Application of siRNA in targeting protein expression in kidney disease

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A B S T R A C T

Although it is one of the major targeted organs by systemically administered siRNA, when compared to other tissues the kidney receives only moderate interest regarding therapeutic siRNA delivery. Here we review recent approaches to target renal protein expression under normal and pathological conditions. Experimental evidence to support the clinical relevance of siRNA administration in the treatment of renal disease is discussed. High-throughput screening using recently available genome-wide RNA interference libraries provides a new, powerful tool that can be applied to conventional and 3D in vitro culture models for lead finding or the identification of signal pathway involvement in renal disease.

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1. Introduction

Since its discovery, disruption of gene transcription based on the mechanism of RNA interference (RNAi) \cite{1} has witnessed a spectacular increase in its use as an experimental tool in molecular and cell biological and in vivo research. RNAi is the mechanism whereby double stranded RNA leads to specific degradation of complementary mRNA sequences, inhibiting translation and subsequently leading to lower expression of the target protein. RNAi represents an important regulatory mechanism of gene transcription. Since the discovery of RNAi, several types of regulatory RNAs have been described, including short interfering RNA (siRNA) derived from longer sequences of double stranded RNA and microRNA (miRNA) \cite{2} (see more details in the review about miRNAs by Kaucsár et al.).
Due to considerable interest from the biotechnology industry, synthetic genome-wide siRNA libraries are now available to identify new targets in disease using functional genomic approaches [2,3]. Therapy using siRNA is being developed for various clinical conditions, including acute kidney injury (AKI) and kidney transplantation [www.clinicaltrials.gov], diabetic nephropathy [4] and primary glomerular diseases [5,6], but mainly focuses on the treatment of tumors and viral infections [7,8]. Despite this relative underrepresentation, renal injury may actually provide an excellent disease target for siRNA therapy as it can benefit from one of the major difficulties encountered in targeted delivery of siRNA: glomerular filtration. Systemic administration of siRNA can lead to a rapid uptake by the kidney yielding a significant decrease of target protein expression [9]. The use of RNAi therefore shows promise as an approach to treat renal disease [10].

Classical pharmacological approaches to treat renal disease rely on post-translation modification of cell signaling pathways by acting on protein interactions. The revolutionary aspect of siRNA therapy is that it acts on disease-associated signaling pathways by down regulating components of the pathway rather than influencing the activity of pathways. This means that siRNA treatment has the potential to prevent injury from occurring in addition to addressing existing injury. This review focuses on recent studies employing approaches for in vivo administration of non-vector delivered siRNA and its uptake by the kidney and examples of the use of viral vector short hairpin RNA (shRNA) delivery aimed at targeting renal protein expression in experimental animal models, for use in vitro screens and also as a strategy to be used under conditions where long-term protein knockdown may be required such as during chronic kidney disease.

Studies that have successfully used RNAi to counteract renal injury in experimental animal models will be highlighted to demonstrate the strong clinical potential of siRNA-mediated therapy in renal disease and injury. We will discuss how in vitro siRNA screens can be used to discover and validate lead targets as well as predict their effect for use in in vivo models.

2. RNA interference (RNAi)

The molecular workings behind RNAi are becoming better understood. For the purpose of this review, it suffices to give a short introduction of the key players and to provide a broad explanation of the current view of the mechanism of action. Double-stranded RNA is cleaved by the ribonuclease Dicer into smaller fragments with a length of 21 to 23 nucleotides. The resulting siRNA fragments are then bound to the RNA-induced silencing complex (RISC). Argonaute 2, a protein component of RISC, unwinds and separates both siRNA strands. Activated RISC, containing the antisense strand (termed guide strand), will then engage in selective degradation of the mRNA that is complementary to the guide strand by cleavage of the mRNA target strand [reviewed in 10,11]. After cleavage of the mRNA, the activated RISC containing the siRNA derived guide strand can continue to degrade additional mRNA fragments. RNAi is a post-transcriptional gene-silencing mechanism: degradation of the target mRNA prevents protein synthesis without any permanent effect on the genome [12]. This effect has been shown to continue for several days up to several weeks, and is dependent on the rate of siRNA “dilution” due to cell proliferation [13,14].

Although pre-synthesized siRNA can be administered in vivo, initial studies found that their effect on protein expression was low due to nuclease degradation of the siRNA [15]. Vectors encoding self complementary mRNA strands are subjected to the same RNAi processing described previously and have also been exploited in in vivo studies. In short, transcription of plasmid DNA encoding both antisense and sense 21 nucleotide sequences of target RNA leads to formation of a short hairpin RNA (shRNA). This can be achieved by designing the construct in a way that a non-coding loop sequence separates the sense and antisense sequence. After transcription, folding of the RNA and self-binding of the complementary 21 nucleotide sequences lead to formation of the shRNA. Cleavage of the loop domain by Dicer gives rise to a ‘regular’ siRNA fragment which may induce RNAi as described previously. In contrast, synthetic siRNAs do not require Dicer-mediated cleavage but are loaded onto the RISC immediately after delivery to cells. Introduction of siRNA to cells that were composed of 25–27 nucleotides were found to undergo Dicer processing and displayed higher silencing potency than 21 nucleotide siRNAs [16]. However, the administration of such Dicer-substrate siRNAs has not been studied with respect to renal targeting or silencing.

Off-target effects of siRNA have been described in a number of papers and manifest themselves by inducing an inflammatory response [17], activating anti-angiogenetic responses [18] or down regulation of non-target proteins [19]. These off-target effects are thought to rely on immune response activation due to recognition of double strand RNA by Toll-like receptors or RISC presentation of the ‘wrong’ siRNA strand as a guide strand, that is presentation of the supposedly non-coding sense strand leading to specific or unintentional effects of transcription of other proteins [20]), or the presence of a ‘seed sequence’ in the siRNA, which has complimentarity to transcripts of unintended genes. Furthermore, siRNA may induce an inflammatory interferon response or translational shut-down of the transfected cell [12]. A few important rules for siRNA designs have become apparent. These have been recently reviewed by Grimm et al. [21] and are applied to correct guide/passenger strand presentation by RISC, lowering immunogenicity and increasing target specificity off-target effects by preventing recognition of non-target mRNA.

3. Delivery of naked siRNA to the kidney

A major concern when using siRNA techniques in vivo has been to achieve effective knockdown at target sites. Potential off-target effects or non-specific side-effects may skew experimental outcome or compromise therapeutic benefit. Although local delivery reduces knockdown effects in non-target tissue and reduces the amount of siRNA needed for treatment, systemic treatment remains the preferred approach due to difficulty achieving local targeting without invasive techniques. Interestingly, together with the liver and spleen, the kidney is the preferential site of nonspecific siRNA accumulation [9]. To study the role of the protein Zag in a
mouse model for ischemia-reperfusion injury, siRNA were diluted in saline and injected daily in a volume of 200 μl via the retro-orbital sinus for three to seven days, leading to renal protein knockdown [23]. The hydrodynamic delivery approach involves the intravenous administration of siRNA compounds in a relatively large volume of vehicle solution. For hydrodynamic delivery, in the original protocol an injection volume was used of 10% of the body weight of the experimental animal in a relatively short time (15–30 s), whereas a volume of 50–100 μl is the generally accepted average for intravenous injections in mouse tail veins. This sudden state of hypervolemia is likely to induce a severe, but transient capillary dysfunction, and may result in capillary ‘leakage’ leading to exposure of parenchymal tissue cells (in the case of the kidney the tubular epithelium) to the siRNA [24]. Hydrodynamic delivery was found to lead to liver damage in most cases [25]. This approach may provide two potential access points for the kidney, namely glomerular filtration resulting in availability of siRNA in the tubular lumen, as well as access from the peritubular interstitium: the basolateral side of the epithelial

### Table 1

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<td>siRNA –</td>
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<td>50 μg</td>
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<td>Xia et al. [34]</td>
<td>Phosphate buffered saline</td>
<td>siRNA –</td>
<td>Intrauretral</td>
<td>Tubules</td>
<td>50 μg</td>
<td>50 μl</td>
<td>–</td>
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Abbreviations: IP, intraperitoneal; IV, intravenous; NA, not applicable.
monolayer. The hydrodynamic approach results in fast uptake of siRNA in the kidney, including cells of the tubular epithelium (Fig. 1a), and has been successfully used in a number of mouse studies. Hydrodynamic delivery of siRNA diluted in PBS in a volume of 1 ml, alternatively followed by a ‘flush’ 0.3 ml of PBS, led to significant target protein knockdown at 24–36 h after administration [26–28]. The feasibility of the hydrodynamic approach in a clinical setting however is very likely to be nil. However, perfusion fluids containing siRNA of tissue grafts prior to transplantation may offer a possible use of this technique. A demonstration of this approach was shown recently in a mouse model for heart transplantation [29].

Comparative studies determining the efficiency of siRNA uptake and retention of different approaches of systemic administration in mice using fluorescent labeled siRNAs have been performed. Although renal siRNA uptake did not give a detectable fluorescence signal 4 h after intraperitoneal and rectal administration, significant uptake was observed following hydrodynamic administration. This uptake was also higher than administration of the labeled siRNA in a smaller, conventional volume at the same time point. 24 h after hydrodynamic administration, kidney cells also showed improved retention of siRNA compared to standard (non-hydrodynamic) intravenous administration [30]. Both non-target and p85α-specific siRNAs were used in this study. These studies are useful in evaluating siRNA targeting and retention by tissue. However target protein levels were not evaluated and there may be a disparity between cellular uptake and functional effect on protein expression. For example, degradation of siRNA can occur, which may result in uptake and retention of the fluorescent probe without achieving specific protein knockdown.

An approach which may rely on the same principal of volume overload, is the technique whereby siRNA is administered intravenously and directly followed by exercising physical pressure of the target organ. Applying pressure on the kidney may result in local capillary dysfunction, and thereby leads to improved, local siRNA uptake when compared to intravenous injection only [31,32]. This approach may only be applied in experimental models using smaller animals.

A combination with in vivo electroporation can enhance siRNA delivery. Electroporation enhanced intra-arterial administration of a transgenic construct in rats resulted in expression in mesengial cells [35]. Administration via the renal artery involves temporary occlusion of the artery itself and thus results in a mild ischemia. Ischemic preconditioning is known to decrease susceptibility to a second ischemic insult [36], which may affect experimental outcome if this approach is used in animal studies.

Intrapelvic injection results in heavy transgene expression within the outer medulla affecting the tubular epithelium, while the interstitial cells and vascular components of the inner medullary region show little or no expression. Furthermore, intraurethral administration of a DNA enzyme followed by electroporation of the kidney led to transgene expression in interstitial cells [37].

Subcapsular administration requires invasive techniques, puncture of the renal capsule and the underlying parenchyma and leads to a mild renal dysfunction (G. Stokman, unpublished observation). Part of the administered volume may be lost via the puncture hole after retraction of the needle. Therefore this approach may have limited practical use.

4. siRNA carriers

The depletion of target proteins by systemic administration of naked, unmodified siRNA is inefficient. For example, administration of siRNA targeted against GFP induced a moderate reduction in expression of renal GFP in GFP-transgenic mice [38]. This weak effect is probably due to neutralization of naked siRNA through degradation by exo- or endonucleases [15]. Spontaneous uptake of siRNA by cells without additional carrier is possible but is reportedly less efficient when compared to strategies employing transfection reagents that complex or encapsulate the siRNA. A large number of approaches have been tested to this respect [7,8]. Here we will specifically discuss the use of siRNA transfection reagents, chemical modification of siRNA and viral vectors encoding shRNAs in targeting in vivo protein expression in the kidney.

4.1. Transfection reagents

The administration of pre-synthesized siRNA using transfection reagents allows the use of pre-designed genome-wide libraries of siRNAs. Several studies have employed commercially available cationic lipid-based transfection reagents that encapsulate the siRNA fragments preventing nuclease degradation and enhance uptake by the tubular epithelium. Liposomes are a widely accepted
means of delivering various biochemical agents. Cationic liposome-based transfection reagents have been successfully employed for in vivo siRNA delivery to the kidney, such as N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) [30,39].

The use of 1,2-dioleoyl-sn-glycerol-3-phosphatidylcholine (DOPC) leads to formation of neutral liposomes. Delivery of fluorochrome-labeled siRNA using DOPC-based liposomes was found to lead to effective uptake of siRNA by renal tubules, as well as by liver and lung. In this study, delivery using DOTAP also resulted in significant parenchymal uptake but was also more associated with uptake by endothelial cells [40]. Indeed, the uptake by endothelial cells in the kidney associated with DOTAP-mediated delivery may reduce effective protein knockdown in other compartments of the kidney such as the tubular epithelium [41].

Novel reagents have been developed specifically to enhance efficiency of siRNA delivery, such as Lipofectamine™ RNAiMax (Invitrogen). To demonstrate its effect on renal targeting the lipofectamine solution was diluted with physiologic saline to an end concentration of 20% as based on pilot study results. Diluted Lipofectamin™ RNAiMax was complexed with siRNA. Renal vein injection of an end volume of 300 μl containing 50 ng (1 nM) fluorescently labeled siRNA (BlockiT™ Alexa Fluor® Red Fluorescent control, Invitrogen) mixed according to the manufacturer’s instructions induced a prominent siRNA uptake within renal tubular cells 20–30 min after delivery (Zs. Rácz and P. Hamar, unpublished results, see Fig. 1b).

Polyethylenimine (PEI), a watersoluble polycationic polymer, is another delivery vehicle for siRNA. PEI forms non-covalent bound complexes with siRNA, not only shielding siRNA from nuclease degradation but also facilitating endocytic uptake. Release from endosomal vesicles into the cytoplasm following cellular uptake is also enhanced following PEI-siRNA administration [42]. A recent study by Malek et al. [43] showed that PEI-siRNA complexes display lower renal targeting compared to naked siRNA. However, naked siRNA taken up in the kidney was mostly degraded, whereas renal accumulation of PEI-siRNA resulted in a significantly higher proportion of intact siRNA. In a further effort to enhance PEI-siRNA stability, the authors studied surface modifications of PEI using polyethylene-polyglycol (PEG). So-called PEGylation should reduce non-specific interactions of the PEI-siRNA complex [44], but was also found to reduce siRNA stability in renal tissue [43]. In addition, increased molecular weight and charge neutrality may make PEI carriers less compatible with glomerular filtration, which is thought to separate based on both particle charge and molecular size (>50 kDa). Liposomal and polymer-complexation delivery techniques are, dependent on endocytosis for cellular uptake. An important benefit of PEI complexation is that it can induce endosomal disruption, resulting in release of the siRNA into the cytoplasm. This disruptive property is thought to depend on extensive protonation of PEI leading to swelling and rupture of the endosomal compartment while avoiding increased acidity thereby reducing siRNA digestion [45].

One novel approach aims at glomerular protein knockdown using PEG-poly-L-lysine copolymer-based nanocarriers while avoiding size-selective restraints of the glomerular filter. Shimizu et al. [6] report that these polymer complex (PIC) nanocarriers have enhanced delivery and retention in the kidney compared to naked siRNA following intraperitoneal administration, more specifically to cells of the glomerulus. When compared to siRNA delivery using a Hemaglutinating virus of Japan (HVJ) envelope vector, a so-called pseudovirion vehicle which is a viral vector lacking viral coding sequences, the siRNA-PIC nanocarrier complex proved superior with respect to glomerular targeting. Importantly, siRNA delivery using this nanocarrier approach specifically reduced glomerular target protein expression which indicates the suitability of this approach when considering glomerular targeting versus the tubular uptake of naked siRNA [6].

Cell-type specific delivery using antibody-mediated targeting offers an exciting approach to reduce non-target cell protein knockdown [46]. In their recent study in which they aimed at siRNA delivery to podocytes, Hauser et al. [47] made use of this strategy. After cleavage of the disulfide bridge, monovalent podocyte-specific sheep IgG antibody fragments were conjugated with neutravidin followed by labeling with biotinylated-protamine and finally complexed with siRNA. Complex formation is thought to depend on charge interactions between protamine and siRNA. In vivo analysis showed that the antibody predominantly bound podocytes but not tubular cells and did not trigger glomerular complement deposition which would result in glomerulosclerotic injury. This approach yielded specific target protein knockdown in podocytes and induced a specific phenotype associated with this knockdown. In addition, podocytes may be more relevant target cells using antibody-mediated endocytosis as they employ a mechanism for removal of bound IgG from the basement membrane whereas the tubular epithelium does not [47,48]. Beside podocyte targeting, the antibody was also detected in the spleen, but not in lung, liver, muscle or colon tissue [47].

Alternatives to protamine-siRNA complexation based on electrostatic interactions have been developed and include 3′-biotinylated siRNA binding to streptavidin-conjugated antibodies [49], monovalent antibody fragments labeling of PEGylated liposomes containing siRNA-carrier complexes [50,51] and antibody-conjugation to cationic lipid-siRNA complexes [52]. However, these strategies have not yet been examined for their potential in renal targeting.

Interesting new approaches however are those whereby siRNA is delivered in biodegradable hydrogels resulting in prolonged in situ release [53]. Such degradable polymer gels have been studied for their capacity to provide extended release of plasmid DNA in tissues, but may equally be used as a carrier system for siRNA delivery [54]. Although not studied in the context of siRNA, this approach may hold promise to result in long-term targeting of the kidney when applied subcapsular, and thereby allowing effective protein knockdown for longer periods of time.

Several approaches have been developed to assist uptake by the tubular epithelium, augmenting or bypassing active uptake. Although micro-injection into the cell has been suggested, the clear practical disadvantages for its use in the kidney do not need to be argued. Electroporation is a standard procedure to induce transient transduction of cells in vitro. For in vivo applications, electroporation has been used for delivery of siRNA to skin and muscle [55,56]. Some studies report the successful use of electroporation of siRNA delivery to renal tissue. In rats, injection of siRNA into the renal artery followed by electroporation led to predominant knockdown of the target protein in the glomeruli, more specifically according to the authors in the mesangial cells [5,33]. However, electroporation itself can induce cell stress signaling [57]. Thus, a feasible alternative could be sonoporation in order to enhance pore-opening and siRNA uptake similar to electroporation [58].

A relatively new approach is ultrasound enhanced nucleotide transfer. Liposomal carriers designed to carry ultrasound imaging gas were studied for their usefulness in vivo siRNA delivery during exposure to ultrasound waves. Although this approach proved successful for siRNA delivery to the skin, delivery to the kidney was not effective [59]. Further optimization of this technique may enhance the capacity to deliver siRNA to the kidney, yet the specialist aspects of the technique may limit its widespread practical use. Both approaches do require exposure of the kidney and thus surgery, although the clear advantage is the passive uptake of siRNA and its direct presence in the cytosol.

4.2. Chemical modification of siRNA

The administration of naked siRNA is certainly feasible, but is subjected to conditions that destabilize the siRNA as unmodified
phosphodiester linkages are very sensitive to serum and cellular nucleases. Chemical modification of siRNA can enhance the stability of the siRNA in the circulation and improve renal targeting. A number of approaches have been developed to this end and many strategies that apply to antisense oligonucleotide (ASO) stabilization or nucleotide analogue alternatives can be considered for use in siRNA approaches (see also recent reviews by Shukla et al. [60] and Ge et al. [61]).

Due to the sensitivity of unmodified phosphodiester linkages to serum and cellular nucleases, chemical modifications of phosphodiester linkages of ASOs may improve stability. Phosphodiester molecules with phosphorothioate or methylphosphonate blocked ends are protected from degradation by exonucleases [62]. A phosphorothioate linkage is formed by replacement of a nonbridging oxygen in the backbone with a sulphur atom, resulting in a prolonged half-life (Fig. 2, center). Phosphorothioate stabilization of the oligonucleotide backbone has previously been used to increase biostability of antisense oligonucleotide fragments [63]. Antisense phosphorothioate oligodeoxynucleotides (PS-ODNs) are widely applied and have been tested in a variety of in vitro and in vivo systems [64]. In addition, PS-ODNs demonstrated broad distribution and low toxicity after systemic delivery in mice, rats and monkeys [65] although high dose phosphorothioates have been implicated in renal toxicity [66] possibly due to aspecific membrane protein binding [67]. Introduction of phosphorothioate containing linkages at the 3′-end of the siRNA strands has also been shown to reduce nuclease-mediated degradation with minimal cytotoxicity [60]. Previous studies revealed that PS-ODNs primarily accumulate in the liver (Kupffer and endothelial cells), spleen (red pulp), skin (dendritic cells, fibroblasts), kidney (proximal tubules) and bone marrow [68,69]. Thus, this strategy of chemical modification to improve nucleotide half-life may be applied in RNAi as well.

Complexing PS-ODNs with cationic lipids is commonly applied to facilitate uptake in cell cultures, but it is not suitable in all in vivo conditions [70]. Although complexing oligonucleotides with cholesterol increases plasma half-life [71], and improves accumulation in some organs, only a small fraction accumulates in the kidney and our observations showed that in vivo silencing efficiency in mouse kidneys is diminished with this approach (P. Hamar, unpublished findings).

The tubular epithelium expresses several Toll-like receptors (TLR) types including TLR3 but not TLR7 [72], which bind single or double strand RNA molecules and function to signal the possibility of viral infection [73]. These may underlie the activation of the innate immune response pathways by siRNA [74]. 2′-O ribose methylation of siRNA reduces not only TLR mediated activation of the immune system [75,76] but also endonuclease degradation. Combined phosphorothioate and 2′-O methylation modification of siRNA was found to have increased nuclease resistance compared to single modifications [77].

Administration of PS-ODNs can result in immune responses after i.v. administration and is characterized by direct immune cell activation. Immune stimulation could be markedly reduced by 5-methyl cytosine or 2′-methoxyethyl modifications, without significant alterations in organ targeting [78].

Immunogenic reactions may also be reduced by PEG complexing. PEG is nontoxic and soluble. PEG conjugated ODNs have longer half-life, higher stability against exonucleases and PEG increased cellular uptake. PEG coupling to an ASO induced more than 10-fold increase in exonuclease stability and prolongation of plasma half-life [79], but did not affect the ability of ODN hybridization [80]. Furthermore, the cellular uptake of ODNs can also benefit from PEGylation [81]. PEGylation shields the negative charge of the ODN, making the molecule more hydrophobic, thereby facilitating cellular uptake of the conjugated drug. Additionally, PEGylation could potentially diminish the immunostimulatory effects of the ODNs [82,83]. PEGylated ODNs can be combined with positively charged lipids, forming micelles with ODNs. Such micelles could significantly improve the stability of ODNs against serum nucleases [84]. Furthermore, different targeting ligands such as folic acids can be attached at the distal terminus of PEG to achieve receptor-mediated targeted delivery of ODNs [85]. However, as discussed previously, PEGylation can reduce targeting of siRNA to the tubular epithelium and may possibly lead to increased uptake by glomerular cells.

Locked nucleic acids (LNAs) are a class of nucleic acid analogues, with strong RNA binding ability [86]. The bicyclic furanose ring in the sugar — phosphate backbone is locked in an RNA mimicking conformation by the introduction of a 2′-O,4′-C methylene (2′-OM) bridge. LNAs are easy to synthesize, have good aqueous solubility and can be combined with different linkages such as to DNA, RNA, phosphorothioates or phosphodiasters. Similar to the 2′-OM oligonucleotide approach, LNA containing antisense oligonucleotides form highly stable duplexes with high specificity for complementary RNAs [87]. A few pioneering studies using LNA modified siRNA show that systemic administration of naked LNA-siRNA is feasible, has improved resistance to serum degradation and leads to effective decrease in target protein expression [88,89]. A single dose of tritium-labeled LNA-siRNA in mice (0.15 mg/kg) resulted in high renal scintillation count, suggesting that LNA-siRNA localized in the kidney after administration [89].

Morpholino oligonucleotides (MOs) are nonionic DNA analogs (Fig. 2, right) with backbones resistant to nuclease digestion. MOs were first delivered into the cytoplasm of cells by Summerton and colleagues in 1996 [90]. A significant advantage of MOs is that RNA-MO hybrids are not substrates for RNase H. MOs targeted against the 5′-untranslated region (UTR) or the start codon prevent the translation machinery from binding [91]. Anti-GFP MOs injected into GFP expressing zebra fish embryos blocked GFP expression with unchanged mRNA level [92]. MOs can block microRNA maturation at the step of Drosha or Dicer cleavage, and they can inhibit the activity of the mature microRNA, in vitro and in vivo [93,94]. Modification of siRNA by 3′ end MO substitution increases construct stability and has prolonged activity at lower dosage concentrations compared to unmodified siRNA [95]. Both LNA and MO-modifications to siRNA may improve in vivo stability with lower off-target effects [96].

Commercially available, chemically modified siRNA (siSTABLE™) has been used in studies targeting the kidney [28,30]. The exonuclease enhanced RNAi-1 (ERI-1) preferentially cleaves siRNAs. ERI-1 mutant C. elegans worms have enhanced RNAi responses, indicating that siRNAs are more stable in ERI-1 mutants [97]. ERI-1 is highly expressed in kidney tissue and may underlie the lower effectiveness of non-stabilized siRNA; siSTABLE siRNA was found to be resistant to ERI-1 degradation and significantly prolonged the siRNA response [5].

4.3. Viral vector delivery shRNA

An alternative to siRNA administration is the delivery of vectors containing expression cassettes coding for shRNA precursors. This
approach has successfully been used in the kidney employing plasmid DNA (pDNA) vectors [33]. Transfection of tissue using pDNA vectors is similarly subject to delivery obstacles as discussed previously for siRNA. In addition, pDNA vectors are also required to enter the nucleus in order for transcription to occur.

Compared to siRNA, pDNA vector transfection is also transient (although the duration of active RNAi may vary between both approaches) but design of the shRNA coding sequence, choice of vector and promoter sequence as well as optimization of mode of delivery may be laborious and time-consuming compared with the relative ease of siRNA synthesis and treatment. Nevertheless, long-term or stable expression of shRNA may be required. Strategies employed for use in gene therapy can be readily adapted for use in RNAi. Depending on the type of vector used, viral vectors may have advantages over pDNA vectors that include stable genomic integration of the shRNA expression cassette and more efficient, endocytosis-independent transfection. For strategies employing pDNA containing shRNA coding sequences the reader is referred to the review by Takahashi et al. [98]. Targeting of the kidney using viral vectors for shRNA delivery has not been used extensively, but studies that describe transgene delivery to the kidney using viral vectors rely on the same principle. Here we will focus on the possible use of viral vector delivery for *in vitro* screening and provide a few examples for its in vivo use in experimental animal models.

Intraparenchymal delivery of replication-deficient adenovirus in rats led to prominent transgene expression in tubules and glomeruli located in the renal cortex [59], whereas injection specifically aimed at the medullary interstitium of the kidney favored expression by tubular epithelium located in the outer medulla [100]. Adenoviral vectors have been reported to induce side-effects that severely limit their potential use for shRNA delivery in kidney disease as they are found to be immunogenic [101]. As these vectors are easily produced, their use for *in vitro* screening assays may currently be of more interest. Culture techniques in which cells are maintained in gel-like protein scaffolds of extracellular matrix components have been used to mimic the *in vivo* environment in an *in vitro* cell assay. Kidney cell lines, such as the Madin–Darby canine kidney (MDCK) or renal carcinoma RCC10 cell line, readily form polarized structures under these culture conditions that can be regarded as proto-tubular structures. These so-called 3D cell culture assays can be used to study processes such as cell polarization, lumen formation, and branching morphogenesis. *In vitro* screens using 3D culture techniques are incompatible with current transfection approaches for siRNA or pDNA shRNA delivery. Adenoviral vector delivery has successfully been used to circumvent this issue and genome-wide adenoviral shRNA libraries are commercially available.

Adeno-associated virus (AAV) vectors have successfully been used to deliver shRNA constructs *in vivo* to various tissues (reviewed in [102]). Adenoviral vectors differ from AAV vectors in life cycle, the longevity of transgene expression [103], activation of the immune system [104] and in tissue specificity [105,106]. In mice, intraparenchymal delivery of recombinant AAV induced transgene expression by epithelium of the tubules that were located near the injection site, but not by endothelial or glomerular cells [107]. Intrapelvic delivery of AAV in mice has also been demonstrated to be effective, inducing transgene expression mainly in the medullary area of the kidney. Following urethral ligation, transgene expression seemed to be increased compared to non-ligated animals. Interestingly, expression of the transgene was detectable at 7 days after AAV administration in control mice but already after 4 days in kidneys of mice with urethral ligation [108]. Retargeting of AAV to modify tropism characteristics can be performed by pseudotyping, AAV-2 delivery of shRNA specific for the mineralocorticoid receptor induced significant down regulation of the receptor up to three weeks after infection and was found to prevent loss of renal function in a rat model for hypertension-induced kidney injury [109].

Lentiviral vectors have the capability to stably transfect a wide variety of dividing and non-dividing cells by genomic integration. Retrograde urethral infusion of VSV-G pseudotyped lentivirus successfully delivered the gene coding for enhanced GFP to tubular epithelial cells in mice [110]. Intraparenchymal delivery of lentivirus particularly induced transgene expression in the cortical and corticomedullary area of the kidney with lower expression in the medullary part [111]. In a rat model for renal transplantation, perfusion of the donor kidney with lentivirus harboring a construct for SHARP-2 shRNA induced significant gene silencing [112]. From a safety perspective, the use of lentiviral vectors may lead to unwanted insertion of the construct in vital gene regions. These issues are reviewed by Manjunath et al. [113].

5. siRNA therapy in disease

5.1. Acute kidney injury

In ischemic or toxic acute kidney injury (AKI) cell stress pathways are activated in the tubular epithelium which may lead to expression of pro-inflammatory factors or induction of apoptosis. Renal tissue injury is often exacerbated by infiltrating immune cells that damage renal tissue by production of reactive oxygen species such as neutrophil-derived myeloperoxidase [114] or by mediating Fas/Fas ligand (FasL) interactions leading to renal cell death via apoptosis [115]. Therefore, potential targets of siRNA-mediated down regulation of transcription include protein stress mediators, proteins regulating the epithelial inflammatory response or proteins involved in the cellular apoptosis machinery.

Nuclear translocation of the NF-κB complex is a prerequisite for expression of pro-inflammatory factors by cells of the tubular epithelium and immune cell infiltration during AKI [116,117]. Sepsis-induced AKI in mice was found to result in increased expression of the pro-inflammatory cytokines TNF-α and IL-1β. This could be counteracted by administration of siRNA specific for p105, a precursor subunit of the NF-κB complex [28].

Complement activation during ischemia-reperfusion injury is an important activator of the innate immune response. Release of the complement factor C5a and binding to its receptor C5aR expressed by the tubular epithelium induces expression and secretion of pro-inflammatory cytokines, mediating influx of neutrophils [118]. By hydrodynamic tail vein injection of shRNA to C5aR the inflammatory response following ischemia could be decreased and renal function preserved [119].

In a rat model, targeting expression of p53, a master switch of apoptosis, intravenous administration of specific siRNA was shown to significantly reduce renal dysfunction and tissue injury following renal ischemia and cisplatin-induced nephrotoxicity [120]. In this study, the differential effect of prolylact versus therapeutic (that is, after induction of injury) administration and the effect of multiple siRNA administrations was also studied. The cumulative effect of multiple low doses of siRNA on renal function following ischemia was established. In addition, protection of renal function could be achieved when siRNA was administered in a single dose and was found to be effective only when administered between 16 h prior to ischemia up to 8 h following ischemia with optimal effects when given at 2 or 4 h after ischemia.

Infiltration of CD8 expressing cytotoxic T cells (CTL) into donor tissue following renal transplantation can lead to Fas/FasL induced induction of apoptosis. In one of the first reports studying RNAi approaches for its potential in renal medicine, siRNA mediating epithelial Fas knockdown by hydrodynamic tail vein and local renal vein injection significantly reduced renal failure and apoptosis of the tubular epithelium following ischemia in an animal model mimicking transplantation associated injury [26]. Later, inhibition of complement-3 (C3) and caspase 3 by siRNA, alone [121] or in combination...
and renal disease or injury target leads can be used to predict signal pathway involvement in in vitro available for use in high-throughput lead screening. The combination of shRNA knockdown screens with image analysis of cellular responses has been used to provide key insight into complex signaling pathway interactions. For example, using protein kinase, lipid kinase and phosphatase-specific siRNA libraries, Winograd-Katz et al. [131] integrated high-content image analysis and siRNA knockdown screening to study disruption of cell adhesion complexes and cell morphology to identify pathways involved in the formation of cellular adhesions. Similarly, high-content siRNA screens have been performed to establish candidate kinases that mediate phosphorylation of tau protein with respect to development of Alzheimer’s disease [132] or kinases and phosphatases involved in regulation of cell proliferation by the mTOR pathway [133].

Optimization and configuration of automated sampling handling, imaging equipment, data management and data analysis are among the obstacles encountered that generally fall outside the field of expertise for most biologists, but which need careful consideration and attention [134]. Here we will highlight features regarding experimental design such as selection of an appropriate model and read-out parameter.

6.1. Model selection

Efforts to study renal disease using in vitro models have produced a vast array of established approaches that incorporate one or more relevant characteristics of the pathology. It is essential to minimize experimental handling steps while maintaining features that allow extrapolation to an in vivo situation. This means that existing models may need to be stripped down and limited to single or twin read-out parameters only.

Screening for protein involvement in regulation of apoptosis such as following toxicant-induced nephrotoxicity can be measured by determining caspase activity, annexin-V surface display or cell viability, all of which are compatible with high-throughput screening approaches. Recent developments aim at the detection of apoptosis by automated live-cell imaging screening approaches for example using caspase activation of fluorogenic substrates [135] or annexin-V binding [136].

To determine protein involvement in cytokine-induced fibrogenesis for example following TGF-β1 stimulation, luciferase- or GFP-reporter assays that are activated upon marker protein expression such as α-smooth muscle actin may be used [137]. Changes in expression can be quantitatively measured using changes in luminescence or fluorescence intensity. Alternatively, spectrophotometric detection of picrosirius red, more commonly used for histochemical staining of collagen, has been used to determine matrix deposition in a screening study to establish anti-fibrotic properties of compounds [138].

6.2. Multi-parameter analysis of morphological changes

Most in vitro cell assays make use of monolayer cell cultures. A three dimensional environment that simulates the physical and chemical properties of native tissues is required for the development of normal cell–cell and cell–matrix interactions, differentiation and functional characteristics. Branching morphogenesis or tubulogenesis, where tubules form from cysts of polarized epithelial cells, also requires a 3D culture environment for in vitro modeling and depends on functional cell–matrix interactions to perform cell polarization, lumen formation and tissue invasion [139,140]. These 3D cultures are also amenable to RNAi screening, but where extended periods of culture are required for formation of more complex tissue structures, viral transduction with shRNA may be more appropriate than knockdown mediated by naked siRNA. Various functional read-outs can be determined from fluorescent or luciferase reporters although labeling — particularly immunolabeling — can be more challenging. Analysis of 3D morphology requires confocal microscopy or
deconvolution of wide-field images to construct flat images of 3D objects. Image analysis software can extract multiple parameters from relatively complex cell structures, giving read-outs on proliferation and morphological characteristics, such as extent of branching. An example of this approach is given in Fig. 3. RCC10 cells spontaneously form a network of tubule-like branches when grown under the appropriate 3D culture conditions. Adenoviral delivery of shRNA specific for luciferase and GFP (both not expressed by this cell line) did not alter the branching morphology of the cells. In contrast, shRNA to FosB and matrix metalloproteinase 9 (MMP9) reduced branching (L. Price, B van de Water and Galapagos NV, manuscript in preparation). Automated image analysis of the resulting structures provides information on branch formation, extension and number of objects. These data reveal clues on the functional significance of the target protein or pathway that is involved. By this approach, predictions on in vivo signaling pathway involvement can be made in the context of renal fibrosis, nephrotoxicity or tissue regeneration responses, from which lead selection can be established for further investigation.

7. Conclusions and future perspectives

The kidney is an organ that is comparatively easily targeted by siRNA. Research into targeting of other tissues, such as brain, muscle or tumor cells, focuses on enhancement of specific uptake by that tissue by preventing renal filtration. Tubular targeting does not seem to benefit from changes made to delivery vehicles such as PEGylation. However, it is likely that enhanced stabilization, low immunogenicity and resistance to nuclease degradation prove highly useful for therapeutic applications of siRNA in renal injury.

Recent studies demonstrate that both tubular epithelium and especially cells from the glomerulus can be preferentially targeted. Targeting of the tubular epithelium appears to occur when siRNA with minor or no structural modifications, combined with an appropriate transfection reagent, are administered systemically and are delivered to the target cells via renal filtration. Glomerular targeting requires additional modification of siRNA carriers to either prevent filtration, improve siRNA uptake (for example by electroporation), or by using antibody-mediated targeting of siRNA carriers to glomerular-specific epitopes.

A few issues remain relatively unanswered at the moment. Current approaches to use siRNA in experimental renal injury models have mainly focused on prophylactic aspects; prevention of target protein expression by administration prior to induction of injury. However, it is unclear how established renal injury will affect the efficiency of siRNA uptake and reduction of protein translation. Indeed, the therapeutic benefit of post-injury siRNA administration may be effective within a certain time range only [120]. For example, renal injury may impair endocytotic uptake whereas epithelial depolarization may reduce specific uptake in strategies aimed at receptor-mediated internalization of vehicles or carriers. Nevertheless, several experimental studies demonstrate that siRNA-mediated therapy offers high potential to treat renal disease. When comparing pharmacological treatment to siRNA therapy, an important benefit is the extended mode of action of siRNA that has been found to be present for up to several days following siRNA administration.

One of the major advantages of siRNA therapy is its high target specificity. It is possible to reduce expression of cell-type specific mediators of injury or disease thereby blocking activation of

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**Fig. 3.** Effect of shRNA mediated protein knockdown on branching morphology. RCC10 kidney carcinoma cells were cultured in a 3D extracellular matrix protein gel in the presence of adenoviruses encoding the indicated shRNAs (provided by Galapagos NV, The Netherlands). In the presence of control knockdown viruses (against non-expressed proteins GFP and luciferase) tumour cells form invasive multicellular structures. To visualize cellular structures the actin cytoskeleton was stained. Automated image analysis detected structures are outlined in red. Invasion is inhibited by knockdown of genes which are essential for tumour cell invasion, such as the FosB transcription factor and the metalloprotease MMP9 (L. Price, B van de Water and Galapagos NV, manuscript in preparation).
downstream signaling pathways. This presents researchers with a new challenge; identification of key players in renal injury that are preferentially cell-type specific, crucial injury mediators and amendable to siRNA knockdown. Screening of siRNA or shRNA libraries in (patho)physiologically relevant cell models will help identify new candidates and predict their in vivo function.

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