Postprint: Methods for Efficient Delivery of miR-483-5p to the Kidney

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Abstract

Objective: To investigate methods for efficiently delivering the target gene miR-483-5p into the kidney. Methods: Renal cortical injection of miR-483-5p lentivirus: Thirty-five C57BL/6J mice were randomly divided into blank control group, low-dose lentivirus group (5 L lentivirus injected into each renal cortex), and high-dose lentivirus group (20 L lentivirus injected into each renal cortex), and sacrificed at 7 d and 21 d after injection; construction of transgenic mice with inducible systemic overexpression of miR-483-5p; construction of transgenic mice with renal tubule-specific overexpression of miR-483-5p using the cre-loxp system. In the three model mice, serum blood urea nitrogen (BUN) levels were measured using an automatic biochemical analyzer to assess renal function, kidney tissue structure was observed by HE staining of tissue sections, and renal cell apoptosis was detected by TUNEL assay. Real-time qPCR was used to detect miR-483-5p expression in the kidney. Results: Renal function was normal in all three types of miR-483-5p overexpressing mice, with no obvious changes in kidney tissue structure and no apoptosis in kidney cells. The highest expression was observed 21 d after injection of 20 L LV3-miR-483-5p into the renal cortex (1.2±0.43 vs 8.6±1.09, $P<0.001$). The inducible systemic overexpression transgenic animals showed low expression efficiency in the kidney (0.9±0.09 vs 1.7±0.19, $P<0.05$), while Creloxp transgenic mice achieved specific expression in the kidney with high efficiency (1.6±1.13 vs 12.36±3.89, $P<0.05$). Conclusion: For the first time, a transgenic mouse model with renal tubule-specific overexpression of miR-483-5p was constructed, achieving specific and efficient expression in renal tubules without affecting kidney structure and function, which can serve as an excellent model for studying the role and mechanism of miR-483-5p in the kidney; injection of 20 L LV3-miR-483-5p into each renal cortex of C57BL/6J mice resulted in high efficiency of miR-483-5p overexpression after 21 d, without affecting renal function or causing damage to renal tissue, and with a relatively short model construction time, providing a good

experimental model for studying the role and mechanism of miR-483-5p in the kidney.

Full Text

Preamble

A Method for Efficient Transduction of miR-483-5p in the Kidney of Mice

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Abstract

Objective: To establish methods for efficient delivery of miR-483-5p into murine renal tissue. **Methods:** Thirty-five C57BL/6J mice were randomly divided into a blank control group, a low-dose lentivirus group (5 L lentivirus injected into each renal cortex), and a high-dose lentivirus group (20 L lentivirus injected into each renal cortex). Tissues were harvested at 7 and 21 days post-injection. Additionally, we generated transgenic mice with inducible systemic overexpression of miR-483-5p and transgenic mice with renal tubule-specific overexpression using the Cre-loxp system. Renal function was assessed by measuring blood urea nitrogen (BUN) levels with an automatic biochemical analyzer. Renal histology was examined via HE staining, and apoptosis was detected using TUNEL assay. miR-483-5p expression in kidney tissue was quantified by real-time qPCR. **Results:** All three mouse models exhibited normal renal function, with no obvious histological changes or apoptosis in kidney tissue. The highest miR-483-5p expression was achieved 21 days after injection of 20 L LV3-miR-483-5p into the renal cortex (1.2±0.43 vs 8.6±1.09, $P<0.001$). The inducible systemic overexpression model showed relatively low efficiency in kidney (0.9±0.09 vs 1.7±0.19, $P<0.05$), whereas the Cre-loxp transgenic mice achieved specific and efficient expression in renal tubules (1.6±1.13 vs 12.36±3.89, $P<0.05$). **Conclusion:** We successfully established, for the first time, a transgenic mouse model with renal tubule-specific overexpression of miR-483-5p that achieves high-level expression without affecting renal structure or function, providing an excellent model for studying the role and mechanisms of miR-483-5p in the kidney. Additionally, injection of 20 L LV3-miR-483-5p into each renal cortex of C57BL/6J mice results in efficient overexpression of miR-483-5p after 21 days without impairing renal function or causing tissue damage, offering another effective model with a shorter construction time for investigating miR-483-5p function in the kidney.

Keywords: lentivirus; renal tissue; transgenic technology; miR-483-5p

Introduction

MicroRNAs (miRNAs) are a class of small, single-stranded RNAs approximately 18-24 nucleotides in length that play crucial regulatory roles in various biological processes. Recent studies have demonstrated that miRNAs are involved in the pathogenesis of numerous diseases, including renal disorders, and their expression levels can serve as auxiliary diagnostic biomarkers for acute kidney injury, holding significant clinical importance. miR-483 is located within intron 2 of the IGF2 gene on autosomal chromosomes and gives rise to two major miRNAs: miR-483-5p and miR-483-3p. Accumulating evidence indicates that miR-483 participates in various pathological processes, including tumorigenesis, osteoarthritis, lipid metabolism, and cardiovascular diseases. Regarding the relationship between miR-483 and kidney disease, previous reports have shown that miR-483 is expressed in renal interstitial cells and is associated with kidney development and fibrosis. Notably, Sui et al. found that miR-483-5p was significantly upregulated in the serum of 15 patients with acute rejection following renal transplantation, suggesting its potential involvement in rejection-induced kidney injury. However, methods for efficiently delivering miR-483-5p into the kidney to deeply investigate its functional roles and mechanisms remain unreported. Therefore, this study employed three approaches—renal cortex injection of miR-483-5p lentivirus, generation of inducible systemic miR-483-5p overexpression transgenic mice, and construction of renal tubule-specific miR-483-5p overexpression transgenic mice—to explore efficient methods for miR-483-5p delivery into the kidney, aiming to provide robust experimental models for future functional and mechanistic studies.

Materials and Methods

Animals and Reagents

Thirty-five 8-week-old C57BL/6J mice were purchased from the Laboratory Animal Center of Southern Medical University (Animal Use License No. SYXK-Yue 2006-0015). Transgenic mice for inducible systemic overexpression of miR-483 were constructed by Cyagen Biosciences using the Tet-on system to drive pre-miR-483 expression. rtTA mice were obtained from Jackson Laboratory (Stock No. 006965). Renal tubule-specific miR-483-5p transgenic mice were generated using the Cre-loxp system by crossing loxp-loxp transgenic mice (constructed by Cyagen Biosciences) with PEPCK-Cre transgenic mice (kindly provided by Professor Jing Nie from the Department of Nephrology, Nanfang Hospital).

LV3-miR-483-5p and LV3-NC lentiviruses, Mmu-miR-483-5p hairpin-it real-time PCR kits, and U6 snRNA real-time PCR kits were obtained from GenePharma (Suzhou). 33-gauge needles and microsyringes were purchased from Hamilton. The TUNEL apoptosis detection kit was from Promega.

An inverted fluorescence microscope (Nikon, Japan) and ABI StepOnePlus real-time PCR system were used for imaging and quantification.

Experimental Procedures

Renal Cortex Injection of miR-483-5p Lentivirus Thirty-five C57BL/6J mice were randomly divided into seven groups: blank control group (Group 7), low-dose lentivirus groups (5 L per kidney, Groups 1-3), and high-dose lentivirus groups (20 L per kidney, Groups 4-6). The specific group assignments were: Group 1: 5 L LV3-NC injection for 21 days; Group 2: 5 L LV3-miR-483-5p injection for 7 days; Group 3: 5 L LV3-miR-483-5p injection for 21 days; Group 4: 20 L LV3-NC injection for 21 days; Group 5: 20 L LV3-miR-483-5p injection for 7 days; Group 6: 20 L LV3-miR-483-5p injection for 21 days; Group 7: sham operation without virus injection. All lentiviruses had a titer of 1×10 TU/mL. After anesthesia, a <1 cm incision was made on each side of the dorsal back to expose the kidneys. Four injection sites were selected in each renal cortex as shown in [Figure 1: see original paper]. The needle was held perpendicular to the kidney surface and inserted rapidly to minimize tissue damage. Upon feeling the capsule puncture, virus injection was initiated, taking care not to insert too deeply to avoid hemorrhage. After injection, the needle was held in place for 1-2 seconds before withdrawal to prevent virus leakage. Wounds were then sutured closed.

Inducible Systemic Overexpression miR-483-5p Transgenic Mouse Model Systemic miR-483 overexpression was regulated by the Tet-on system ([Figure 2: see original paper]A), enabling temporal control through the rtTA-doxycycline (Dox) induction system. In this study, double-positive mice carrying both the pri-miR-483 gene and the rtTA allele were designated as TG483 mice (experimental group), while single-positive littermates for either pri-miR-483 or rtTA served as negative controls. Starting at six weeks of age, TG483 mice received drinking water containing 2 mg/mL Dox to induce miR-483 expression.

Renal Tubule-Specific miR-483-5p Expression Transgenic Mouse Model Using the Cre-loxp system ([Figure 2: see original paper]B), PEPCK-Cre mice were crossed with loxp-loxp mice. In stable F2 generation positive offspring, PEPCK-Cre recombination excised the loxp-flanked SV40 polyadenylation signal, enabling robust replication of miR-483-5p precursors specifically in renal tubular cells.

Serum Biochemical Analysis Under anesthesia, the thoracic cavity was opened along the sternal midline without disrupting the inner wall to maintain negative pressure. Blood was collected via cardiac puncture, allowed to clot at room temperature for 2 hours, then centrifuged at 3000×g for 15 minutes. Serum BUN levels were measured using an Olympus AU5400 automatic biochemical analyzer.

Hematoxylin-Eosin (HE) Staining Unilateral kidneys were harvested and fixed in 4% paraformaldehyde at room temperature for 24 hours, followed by routine dehydration, clearing, paraffin infiltration, and embedding. Intact paraffin sections were baked at 65°C for 30 minutes, deparaffinized, and rehydrated. After three PBS washes (5 minutes each), sections were stained with hematoxylin for 2 minutes, rinsed in tap water, differentiated in 1% hydrochloric acid alcohol for 3 seconds, rinsed again, then counterstained with eosin for 20 seconds. Following dehydration and clearing, sections were mounted with neutral resin and examined under a microscope.

TUNEL Assay for Apoptosis Detection Apoptosis was detected using a fluorometric TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay. Intact paraffin sections were baked at 65°C for 30 minutes and deparaffinized. After three PBS washes (5 minutes each), sections were fixed in fresh 4% paraformaldehyde for 15 minutes, washed twice in PBS (5 minutes each), then permeabilized with proteinase K (1:500 in PBS) at 37°C for 30 minutes. Following a PBS wash, sections were refixed in fresh 4% paraformaldehyde for 5 minutes, washed again in PBS, then incubated with TdT reaction equilibrium buffer at 37°C for 10 minutes. The buffer was removed and replaced with TdT reaction mixture (equilibrium buffer:nucleotide mix:rTdT enzyme = 45:5:1) for 60 minutes at 37°C. The reaction was terminated by incubating with 2×SSC (diluted from SSC stock) for 15 minutes at 37°C. After three PBS washes (5 minutes each), sections were mounted with DAPI-containing mounting medium and examined by fluorescence microscopy.

Renal Tissue RNA Extraction and Real-Time qPCR Under anesthesia, the abdominal cavity was opened along the midline and unilateral kidneys were harvested. Fifty milligrams of renal cortex were homogenized in 1 mL TRIzol reagent. Total RNA was extracted according to the manufacturer' s protocol. miRNA detection was performed using GenePharma kits with U6 as internal control. Ct values were recorded and relative expression was calculated using the formula: miR-483-5p relative expression = 2^-(miR-483-5p Ct - U6 Ct).

In Situ Hybridization The digoxigenin-labeled probe sequence for in situ hybridization was CTCCCTTCTTTCCTCCCGTCTT. All procedures were performed in an RNase-free environment using RNase-treated reagents and consumables. Intact paraffin sections were deparaffinized, rehydrated, and washed three times in PBS (5 minutes each). Sections were treated with 20 g/mL proteinase K for 30 minutes, washed in PBS, then fixed in fresh 4% paraformaldehyde for 10 minutes. Pre-hybridization solution was applied at 60°C for 2 hours. The probe was diluted 1:100 in hybridization solution, denatured at 95°C for 8 minutes, chilled on ice for 5 minutes, then applied to sections and hybridized overnight at 60°C (16 hours). Post-hybridization washes were performed three times with 50% formamide/50% 2×SSC at hybridization temperature (30 minutes each). Sections were incubated with digoxigenin-specific fluorescent secondary

antibody (1:800 dilution) at 37°C for 60 minutes, washed seven times in PBS (5 minutes each), then mounted with DAPI and examined microscopically.

Statistical Analysis All data are presented as mean ± standard deviation. Statistical analysis was performed using SPSS 13.0 software. Differences between two groups were analyzed using independent samples t-test. $P<0.05$ was considered statistically significant.

Results

Renal Cortex Injection of miR-483-5p Lentivirus Mouse Model

Lentivirus injection into the renal cortex of C57BL/6J mice was successfully completed without intraoperative mortality or postoperative complications. Low-dose injection (5 L per renal cortex) of miR-483-5p lentivirus did not affect renal function at either 7 or 21 days post-injection, with serum BUN levels showing no significant difference compared to the blank control group (BUN levels: LV3-NC 5 L 21 days, P=0.823; LV3-miR-483-5p 5 L 7 days, P=0.778; LV3-miR-483-5p 5 L 21 days, P=0.627), all remaining within normal ranges ([Figure 3: see original paper]A). HE staining revealed no abnormal renal architecture or pathological lesions ([Figure 3: see original paper]B), and TUNEL assay showed no significant apoptosis in renal tubular cells within the cortex ([Figure 3: see original paper]B). These results demonstrate that 5 L lentivirus does not affect renal morphology or function and does not induce apoptosis in tubular cells. However, miR-483-5p levels in the renal cortex showed no significant increase at either 7 or 21 days after injection (miR-483-5p relative expression: LV3-miR-483-5p 5 L 7 days, P=0.830; LV3-miR-483-5p 5 L 21 days, P=0.94; [Figure 3: see original paper]C).

High-dose injection (20 L per renal cortex) of miR-483-5p lentivirus similarly did not impair renal function at 7 or 21 days, with BUN levels showing no significant difference from controls (BUN levels: LV3-NC 20 L 21 days, P=0.483; LV3-miR-483-5p 20 L 7 days, P=0.069; LV3-miR-483-5p 20 L 21 days, P=0.252), all within normal ranges ([Figure 4: see original paper]A). qPCR analysis showed no upregulation of KIM-1, an early marker of kidney injury (KIM-1 relative expression: LV3-miR-483-5p 20 L 21 days, P=0.882; [Figure 4: see original paper]B). HE staining revealed normal renal architecture ([Figure 4: see original paper]C), and TUNEL assay confirmed no significant apoptosis in cortical tubular cells ([Figure 4: see original paper]C). These data indicate that 20 L lentivirus also does not affect renal morphology or function or induce tubular cell apoptosis. Notably, miR-483-5p levels increased 3-fold by day 7 and reached 8.6-fold over baseline by day 21 (miR-483-5p relative expression: LV3-miR-483-5p 20 L 7 days, P=0.01; LV3-miR-483-5p 20 L 21 days, P=0.0001; [Figure 4: see original paper]D).

These results demonstrate that uniform injection of 20 L miR-483-5p lentivirus into each renal cortex of C57BL/6J mice using a 33-gauge needle achieves ro-

bust overexpression of miR-483-5p (8.6-fold) after 21 days without compromising renal morphology or function, representing a safe and efficient method for delivering miR-483-5p to the renal cortex.

Inducible Systemic Overexpression miR-483-5p Mouse Model

Systemic miR-483 overexpression was regulated by the Tet-on system, enabling temporal control through rtTA-doxycycline induction. Genotyping results are shown in [Figure 5: see original paper]A. TG483 mice (positive) exhibited normal vital signs and showed no renal morphological or functional abnormalities (data not shown). Real-time qPCR analysis revealed a 1.7-fold increase in renal miR-483-5p expression (0.9±0.09 vs 1.7±0.19, P=0.004; [Figure 5: see original paper]B).

Renal Tubule-Specific miR-483-5p Expression Model

Renal tubule-specific miR-483-5p transgenic mice were generated using the Cre-loxp system. Stable F2 generation positive and negative offspring showed normal vital signs and renal structure and function (genotyping shown in [Figure 6: see original paper]A; other data not shown). Fluorescence in situ hybridization confirmed specific expression of miR-483-5p in renal tubular cells within the cortex ([Figure 6: see original paper]B). Real-time qPCR demonstrated a dramatic 12.35-fold upregulation of miR-483-5p in kidneys of positive versus negative mice (1.6±1.13 vs 12.35±3.89, P=0.01; [Figure 6: see original paper]D), while expression in other tissues such as heart remained unchanged (0.9±0.35 vs 0.8±0.18, P=0.694; [Figure 6: see original paper]C).

Discussion

Various methods have been reported for efficient gene delivery to the kidney, each with distinct advantages and limitations. While miR-483-5p was found to be upregulated in serum of patients with acute rejection following renal transplantation, suggesting involvement in rejection-induced kidney injury, our study demonstrates that overexpression of miR-483-5p in normal mouse kidneys does not cause acute kidney injury. However, our subsequent experiments confirmed that miR-483-5p is indeed a key molecule involved in acute kidney injury under pathological conditions (data not shown). To deeply investigate the functional roles and mechanisms of miR-483-5p in regulating kidney function, efficient delivery methods are urgently needed. Given the limited research on miR-483-5p in the kidney, no previous studies have reported optimal delivery methods. This study systematically evaluated lentivirus injection and transgenic approaches for safe and efficient miR-483-5p delivery to the kidney.

Lentiviral vectors, derived from HIV-1, represent highly efficient gene transfer systems capable of infecting both dividing and non-dividing cells with high transduction efficiency and excellent targeting properties. Compared to other viral vectors such as adenovirus, lentiviral vectors offer superior transduction

efficiency, stability, and capacity for foreign gene insertion. However, achieving stable expression in renal cells remains challenging, with various injection routes attempted including renal artery injection, retrograde ureteral injection, and tail vein injection, each presenting limitations. Renal artery injection of adenovirus requires clamping renal vessels for 45 minutes, causing ischemic tubular injury and mononuclear infiltration, making it unsuitable for kidney injury studies. Retrograde ureteral perfusion with viral vectors resulted in limited gene expression, predominantly in the renal medulla rather than cortex. Tail vein injection, while non-invasive, yields poor renal expression and is not a feasible approach for kidney-specific delivery.

In our study, we employed multi-site uniform injection of lentiviral vectors into both renal cortices. The 33-gauge Hamilton microsyringe, commonly used in stereotactic brain injections in neurosurgical research, effectively minimized hemorrhage and tissue damage. Through optimization of dose and time course, we determined that injection of 20 L miR-483-5p lentivirus into each renal cortex of C57BL/6J mice achieves safe, efficient overexpression (8.6-fold) after 21 days without affecting renal morphology or function.

Transgenic technology has revolutionized basic research, enabling precise investigation of single gene functions in disease pathophysiology. In exploring miR-483-5p transgenic models, we first generated inducible systemic overexpression mice using the Tet-on system. While this approach offered temporal control via doxycycline induction, the highest expressing line showed only 1.7-fold upregulation in kidney—lower than the lentivirus model—and lacked tissue specificity. Although TG483 mice displayed normal vital signs and metabolic parameters, the non-specific expression pattern limited its utility for kidney-specific studies. Subsequently, we successfully established, for the first time, a renal tubule-specific miR-483-5p transgenic mouse using the Cre-loxp system. This model achieved both high specificity and remarkable expression efficiency (12.35-fold), surpassing the other two approaches.

We conclude that the renal tubule-specific miR-483-5p transgenic mouse represents an excellent model for efficient, kidney-specific miR-483-5p delivery without compromising renal structure or function. Additionally, bilateral renal cortex injection of 20 L miR-483-5p lentivirus in C57BL/6J mice provides another effective model with the advantages of rapid construction, low cost, and minimal surgical complexity, despite slightly lower specificity and expression efficiency compared to the transgenic model. The lentivirus approach is particularly suitable for preliminary functional studies, whereas the transgenic model, though time-consuming and expensive to generate, offers unparalleled stability and convenience for long-term mechanistic investigations. These optimized delivery methods not only provide robust experimental models for elucidating the role and mechanisms of miR-483-5p in kidney function but also lay the groundwork for potential future clinical applications.

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Note: Figure translations are in progress. See original paper for figures.