Naked caspase 3 small interfering RNA is effective in cold preservation but not in autotransplantation of porcine kidneys

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ABSTRACT

Background: Caspase 3 associated with apoptosis and inflammation plays a key role in ischemia–reperfusion injury. The efficacy of naked caspase 3 small interfering RNA (siRNA) has been proved in an isolated porcine kidney perfusion model but not in autotransplantation.

Materials and methods: The left kidney was retrieved from mini pigs and infused with the University of Wisconsin solution with or without 0.3 mg of caspase 3 siRNA into the renal artery with the renal artery and vein clamped for 24-h cold storage (CS). After right nephrectomy, the left kidney was autotransplanted into the right for 48 h without systemic treatment of siRNA.

Results: Fluorescent dye–labeled caspase 3 siRNA was visualized in the post-CS kidneys but was weakened after transplantation. The expression of caspase 3 messenger RNA and precursor was downregulated by siRNA in the post-CS kidneys. In the siRNA-preserved posttransplant kidneys, however, the caspase 3 messenger RNA and active subunit were upregulated with further decreased precursor but increased active caspase 3+ cells, apoptotic cells, and myeloperoxidase+ cells. Moreover, the renal tissue damage was aggravated by siRNA, whereas the renal function was not significantly changed.

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Conclusions: Naked caspase 3 siRNA administered into the kidney was effective in cold preservation but not enough to protect posttransplant kidneys, which might be because of systemic complementary responses overcoming local effects.

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1. Introduction

Transplantation is the best treatment for end-stage renal failure patients, although many problems still need to be resolved, such as ischemia–reperfusion injury (IRI) and donor preservation [1]. IRI, inevitable in transplantation and associated with graft survival [2], causes microcirculatory failure and inflammation, followed by ultimate cell death through apoptosis or necrosis [3]. Caspase 3, a major effector enzyme, is upregulated by IRI mediating apoptosis and inflammation [4,5].

Pharmacologic inhibitors and genetic alterations have been used to modulate the caspase 3 expression. Pan caspase inhibitors protect against liver IRI and prolong posttransplant survival [6,7] but have potential limitations because of toxicities. The development of specific caspase 3 inhibitors has also been hampered by the high homology of caspase family members [8]. Nevertheless, caspase 3 knockout (casp3−/−) mice were protected from developing diabetes by reducing apoptosis and lymphocyte infiltration in the islets [9]. But bewilderingly, caspase 3 deficiency increases the vulnerability of developing brain to hypoxic–ischemic injury through caspase 3–independent pathways [10]. Therefore, it is imperative to explore specific, transient, and safe strategies targeting caspase 3 against acute injury.

RNA interfering using small interfering RNA (siRNA), 21 nucleotide duplexes, has provided one such strategy [11,12]. siRNA posttranscriptionally silences genes by degrading the complementary sequence of messenger RNA (mRNA) precisely to one nucleotide mismatch. Administering siRNA directly to cells or organs has been proved to be specific to one nucleotide mismatch. Administering siRNA posttranscriptionally silences gene by degrading nucleotide duplexes, has provided one such strategy [11,12]. The in vivo ready custom caspase 3 siRNA (Silencer) was then verified in our ex vivo study [4]. The fluorescent Alexa Fluor 555 dye–labeled caspase 3 siRNA (Life Technologies) was used to monitor its localization in a few additional post-CS and posttransplant kidneys by a laser scanning confocal microscope (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany).

2. Materials and methods

2.1. Caspase 3 siRNA preparation

Three pairs of siRNA, targeting porcine caspase 3 mRNA (National Center for Biotechnology Information CoreNucleotide Accession No. AB029345), were designed (Life Technologies, Paisley, UK). The most effective sequences, 5′-GGGAGACCUCU-CACAAACUUt-3′ and 5′-AAGUUGUGAGGUCUCCGt-3′, were selected in LLC-PK1 cells [25]. The in vivo ready custom caspase 3 siRNA (Silencer) was then verified in our ex vivo study [4]. The most effective sequences, 5′-GGGAGACCUCU-CACAAACUUt-3′ and 5′-AAGUUGUGAGGUCUCCGt-3′, were selected in LLC-PK1 cells [25]. The in vivo ready custom caspase 3 siRNA (Silencer) was then verified in our ex vivo study [4].

2.2. Animals

Under the regulation layout by the Chinese animal welfare authority, male mini pigs weighing 25–30 kg were used. They were housed with air condition, straw–saw dust beds, and free access to water and fed with wetted granulated full fodder.

2.3. Anesthetic protocol

The animals were premedicated with 0.5 mg/kg of diazepam and 5 mg/kg of ketamine hydrochloride intramuscularly, followed by general anesthesia using 1 mg/kg of propofol (Fresenius Kabi, Bad Homburg, Germany) intravenously (i.v.), and maintained with a mix solution of 0.25 mg/kg/h of diazepam, 2.5 mg/kg of ketamine hydrochloride, and 0.0125 ml/kg/h of compound detomidine hydrochloride or 0.5 mg/kg/h of propofol i.v. in turn. The respiration was supported by a ventilator (Dräger, Lübeck, Germany) through an inserted trachea cannula. Five hundred milliliters of 5% glucose and 0.9% sodium chloride and 500 ml of hydroxyethyl starch 130/0.4 and sodium chloride injection (Fresenius Kabi, Bad Homburg, Germany) were also administered i.v. In addition, 100 ml of 0.3 g of levofoxacin lactate and 2 million units of benzylpenicillin were given i.v. 30 min before surgery. The same anesthetic protocol was used for donor retrieving and transplantation.

2.4. Donor kidney retrieving and preservation

The left kidney was mobilized and removed with minimal warm ischemia (about 1 min) after ligating the renal artery near the abdominal aorta, renal vein near the inferior vena cava, and ureter. The isolated kidney was flushed immediately with 200 ml of precooled Ringer solution with 1000 IU of heparin at 100 cm H2O hydrostatic pressure until the kidney became pale and then followed by 200 ml of the University of Wisconsin (UW; Bristol-Myers Squibb, New York, NY) solution. At last, the half of 40 ml of precooled UW solution...
with (the treatment group, \( n = 6 \)) or without (the negative control, \( n = 6 \)). 0.3 mg of siRNA was infused into the renal artery to push out the remaining UW solution; the renal vein was then clamped and another 20 mL of the solution was infused, and the renal artery was finally clamped. The kidney was preserved on ice for 24 h.

2.5. Right kidney nephrectomy and autotransplantation

Next day, the right kidney was resected (post-nephrectomy, the baseline control) after ligating the right renal artery and vein, as well as the ureter, close to the renal hilum. The left kidney was orthotopically autotransplanted into the right for 48 h. In addition, a double lumen cuffed silicone vascular access catheter (Arrow International, Reading, PA) was placed in the left internal jugular vein. The lumens of the central line were fixed behind the ear and blocked with heparin.

2.6. Sample collection

Blood samples were taken before donor retrieving, pretransplant, and 48-h posttransplant to collect serum. Renal biopsies were also taken 24-h post-CS. At 48 h posttransplant, the animal was anesthetized and killed after harvesting the graft. Some renal tissues were fixed with 10% buffered formalin, and the others were snap-frozen.

2.7. Histologic assessment

The renal histologic damage was assessed in hematoxylin- and eosin-stained sections by three researchers blinded to the coding. The semiquantitative score system comprised four criteria, including loss of tubular epithelium; tubular vacuolation and nuclear loss; protein casts; and interstitial edema, expansion, and cellular infiltration. Each criterion was graded from mild to severe by the percentage of injury: 0 (<1%); 1 (1%–25%); 2 (26%–50%); 3 (51%–75%); and 4 (>75%).

2.8. Caspase 3 mRNA expression

Total RNA was extracted from renal tissues with Trizol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was transcribed into complementary DNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD). Real-time quantitative polymerase chain reaction (QPCR) was performed using the TaqMAN premix (Takara Bio Inc, Otsu, Japan), primers, and probes for porcine caspase 3 and \( \beta \)-actin (Life Technologies) in a Rotor-Gene Q system (Qiagen, Germantown, MD). After a hot start (2 min at 50\(^\circ\)C and 10 min at 95\(^\circ\)C), amplification was performed for 50 cycles (15 s at 95\(^\circ\)C and 60 s at 60\(^\circ\)C). The expression of caspase 3 mRNA normalized with \( \beta \)-actin was calculated against relative non-IRI kidneys (randomly selected six of the 12 post-nephrectomy kidneys) using a \( 2^{-\Delta\Delta Ct} \) method.

2.9. Caspase 3 protein expression detected by Western blotting

Twenty micrograms of protein from kidney homogenate were separated on 15% (wt/vol) polyacrylamide denaturing gels and electroblotted onto Hybond-C membranes. These membranes were blocked with 5% (wt/vol) milk, probed with anti–full-length caspase 3 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-\( \beta \)-actin antibody (1:10,000 dilution; Abcam, Cambridge, UK) and then incubated with peroxidase-conjugated secondary antibodies (1:10,000 dilution; Jackson Immunoresearch, West Grove, PA) at room temperature for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence substrate (Thermo Fisher Scientific, Rockford, IL) and a Bio-Image Analysis System (Bio-Rad Laboratories, Hertfordshire, UK). The semiquantitative analysis results were expressed as optical volume density (OD \( \times \) mm\(^2\)) of caspase 3 corrected by \( \beta \)-actin for loading (Quantity one 4.2, Bio-Rad Laboratories).

2.10. Active caspase 3 immunostaining

Active caspase 3 immunostaining, recognizing 17-kd subunit, was undertaken on 4-\( \mu \)m paraffin sections using a DAKO ChemMate EnVision Detection Kit (DAKO, Carpinteria, CA). Antigen retrieval was performed using 10 mM of sodium citrate buffer, pH 6.0, in a steam bath maintained by high-power microwave for 20 min. The sections were blocked and labeled by an anti-active caspase 3 antibody (1:100 dilution; R&D System, Minneapolis, MN) at 4\(^\circ\)C overnight. The antibody binding was revealed by 3'-amino-9-ethylcarbazole (AEC, dark red color). The active caspase 3+ cells in the renal cortex were semiquantitatively scored in 20 fields at \( \times 400 \) magnification.

2.11. In situ end-labeling apoptotic cells

Paraffin sections were used for in situ end-labeling (ISEL) fragmented DNAs with digoxigenin-deoxyuridine by terminal deoxynucleotidyl transferase using an Apoptosis Detection Kit (Millipore, Billerica, MA). Briefly, the sections were digested by 40 \( \mu \)g/mL of proteinase K for 15 min at 37\(^\circ\)C, incubated with terminal deoxynucleotidyl transferase and digoxigenin-deoxyuridine at 37\(^\circ\)C for 60 min, and transferred to wash or stop buffer for 30 min. After adding anti-digoxigenin peroxidase complex for 30 min, these sections were developed by AEC substrate. Apoptotic cells were examined at \( \times 400 \) magnification over 20 fields of tubulointerstitial areas.

2.12. Myeloperoxidase immunostaining

Immunostaining of myeloperoxidase (MPO), a marker mainly for neutrophil granulocytes, was undertaken on paraffin sections using the same kit with active caspase 3 staining. The sections were digested by the same method with ISEL, blocked by peroxidase-blocking reagent, and labeled by anti-MPO antibody (1:600 dilution; DAKO) at 4\(^\circ\)C overnight. The antibody binding was revealed by AEC. MPO+ cells in the renal cortex were semiquantitatively scored in 20 fields at \( \times 400 \) magnification.

2.13. Statistical analysis

Results are expressed as mean \( \pm \) standard error of the mean. Normality tests were performed and statistical differences between two groups were assessed by unpaired \( t \)-test.
3. Results

3.1. Localization of caspase 3 siRNA

The fluorescent Alexa Fluor 555 dye–labeled caspase 3 siRNA was examined by a confocal microscope in a few additional post-CS and posttransplant kidneys. The specific pattern of fluorescence (red color in cytoplasm; Fig. 1A and B) was revealed in the siRNA-preserved post-CS kidneys with or without 4′, 6-diamidino-2-phenylindole count staining (blue nuclei), in contrast to the UW only–preserved post-CS negative control kidneys (Fig. 1C). After transplantation, the fluorescent pattern was weakened in the kidney preserved by caspase 3 siRNA (Fig. 1D) but also visible in the spleen (Fig. 1E), as well as the intestine, liver, and lung tissues (data not shown). There was no specific fluorescence in the matched negative control kidneys (Fig. 1F) and other organs (data not shown).

3.2. Caspase 3 mRNA and protein in the post-CS kidneys

The expression of caspase 3 mRNA and protein in the post-CS kidneys was assessed by real-time QPCR and Western blotting. With caspase 3 siRNA treatment, the level of caspase 3 mRNA (Fig. 2A) was significantly decreased in the post-CS kidneys compared with that in the UW only–preserved negative control kidneys. The 32-kD precursor of caspase 3 was predominantly revealed in the representative blots and also significantly downregulated by caspase 3 siRNA in the post-CS kidneys (Fig. 2B).

3.3. Caspase 3 mRNA and protein expression in the posttransplant kidneys

The expression of caspase 3 mRNA and protein in the postnephrectomy and posttransplant kidneys was detected by real-time QPCR and Western blotting. Caspase 3 mRNA was increased in the caspase 3 siRNA–treated posttransplant kidneys compared with that in the UW only–preserved negative kidneys.

Fig. 1 - Localization of fluorescent dye–labeled caspase 3 siRNA in a few additional (A–C) post-CS and (D and E) posttransplant kidneys, as well as (F) the spleen. The specific fluorescent pattern of Alexa Fluor 555 (red color in cytoplasm) was visualized in the (A and B) post-CS kidney preserved by UW with caspase 3 siRNA with or without 4′, 6-diamidino-2-phenylindole count staining (blue nuclei) but was not revealed in the (C) post-CS kidney preserved by UW only. After transplantation, the fluorescent pattern was weakened in the (E) kidney preserved by siRNA and also visible in other organs such as (F) the spleen but was not seen in the (F) matched negative control kidneys preserved by UW only. C3siRNA = caspase 3 siRNA; PostTx = posttransplant. (Color version of figure is available online.)
control kidneys (Fig. 3A). In the siRNA-preserved posttransplant kidneys, the 17-kD caspase 3 active subunit was significantly increased in contrast to further decreased precursor (Fig. 3B). The caspase 3 precursor was also significantly increased in the negative control posttransplant kidneys compared with that in the post-nephrectomy kidneys (Fig. 3B).

3.4. Active caspase 3+ cells

Active caspase 3+ cells in the kidney of post-nephrectomy, post-CS, and posttransplant were examined by immunostaining. The active caspase 3+ cells were few in post-nephrectomy kidneys (Fig. 4A) but gradually increased by CS
The most active caspase 3+ cells located in tubulointerstitial areas and tubular lumens (Fig. 4B–F) with few in glomerular areas (Fig. 4E), which either demonstrated apoptotic morphologic features (Fig. 4F) or shed into tubular lumens (Fig. 4B and C). The semi-quantitative analysis revealed much less active caspase 3+ cells in the siRNA-treated post-CS kidneys, but there was a 1.3-fold increase in the posttransplant kidneys preserved by siRNA compared with those preserved by UW only. The number of active caspase 3+ cells was also increased by transplantation with or without siRNA treatment in comparison with that in the post-nephrectomy kidneys (Fig. 4G).

Fig. 3 – The expression of caspase 3 mRNA and protein in post-nephrectomy and posttransplant kidneys measured by QPCR and Western blotting. (A) In the siRNA-treated posttransplant kidney, caspase 3 mRNA was significantly increased compared with that in the UW only–preserved kidneys. (B) The 17-kD caspase 3 active subunit was also significantly increased by siRNA treatment as showed in the representative blots with further decreased precursor, whereas the caspase 3 precursor was significantly increased in the UW only–treated posttransplant kidneys compared with that in the post-nephrectomy kidneys. The mRNA data are expressed as $2^{-\Delta\Delta CT}$ normalized with β-actin relative to the non-IRI kidneys (randomly selected six of the 12 post-nephrectomy kidneys) of each group (mean ± standard error of the mean; n = 6), whereas the protein data are expressed as corrected volume density against the loading control of 42-kD β-actin. PostN = post-nephrectomy; PostTx = posttransplant. *P < 0.05; **P < 0.01.
3.5. Apoptotic cells

Apoptotic cells in the kidney were examined by ISEL fragmented DNAs. Apoptotic cells were hardly seen in the post-nephrectomy (Fig. 5A) and post-CS kidneys (Fig. 5B and C). After transplantation, apoptotic cells were increased, mainly located in tubulointerstitial areas, and some were shedding into tubular lumens (Fig. 5D–F). With the treatment of siRNA, apoptotic cells were significantly decreased in the post-CS kidneys but increased in the posttransplant kidneys. The number of apoptotic cell was also significantly higher in the posttransplant kidneys regardless of the siRNA treatment compared with that in the post-nephrectomy kidneys (Fig. 5G).
3.6. MPO$^+$ cells in the kidney

The distribution of MPO$^+$ cells in the kidney was detected by immunohistochemistry staining. MPO$^+$ cells were scattered in the post-nephrectomy (Fig. 6A) and post-CS kidneys (Fig. 6B and C). Most MPO$^+$ cells in the posttransplant kidneys located in vascular lumens and interstitial areas (Fig. 6D–F) and some also in glomerular areas (Fig. 6D and F). The MPO$^+$ cells penetrated through tubular areas (Fig. 6B), or demonstrated morphologic features of apoptosis such as condensed nuclei (Fig. 6D), were also seen. The number of MPO$^+$ cells in the posttransplant kidneys was increased by siRNA treatment. The MPO$^+$ cells in the posttransplant kidneys were also significantly higher than those in the post-nephrectomy kidneys.

Fig. 5 — Apoptotic cells were detected by ISEL in (A) post-nephrectomy, (B and C) post-CS, and (D–F) posttransplant kidneys. The apoptotic cells are showed in (D–F) tubulointerstitial areas and tubular lumens; some of them had (F) polymorphic nuclei. The number of apoptotic cells was significantly increased in the posttransplant kidneys preserved by caspase 3 siRNA. (G) There was no significant difference between the two post-CS groups. Compared with the post-nephrectomy kidneys, the apoptotic cells were significantly increased by transplantation regardless of the siRNA. Data are expressed as mean number per high-power field of each group (mean ± standard error of the mean; n = 6). PostN = post-nephrectomy; PostTx = posttransplant; TUNEL = terminal dUTP nick end labeling. **P < 0.01. (Color version of figure is available online.)
kidneys. There was no significant difference between the two post-CS groups (Fig. 6G).

3.7. Score of renal tissue damage

Renal histologic injury was assessed in hematoxylin- and eosin-stained sections. There was mild tubular dilation and interstitial edema in the post-CS kidneys (Fig. 7A and B). Lots of tubular vacuolation and detachment, as well as protein casts, interstitial expansion, and cellular infiltration, were seen in the posttransplant kidneys (Fig. 7C and D). The semiquantitative analysis revealed that the tissue damage was significantly more severe in the siRNA-preserved posttransplant kidneys, although it was marginally better in the

Fig. 6 – MPO+ cells were detected by immunostaining in (A) post-nephrectomy, (B and C) post-CS, and (D–F) transplant kidneys. MPO+ cells were seen in the (D) vascular lumen and (D–F) interstitial areas and also revealed in (D and F) the glomerular area; some demonstrated morphologic features of apoptosis such as (F) condensed nuclei or penetrated through the (B) tubular area. MPO+ cells were significantly increased by caspase 3 siRNA after transplantation without significant difference between the two post-CS groups. (G) The number of MPO+ cells was significantly increased in both posttransplant groups compared with that in post-nephrectomy kidneys. Data are expressed as mean number in the high-power field of each group (mean ± standard error of the mean; n = 6). PostN = post-nephrectomy; PostTx = posttransplant. *P < 0.05. (Color version of figure is available online.)
siRNA-treated post-CS kidneys. The tissue damage in the posttransplant kidneys was also worse than that in the post-CS kidneys preserved by siRNA (Fig. 7E).

3.8. Renal function

The serum creatinine and urea nitrogen were examined at pre-nephrectomy, pretransplantation, and 48-h posttransplantation. After 48-h transplantation, the serum creatinine and urea nitrogen were only numerically increased by 30% and 43%, respectively, in the siRNA-treated group. There was no significant difference between the two groups at any time point (Fig. 8A and B).

4. Discussion

The donor preservation has significant impacts on graft function and survival [1]. For the first time, the effects of naked caspase 3 siRNA, delivered directly into the isolated
and inflammation, and tubulointerstitial damage [5]. Caspase 3 overexpression resulted in increased myocardial damage and infarction but decreased cardiac function during IRI [26]. In contrast, caspase 3 knockdown by siRNA transferred locally into the atrial suppressed apoptosis and the onset of persistent atrial fibrillation [27]. Up to date, siRNAs have been used in a wide range of biological models including IRI [16,25,28], although using a synthetic siRNA represents a kind of novel nonviral approach. Naked siRNAs offer transient, safe, and adequate effects for acute injuries. It has been also reported that caspase 8 and caspase 3 siRNAs successfully decreased mice liver IRI [16]; complement 3, caspase 3, caspase 8, or complement 5a receptor siRNAs prevented apoptosis and improved renal IRI in mice [17,28].

In our previous study, administered naked caspase 3 siRNA into both isolated kidneys and hemoperfusates during CS suppressed caspase 3 expression, reduced apoptosis and inflammation, and improved oxygenation and acid–base homeostasis after 3-h reperfusion of the isolated kidneys. Therefore, it was hypothesized that caspase 3 siRNA should have a similar effect in vivo. It is also worthy to know whether applying caspase 3 siRNA in local preservation only could be effective or an additional systemic treatment was essential in vivo. The effect of caspase 3 siRNA delivered into the renal artery during CS, therefore, was first investigated in this porcine autotransplantation model. The expression of caspase 3 mRNA was markedly downregulated in the siRNA-treated post-CS kidneys, although the caspase 3 precursor was also significantly decreased as a result. These effects were attributed to the degradation of existing mRNA caused by caspase 3 siRNA and also proved that the local delivery of siRNA was effective. The CS might stabilize caspase 3 siRNA and hydraulic pressure inside the clamped vessels might favor siRNA penetrating into renal cells, although low temperature might restrict the function of siRNA.

In contrast to the previous favorable results, worse tissue damage together with higher level of caspase 3 activation, apoptosis, and MPO+ cells was shown in the siRNA-preserved transplanted kidneys. On reperfusion, the siRNA in the preserved kidney was flushed away as the specific fluorescent pattern was weakened in the posttransplant kidneys but visible in other organs, whereas the local destruction of siRNA to existing mRNA in the post-CS kidneys might also quickly disappear because a surge of blood flow into the kidney caused siRNA degeneration. The poor stability of naked siRNA in vivo is well known [29], although this characteristic could be beneficial in avoiding any side effects. The warm recovered enzyme activity that was inhibited during CS, and the reperfusion recruited even more enzymes from the blood, both of which could accelerate the degeneration of siRNA.

After transplantation, a series of in vivo complementary responses to the lower level of caspase 3 mRNA in the post-CS kidneys was initiated, which first led an increase in caspase 3 mRNA synthesis. As the consequence of siRNA degeneration and caspase 3 mRNA synthesis, the level of caspase 3 mRNA was increased in the siRNA-preserved posttransplant kidneys. However, increased caspase 3 mRNA did not result in an increase in caspase 3 precursor but a further decrease. In fact, 17-kD activated caspase 3 was remarkably increased,
which indicates a downstream demanding in activated caspase 3, so caspase 3 precursor cleavage exceeded its translation from increased mRNA. Taken together, a post-transplant feedback loop was functioned by effective delivery of caspase 3 siRNA during CS, which led to an increased mRNA synthesis after transplantation. Because of the instability of siRNA in vivo, caspase 3 mRNA accumulated and subsequently translated to the caspase 3 precursor. Most newly generated precursor, however, was cleaved into active caspase 3, which further involved in downstream biological events such as enhanced apoptosis and inflammation, and the renal tissue damage was finally exacerbated in the transplanted kidneys. The toxicity of caspase 3 siRNA, of course, needs to be ruled out using a negative control siRNA. There is, however, no such proper negative control siRNA available so far as the genome of porcine is not fully established.

In conclusion, administering caspase 3 siRNA directly into the kidney was effective in cold preservation, but failed to protect transplanted kidneys in a porcine autotransplantation model, and even enhanced renal IRI, which may be because of the instability of siRNA and systemic complementary responses. Before applying siRNA therapy to human, its clinical feasibility and optimizing effect need to be further investigated using more stabilized siRNA administering locally and systemically in a long-term study.

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