miR-205 Inhibits Renal Tubular Epithelial Cell Transdifferentiation via Down-Regulating ZEB1 and ZEB2 Expression Post-print

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Abstract

Objective To investigate the mechanism of miR-205 in renal tubular epithelial cell transdifferentiation. Methods miR-205 mimics and scrambled control were transfected into HK-2 transdifferentiated cell lines, respectively. Real-time qPCR was utilized to detect the expression levels of miR-205 and ZEB1, E-cadherin, -SMA mRNA; Western blotting was employed to detect the expression levels of ZEB1, ZEB2, E-cadherin, and -SMA. Cellular immunohistochemistry was performed to detect the ectopic expression of -catenin and the expression of E-cadherin. Results Following transfection of miR-205 mimics into HK-2 transdifferentiated cell lines, the expression levels of ZEB1 and ZEB2 were significantly downregulated compared with the high glucose group ($P<0.01$), while the expression level of E-cadherin was markedly increased compared with the high glucose group ($P<0.01$). Concurrently, the expression level of the mesenchymal marker -SMA was significantly decreased ($P<0.01$). miR-205 mimics also significantly inhibited the ectopic expression of -catenin during HK-2 transdifferentiation and played an important role in maintaining epithelial cell morphology. Conclusion miR-205 can inhibit the progression of renal tubular epithelial-mesenchymal transdifferentiation by downregulating the expression of ZEB1 and ZEB2.

Full Text

Preamble

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The MicroRNA miR-205 Inhibits Epithelial-Mesenchymal Transition in HK-2 Cells by Down-regulating ZEB1 and ZEB2 Expression

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Abstract

Objective: To investigate the mechanism of miR-205 in regulating epithelial-mesenchymal transition (EMT) in renal tubular epithelial cells. **Methods:** HK-2 cells were transfected with miR-205 mimics or scrambled control. Real-time qPCR was used to detect the expression levels of miR-205, ZEB1, E-cadherin, and -SMA mRNA. Western blotting was performed to measure protein levels of ZEB1, ZEB2, E-cadherin, and -SMA. Immunocytochemistry was employed to examine ectopic -catenin expression and E-cadherin expression. **Results:** Transfection with miR-205 mimics significantly down-regulated ZEB1 and ZEB2 expression compared with the high glucose group (P<0.01), while markedly increasing E-cadherin expression (P<0.01) and reducing the mesenchymal marker -SMA (P<0.01). miR-205 mimics also significantly inhibited ectopic -catenin expression during HK-2 cell transdifferentiation and played an important role in maintaining epithelial cell morphology. **Conclusion:** miR-205 can inhibit the process of renal tubular epithelial-mesenchymal transition by down-regulating ZEB1 and ZEB2 expression.

Keywords: miR-205; epithelial-mesenchymal transition; HK-2 cell line; ZEB1

Introduction

Epithelial-mesenchymal transition (EMT) of renal tubular epithelial cells and the resulting accumulation of extracellular matrix in the renal interstitium represent the primary pathological mechanism underlying chronic renal fibrosis. The EMT process involves phenotypic changes in epithelial cells, including loss of cell adhesion and polarity, and acquisition of migratory mesenchymal cell characteristics. Studies in mouse models of renal fibrosis have shown that approximately 12% of fibroblasts originate from bone marrow, while about 30% are derived from the transdifferentiation of renal tubular epithelial cells. These fibroblasts constitute the main source of extracellular matrix produced during transdifferentiation. Fluctuating high glucose levels significantly promote fibrosis progression in endothelial and myocardial cells. The potential mechanism involves high glucose-induced reactive oxygen species production, which activates various growth factors and hormonal factors, leading to extracellular matrix deposition. Our research group previously established a fluctuating high glucose-induced EMT model in renal tubular epithelial cells, which has been validated in subsequent experiments.

MicroRNAs (miRNAs) are small non-coding RNAs consisting of approximately 18-24 nucleotides that lack an open reading frame. Although they do not encode proteins, miRNAs participate in various critical physiological and pathological processes by binding to complementary sequences in the 3' untranslated region (3' UTR) of target mRNAs, causing mRNA degradation or translational repression and resulting in specific gene silencing. In early diabetic nephropathy, miR-21 expression is down-regulated, and overexpression of miR-21 can inhibit mesangial cell proliferation and reduce 24-hour urinary protein excretion in model mice. In high glucose environments, miR-29a expression decreases in renal tubular epithelial cells; since miR-29a regulates collagen type I gene transcription by targeting the 3' UTR site, its down-regulation reduces collagen type I protein expression and ameliorates renal fibrosis. Overexpression of miR-192 promotes TGF- 1-induced renal fibrosis, while miR-192 knockdown inhibits fibrosis progression. miR-200a, miR-21, and miR-8 are closely associated with the Wnt/ -catenin signaling pathway, and miR-146a, miR-145, and miR-217 also play important roles in renal fibrosis. During EMT, miR-205 expression decreases while ZEB1 expression increases, which suppresses the epithelial adhesion molecule E-cadherin and promotes EMT progression. The miR-200 family (miR-200a, -200b, -200c, -141, -429) and miR-205 are down-regulated more than 100-fold during EMT. Previous studies have demonstrated that differential expression of specific miRNAs plays important roles in renal tubular epithelial cell transdifferentiation, but the regulatory mechanism of miR-205 in this process remains unreported. Through target gene scanning, we identified binding sites for miR-205 in the 3' UTR regions of ZEB1 and ZEB2. Our study reveals that miR-205 can inhibit renal tubular epithelial cell transdifferentiation by down-regulating ZEB1 and ZEB2 expression.

Materials and Methods

1.1 Materials

The human renal proximal tubular epithelial cell line HK-2 was purchased from the American Type Culture Collection (ATCC). Real-time qPCR kits were obtained from TaKaRa. D-MEM/F12 culture medium, trypsin, and fetal bovine serum were purchased from Gibco. D-glucose was from Sigma, Lipofectamine 2000 from Thermo, and DAB chromogenic reagent kit and hematoxylin staining solution from Beyotime Biotechnology. miRNeasy mini kits were from Qiagen. Antibodies against ZEB1, ZEB2, E-cadherin, -catenin, and -SMA were from Abcam, while internal reference standards and secondary antibodies were from Santa Cruz. Real-time PCR primers were synthesized by Shanghai GenePharma.

The Applied StepOnePlus PCR system was from ABI (USA), NanoDrop-1000 from NanoDrop Technologies (Germany), 25 cm^2 culture flasks and plates from Corning (USA), and the research-grade inverted microscope from Olympus

(Japan).

1.2 Cell Culture

HK-2 cells were cultured in D-MEM/F12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin at 37°C in a 5% CO incubator. Medium was changed every 1-2 days, and cells were passaged with trypsin as needed. A fluctuating high glucose model was used to induce HK-2 transdifferentiation. Cells in good condition were seeded evenly in 6-well plates. When cell confluence reached approximately 60%, cells were starved in serum-free medium for 12 hours, then divided into normal and fluctuating high glucose groups. The fluctuating high glucose group was alternately cultured with 5.5 mmol/L and 25 mmol/L D-glucose every 12 hours. After 48 hours, model establishment was evaluated, and both normal and successfully modeled groups proceeded to subsequent experiments.

1.3 Cell Grouping and Transfection

Successfully modeled cells were divided into three groups: fluctuating high glucose control (HG), negative control (SC, 25 nmol/L scrambled control), and miR-205 intervention (MG, 25 nmol/L miR-205-5p mimics). According to the Invitrogen protocol, miRNA oligomers and Lipofectamine 2000 were mixed evenly, incubated at room temperature for 20 minutes, then added to cell culture plates. After 6 hours, complete medium was replaced. All three groups were cultured under fluctuating high glucose conditions. The normal group (NG) was cultured with 5.5 mmol/L D-glucose throughout. All groups were maintained at 37°C in a 5% CO incubator for 3 days, with cells collected at 48 and 72 hours.

1.4 Quantitative PCR Detection

At 48 hours post-transfection, cells were collected and total RNA was extracted. RNA quality was quantified using a spectrophotometer. RNA samples were reverse-transcribed into cDNA, which served as template for quantifying miR-205, ZEB1, E-cadherin, -SMA, and GAPDH expression. miR-205 and U6 snRNA were detected using TaKaRa SYBR PrimeScript™ miRNA RT-PCR Kit, while non-miRNA targets were detected using TaKaRa SYBR Fast qPCR Mix Kit. All amplifications were performed on the Applied StepOnePlus Real-Time PCR System. Specific primer sequences are listed in Table 1 . U6 snRNA served as internal control for miR-205, and GAPDH for other genes. Relative expression levels were calculated using the 2^(-ΔΔCT) formula. All experiments were independently repeated three times.

Table 1 Primers used in real-time qPCR

Genes	Forward Primer	Reverse Primer
miR-205	5’ - CATACCTCCTTCATTCCACCG-3’	5’ - GTATGGTTTTGACGACTGTGTGAT-3’
U6 snRNA	5’ - ATTGGAACGATACAGAGAAGATT-3’	5’ - GGAACGCTTCACGAATTTG-3’
E-cadherin	5’ - TTCAAACCCATAGTGGTTGCTC-3’	5’ - TGGGAGATACCAAACCAACTG-3’
-SMA	5’ - CCCACCACGTACAAGGGTC-3’	5’ - ATGCCATCGTTGTTCACTGGA-3’
GAPDH	5’ - GAAGAAGAGGACAGCACTG-3’	5’ - TCCCATTCCCACCATCAC-3’

1.5 Immunocytochemical Detection of -catenin and E-cadherin

Sterile coverslips pre-treated with poly-L-lysine were placed in 6-well plates. Cells were treated according to experimental groups for 48 hours, then fixed with 4% paraformaldehyde. Endogenous peroxidase was inactivated with 3% H O at room temperature for 10 minutes. Cells were permeabilized with 0.1% Triton X-100 for 20 minutes and blocked with 10% BSA. Primary antibodies against -catenin and E-cadherin (1:50) were added and incubated overnight at 4°C (PBS served as negative control). After washing, secondary antibody (1:50) was added and incubated for 2 hours, followed by DAB chromogenic reaction and hematoxylin counterstaining. Images were captured using an Olympus IX71 inverted research microscope (×400 magnification).

1.6 Western Blot Detection of ZEB1, ZEB2, E-cadherin, and -SMA

Cells were collected at 72 hours and washed three times with ice-cold PBS. RIPA lysis buffer with protease inhibitors was added, and cells were incubated on ice for 30 minutes with agitation every 10 minutes. Lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C to extract total protein. Protein concentration was determined by BCA assay. Thirty micrograms of protein were separated by 10% SDS-PAGE for 2 hours and transferred to PVDF membranes (200 mA, 1 hour). Membranes were blocked with 5% skim milk for 2 hours, then incubated overnight at 4°C with primary antibodies against E-cadherin, -SMA, ZEB1, and ZEB2. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) at 37°C for 1 hour. Following washing, ECL reagent was added and membranes were imaged using a Bio-Rad chemiluminescence imaging system. Each experiment was independently repeated three times, and band intensities were quantified using Image

J software relative to internal controls.

Statistical Analysis

Experimental data are presented as mean ± standard deviation. Statistical analysis was performed using SPSS 13.0 software. Differences were considered statistically significant at $P<0.05$.

Results

2.1 Establishment of Fluctuating High Glucose-Induced HK-2 Cell Transdifferentiation Model

Cells in the HG group exhibited morphological transformation into spindle-shaped mesenchymal cells [Figure 1: see original paper]. E-cadherin expression was significantly reduced while -SMA expression was markedly increased [FIGURE:2, FIGURE:4]. Concurrently, ectopic nuclear expression of -catenin was significantly enhanced [Figure 3: see original paper]. These findings confirmed successful establishment of the HK-2 transdifferentiation model using fluctuating high glucose conditions.

2.2 Effect of miR-205 on Maintaining HK-2 Epithelial Cell Morphology

After 72 hours of culture, inverted microscopy revealed that HG group cells showed obvious morphological changes compared with NG group cells. NG group cells maintained normal oval epithelial morphology, whereas HG group cells transformed into spindle-shaped mesenchymal cells. However, the proportion of spindle-shaped cells in the MG group was significantly lower than in both HG and SC groups [Figure 1: see original paper]. These results demonstrate that miR-205 mimics play a positive role in maintaining HK-2 epithelial cell morphology.

2.3 Effects of miR-205 Mimics on ZEB1, E-cadherin, and -SMA mRNA Expression

TargetScan analysis identified ZEB1 and ZEB2 as potential targets of miR-205 during transdifferentiation. Both ZEB1 and ZEB2 function as transcriptional repressors of E-cadherin during this process. Real-time qPCR analysis showed that E-cadherin mRNA expression in the HG group was significantly decreased compared with the NG group ($P<0.01$), while -SMA mRNA expression was markedly increased ($P<0.01$), consistent with the characteristics of HK-2 cell transdifferentiation. miR-205 expression in the HG group was substantially reduced compared with the NG group ($P<0.01$), whereas MG group showed significantly elevated miR-205 expression compared with the HG group ($P<0.01$) [Figure 2B: see original paper]. Furthermore, ZEB1 mRNA expression in the MG

group was significantly down-regulated compared with the HG group (P<0.01), E-cadherin mRNA was up-regulated (P<0.01), and -SMA mRNA was reduced (P<0.01). These findings indicate that miR-205 plays an important regulatory role in HK-2 cell transdifferentiation and that enhanced miR-205 expression effectively inhibits this process.

2.4 miR-205 Mimics Inhibit Ectopic -catenin Expression and Maintain Epithelial Cell Polarity

Immunocytochemical results [Figure 3: see original paper] showed that in the fluctuating high glucose HG group, -catenin expression accumulated in the nucleus while E-cadherin expression decreased compared with the NG group, consistent with epithelial transdifferentiation characteristics. However, miR-205 mimic intervention in the MG group significantly reduced -catenin expression and enhanced E-cadherin expression. These results demonstrate that miR-205 participates in suppressing ectopic -catenin expression and plays an important role in preventing E-cadherin down-regulation during renal tubular epithelial-to-mesenchymal transdifferentiation.

2.5 miR-205 Inhibits HK-2 Cell Transdifferentiation by Down-regulating ZEB1 and ZEB2

Transfection of HG group cells with miR-205 mimics maintained oval epithelial morphology [Figure 1: see original paper], enhanced expression of the epithelial adhesion molecule E-cadherin [FIGURE:2, FIGURE:4], reduced expression of the mesenchymal marker -SMA [FIGURE:2, FIGURE:4], and significantly down-regulated ZEB1 and ZEB2 expression compared with the HG group [FIGURE:2, FIGURE:4]. ZEB1 and ZEB2 are transcriptional repressors of E-cadherin during epithelial-mesenchymal transdifferentiation, and their increased expression inhibits E-cadherin. Our study demonstrates that elevating miR-205 levels via miR-205 mimics significantly suppresses ZEB1 and ZEB2 expression, thereby promoting E-cadherin expression and inhibiting HK-2 cell transdifferentiation.

Discussion

miRNAs function by binding to complementary sequences in the 3' UTR region of target mRNAs, leading to mRNA degradation or translational repression and resulting in specific gene silencing. This mechanism plays crucial regulatory roles in organism growth, development, and various diseases, particularly tumor initiation and progression. miRNAs modulate multiple biological signaling pathways, and bioinformatics data indicate that each miRNA can regulate hundreds of target genes, suggesting that miRNAs may influence all signaling pathways. For instance, progressive increase in miR-205 expression in congenital obstructive nephropathy mouse renal epithelial cells correlates positively with

hydronephrosis severity. Additionally, miR-205 participates in TGF- 1 pathway regulation via SMAD2/SMAD7 modulation, acts as an oncogene in hematopoietic cells, and functions as a tumor suppressor in osteosarcoma. miR-205 and Krüppel-like factors show potential as diagnostic biomarkers for basal-like breast cancer, and miR-205 may serve as a therapeutic target for breast and inflammatory breast cancers. These studies demonstrate that miR-205 plays important roles in EMT and tumorigenesis, and in-depth investigation of miR-205 function holds significant clinical implications for disease diagnosis and treatment. However, the function of miR-205 in renal fibrosis has not been previously reported.

In this study, TargetScan analysis identified miR-205 binding sites in the ZEB1 3' UTR region (817-824) and ZEB2 3' UTR regions (561-567 and 622-628). ZEB1 and ZEB2 are transcriptional repressors of E-cadherin, which plays a critical role in maintaining epithelial cell adhesion and polarity during EMT and serves as a hallmark molecule of the process. After transfecting HG group cells with scrambled control or miR-205 mimics, the SC group showed no statistically significant differences from the HG group. In contrast, the MG group exhibited significantly down-regulated ZEB1 and ZEB2 expression ($P<0.01$), up-regulated E-cadherin ($P<0.01$), and reduced -SMA expression ($P<0.01$) [FIGURE:2, FIGURE:4]. miR-205 mimics also significantly inhibited ectopic -catenin expression in the MG group [Figure 3: see original paper] and played an important role in maintaining epithelial cell morphology [Figure 1: see original paper].

Previous studies have shown that the miR-200 family targets ZEB1 and ZEB2, which inhibit expression of many epithelial genes including E-cadherin. Other research has confirmed that miR-205 overexpression can restore E-cadherin expression. Our findings demonstrate that miR-205 mimic-mediated inhibition of ZEB1 and ZEB2 expression also promotes E-cadherin restoration during HK-2 cell transdifferentiation inhibition.

Early-stage renal fibrosis presents with subtle clinical symptoms that are difficult to detect. Therefore, identifying a non-invasive, easily monitored biomarker that accurately reflects fibrosis progression is crucial for early diagnosis and prevention of chronic kidney disease. Changes in miRNA expression precede disease onset, and miRNAs exhibit tissue-specific expression patterns with differential expression under disease conditions, providing a theoretical basis for their use as renal fibrosis diagnostic markers. Recent studies have further validated the potential of miRNAs as disease diagnostic tools.

This study investigated potential targets of miR-205 during HK-2 cell transdifferentiation, demonstrating that miR-205 overexpression down-regulates ZEB1 and ZEB2 by binding to specific sites in their 3' UTR regions, thereby inhibiting renal tubular epithelial-mesenchymal transition. In summary, miR-205 can inhibit renal tubular epithelial cell transdifferentiation by down-regulating ZEB1 and ZEB2 expression, providing a novel therapeutic target for renal interstitial fibrosis. Future studies will investigate differential miR-205 expression in body fluids of renal fibrosis patients to explore non-invasive early diagnostic methods, which will provide scientific evidence and practical foundations for early clinical

diagnosis of renal fibrosis.

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