectives

As we have seen the gene therapy field is developing fast, providing many different approaches to tackle complex diseases. Although not applied in the clinic yet, there are several promising *in vivo* examples in which gene therapy can be used to treat genetic kidney diseases, halt inflammation and fibrosis in chronic kidney disease, or stimulate regeneration of damaged kidneys. In a mouse model of Fabry disease adenoviral delivery of α-gal A through a single intravenous injection resulted in stable α-gal A expression and normalization of globotriaosylceramide levels for more than 6 months (Park et al., 2003). In another genetic kidney disease, *ex-vivo* gene therapy showed that overexpression of the *CTNS* gene product in hematopoietic stem cells was able to prevent kidney function loss in a cystinosis mouse model (Harrison et al., 2013). Furthermore, hepatic expression of a IL-6 receptor fusion protein was able to block IL-6 signaling and improve kidney function in an ischemia and reperfusion injury mouse model (Gortz et al., 2015), and the expression of recombinant hepatocyte growth factor reduced allograft scarring and improved renal

function in a rat transplantation model (Herrero-Fresneda et al., 2006). Another approach involved the exogenous expression of recombinant BMP7, which could not only inhibit progression of experimental kidney fibrosis but also facilitated the reversal of established fibrotic lesions in mice (Zeisberg et al., 2005).

For some therapies it is important to directly target the kidney cells. Efficient transgene delivery to the kidneys has been obtained using viral vectors (Rocca et al., 2014), gold nanoparticles (Choi et al., 2011), hydrodynamic injection into the vena cava (Wu et al., 2005), electroporation (Sandovici et al., 2010) and ultrasound mediated release of oligonucleotides from microbubbles (Deelman et al., 2010; Zhong et al., 2013). However, in many cases expression of recombinant genes from muscles or liver cells will be easier and more effective.

Of course gene therapies also have their limitations. For instance, when a disease has resulted in irreversible damage before diagnosis. Even if we are able to restore cell function, the complex micro-architecture required for kidney function may be lost. In that case, other approaches will be required, such as dialysis, kidney transplantation, kidney progenitor cells, or bioartificial kidneys. Therefore, gene therapy can be a powerful preventive strategy but still depends on early detection of the disease. Gene therapy is also in many cases still very expensive. However, as it has the potential of providing curative treatments it may be more cost effective in the long run than for instance a life-long supply of recombinant protein or kidney dialysis and transplantation (Yla-Herttuala, 2015). It will be interesting to see how gene therapy will develop in the future and if it can be used to improve patient life.

Acknowledgments

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