

## Original Article

# miR-302b suppresses the high-sucrose induced epithelial-mesenchymal transition in diabetic nephropathy

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**Abstract:** Objective: Diabetic nephropathy (DN) is a kind of vascular complication for diabetes, and its pathogenesis remains complicate. This study aimed to investigate the potential role of miR-302b in the high-sucrose induced epithelial-mesenchymal transition (EMT) during the development of DN and to reveal its possible molecular mechanism. *Methods:* The podocytes and epithelial cells were treated with the high-sucrose (30 mM) to construct the DN model. The overexpressed vector of miR-302b was packaged by the virus packaging method and then was transfected into the podocytes and epithelial cells. Results: The EMT-related protein including Slug, snail, vimentin, and fibronectin was significantly increased, whereas E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin were significantly decreased by the overexpression of miR-302b. *Conclusion:* Taken together, our study revealed that the overexpression of miR-302b functioned as a protector in the development of DN by suppressing the high-sucrose induced EMT process.

**Keywords:** Diabetic nephropathy, miR-302b, podocytes, epithelial cells, epithelial-mesenchymal transition

## Introduction

Diabetic nephropathy (DN) remains to be one of the vascular complications of diabetes, which is the main cause for the deaths caused by diabetes [1]. Previous studies have reported that the pathological changes for DN are mainly presented as the glomerular mesangial cell proliferation and the glomerular basement membrane thickness [2, 3]. The pathological changes for DN are mainly presented as the glomerular mesangial cell proliferation and the glomerular basement membrane thickness [4]. Therefore, to explore the potential pathogenesis for DN will be of great significance for the clinical treatment of DN.

microRNAs (miRNA) are some 20-22 nt in length that play pivotal roles in various biological processes at the transcriptional and post-transcriptional level by targeting the 3'-UTR of genes [5]. Increasing evidence has reported

that there are many miRNAs involving in the biology of DN [6]. For example, serum miR-93 was down-regulated in DN cells and can regulate the progression of DN by regulating the downstream genes [7]. miR-192 is up-regulated in DN, and suppression for miR-192 promotes the improvement of renal fibrosis [8]. In addition, it has been reported that miR-302b is often down-regulated in variety kinds of tumors, such as hepatocellular cancer, ovarian cancer, and esophagus cancer [9, 10], and can suppress the inflammation during infection [11].

Recent evidence revealed that the tubular EMT remains to be one of the most pathogenesis for DN [12]. A various miRNAs are involved in the tubular epithelial-mesenchymal transition [13, 14]. It has been reported that miR-302b is involved in the apoptosis biological process in the EMT of embryonic stem cells [15]. However, few have demonstrated the correlation between miR-302 expression in the tubular EMT of DN.

In the present study, we constructed the DN model by high-sucrose treatment using the podocytes and epithelial cells *in vitro* and analyzed the expression of miR-302b to assess the effects of miR-302b in protecting DN. Further experimental studies were conducted to analyze the ETM-related protein expression. This study aimed to investigate the possible roles of miR-302b in protecting DN and to elucidate its potential molecular mechanism.

### Materials and methods

#### *Cell preparation and treatment*

The mice glomerular podocytes and the epithelial cells were purchased from the FuDan IBS Cell Central (Shanghai, China), and were cultured in the RPMI 1640 medium containing 10% fetal bovine serum (FBS, Sigma-Aldrich, USA).

For cell treatment, cells were treated with high-sucrose at the concentration of 30 mM with different time points at 6 h, 12 h, 24 h, 48 h, and 72 h. Cells treated with the low-sucrose at concentration of 5.5 mM were considered as the control.

#### *qRT-PCR analysis*

Total RNA was isolated from the cells according to the manufacturer's instructions. Complementary DNA (cDNA) was produced using the reverse transcriptase (iScript™ cDNA Synthesis Kit; Bio-Rad Laboratories). Expression for the targets was detected by the SYBR green-based quantitative RT-PCR (SYBR Green Master mix; Thermo Scientific, Waltham, MA, USA). U6 served as the internal control. The primers used for the target gene amplification were as follows: miR-302b: 5'-GGGUCUCCCAACCCUUGUA-3', and U6: 5'-CGCAAGGATGACACGCAAA-TTC-3', and the universal reverse primer was 5'-5'-CAGTGGTGTCTGGAGT-3'.

#### *Cell transfection*

The coding sequence of miR-302b was transfected into the pEGFP to produce the vector of pEGFP-miR-302b (GENEchem, Shanghai, China). The pEGFP vectors are served as the control. Vectors were transfected into the 293FT cells using the virus packaging method. Briefly, 293FT cells were cultured in the 96 well plates till they were 80% confluent. Then the vectors

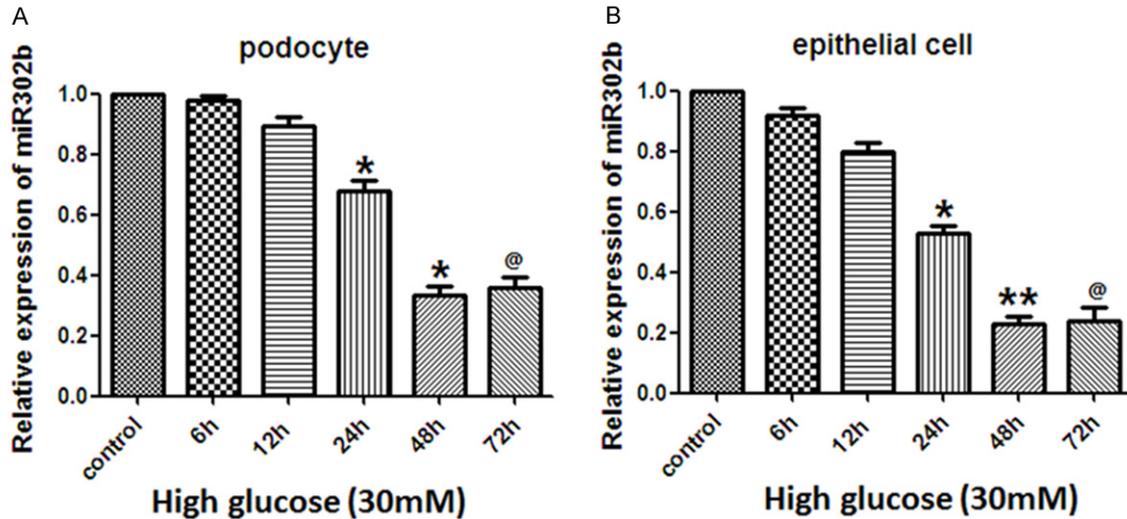
were packaged using the 3rd generation lentiviral packaging system (purchased from Addgene; <http://www.addgene.org/>). Followed by the transfection into 293FT cells using the Lipofectamine 2000 (Life Technologies, Invitrogen, USA). After 48 h of incubation, the supernatant containing virus was collected, cleared by centrifugation, filtered by 0.45 μm millipore filter, concentrated with PEG-it™ Virus Precipitation Solution, and then proceeded to titration. For infection, a final concentration of 8 μg/mL polybrene (Millipore, TR-1003-G) was added together with the viral supernatant into MSCs in passage 4 at 30%-40% confluence. The cells were infected with MOI=30. The efficiency of gene transduction was evaluated by immunofluorescence, PCR and Flow-cytometry.

#### *Western blotting*

Cells were lapped with the radioimmunoprecipitation (RIPA) assay (Sangon, Shanghai, China) containing phenylmethanesulfonyl fluoride (PMSF, Sigma), and then were centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant was collected for the measurement of protein concentrations using BCA protein assay kit (Pierce, Rochford, IL). For Western blotting, protein sample was subjected onto a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferred onto the polyvinylidene difluoride (PVDF) membranes (Mippore). Then the PVDF membranes were blocked in Tris-Buffered Saline Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. Consequently, the membrane was incubated with rabbit anti-human antibodies (Slug, snail, vimentin, fibronectin, E-cadherin, β-catenin and α-catenin, 1:100 dilution, Invitrogen, USA) and overnight at 4°C. Then membrane was incubated with horseradish peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed 3 times with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

#### *Statistical analysis*

All the experiments were conducted 3 times independently in this study. Data were expressed



**Figure 1.** Influence of high-sucrose treatment on the miR-302b expression in the diabetic nephropathy (DN)-related cells. A: High-sucrose (30 mM) treatment resulted in a significant low expression of miR-302b in podocytes at 24 h till to 48 h; B: High-sucrose (30 mM) treatment resulted in a significant low expression of miR-302b in epithelial cells. No significant difference for the miR-302b expression in the two kinds of cells was observed between the 48 h and the 72 h. \*:  $P<0.05$  and \*\*:  $P<0.01$ , compared to the control, and @:  $P<0.05$  compared to cells treated with sucrose at 48 h.

ssed as the mean  $\pm$  standard deviation (SD). Student's t test was used to compare the difference between two groups. Data were analysed by the SPSS 19.0 (Chicago, USA). The  $P<0.05$  were considered as the statistically significant.

## Results

### *High-sucrose treatment on the miR-302b expression*

Both the two kinds of cells including podocytes and epithelial cells were treated with high-sucrose at a concentration of 30 mM to assess the effects of high-sucrose treatment on the miR-302b expression (**Figure 1**). Until 24 h, no significant difference was observed for the expression of miR-302b expression in the two kinds of cells. However, the relative expression of miR-302b in the two kinds of cells was significantly decreased from 12 h till 48 h ( $P<0.05$ ), which indicated the miR-302b expression was affected by the high-sucrose in the podocytes and the epithelial cells. Namely, the diabetes model was successfully constructed.

### *Overexpression of miR-302b in the podocytes and epithelial cells*

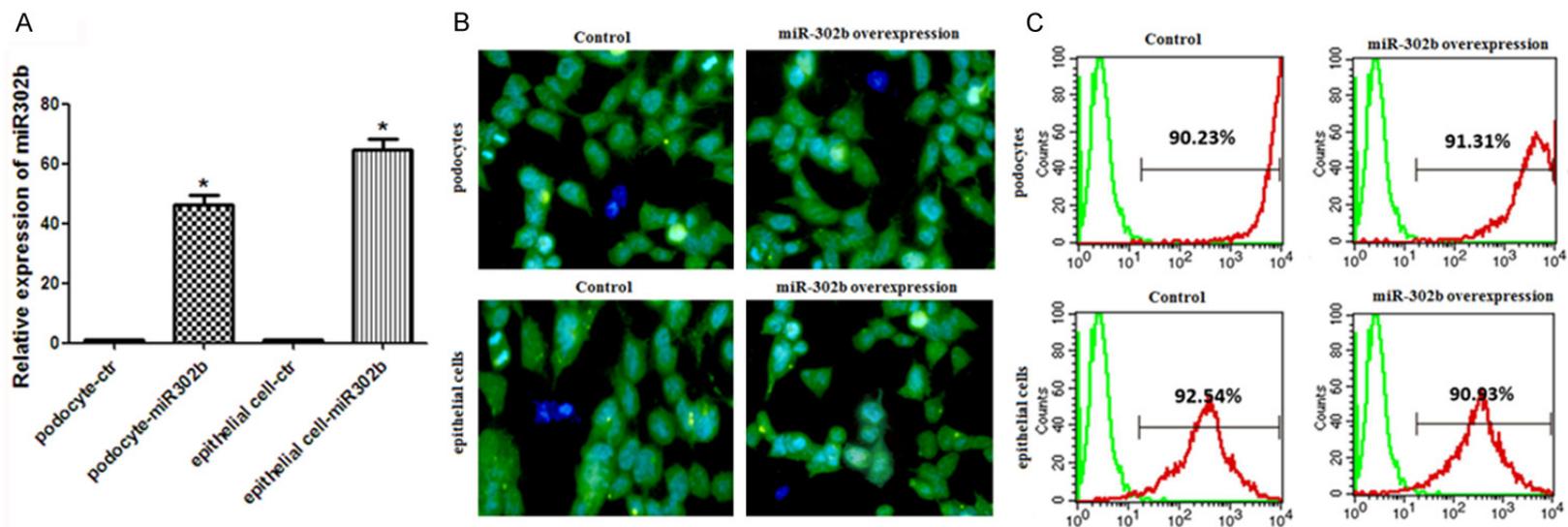
After miR-302b sequence was packaged in the slow virus, virus vector was transfected into the

cells (**Figure 2**). The relative mRNA expression of miR-302b in the podocytes and in the epithelial cells was both significantly increased compared to the control ( $P<0.05$ ). In addition, the flow cytometry and immunofluorescence assay revealed that the virus vector was successfully transfected into the two kinds of cells.

### *miR-302b overexpression promotes the transformation of epithelial cells*

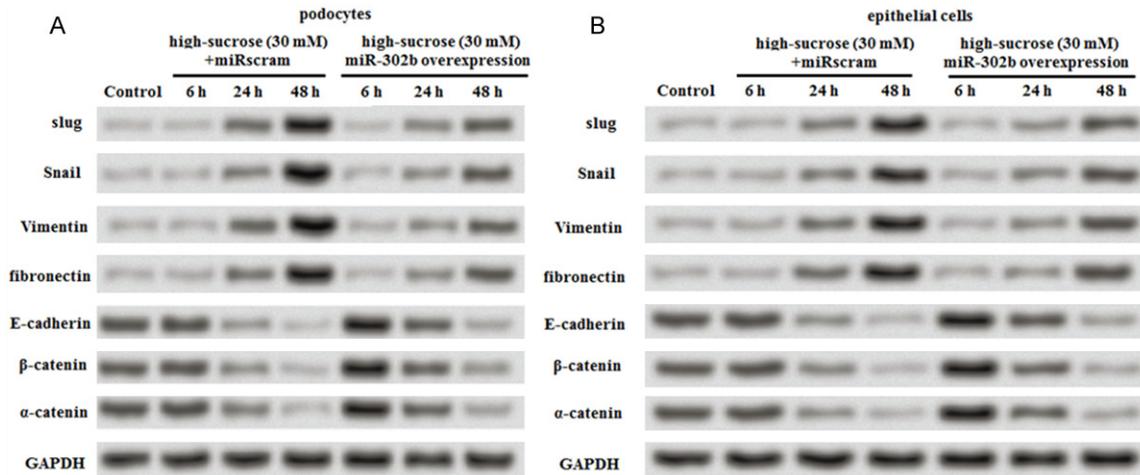
We further analyzed the epithelial cell transformation-related protein expression to investigate the influence of miR-302b expression on podocytes and epithelial cells transformation (**Figure 3**). In the podocytes, the expression of proteins including Slug, snail, vimentin, and fibronectin was significantly decreased compared its control ( $P<0.05$ ), while E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin were all significantly increased with time increasing ( $P<0.05$ , **Figure 3A**). Accordingly, the expression for all the proteins in epithelial cells treated with miR-302b overexpressed vector was the same as that in the podocytes (**Figure 3B**). These results indicated that the overexpressed miR-302b could increase the expression of Slug, snail, vimentin, and fibronectin but decrease the expression of E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin in the two kinds of cells.

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**Figure 2.** Analysis of miR-302b transfection in podocytes and epithelial cells. A: The relative expression of miR-302b was highly expressed in the two kinds of cells after the cell transfection; \*: P<0.05 compared to the control; B: Immunofluorescence assay revealed that the positive cells transfected with the miR-302b overexpressed vector was more than the control; C: Flow cytometry analysis showed that vectors were successfully transfected into the two kinds of cells.

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**Figure 3.** Effects of miR-302b expression on the EMT-related protein expression in DN-related cells. A: In podocytes, the high expression of miR-302b significantly decreased the expression of proteins including slug, snail, vimentin, and fibronectin, but significantly increased the expression of proteins including E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin; B: In epithelial cells, the expression of all the EMT-related proteins were expressed as the same as that in podocytes, indicated the effects of miR-302b expression on these proteins.

### Discussion

Increasing evidence has reported the crucial roles of EMT in the biology of DN [1-3]. Studies have derived that miRNAs play pivotal roles in the pathogenesis of DN, including miR-192, and miR-377 [16, 17]. In this study, we analyzed the expression of miR-302b and investigated the potential effects of miR-302b expression on the EMT-related protein expression in the high-sucrose induced DN using the podocytes and epithelial cells. Our results showed that when the podocytes and epithelial cells were induced by the high-sucrose, the relative expression of miR-302b was significantly declined at 48 h (Figure 1), suggesting that miR-302b may play significant roles in the high-sucrose induced EMT in DN. Accordingly, we further transfected the miR-302b overexpression vector into the cells by virus packaging method (Figure 2), and the results showed that miR-302b was successfully transfected into the two kinds of cells.

Meanwhile, studies have demonstrated the tubular EMT played crucial roles in the development of renal fibrosis through complicate mechanism [18-20]. The loss of epithelial cell adhesion, new expression of  $\alpha$ -SMA, the base membrane damage of tubules, and migration and invasion of cells are the main processes for the EMT during renal fibrosis [21]. Vimentin is an intermediate filament family protein, which

is responsible for maintaining cell shape, integrity of the cytoplasm and stabilizing cytoskeletal interactions [22], while fibronectin is involved in cell adhesion and migration processes including blood coagulation [23]. It has been demonstrated that fibronectin and vimentin are two symbols for the EMT in various kinds of diseases, including tumors and endocrine diseases [24]. In this study, when cells were treated with high-sucrose, the expressions of fibronectin and vimentin were higher than the cells without sucrose treatment both in the podocytes and in the epithelial cells (Figure 3), suggesting the inducement effect of high-sucrose treatment on the EMT in the two kinds of cells. Besides, snail protein is a zinc finger transcriptional repressor which down-regulates the expression of ectodermal genes within the mesoderm, and snail and slug are the transcriptional factors for the EMT [25]. Abnormal expression of snail and slug indicated the occurrence of EMT through  $\beta$ -catenin-T-cell factor 4 [26]. In this study, the expressions of snail and slug were decreased by the overexpressed miR-302b, implying the suppressed influence of miR-302b on the EMT via decreasing the protein expression.

Furthermore, it has been said that down-regulation of E-cadherin is thought to play a fundamental role during early steps of invasion and metastasis of carcinoma cells [27]. In agreement with previous evidence, our study showed that when cells were treated with high-sucrose,

the expression of E-cadherin was down-regulated (**Figure 3**), suggesting the inducement of EMT caused by high-sucrose treatment. The roles of miR-302b expression on the EMT-related protein expression in tubules fibrosis have not been fully revealed. However, previous evidence has demonstrated that miR-302b could regulate the expression of E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin in human corneal endothelial cells [28]. Additionally, E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin were the EMT-related symbols, and were highly expressed in the tubular EMT [29]. Our data showed that when miR-302b was highly expressed in the sucrose treated cells, the expressions of E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin were decreased (**Figure 3**). Therefore, we speculated that miR-302b up-regulation may suppress the EMT progression by down-regulating the proteins including E-cadherin,  $\beta$ -catenin and  $\alpha$ -catenin.

To sum up, our study suggested that miR-302b down-regulation functions as a protector in the high-sucrose induced EMT during DN through resulting in the abnormal expression of EMT-related protein expression. This study may provide theoretical basis for illustrating the role of miR-302b in the pathogenesis of DN and for the clinical treatment of DN. Further studies are still needed to explore the deep molecular mechanism of miR-302b in the EMT in DN at transcriptional level.

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#### Disclosure of conflict of interest

None.

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#### References

- [1] Jerums G, Ekinci EI, Premaratne E, Baker ST, Panagiotopoulos S and Macisaac RJ. Diabetic nephropathy. John Wiley & Sons, Ltd, 2015.
- [2] Sato S, Sasaki Y, Adachi A and Ghazizadeh M. Validation of glomerular basement membrane thickness changes with aging in minimal change disease. *Pathobiology* 2010; 77: 315-319.
- [3] Wichapoon B, Punsawad C, Chaisri U and Viriyavejakul P. Glomerular changes and alterations of zonula occludens-1 in the kidneys of Plasmodium falciparum malaria patients. *Malaria J* 2014; 13: 1-10.
- [4] Jiang P, Xueying LI and Ren Y. Stereologic analysis of the thickness of glomerular basement membrane in 1 to 5 years old children with minimal change disease. *Chinese Journal of Stereology & Image Analysis* 2013.
- [5] Aigner A. MicroRNAs (miRNAs) in cancer invasion and metastasis: Therapeutic approaches based on metastasis-related miRNAs. *J Mol Med* 2011; 89: 445-457.
- [6] Rudnicki M, Beckers A, Neuwirt H and Vandesompele J. RNA expression signatures and posttranscriptional regulation in diabetic nephropathy. *Nephrol Dialysis Transplant* 2015; 30: iv35-iv42.
- [7] Long J, Wang Y, Wang W, Chang BH, Danesh FR. Identification of microRNA-93 as a novel regulator of vascular endothelial growth factor in hyperglycemic conditions. *J Biol Chem* 2010; 285: 23457-23465.
- [8] Sumanth P, Linda L, Guangdong S, Gregory L, Mitsuo K and Rama N. Inhibiting MicroRNA-192 Ameliorates Renal Fibrosis in Diabetic Nephropathy. *J Am Soc Nephrol* 2012; 23: 458-469.
- [9] Oksuz Z, Serin MS, Kaplan E, Dogen A, Tezcan S, Aslan G, Emekdas G, Sezgin O, Altintas E and Tiftik EN. Serum microRNAs; miR-30c-5p, miR-223-3p, miR-302c-3p and miR-17-5p could be used as novel non-invasive biomarkers for HCV-positive cirrhosis and hepatocellular carcinoma. *Mol Biol Rep* 2014; 42: 1-8.
- [10] Ge T, Yin M, Yang M, Liu T and Lou G. MicroRNA-302b Suppresses Human Epithelial Ovarian Cancer Cell Growth by Targeting RUNX1. *Cell Physiol Biochem* 2014; 34: 2209-2220.
- [11] Zhou X, Li X, Ye Y, Zhao K, Zhuang Y, Li Y, Wei Y and Wu M. MicroRNA-302b augments host defense to bacteria by regulating inflammatory responses via feedback to TLR/IRAK4 circuits. *Nat Commun* 2014; 5: 3619.
- [12] Aresu L, Rastaldi MP, Scanziani E, Baily J, Radaelli E, Pregel P and Valenza F. Epithelial-mesenchymal transition (EMT) of renal tubular cells in canine glomerulonephritis. *Virchows Arch* 2007; 451: 937-942.
- [13] Yuriko K and Masaru K. Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (review). *Int J Mol Med* 2008; 22: 271-275.
- [14] Ru P, Steele R, Newhall P, Phillips NJ, Toth K and Ray RB. miRNA-29b suppresses prostate cancer metastasis by regulating epithelial-

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- mesenchymal transition signaling. *Mol Cancer Ther* 2012; 11: 1166-1173.
- [15] Guo WT, Wang XW, Yan YL, Li YP, Yin X, Zhang Q, Melton C, Shenoy A, Reyes NA and Oakes SA. Suppression of epithelial-mesenchymal transition and apoptotic pathways by miR-294/302 family synergistically blocks let-7-induced silencing of self-renewal in embryonic stem cells. *Cell Death Differ* 2014; 22: 1158-1169.
- [16] Wang Q, Wang Y, Aw, Wang J, Shi Q, Li X and Quigg R. MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. *FASEB J* 2008; 22: 4126-4135.
- [17] Aleksandra K, Robert J, Dong Dong L, Aled L, Aled P and Donald F. Loss of MicroRNA-192 promotes fibrogenesis in diabetic nephropathy. *J Am Soc Nephrol Jasn* 2010; 21: 438-447.
- [18] Takayuki A, Mitsumi A, Naoya S, Atsuko KI, Kazuki O, Toshiyuki S, Kurokawa MS, Kazuki O, Kenjiro K and Yugo S. Roles of layilin in TNF- $\alpha$ -induced epithelial-mesenchymal transformation of renal tubular epithelial cells. *Biochem Biophys Res Commun* 2015; 467: 63-69.
- [19] Rastaldi MP. Epithelial-mesenchymal transition and its implications for the development of renal tubulointerstitial fibrosis. *J Nephrol* 2006; 19: 407-412.
- [20] Kalluri R and Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; 119: 1420-1428.
- [21] Fukagawa A, Ishii H, Miyazawa K and Saitoh M. New electron diffraction method to identify the chirality of enantiomorphous crystals. *Acta Crystallographica* 2003; 59: 802-810.
- [22] Byrappa V, Lee AP, Vydianathan R, Maurya AK, Lian MM, Swann JB, Yuko O, Flajnik MF, Yoichi S and Masanori K. Elephant shark genome provides unique insights into gnathostome evolution. *Nature* 2014; 505: 174-179.
- [23] Stine JM, Yizhi S, Geoffrey A, Bowler BE and Klára B. Structure and Unfolding of the Third Type III Domain from Human Fibronectin. *Biochemistry* 2015; 54: 6724-6733.
- [24] Gavert N, Vivanti A, Hazin J, Brabletz T and Ben-Ze'Ev A. L1-mediated colon cancer cell metastasis does not require changes in EMT and cancer stem cell markers. *Mol Cancer Res Mcr* 2011; 9: 14-24.
- [25] Chie KS, Hiromi S, Tadashi T and Yutaka K. Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell* 2009; 15: 195-206.
- [26] Medici D, Hay ED and Olsen BR. Snail and Slug Promote Epithelial-Mesenchymal Transition through  $\beta$ -Catenin-T-Cell Factor-4-dependent Expression of Transforming Growth Factor- $\beta$ 3. *Mol Biol Cell* 2008; 19: 4875-4887.
- [27] Rosivatz E, Becker I, Specht K, Fricke E, Luber B, Busch R, Höfler H and Becker KF. Differential Expression of the Epithelial-Mesenchymal Transition Regulators Snail, SIP1, and Twist in Gastric Cancer. *Am J Pathol* 2002; 161: 1881-1891.
- [28] Zhu YT, Tighe S, Chen SL, John T, Kao WY, Tseng SC. Engineering of Human Corneal Endothelial Grafts. *Curr Ophthalmol Rep* 2015; 3: 207-217.
- [29] Liu Y. New Insights into Epithelial-Mesenchymal Transition in Kidney Fibrosis. *J Am Soc Nephrol Jasn* 2009; 21: 212-222.