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Gelofusine Attenuates Tubulointerstitial Injury Induced by cRGD-Conjugated siRNA by Regulating the TLR3 Signaling Pathway

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Integrin αvβ3, which is selectively targeted by cyclic arginine-glycine-aspartic acid (cRGD) peptides, is significantly upregulated in tumors. Previous studies showed that small interfering RNA (siRNA) modified with cRGD (cRGD-siRNA) could significantly inhibit tumor growth through RNAi with oncogene expression. However, cRGD-siRNA is partially reabsorbed and trapped in the kidneys, causing renal injury in an unpredictable manner. This study aimed to investigate the influence of Gelofusine on tubulointerstitial injury induced by cRGD-siRNA *in vitro* and *in vivo*. The effect of Gelofusine on the distribution of cRGD-siRNA in tumor-bearing nude mice and wild-type mice was also explored. We found that Gelofusine inhibited apoptosis and activation of the innate immune response of human tubular epithelial cells induced by cRGD-siRNA *in vitro*. In addition, co-injection of Gelofusine efficiently reduced renal retention of cRGD-siRNA without affecting its tumor targeting *in vivo*. Further *in vivo* studies indicated that Gelofusine significantly attenuated tubulointerstitial injury induced by cRGD-siRNA through regulating Toll-like receptor 3 (TLR3)-mediated activation of the nuclear factor κ B (NF-κB) and caspase-3 apoptotic pathway. In conclusion, Gelofusine, acting as a novel and effective renal protective agent, could form a compound preparation with siRNA drugs for future clinical applications.

INTRODUCTION

Small double-stranded RNA, called small interfering RNA (siRNA), is a promising therapeutic solution to treat human diseases such as viral infections, cancer, genetic disorders, and autoimmune disorders because of its strong ability to selectively silence target genes as a post-transcriptional gene regulation process.[1,2] However, a key challenge to realizing the clinical application of siRNA is the need for safe and effective delivery systems because naked siRNA is unstable in the bloodstream and does not readily cross membranes; it is also immunogenic.[3] A broad diversity of delivery systems is under clinical trials to address the challenges of *in vivo* delivery, including cationic liposomes, polymeric carriers, and siRNA conjugates.[4] Additionally, these systems have been modified with different targeting ligands, such as the Arg-Gly-Asp (RGD) peptide, aptamer, cholesterol, and antibody, to increase their targeting ability.[5] Recently, cyclic RGD (cRGD)-conjugated siRNA, which specifically silences the expression of vascular endothelial growth factor receptor 2 (VEGFR2) mRNA via targeting the αvβ3 integrin receptor on neovascular endothelial cells, was found to inhibit tumor growth.[6] Similarly, cRGD-epidermal growth factor receptor (EGFR) siRNA conjugate had anti-tumor effects on glioblastoma by silencing the expression of EGFR mRNA through targeting the αvβ3 integrin receptor on glioblastoma cells.[7] In addition, other studies also reported that siRNA nanoparticles based on RGD peptides could significantly inhibit tumor growth through RNAi with oncogene expression.[8,9] Hence, it could be a valuable and promising way to develop novel targeted anti-tumor drugs with RGD-based siRNA delivery systems. However, no matter what kind of RGD-based delivery systems siRNA uses, these materials are partly accumulated and mainly eliminated by the kidneys

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following systemic administration.[6–11] Consequently, such pharmacokinetic characteristics could impair the optimal therapeutic dosing of medicine based on RGD peptides in patients and might cause renal toxicity in an unpredictable manner.[12]

Tubulointerstitial injury is a relatively common cause of both acute kidney injury and chronic kidney disease. Besides drugs, autoimmune and systemic disorders, and metabolic etiologies, infections (including viruses and bacteria) are also an important cause of acute tubulointerstitial nephritis.[13,14] Toll-like receptors (TLRs) are a family of pattern recognition receptors that can trigger innate immunity by recognizing pathogen-associated molecules (including lipopolysaccharide, lipoproteins, microbial DNA, viral double-stranded RNA, and zymosan).[15,16] Tubular epithelial cells, which express TLR1, TLR2, TLR3, TLR4, and TLR6, can contribute to the activation of immune responses, resulting in tubulointerstitial injury.[15,17] For example, TLR3, an important receptor for longer (more than 20 bp) double-stranded RNA (including siRNA), has been shown to be widely expressed on the surface and endosome of tubular epithelial cells.[16,18] When bound via its ligands, TLR3 signal transduction leads to the activation of a series of downstream pathways involved in the cellular response to stress. Common to TLR3 signal transduction pathways is the activation of nuclear factor κB (NF-κB) pathway, a transcription factor that regulates the expression of multiple genes involved in chemoattraction, inflammation, cellular proliferation, and antimicrobial activity.[19] TLR3 activation can lead to the induction of antiviral and proinflammatory cytokines (including interleukin 6 [IL-6] and interferon beta [IFN-β]) by activating the NF-κB pathway in both immune and nonimmune cells.[19–21] In terms of the kidneys, this innate immune response to double-stranded RNA via TLR3 leads to renal inflammation, such as tubulointerstitial nephritis. In addition, many studies indicate that TLR3 activation can trigger apoptotic death in both cancer and normal tissues through caspase-3-dependent pathways.[22–24] This unavoidable phenomenon of apoptosis in response to double-stranded RNA via TLR3 is detrimental in pre-existing renal inflammation such as tubulointerstitial nephritis. It has been reported that 21-nt siRNA duplexes, the standard length for clinically translated RNAi therapeutic agents, could activate TLR3 independent of sequence or target. [25] Nevertheless, direct evidence has rarely been presented regarding the role of TLR3 in siRNA-induced nephritis.[7,26]

After the size-selective properties of glomerular filtration, the primary urine contains proteins of low molecular weight (< 60 kDa), such as peptides, whereas larger proteins are excluded,[27] Following this process, proteins of low molecular weight are reabsorbed by tubular proximal cells, which, at present, is the only documented process for tubular protein clearance.[28] Two receptors, megalin and cubilin, are responsible for the reabsorption of peptides. Megalin and cubilin are receptors of tubular epithelial cells that exist in tandem and form a complex that mediates peptide uptake by proximal tubular epithelial cells.[29] Megalin- and cubilin-mediated uptake of receptor-bound peptides ultimately leads to delivery to lysosomes for proteolytic digestion.[30] Metabolized peptides are released into the bloodstream, whereas not only radiolabeled peptides but also conjugates and nanoparticles constructed with both peptides and other molecules might be retained in the tubular cells, leading to subsequent side effects.[31] Thus, when siRNA delivery systems combined with peptides are reabsorbed and endocytosed into the endosome by tubular epithelial cells, unwanted immune-related nephrotoxicity may be induced by the TLR3 signaling pathway.

A possible approach is to prevent the megalin- and cubilin-mediated endocytosis pathway on the membrane of tubular epithelial cells to reduce the risk of nephrotoxicity of RGD-based siRNA delivery systems induced by renal reabsorption. At present, Gelofusine (B. Braun Medical, Boulogne Billancourt, France), which consists of succinylated bovine gelatin molecules and is used in clinical emergency units as a gelatin-based plasma expander, has been reported to increase the renal excretion of megalin ligands such as peptides.[32] Some reports indicate that Gelofusine infusions efficiently reduce the retention of somatostatin analogs and radiolabeled compounds in cortical proximal tubules.[33,34] Furthermore, Gelofusine significantly induced a more than 50% reduction in renal reabsorption of radiolabeled conjugate based on RGD peptide without affecting tumor uptake.[35] However, the effects of Gelofusine infusions on the reabsorption of siRNA delivery systems are unclear, especially delivery systems based on peptides. Similarly, it is also unknown whether Gelofusine has protective effects on tubulointerstitial injury via interfering with the reabsorption of agents.

In the present study, cRGD-conjugated siRNAs were well constructed and found to significantly inhibit tumor growth by targeting cells expressing the αvβ3 integrin receptor as a specific example of siRNA delivery systems. First, the study confirmed that cRGD-conjugated siRNA directly induces tubulointerstitial injury, depending on the TLR3 signaling pathway, by being reabsorbed and endocytosed by tubular epithelial cells. On the basis of these results, the protective effects of Gelofusine on tubulointerstitial injury by influencing the renal reabsorption of cRGD-conjugated siRNA were studied *in vitro* and *in vivo*. Collectively, these results might probably be beneficial in the development of future RNAi therapeutic agents, especially siRNA conjugates or nanoparticle-based on peptides.

RESULTS

Gelofusine Inhibited the Uptake of cRGD-siRNA by Tubular Epithelial Cells *In Vitro*

cRGD-negative control siRNA (siNC) conjugates were labeled with a fluorophore (indodicarbocyanine-5 [Cy5]), called cRGD-siNC-Cy5, to evaluate the effect of Gelofusine on the uptake and intracellular distribution of cRGD-siNC in HK-2 cells. Flow cytometry results showed that cRGD-siNC-Cy5 was significantly taken up by HK-2 cells at a concentration of 50 μg/mL or 100 μg/mL, whereas Gelofusine (100 μg/mL) treatment led to an approximately 35% decrease in the uptake of cRGD-siNC-Cy5, in accordance with the results of confocal microscopy, suggesting that Gelofusine might affect the binding of cRGD-siNC to tubular epithelial cells (Figure 1A). The cellular uptake levels of cRGD-siRNA treated with different

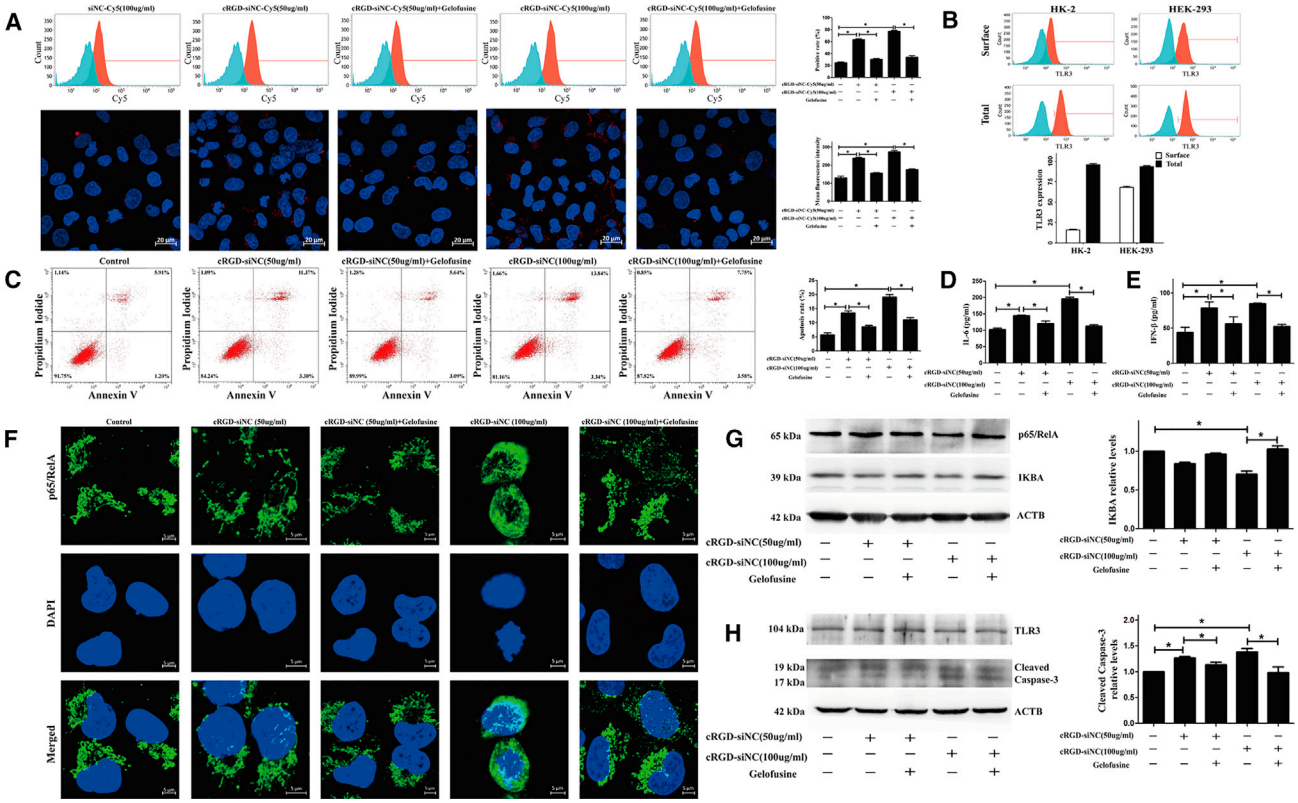

Figure 1. Influence and Mechanism of Action of Gelofusine on Immunogenicity and Apoptosis Induced by cRGD-siRNA *In Vitro*

(A) The cellular uptake levels and distribution of cRGD-siRNA in HK-2 cells. The cells were transfected with different concentrations of cRGD-siNC-Cy5. For the Gelofusine group, before transfection with cRGD-siNC-Cy5, the cells were pretreated with 100 μg/mL Gelofusine. After 24-hr treatment, the cells were collected and measured by flow cytometry or fixed and visualized using confocal laser microscopy. Cell nuclei were counterstained with DAPI (blue), and siRNA was labeled with Cy5 (red). (B) Analysis of TLR3 expression on the surface and total area of HK-2 and HEK293 cells. The cells were collected and measured by flow cytometry. (C) Analysis of apoptosis of HK-2 cells using different treatments. HK-2 cells were transfected with different concentrations of cRGD-siNC. For the Gelofusine group, before transfection with cRGD-siNC, the cells were pretreated with 100 μg/mL Gelofusine. After 48-hr transfection, the cells were collected and analyzed by flow cytometry with Annexin V and propidium iodide. (D and E) Quantitative analysis of IL-6 (D) and IFN-β (E) levels using ELISA. After 24-hr treatment, the cell supernatant was collected and analyzed using ELISA. (F) The distribution of p65/RelA in HK-2 cells when using different treatments. After 24-hr treatment, the cells were fixed and visualized using confocal laser microscopy. Cell nuclei were counterstained with DAPI (blue), and p65/RelA was detected with an antibody conjugated with Alexa Fluor 488 (green). (G and H). Effects on the NF-κB pathway (G) and cleaved caspase-3 expression (H) using different treatments. After 48-hr treatment, the cells were collected for western blot analysis. The expression of protein was calculated compared with the expression of β-actin (ACTB). *$p < 0.05$; n = 3; Scale bars, 20 μm or 5 μm.

concentrations of Gelofusine (0, 25, 50, and 100 μg/mL) was also evaluated, showing a dose effect (Figures S1A–S1C). Moreover, knockdown of megalin reduced the cellular uptake of cRGD-–siNC-Cy5, similar to the influence of Gelofusine treatment (Figures S1A–S1C), indicating that the megalin receptor was involved in the cellular uptake of cRGD-siNC-Cy5. On the other hand, it was strange that the uptake of high-dose siNC-Cy5 (100 μg/mL) was up to approximate 25%, which was close to the uptake of cRGD-siNC-Cy5 with Gelofusine blocking (Figure 1A). This suggested that siNC-Cy5 and part of cRGD-siNC-Cy5 might enter into renal tubular epithelial cells through other ways, in addition to the megalin/cubilin receptors associated with Gelofusine.[12,20,22]

The results also showed that HEK293 and HK-2 cells expressed TLR3 both on the cellular surface and in the cytoplasm, and the expression of TLR3 on the surface of HK-2 cells was approximately 15% (Figure 1B), suggesting that both HEK cells and human proximal tubular cells highly expressed TLR3. Collectively, these data showed that, after treatment with high-dose siRNA, part of the siRNA might bind to TLR3 expressed on the surface of HK-2 cells and was trapped by HK-2 cells.[26]

Gelofusine Reduced Apoptosis of Tubular Epithelial Cells Caused by cRGD-siRNA *In Vitro*

The activity of proximal tubular epithelial cells is necessary to maintain the integrity of renal reabsorption function. Therefore, this study evaluated apoptosis of tubular epithelial cells induced by cRGD-siRNA using flow cytometry and western blot analysis. The data showed that cRGD-siNC could significantly cause apoptosis of tubular epithelial cells (the apoptosis rate was 16.18%

at a concentration of 50 μg/mL and 22.69% at a concentration of 100 μg/mL), whereas cRGD alone did not cause apoptosis (Figures 1C and S2A). However, treatment with Gelofusine efficiently reduced the apoptosis rate of tubular epithelial cells by approximately 10%, similar to the influence of knockdown of TLR3 (Figures 1C and S2A). In addition, western blot analysis showed that HK-2 cells expressed TLR3, which was consistent with the result of flow cytometry (Figure 1H). Caspase-3 protein was significantly activated after stimulation with cRGD-siNC in HK-2 cells, whereas Gelofusine could suppress this activation compared with the control group (Figure 1H). Taken together, these results suggested that Gelofusine could reduce apoptosis of tubular epithelial cells caused by cRGD-siRNA by regulating the TLR3-caspase-3 pathway *in vitro*.

Gelofusine Inhibited Expression of IL-6 and IFN-β Induced by cRGD-siRNA *In Vitro*

It is reported that double-stranded RNA stimulates cells to activate the TLR3-mediated NF-κB signaling pathway, leading to the expression of cytokines, including IL-6 and IFN-β.[20,21] Therefore, IL-6 and IFN-β were used as indicators to evaluate activation of the innate immune response induced by cRGD-siRNA in tubular epithelial cells. After stimulating cells for 24 hr, cRGD-siNC led to the elevation of IL-6 and IFN-β in a concentration-dependent manner, but cRGD alone did not cause this phenomenon; however, Gelofusine could significantly weaken the stimulating effect of cRGD-siNC, similar to the influence of knockdown of TLR3 (Figures 1D, 1E, S2B, and S2C).

NF-κB signaling is one of the downstream pathways mediated by TLR3 transduction, in which the nuclear transport of p65/RelA, regulated by IκBα (IKBA), is the key to activating the NF-κB pathway.[36] Compared with the control group, the results showed that NF-κB signaling could induce transport of p65/RelA protein from the cytoplasm into the nucleus, especially in the high-concentration group of cRGD-siNC (100 μg/mL), which could significantly be inhibited by Gelofusine (Figure 1F). In addition, it was found that NF-κB signaling could significantly reduce the expression of IKBA stimulated by cRGD-siNC, whereas this phenomenon was suppressed by Gelofusine, similar to the influence of knockdown of TLR3 (Figures 1G and S2E–S2G). Thus, these studies suggested that the release of p65/RelA in the cytoplasm was caused by cRGD-siRNA following reduction of IKBA *in vitro*, resulting in nuclear transport of p65/RelA and, thereby, activating the NF-κB signaling pathway by upregulating the expression of IL-6 and IFN-β. Interestingly, activation of the NF-κB pathway induced by cRGD-siRNA could be inhibited by Gelofusine.

Influence of Gelofusine on the Distribution and Reabsorption of cRGD-siRNA *In Vivo*

Previous studies have reported that both conjugates and nanoparticles of siRNA modified with cRGD, which can actively target the αvβ3 integrin receptor expressed on tumor tissues, including glioma, have the ability to inhibit tumor growth.[6–8] Nude mice bearing U87MG tumor xenografts were used to evaluate the influence of Gelofusine on tumor targeting of cRGD-siRNA, which is of vital importance for compound preparation of cRGD-siRNA and Gelofusine in future clinical applications. After 24 hr of intravenous injection of siNC-Cy5, cRGD-siNC-Cy5, or a mixture of cRGD-siNC-Cy5 and Gelofusine, it was found that cRGD-siNC-Cy5 had the ability to target the tumor tissues, whereas Gelofusine had no effect on the accumulation of cRGD-siNC-Cy5 in U87MG tumor xenografts compared with the tumor-free targeting of siNC-Cy5, but Gelofusine could significantly reduce the accumulation of cRGD-siNC-Cy5 in the kidneys (Figures 2A–2C).

However, because the tumor-specific accumulation of cRGD-siRNA in tumor-bearing nude mice has an effect on the accurate evaluation of renal reabsorption of cRGD-siRNA, the influence of Gelofusine on the reabsorption of cRGD-siRNA in wild-type BALB/c mice not bearing tumors was evaluated. After 24 hr of administration of saline, cRGD-siNC-Cy5 or a mixture of cRGD-siNC-Cy5 and Gelofusine, it was found that the Gelofusine could significantly reduce the accumulation of cRGD-siNC-Cy5 in the group with a mixture of cRGD-siNC-Cy5 and Gelofusine compared with the cRGD-siNC-Cy5-only group (Figures 2D and 2F). Gelofusine also reduced the total amount of cRGD-siNC-Cy5 in the renal cortex (Figure 2E), suggesting that it could inhibit the reabsorption of cRGD-siRNA in tubular epithelial cells *in vivo*, consistent with the *in vitro* results.

Gelofusine Attenuated Tubulointerstitial Injury Induced by cRGD-siRNA *In Vivo*

A model of renal tubular injury was established using cRGD-siRNA in wild-type BALB/c mice, similar to the previous study, as follows.[7] The mice were injected intravenously 7 times over a 48-hr interval with saline, cRGD-siNC (5 nmol/20 g), or a mixture of cRGD-siNC (5 nmol/20 g) and Gelofusine (4 mg/20 g). During sustained administration, no significant difference was found in weight changes in each group (Figure 3B). As shown in Figures 3A and 3C, no obvious abnormality was observed in appearance of the kidneys in each group; however, it was found that the kidney weight index of the cRGD-siNC group slightly decreased ($p = 0.162$) compared with the saline group, whereas infusion of Gelofusine can reverse this phenomenon ($p = 0.065$, compared with the cRGD-siNC group), indicating that cRGD-siRNA can cause mild renal injury in this situation instead of severe toxicity such as renal atrophy.

The cRGD-siNC group showed obvious pathological changes in tubulointerstitial nephritis, such as renal edema and interstitial hyperemia, whereas co-injection of Gelofusine led to remission of tubulointerstitial nephritis compared with the saline group (Figure 4A). Inflammatory cells labeled with CD11b, including monocytes and granulocytes, infiltrated the renal cortex following administration of cRGD-siNC, whereas infusion of Gelofusine could alleviate this phenomenon compared with the saline group (Figures 4A and 4D). However, no significant difference was found in the levels of IL-6 and IFN-β in the serum of each group (Figures 4I and 4J), indicating that cRGD-siNC (5 nmol/20 g) did not cause a systemic immune response, which was consistent with the results reported in previous

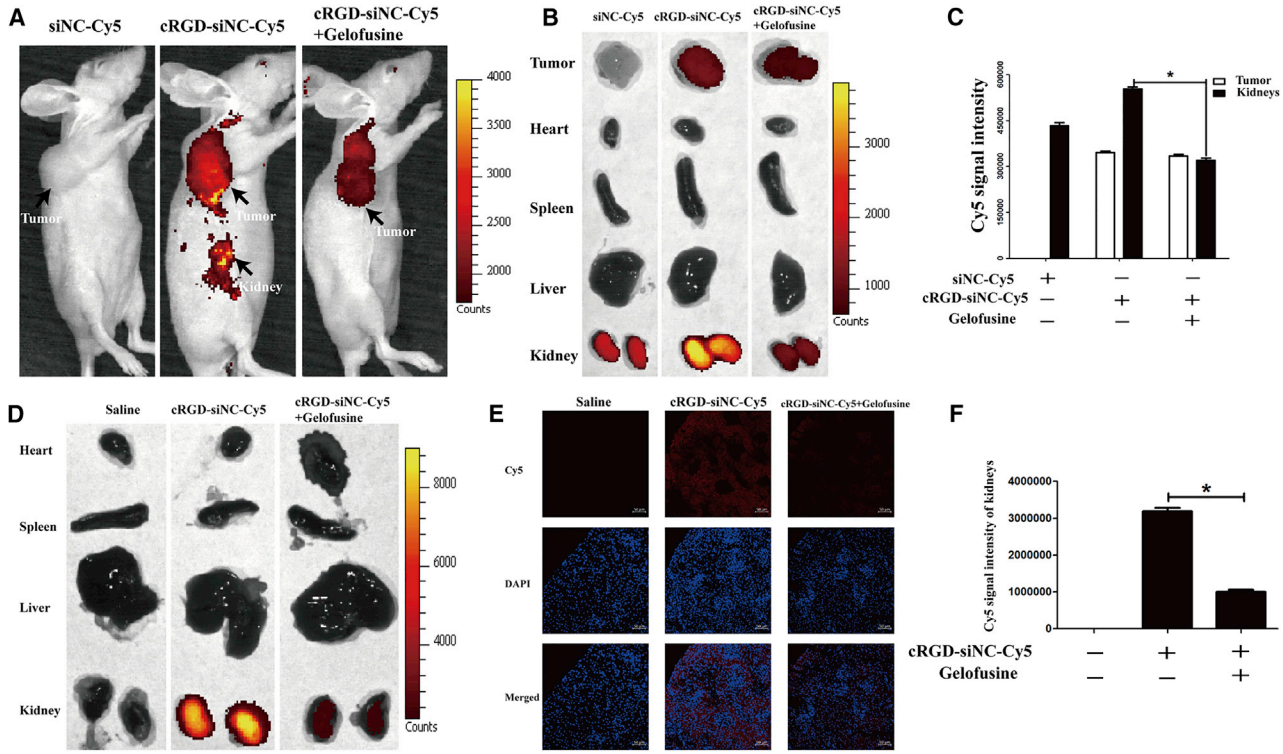

Figure 2. Influence of Gelofusine on the Distribution and Reabsorption of cRGD-siRNA *In Vivo*

(A) Influence of Gelofusine on the distribution of cRGD-siRNA in nude mice bearing U87MG tumor xenografts. Nude mice bearing U87MG tumor xenografts were injected with siNC-Cy5 (1 nmol/20 g), cRGD-siNC-Cy5 (1 nmol/20 g), or a mixture of cRGD-siNC-Cy5 (1 nmol/20 g) and Gelofusine (4 mg/20 g) with a single dose via the tail vein, and fluorescence images of whole animals were taken using the IVIS imaging system at 24 hr. (B) Influence of Gelofusine on the distribution of cRGD-siRNA in isolated organs 24 hr after injection in nude mice bearing U87MG tumor xenografts. (C) Analysis of Cy5 signal intensity of isolated tumors and kidneys in different groups in nude mice bearing U87MG tumor xenografts. (D) Influence of Gelofusine on the distribution of cRGD-siRNA in isolated organs 24 hr after injection in wild-type BALB/c mice. Wild-type BALB/c mice were injected with saline, cRGD-siNC-Cy5 (1 nmol/20 g), or a mixture of cRGD-siNC-Cy5 (1 nmol/20 g) and Gelofusine (4 mg/20 g) with a single dose via the tail vein, and fluorescence images of whole animals were taken using the IVIS imaging system at 24 hr. (E) Frozen sections of kidneys with different treatments 24 hr after injection in wild-type BALB/c mice. The nuclei were stained with DAPI (blue), and siRNA was labeled with Cy5 (red). (F) Analysis of Cy5 signal intensity of isolated kidneys in different groups in wild-type BALB/c mice. All images were scaled to the same minimum and maximum color values. *$p < 0.05$. Scale bars, 50 μm. *$p < 0.05$.

studies.[6,7,37] In addition, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to label apoptotic cells for evaluating renal injury. As shown in Figures 4A, 4B, 4E, and 4G, the cRGD-siNC group displayed obvious tubular cell apoptosis with a higher apoptotic index, accompanied by activation of caspase-3, whereas co-injection of Gelofusine could alleviate this phenomenon compared with the saline group.

In the study of the tubulointerstitial injury mechanism induced by cRGD-siRNA, the TLR3 signaling pathway was selected for further studies in line with *in vitro* research. As displayed in Figure 4B, tubular epithelial cells of mice widely expressed TLR3, consistent with the results found in HK-2 cells. The expression of IKBA in the cRGD-siNC group significantly decreased, followed by nuclear translocation of p65/RelA, whereas infusion of Gelofusine could inhibit this reduction and translocation compared with the saline group (Figures 4B, 4C, 4F, and 4H). The signaling mechanism by which Gelofusine attenuated tubulointerstitial injury induced by cRGD-siRNA is depicted in Figure 5. Taken together, these results indicated that tubulointerstitial injury occurred in the renal cortex, accompanied by apoptosis of tubular epithelial cells induced by cRGD-siRNA through TLR3-mediated activation of the NF-κB pathway and caspase-3 apoptotic pathway in mice. However, Gelofusine had the ability to attenuate tubulointerstitial injury as a promising kidney-protective agent *in vivo*.

DISCUSSION

siRNA, as a new therapeutic agent, offers immense therapeutic potential because of higher potency and more specificity and flexibility than conventional drugs, which allows them to be designed more easily for disease treatment compared with conventional drugs.[38] However, the clinical application of siRNA as a therapeutic agent inevitably faces some obstacles, including off-target effects, instability under physiological conditions, and possible immunogenicity via activation of TLR.[39] Therefore, clinically suitable drug delivery systems are required to bring siRNA to its site of action with safe and effective

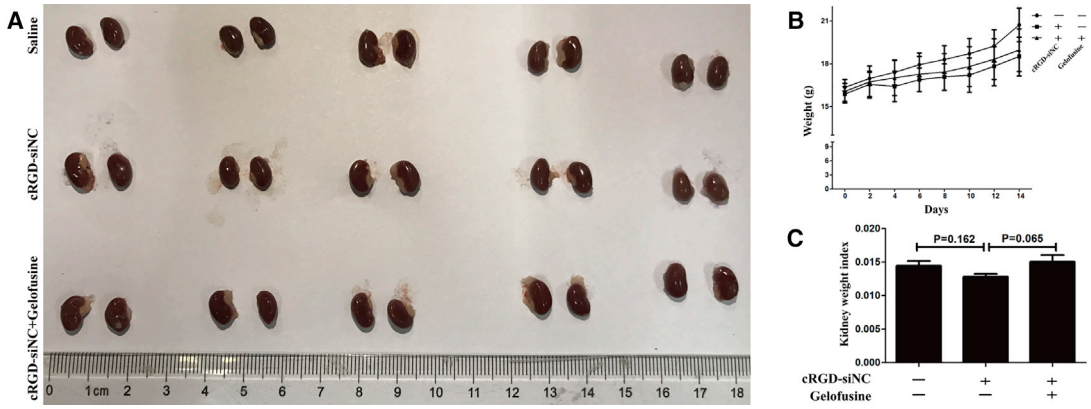

Figure 3. Weight Curves and Renal Morphology Influenced by cRGD-siRNA *In Vivo*

Wild-type BALB/c mice were injected with saline, cRGD-siNC (5 nmol/20 g), or the mixture of cRGD-siNC (5 nmol/20 g) and Gelofusine (4 mg/20 g). The mice were administered different treatments continuously seven times by intravenous injection into the tail vein at an interval of 48 hr. The mice were euthanized 12 hr after the last treatment, and their kidneys were removed surgically, weighed, and fixed. Serum was collected for immunological evaluation. (A) Gross observation of renal size. (B) The weight curves of mice during administration. (C) Kidney weight index of different groups.

characteristics. Some delivery systems have been modified with different targeting ligands, including the RGD peptide, to obtain targeting ability. Previous studies have reported that both conjugates and nanoparticles of siRNA modified with cRGD, which can actively target the αvβ3 integrin receptor expressed on tumor tissues, have the ability to inhibit tumor growth by specifically silencing the expression of tumor proliferation-related genes.[6–8] However, following systemic administration, RGD-modified siRNA delivery systems are inevitably accumulated and eliminated by the fragile kidneys, causing unpredictable tubulointerstitial injury via the immunogenicity of siRNA.

Gelofusine in the Regulation of cRGD-siRNA Uptake by Proximal Tubular Epithelial Cells

Following proteins of low molecular weight (< 60 kDa), such as peptides filtered by glomeruli, peptides are mainly reabsorbed and endocytosed by proximal tubular epithelial cells into lysosomes for proteolytic digestion in the renal cortex.[31] The two interacting receptors expressed on renal tubular epithelial cells, megalin and cubilin, are key receptors in this process. Megalin is a large transmembrane protein (approximately 600 kDa) belonging to the low-density lipoprotein receptor family. Cubilin is a peripheral membrane protein (approximately 460 kDa), which was an intrinsic factor cobalamin receptor with no transmembrane domain. Megalin mediates the internalization of cubilin-ligand complexes by binding to cubilin.[29] After glomerular filtration, most proteins, such as vitamin D-binding protein, albumin, β2- and α1-microglobulin, and several hormones, are reabsorbed as ligands of megalin/cubilin receptors.[40] One possible approach is to interfere with the megalin/cubilin receptor-mediated reabsorption pathway to reduce the reabsorption and accumulation of peptides in the renal cortex.[29] It was found *in vitro* that cRGD-siRNA could be taken up by human renal tubular epithelial cells, whereas it could be reduced significantly by Gelofusine pretreatment. *In vivo*, first immunodeficient nude mice bearing U87MG tumor xenografts were selected for evaluating the effect of Gelofusine on tumor targeting of cRGD-siRNA. It was found that Gelofusine had no influence on the accumulation of cRGD-siRNA in tumor tissue via intravenous administration. However, because the tumor-specific accumulation of cRGD-siRNA in immunodeficient nude mice has an effect on the evaluation of accurate renal reabsorption and immunogenicity of cRGD-siRNA, immunocompetent wild-type BALB/c mice were chosen instead for studying tubulointerstitial injury and its mechanisms induced by cRGD-siRNA. After administration of cRGD-siRNA, it was found that cRGD-siRNA mainly accumulated in the renal cortex, suggesting that it was mainly excreted and reabsorbed by the kidneys. Surprisingly, Gelofusine infusions led to a significant decrease in the retention of cRGD-siRNA in the cortical proximal tubules, suggesting that Gelofusine might influence the renal absorption of cRGD-siRNA. It has been reported that charges of ligands may play a key role in the binding to megalin/cubilin receptors because ligands, including peptides, interact with megalin through a negatively charged calcium-binding area in megalin.[41] Renal reabsorption of peptides can be reduced by co-administering cationic compounds such as lysine or a mixture of positively charged amino acids.[31] cRGD-siRNA is a conjugate of siRNA modified with cyclo(RGDfK) oligopeptide, wherein the cRGD oligopeptide contains cationic amino acids such as arginine and lysine. It can be identified by the negatively charged binding site on the megalin/cubilin receptors to be reabsorbed into tubular epithelial cells, resulting in renal retention. Gelofusine, a gelatin-based plasma expander, provides a large number of cationic amino acids that competitively mask the binding site of cRGD-siRNA with megalin/cubilin receptors, reducing the reabsorption of cRGD-siRNA.[32,42,43] Thus, according to this principle, Gelofusine has the ability to reduce the retention of cRGD-siRNA in the renal cortex without affecting its tumor targeting.

Gelofusine in the Regulation of Activation of the TLR3 Pathway Induced by cRGD-siRNA

TLR3 is a pattern recognition receptor that specifically recognizes double-stranded siRNA, and its activation leads to the activation of

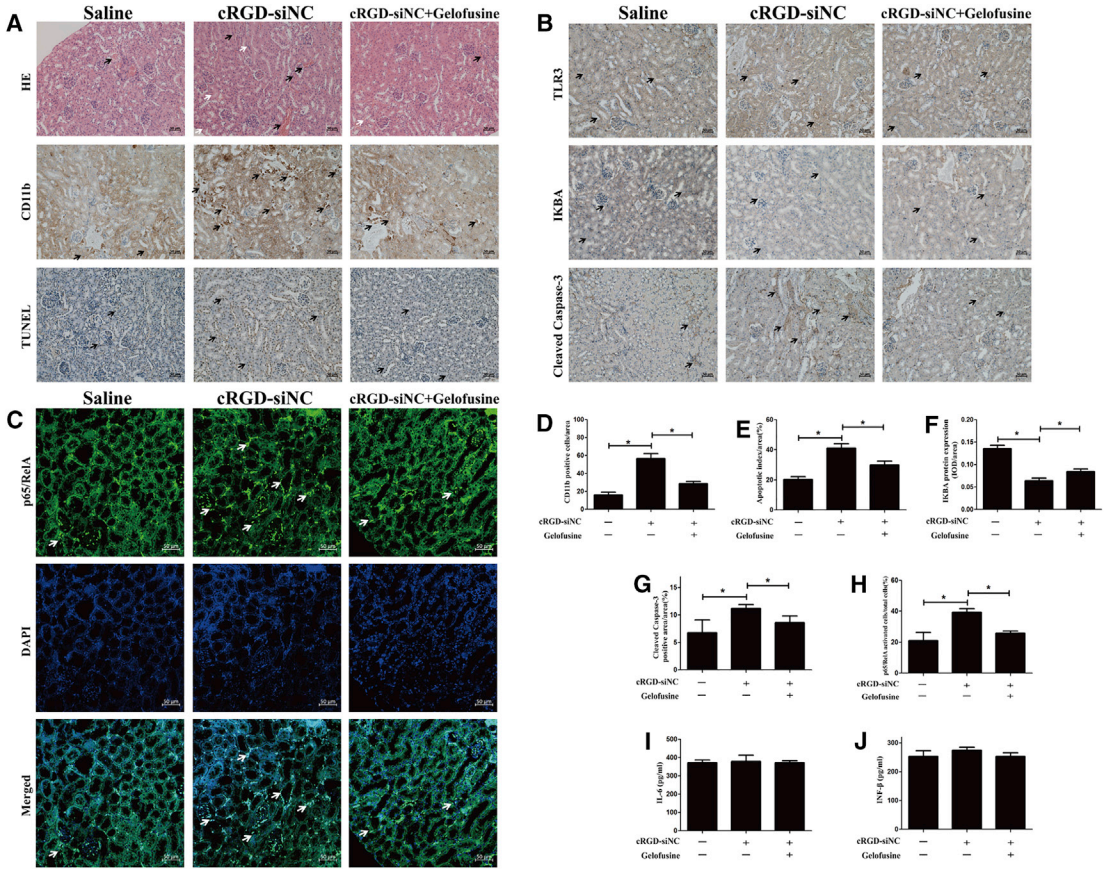

Figure 4. Influence and Mechanism of Action of Gelofusine on Tubulointerstitial Injury Induced by cRGD-siRNA *In Vivo*

(A) Analysis of H&E-stained sections, CD11b immunohistochemical staining, and TUNEL staining of kidneys. The extent of tubulointerstitial injury was evaluated using H&E staining. Black arrows indicate interstitial hyperemia, and white arrows indicate renal edema. The degree of infiltration of monocytes and granulocytes was evaluated using CD11b immunohistochemistry, with brown-stained cells indicated by black arrows. Apoptosis in kidneys was analyzed using TUNEL staining, with brown-labeled cells indicated by black arrows. (B) Analysis of the expression of TLR3, IKBA, and cleaved caspase-3 via immunohistochemical staining in kidneys. Positive expression of cells or areas is shown as brown-stained cells, indicated by black arrows. (C) The distribution of p65/RelA in the renal cortex. The nuclei were stained with DAPI (blue), whereas p65/RelA was labeled with an antibody conjugated with Alexa Fluor 488 (green). p65/RelA enrichment in the nucleus indicated that the NF-κB pathway was activated, as shown by white arrows. (D) Analysis of the infiltration of monocytes and granulocytes by CD11b-positive cells/area. (E) Analysis of apoptosis in kidneys using an apoptotic index with TUNEL staining. (F) The relative expression level of IKBA protein was evaluated using mean optical density (integral optical density [IOD]/area). (G) Analysis of the degree of caspase-3 activation using cleaved caspase-3-positive area/total area. (H) Analysis of the degree of NF-κB pathway activation in the renal cortex using p65/RelA-activated cells/area. (I and J) Quantitative analysis of IL-6 (I) and IFN-β (J) in the serum of wild-type BALB/c mice was measured using ELISA. *$p < 0.05$, scale bars, 50 μm.

a series of downstream pathways involved in the cellular response to stress, such as the NF-κB inflammatory pathway and caspase-3 apoptotic pathway.[23,44] The present study found that the HEK293 cell line and the human tubular epithelial cell line HK-2 both expressed the TLR3 receptor, suggesting that cRGD-siRNA might inevitably bind to the TLR3 receptor through the intravenous administration route.

Following TLR3 signal transduction, the transcription factor NF-κB is activated, which controls important pathophysiological processes such as inflammatory response, immune response, cell proliferation, transformation, and apoptosis.[36] The results showed that stimulation with cRGD-siRNA in human renal tubular epithelial cells could increase IFN-β and IL-6 expression *in vitro*, whereas Gelofusine pretreatment could suppress this phenomenon. *In vivo*, after continuous administration of cRGD-siRNA (5 nmol/20 g) for 14 days in wild-type BALB/c mice, it was found that tubulointerstitial injury occurred in the renal cortex, such as renal edema and interstitial hyperemia, with infiltration of inflammatory cells, including monocytes and granulocytes. Similar to the *in vitro* study, tubulointerstitial nephritis was greatly reduced with Gelofusine treatment. However, no significant difference was found in IFN-β and IL-6 serum levels among different treatment groups, indicating that cRGD-siRNA did not cause a systemic immune response under such administration conditions. In addition to NF-κB pathway activation, the caspase-3-dependent apoptotic pathway is also activated following recognition of double-stranded siRNA by TLR3, which might be detrimental in pre-existing renal inflammation, such as tubulointerstitial

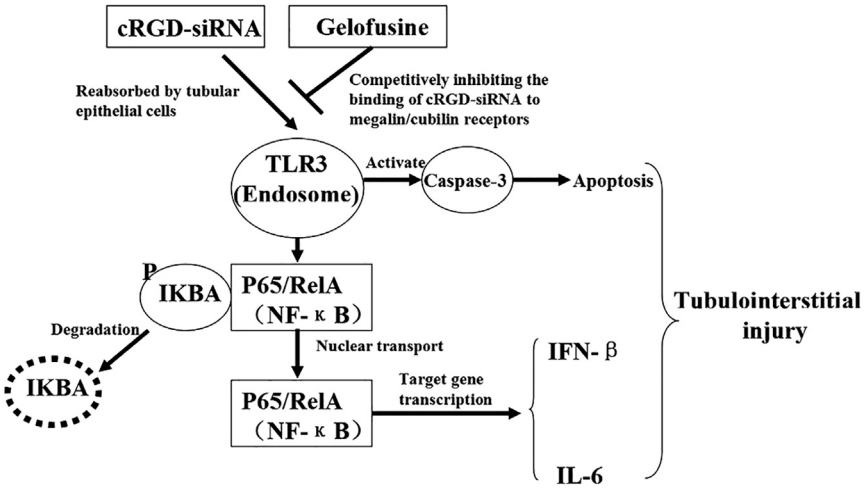

Figure 5. The Mechanism by which Gelofusine Attenuated Tubulointerstitial Injury Induced by cRGD-Conjugated siRNA

cRGD-siRNA was reabsorbed and endocytosed into endosomes by tubular epithelial cells through binding to megalin/cubilin receptors, followed by TLR3 activation in endosomes. The activated TLR3 resulted in IKBA degradation and subsequent NF-κB translocation to the nucleus, where it induced the transcription of multiple genes, including IL-6 and IFN-β, to promote infiltration of inflammatory cells and inflammatory injury. In addition, TLR3 activation could also trigger apoptosis, aggravating the existing tubulointerstitial injury via activating caspase-3. However, Gelofusine could competitively inhibit the binding of cRGD-siRNA to megalin/cubilin receptors, avoiding TLR3 activation in endosomes.

nephritis.[23–25] This study showed that the proportion of apoptosis of human renal tubular epithelial cells increased with activation of caspase-3 protein after being stimulated with cRGD-siRNA, whereas Gelofusine could reduce the apoptosis of cells. Similarly, Gelofusine infusions also led to a significant decrease in the proportion of apoptosis caused by cRGD-siRNA in cortical proximal tubules *in vivo*. Taken together, Gelofusine can reduce the reabsorption of tubular epithelial cells by competitively inhibiting the binding of cRGD-siRNA to megalin/cubilin receptors, avoiding TLR3 receptor recognition in endosomes. Thus, Gelofusine has protective effects on the inflammatory response and apoptosis damage induced by cRGD-siRNA in tubular epithelial cells *in vitro* and *in vivo*.

Clinical Application Prospects of Gelofusine in siRNA Drugs Modified with Polypeptides

At present, several RNAi-based approaches, including siRNA drugs, are undergoing assessment in phase I, II, and even phase III clinical trials.[2] RNAi therapeutic approaches have several advantages compared with traditional medicines, such as the ability to harness a natural pathway, the ability to target virtually any protein, and simplifying the discovery of drug candidates.[45] However, the challenges of RNAi-based drugs in clinical trials are immune stimulation, in particular immunogenicity in metabolic organs, accumulation in nontarget tissues, and off-target effects within cells.[46] Off-target effects in nontarget tissues can be solved by constructing an appropriate delivery system, such as RGD-based siRNA delivery systems, and intracellular off-target effects can be reduced by sequence screening and backbone modification of siRNA.[3] Although the immunogenicity of siRNA drugs can be also mitigated by backbone modification and sequence screening, potency can be significantly decreased compared with natural siRNA structure. For example, although 19-nt blunt-ended siRNA structure has the ability to avoid TLR3 activation, it weakens the performance of RNAi compared with 2-nt overhang, 21-nt siRNAs, which is detrimental to the efficacy of siRNA drugs, especially drugs with the purpose of inhibiting tumors.[47] Thus, RNAi-associated immunogenicity might act as a hurdle to safe and effective RNAi, particularly in clinical applications. In previous studies, conjugates and nanoparticles of 21-nt standard siRNA structure modified with cRGD were constructed and have shown good application prospects in the field of cancer therapy.[6–8] However, this study found that 21-nt standard siRNA structure modified with cRGD caused unpredictable tubulointerstitial injury by activating the TLR3 signaling pathway in the renal cortex following continuous administration for 14 days. Therefore, a feasible way to reduce the accumulation of cRGD-siRNA in the kidneys should be used by accelerating the excretion of cRGD-siRNA to reduce or avoid kidney injury without affecting the performance of siRNA drugs. A new method was introduced in this study to achieve this objective. The present study found that infusion of Gelofusine with siRNA drugs modified with cRGD could attenuate tubulointerstitial injury with a decrease in the retention of siRNA drugs by competitively masking the binding site in megalin/cubilin receptors. Therefore, Gelofusine, as a promising kidney-protective agent, can form a compound preparation with siRNA drugs modified with cRGD or other polypeptides for future clinical application, greatly facilitating the development of preclinical and clinical trials of siRNA drugs.

MATERIALS AND METHODS

Cell Culturing

The U87MG human malignant glioblastoma multiforme cell line (ATCC number HTB-14) and the HEK293 cell line (ATCC number CRL-1573) were purchased from the ATCC. The HK-2 human proximal tubular cell line was kindly provided by the National Clinical Research Center for Kidney Disease, State Key Laboratory of Organ Failure Research, Nanfang Hospital, Southern Medical University (Guangzhou, China). The cells were grown and cultured using supplier-recommended reagents and media according to standard protocols and procedures.

Preparation of cRGD-siRNA

Cyclic RGD was covalently conjugated to the 5′ end of the siRNA sense strand using a thiol-maleimide linker to prepare cRGD-siRNA

conjugates. The synthetic process and quality control were performed as described previously.[6] siRNA sequences and modifications for this study were as follows: siNC sense strand, 5′-(mC)(mG)(mU)GAUUGCGAGACUC(mU)(mG)(mA)dTdT-3′; siNC anti-sense strand, 5′-(mU)(mC)(mA)GAGUCUCGCAAUC(mA)(mC)(mG)dTdT-3′. mN represents 2′-*O*-methyl sugar-modified RNA nucleosides. siNC was the negative control siRNA that had no homologous sequence in human and mouse transcriptomes, and Cy5-labeled siNC (siNC-Cy5) was also prepared. cRGD-siNC was connected by cRGD oligopeptide and siNC. cRGD-siNC-Cy5 was connected by cRGD oligopeptide and siNC-Cy5. All of the aforementioned siRNAs were purchased from Guangzhou RiboBio.

Establishment of the *In Vitro* Assay

The cells were seeded into different types of plates (Corning Life Sciences, California, USA) at the recommended density of cells per well and allowed to attach for 24 hr. The processing of different groups was as follows. For the control group, the processing condition was the same as for other groups without any treatment. For the cRGD-siNC or cRGD-siNC-Cy5 group, cRGD-siNC or cRGD-siNC-Cy5 at concentrations of 50 μg/mL or 100 μg/mL was added into different wells and incubated with cells for 24 hr in Opti-MEM without serum. For the Gelofusine group, before cRGD-siNC or cRGD-siNC–Cy5 treatment, Gelofusine (100 μg/mL) was added into different wells and incubated with the cells for 2 hr in Opti-MEM without serum. At this point, cRGD-siNC at a concentration of 50 μg/mL or 100 μg/mL was added into wells and continually incubated with the cells for 24 hr. For the cRGD peptide group, cRGD peptide at a concentration of 100 μg/mL was added into wells and continually incubated with the cells for 24 hr in Opti-MEM without serum. Next, 10% serum was added, and the incubation continued to different time points for different assays, such as cellular uptake, intracellular distribution, and ELISA, without continuous incubation, whereas western blot and apoptosis analyses were performed within 36 hr.

In the RNA knockdown assay, before receiving different treatments, the cells were transfected with siRNAs (100 nM) to silence TLR3 or megalin, respectively, with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The siRNA sequences for the experiments were as follows: human TLR3 siRNA (siTLR3) sense strand, 5′-GGUAUAGCCAGCUAACUAGdTdT-3′; human siTLR3 anti-sense strand, 5′-CUAGUUAGCUGGCUAUACCdTdT-3′; human megalin siRNA (siMegalin), sense strand, 5′-GCAGCUUACUUGUGACAAUdTdT-3′; human siMegalin anti-sense strand, 5′-AUUGUCACAAGUAAGCUGCdTdT-3′.

Animal Handling

BALB/c nude mice (female, 4–6 weeks old, ∼20 g) and BALB/c mice (female, 4–6 weeks old, ∼20 g) were purchased from the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China) and maintained in a sterile environment according to standardized animal care guidelines. The experiments were carried out according to national regulations.

Assessment of *In Vivo* Distribution of Tumor-Bearing Nude Mouse Model and Wild-Type Mice

Nude mice were inoculated subcutaneously on the right back with 5×10^6 U87MG cells. Tumor volumes were measured using the following formula: volume = 1/2 × length × (width),[2] where length represented the longest tumor diameter, and width represented the shortest tumor diameter. When the tumor volume reached 300 mm^3, the mice were randomized into different groups as follows. Group 1 was co-injected with siNC-Cy5 (1 nmol/20 g) and saline, group 2 was co-injected with cRGD-siNC-Cy5 (1 nmol/20 g) and saline, and group 3 was co-injected with cRGD-siNC-Cy5 (1 nmol/20 g) and Gelofusine (4 mg/20 g). BALB/c mice were randomized into different groups as follows. Group 1 was co-injected with siNC-Cy5 (1 nmol/20 g) and saline, group 2 was co-injected with cRGD-siNC-Cy5 (1 nmol/20 g) and saline, and group 3 was co-injected with cRGD-siNC-Cy5 (1 nmol/20 g) and Gelofusine (4 mg/20 g). The volume of each injection was 150 μL/20 g. The subsequent biodistribution was detected at 24 hr using the Xenogen IVIS Spectrum imaging system (PerkinElmer, Massachusetts, USA) at the appropriate wavelength (Cy5, λex = 640 nm and λem = 680 nm). The animals were euthanized, and the tumors as well as major organs were excised and imaged. The organs were kept at –80°C for subsequent immunofluorescence assays.

Establishment of the *In Vivo* Renal Injury Experiment

Fifteen BALB/c mice were randomized into three groups (n = 5/group). All animals were injected intravenously seven times over a 48-hr interval with one of following treatments similar to the previous study[7]: group 1, saline; group 2, co-injected with cRGD-siNC (5 nmol/20 g) and saline; group 3, co-injected with cRGD-siNC (5 nmol/20 g) and Gelofusine (4 mg/20 g). The volume of each injection was 150 μL/20 g. The mice were euthanized 12 hr after the last treatment. The serum was collected, and kidneys were removed surgically, weighed, fixed, paraffin-embedded, and sectioned for further analysis. The weight curves and kidney weight index were plotted.

The level and mechanism of tubulointerstitial injury were determined by H&E staining, immunohistochemistry, immunofluorescence, ELISA, and TUNEL for measuring apoptosis.

Expression Level of TLR3

A cell suspension of HK-2 or HEK293 cells (1×10^4) was mixed with 5 μL of anti-TLR3 antibodies (eBioscience, California, USA) at 4°C for 30 min. Then cells were collected, washed twice with PBS, and resuspended in 0.1 mL of PBS. Fluorescence data were collected using the FACSCalibur cell sorting system (BD Biosciences, New Jersey, USA) and analyzed using Cell Quest software.

Cellular Uptake and Intracellular Distribution Assay

The cellular uptake and intracellular distribution assay was performed as described previously.[6,7] For cellular uptake analysis, the cells were collected, and the assay was performed using the BD FACSCalibur cell sorting system. Data were obtained and analyzed using Cell Quest software. For intracellular distribution analysis,

the cells were observed using confocal microscopy (LSM 800, Zeiss, Germany; Cy5 excitation = 640 nm, emission = 680 nm).

ELISA

ELISA kits (Bio-Swamp, Shanghai, China) for mouse IL-6 and IFN-β were used to detect cytokines from mouse serum and human IL-6 and IFN-β from cell culture medium according to the manufacturer's instructions.

qRT-PCR and Western Blot Analysis

qRT-PCR and western blot analysis were conducted as described previously.[48] Primer sequences for qRT-PCR analysis were as follows: human TLR3 forward primer, 5′-AGCCTTCAACGACTGA TGCT-3′; human TLR3 reverse primer, 5′-TTTCCAGAGCCGTGC TAAGT-3′; human megalin forward primer, 5′-GTTCAGATGAC GCGGATGAAA-3′; human megalin reverse primer, 5′-TCACAG TCTTGATCTTGGTCACA-3′; human β-actin (ACTB) forward primer, 5′- CATGTACGTTGCTATCCAGGC-3′; human ACTB reverse primer, 5′- CTCCTTAATGTCACGCACGAT-3′. The antibodies used in this experiment were as follows: anti-TLR3 (ab62566, Abcam, MA, USA), cleaved caspase-3 (9664, Cell Signaling Technology, MA, USA), anti-p65/RelA (10745-1-AP, Proteintech, Wuhan, China), anti-IKBA (10268-1-AP, Proteintech), anti-ACTB (HRP-60008, Proteintech), and anti-megalin (19700-1-AP, Proteintech).

Apoptosis Analysis

Adherent and floating cells were collected according to the manufacturer's instructions, stained with fluorescein isothiocyanate-labeled Annexin V (eBioscience) and propidium iodide (eBioscience), and analyzed on the BD FACSCalibur cell sorting system.

Immunofluorescence Analysis

The immunofluorescence analysis of cells was performed as described previously.[49] Kidneys from the assessment of *in vivo* distribution were analyzed for subsequent frozen section fluorescence, which was performed as described previously.[50] Kidneys from the *in vivo* renal injury experiment were analyzed for subsequent paraffin-embedded section immunofluorescence, which was performed as described previously.[51] The antibodies or reagents used in this analysis were as follows: anti-p65/RelA (10745-1-AP, Proteintech), Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (SA00006-2, Proteintech), and DAPI (Roche, Switzerland). Images were viewed and captured using a confocal laser-scanning microscope (LSM 800, Zeiss, Germany).

H&E Staining, Immunohistochemistry, and TUNEL Analysis

Kidneys from the *in vivo* renal injury experiment were fixed immediately using 4% paraformaldehyde. All sections were stained with H&E. Immunohistochemistry using a microwave-based antigen retrieval technique was performed on deparaffinized sections.[52] The primary antibodies used in the present study included anti-TLR3 (ab62566, Abcam), cleaved caspase-3 (9664, Cell Signaling Technology), anti-p65/RelA (10745-1-AP, Proteintech), anti-IKBA (10268-1-AP, Proteintech), and anti-CD11b (ab133357, Abcam). Tissue sections were processed for TUNEL analysis using an *in situ* cell death detection kit (POD, Roche) as a measure of apoptosis according to the manufacturer's protocol. Images were viewed and captured using a light microscope (Zeiss, Germany). Percentages of positive staining area were quantified using the Image-Pro Plus software (Media Cybernetics, Maryland, USA).

Statistical Analysis

Data were expressed as mean ± SE. A homogeneity of variance test was conducted first. The data were statistically analyzed through one-way ANOVA with a least significant difference or Dunnett T3 test for intergroup comparisons (SPSS software, version 19.0, SPSS, IL, USA). p values of less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.03.006.

AUTHOR CONTRIBUTIONS

A.J. conceived the study, supervised experiments, and analyzed the data. B.C. designed the experiments and wrote the manuscript. W.L., W.W., and Z.W. performed cellular experiments. Y.W. and W.H. conducted animal experiments. L.G. and S.H. provided the samples and performed the pathological analysis. X.L. and X.P. analyzed the data and revised the manuscript.

CONFLICTS OF INTEREST

The authors have no conflict of interest.

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REFERENCES

1. Sullenger, B.A., and Nair, S. (2016). From the RNA world to the clinic. Science *352*, 1417–1420.
2. Ozcan, G., Ozpolat, B., Coleman, R.L., Sood, A.K., and Lopez-Berestein, G. (2015). Preclinical and clinical development of siRNA-based therapeutics. Adv. Drug Deliv. Rev. *87*, 108–119.
3. Ku, S.H., Jo, S.D., Lee, Y.K., Kim, K., and Kim, S.H. (2016). Chemical and structural modifications of RNAi therapeutics. Adv. Drug Deliv. Rev. *104*, 16–28.
4. Lee, S.H., Kang, Y.Y., Jang, H.E., and Mok, H. (2016). Current preclinical small interfering RNA (siRNA)-based conjugate systems for RNA therapeutics. Adv. Drug Deliv. Rev. *104*, 78–92.
5. Lee, S.J., Kim, M.J., Kwon, I.C., and Roberts, T.M. (2016). Delivery strategies and potential targets for siRNA in major cancer types. Adv. Drug Deliv. Rev. *104*, 2–15.

6. Liu, X., Wang, W., Samarsky, D., Liu, L., Xu, Q., Zhang, W., Zhu, G., Wu, P., Zuo, X., Deng, H., et al. (2014). Tumor-targeted in vivo gene silencing via systemic delivery of cRGD-conjugated siRNA. Nucleic Acids Res. *42*, 11805–11817.
7. He, S., Cen, B., Liao, L., Wang, Z., Qin, Y., Wu, Z., Liao, W., Zhang, Z., and Ji, A. (2017). A tumor-targeting cRGD-EGFR siRNA conjugate and its anti-tumor effect on glioblastoma in vitro and in vivo. Drug Deliv. *24*, 471–481.
8. Liu, L., Liu, X., Xu, Q., Wu, P., Zuo, X., Zhang, J., Deng, H., Wu, Z., and Ji, A. (2014). Self-assembled nanoparticles based on the c(RGDfk) peptide for the delivery of siRNA targeting the VEGFR2 gene for tumor therapy. Int. J. Nanomedicine *9*, 3509–3526.
9. Huang, Y., Wang, X., Huang, W., Cheng, Q., Zheng, S., Guo, S., Cao, H., Liang, X.J., Du, Q., and Liang, Z. (2015). Systemic Administration of siRNA via cRGD-containing Peptide. Sci. Rep. *5*, 12458.
10. Sakurai, Y., Hatakeyama, H., Sato, Y., Hyodo, M., Akita, H., Ohga, N., Hida, K., and Harashima, H. (2014). RNAi-mediated gene knockdown and anti-angiogenic therapy of RCCs using a cyclic RGD-modified liposomal-siRNA system. J. Control. Release *173*, 110–118.
11. Schiffelers, R.M., Mixson, A.J., Ansari, A.M., Fens, M.H., Tang, Q., Zhou, Q., Xu, J., Molema, G., Lu, P.Y., Scaria, P.V., et al. (2005). Transporting silence: design of carriers for siRNA to angiogenic endothelium. J. Control. Release *109*, 5–14.
12. Liu, D., Wen, Y., Tang, T.T., Lv, L.L., Tang, R.N., Liu, H., Ma, K.L., Crowley, S.D., and Liu, B.C. (2015). Megalin/Cubulin-Lysosome-mediated Albumin Reabsorption Is Involved in the Tubular Cell Activation of NLRP3 Inflammasome and Tubulointerstitial Inflammation. J. Biol. Chem. *290*, 18018–18028.
13. Krishnan, N., and Perazella, M.A. (2015). Drug-induced acute interstitial nephritis: pathology, pathogenesis, and treatment. Iran. J. Kidney Dis. *9*, 3–13.
14. Raghavan, R., and Eknoyan, G. (2014). Acute interstitial nephritis - a reappraisal and update. Clin. Nephrol. *82*, 149–162.
15. Anders, H.J., and Schlöndorff, D. (2007). Toll-like receptors: emerging concepts in kidney disease. Curr. Opin. Nephrol. Hypertens. *16*, 177–183.
16. Patole, P.S., Gröne, H.J., Segerer, S., Ciubar, R., Belemezova, E., Henger, A., Kretzler, M., Schlöndorff, D., and Anders, H.J. (2005). Viral double-stranded RNA aggravates lupus nephritis through Toll-like receptor 3 on glomerular mesangial cells and antigen-presenting cells. J. Am. Soc. Nephrol. *16*, 1326–1338.
17. Anders, H.J., Banas, B., and Schlöndorff, D. (2004). Signaling danger: toll-like receptors and their potential roles in kidney disease. J. Am. Soc. Nephrol. *15*, 854–867.
18. Meng, Z., and Lu, M. (2017). RNA Interference-Induced Innate Immunity, Off-Target Effect, or Immune Adjuvant? Front. Immunol. *8*, 331.
19. Kawai, T., and Akira, S. (2007). Signaling to NF-kappaB by Toll-like receptors. Trends Mol. Med. *13*, 460–469.
20. Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature *413*, 732–738.
21. Jiang, Z., Mak, T.W., Sen, G., and Li, X. (2004). Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. Proc. Natl. Acad. Sci. USA *101*, 3533–3538.
22. Estornes, Y., Toscano, F., Virard, F., Jacquemin, G., Pierrot, A., Vanbervliet, B., Bonnin, M., Lalaoui, N., Mercier-Gouy, P., Pachéco, Y., et al. (2012). dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. Cell Death Differ. *19*, 1482–1494.
23. Kleinman, M.E., Kaneko, H., Cho, W.G., Dridi, S., Fowler, B.J., Blandford, A.D., Albuquerque, R.J., Hirano, Y., Terasaki, H., Kondo, M., et al. (2012). Short-interfering RNAs induce retinal degeneration via TLR3 and IRF3. Mol. Ther. *20*, 101–108.
24. Salaun, B., Coste, I., Rissoan, M.C., Lebecque, S.J., and Renno, T. (2006). TLR3 can directly trigger apoptosis in human cancer cells. J. Immunol. *176*, 4894–4901.
25. Kleinman, M.E., Yamada, K., Takeda, A., Chandrasekaran, V., Nozaki, M., Baffi, J.Z., Albuquerque, R.J., Yamasaki, S., Itaya, M., Pan, Y., et al. (2008). Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature *452*, 591–597.
26. Molitoris, B.A., Dagher, P.C., Sandoval, R.M., Campos, S.B., Ashush, H., Fridman, E., Brafman, A., Faerman, A., Atkinson, S.J., Thompson, J.D., et al. (2009). siRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury. J. Am. Soc. Nephrol. *20*, 1754–1764.
27. Maack, T., Johnson, V., Kau, S.T., Figueiredo, J., and Sigulem, D. (1979). Renal filtration, transport, and metabolism of low-molecular-weight proteins: a review. Kidney Int. *16*, 251–270.
28. Christensen, E.I., Verroust, P.J., and Nielsen, R. (2009). Receptor-mediated endocytosis in renal proximal tubule. Pflugers Arch. *458*, 1039–1048.
29. Christensen, E.I., and Birn, H. (2001). Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. Am. J. Physiol. Renal Physiol. *280*, F562–F573.
30. Amsellem, S., Gburek, J., Hamard, G., Nielsen, R., Willnow, T.E., Devuyst, O., Nexo, E., Verroust, P.J., Christensen, E.I., and Kozyraki, R. (2010). Cubilin is essential for albumin reabsorption in the renal proximal tubule. J. Am. Soc. Nephrol. *21*, 1859–1867.
31. Rolleman, E.J., Valkema, R., de Jong, M., Kooij, P.P., and Krenning, E.P. (2003). Safe and effective inhibition of renal uptake of radiolabelled octreotide by a combination of lysine and arginine. Eur. J. Nucl. Med. Mol. Imaging *30*, 9–15.
32. ten Dam, M.A., Branten, A.J., Klasen, I.S., and Wetzels, J.F. (2001). The gelatin-derived plasma substitute Gelofusine causes low-molecular-weight proteinuria by decreasing tubular protein reabsorption. J. Crit. Care *16*, 115–120.
33. van Eerd, J.E., Vegt, E., Wetzels, J.F., Russel, F.G., Masereeuw, R., Corstens, F.H., Oyen, W.J., and Boerman, O.C. (2006). Gelatin-based plasma expander effectively reduces renal uptake of 111In-octreotide in mice and rats. J. Nucl. Med. *47*, 528–533.
34. Veldman, B.A., Schepkens, H.L., Vervoort, G., Klasen, I., and Wetzels, J.F. (2003). Low concentrations of intravenous polygelines promote low-molecular weight proteinuria. Eur. J. Clin. Invest. *33*, 962–968.
35. Briat, A., Wenk, C.H., Ahmadi, M., Claron, M., Boturyn, D., Josserand, V., Dumy, P., Fagret, D., Coll, J.L., Ghezzi, C., et al. (2012). Reduction of renal uptake of 111In-DOTA-labeled and A700-labeled RAFT-RGD during integrin αvβ3 targeting using single photon emission computed tomography and optical imaging. Cancer Sci. *103*, 1105–1110.
36. Hayden, M.S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. Cell *132*, 344–362.
37. Whitehead, K.A., Dahlman, J.E., Langer, R.S., and Anderson, D.G. (2011). Silencing or stimulation? siRNA delivery and the immune system. Annu. Rev. Chem. Biomol. Eng. *2*, 77–96.
38. Bakhtiyari, S., Haghani, K., Basati, G., and Karimfar, M.H. (2013). siRNA therapeutics in the treatment of diseases. Ther. Deliv. *4*, 45–57.
39. Whitehead, K.A., Langer, R., and Anderson, D.G. (2009). Knocking down barriers: advances in siRNA delivery. Nat. Rev. Drug Discov. *8*, 129–138.
40. Baines, R.J., and Brunskill, N.J. (2008). The molecular interactions between filtered proteins and proximal tubular cells in proteinuria. Nephron, Exp. Nephrol. *110*, e67–e71.
41. Fass, D., Blacklow, S., Kim, P.S., and Berger, J.M. (1997). Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. Nature *388*, 691–693.
42. Rolleman, E.J., Melis, M., Valkema, R., Boerman, O.C., Krenning, E.P., and de Jong, M. (2010). Kidney protection during peptide receptor radionuclide therapy with somatostatin analogues. Eur. J. Nucl. Med. Mol. Imaging *37*, 1018–1031.
43. Wang, F., Li, Y., Shen, Y., Wang, A., Wang, S., and Xie, T. (2013). The functions and applications of RGD in tumor therapy and tissue engineering. Int. J. Mol. Sci. *14*, 13447–13462.
44. Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. *11*, 373–384.
45. Barata, P., Sood, A.K., and Hong, D.S. (2016). RNA-targeted therapeutics in cancer clinical trials: Current status and future directions. Cancer Treat. Rev. *50*, 35–47.
46. Gavrilov, K., and Saltzman, W.M. (2012). Therapeutic siRNA: principles, challenges, and strategies. Yale J. Biol. Med. *85*, 187–200.
47. Ghosh, P., Dullea, R., Fischer, J.E., Turi, T.G., Sarver, R.W., Zhang, C., Basu, K., Das, S.K., and Poland, B.W. (2009). Comparing 2-nt 3′ overhangs against blunt-ended siRNAs: a systems biology based study. BMC Genomics *10* (*Suppl 1*), S17.

48. Gu, Y., Wang, Q., Guo, K., Qin, W., Liao, W., Wang, S., Ding, Y., and Lin, J. (2016). TUSC3 promotes colorectal cancer progression and epithelial-mesenchymal transition (EMT) through WNT/β-catenin and MAPK signalling. J. Pathol. *239*, 60–71.

49. Lim, D.M., Narasimhan, S., Michaylira, C.Z., and Wang, M.L. (2009). TLR3-mediated NF-kappaB signaling in human esophageal epithelial cells. Am. J. Physiol. Gastrointest. Liver Physiol. *297*, G1172–G1180.

50. Chen, J., Shetty, S., Zhang, P., Gao, R., Hu, Y., Wang, S., Li, Z., and Fu, J. (2014). Aspirin-triggered resolvin D1 down-regulates inflammatory responses and protects against endotoxin-induced acute kidney injury. Toxicol. Appl. Pharmacol. *277*, 118–123.

51. Procino, G., Carmosino, M., Milano, S., Dal Monte, M., Schena, G., Mastrodonato, M., Gerbino, A., Bagnoli, P., and Svelto, M. (2016). β3 adrenergic receptor in the kidney may be a new player in sympathetic regulation of renal function. Kidney Int. *90*, 555–567.

52. Lan, H.Y., Mu, W., Nikolic-Paterson, D.J., and Atkins, R.C. (1995). A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: use of microwave oven heating to block antibody crossreactivity and retrieve antigens. J. Histochem. Cytochem. *43*, 97–102.