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MicroRNA-140-5p Suppresses Hypoxia-induced Nephrotoxicity and Renal Fibrogenesis in *in vitro* Conditions

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ABSTRACT

Background: miR-140-5p is frequently dysregulated in different types of tumors and has been found to suppress inflammation through TLR4 in lung injury. In the current investigation, we try to decipher the possible function of miR-140-5p in hypoxia-induced nephrotoxicity and renal fibrogenesis in human renal cells.

Methods: Hypoxia in HK-2 cells was produced using cobalt chloride ($CoCl_2$). Hypoxia-induced cellular viability and apoptosis were determined by cell viability assay and ELISA-based apoptosis assay, respectively. Western blotting was carried out to measure expression levels of pro-fibrotic and apoptosis-related proteins.

Result: The present study observed that upregulation of miR-140-5p significantly decreases apoptosis and increases cell viability in hypoxia challenged HK-2 cells. Furthermore, miR-140-5p over expression decreased expression of pro-apoptotic proteins like Bad and Bax and pro-fibrotic proteins like Collagen I, CTGF and fibronectin. While as, expression of anti-apoptotic protein, namely Bcl-2, was increased in hypoxia-insulted HK-2 cells. Moreover, the luciferase reporter assay confirmed that miR-140-5p targets TLR4. TLR4 knockdown prevents miR-140-5p from performing its suppressive role in hypoxia linked to apoptosis and fibrosis.

Key words: Apoptosis, Fibrogenesis, Hypoxia, MiR-140-5p, TLR4.

INTRODUCTION

MicroRNAs (miRNAs) are responsible for inducing mRNA degradation or blocking translation (Romaine *et al*. 2015). MiRNAs participate in a broad array of cellular processes, including cell growth, differentiation and death in part, by regulating multiple genes and their downstream networks (Yang *et al*. 2015). In recent years, miRNAs have been recognized as important modulator of diabetic nephropathy (DN) pathophysiology, including extracellular matrix protein production (Wang *et al*. 2008), cell death (Kato and Natarajan, 2015), glomerulosclerosis and tubulointerstitial fibrosis (Kato and Natarajan, 2015). MiR-140-5p is frequently dysregulated in different tumor cells, including colorectal cancer and ovarian cancer (Zhai *et al*. 2015a). Earlier, miR-140-5p has been found to suppress inflammation through TLR4 in lung injury (Yang *et al*. 2018).

Renal cells are prone to injury associated with hypoxia due to their more oxygen demand high metabolic activity (Louis and Hertig, 2015). In response to persistent hypoxia insult, tubular epithelial cells initiate an inflammatory and fibrogenic cytokine response, ultimately activating fibroblasts and tubular epithelial cells. Fibroblasts produce excessive extracellular matrix proteins (ECM), while activated tubular epithelial cells undergo apoptosis. Furthermore, transition of epithelial cells from epithelial-to-mesenchymal stage contributes to tubular atrophy. On the other hand, excessive production of ECM results in endothelial dysfunction and promotes hypoxia. Taken together, all these changes in response to persistent hypoxia causes tissue destruction (Zafrani and Ince, 2015).

Toll-like receptor 4 (TLR4) encodes a transmembrane protein that contains leucine-rich repeat (LRR)-motifs in its extracellular portions involved in recognizing various biological molecules (Seifert *et al*. 2015). TLR4 regulates complex cellular processes like apoptosis, proliferation, Wnt signaling pathway and immunosuppressive cytokines (Yi *et al*. 2012). A previous study strongly implicates TLR4 in protecting against pulmonary fibrosis (Liang *et al*. 2016). Experimental evidence also supports the role of TLR4 in fibrosis of the kidney, skin and lungs (Bhattacharyya *et al*. 2018). Importantly, TLR4 can promote fibrosis and inhibits tubular damage in the context of renal injury (Pulskens *et al*. 2010a). A good number of previous studies have reported an association between TLR4 and miR-140-5p (Wang *et al*. 2019). Additionally, targeting TLR4 by miR-140-5p has been demonstrated to be linked with the pathogenesis of atrophic nonunion (Guo *et al*. 2019), acute lung injury (Yang *et al*. 2018), respiratory syncytial virus disease (Zhang and Shao, 2018), intervertebral disc degeneration (Zhang *et al*. 2018), and pulmonary arterial hypertension (Li *et al*. 2017).

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MATERIALS AND METHODS

Cell culture and treatment

HK-2 cell line was provided by ATCC and grown in DMEM (GBICO, USA) with 10% FBS and antibiotic in CO_2 incubator at 37°C overnight. For hypoxia studies, HK-2 cells were stimulated with $CoCl_2$ (200 µM) for 12 h, as described by Waza and colleagues (Waza *et al*. 2018).

All the experimental work was carried out in Ningbo Yinzhou No.2 Hospital, Ningbo, (China) from 2019 to 2021.

Cell transfection

The miR-140-5p mimic, si-TLR4, si-NC and miR-NC vectors were used. The coding sequence of TLR4 was amplified from HK-2 cells and inserted in pcDNA3.1 expression vector (pcDNA3.1-TLR4). The empty pcDNA3.1 plasmid was used as negative control (NC). Transfection of different vectors was performed using Lipofectamine. After transfection, HK-2 cells were induced with hypoxia for 12 h. Transfection efficiency was determined by qRT-PCR.

qRT-PCR

HK-2 cells were lysed to obtain total RNA using QIAzol Lysis Reagent and then reversed to cDNA. It was followed by mRNA quantification using SYBRGreen (Takara) with appropriate primers against MiR-140-5p, Bad, Bax, Bcl-2, CTGF, Fibronectin, Collagen I, TLR4 and GAPDH. The mRNA level of the target gene was determined by $2^{-\Delta\Delta CT}$, using GAPDH as control.

Cell viability assay

Cell viability was analyzed in HK-2 cells using CCK-8 assay. In brief, HK-2 cells (3×10^3 cells per well) were grown in 96-well plates for 24 h at the normal conditions. It was followed by transfection and hypoxia studies, as described above. After completion of experimental time, 10 µl of CCK-8 was added to each well. Absorbance was taken at 450 nm, followed by cell viability calculations.

Cell apoptosis assay

HK-2 cells were grown in 96-well plates and were either treated with hypoxia alone or transfections followed by hypoxia. After completion of experimental time, ELISA-based cell death assay kit (Roche, Germany) was used to determine apoptosis by following manufacturer's instructions.

Luciferase reporter assay

3′UTR sequences of TLR4 containing miR-140-5p target site [wild or mutant type] were cloned in pmir-GLO report luciferase vector to generate TLR4-WT or TLR4-MUT reporter plasmid, respectively. Subsequently, cells were grown and co-transfected with either miR-140-5p mimic with WT TLR4 or MUT TLR4 reporter plasmid. Calculation of relative luciferase activities was performed post-transfection (48 h).

Protein extraction

Preparation of HK-2 cell lysate was achieved using lysis buffer (NP-40). To prevent proteolysis of cell lysate, Halt Protease Inhibitor Cocktail (Thermo Scientific) was used. Centrifugation (3000 rpm for 10 min) of cell lysate was performed to obtain supernatant. Bradfords assay was used to determine protein concentration.

Western blotting

Preparation of protein samples was carried out as performed by (Waza *et al*. 2012). Detection of target proteins was determined using specific antibodies; anti-Bad, anti-Bax, anti-Bcl-2, anti-CTGF, anti-Fibronectin, anti-Collagen I, anti-TLR4 and anti-GAPDH. LI-COR system was used for secondary detection.

RESULTS AND DISCUSSION

Decreased expression of miR-140-5p in hypoxia-insulted HK-2 cells

Initially, miR-140-5p levels in hypoxia-induced HK-2 cells were determined using qRT-PCT. The induction of hypoxia

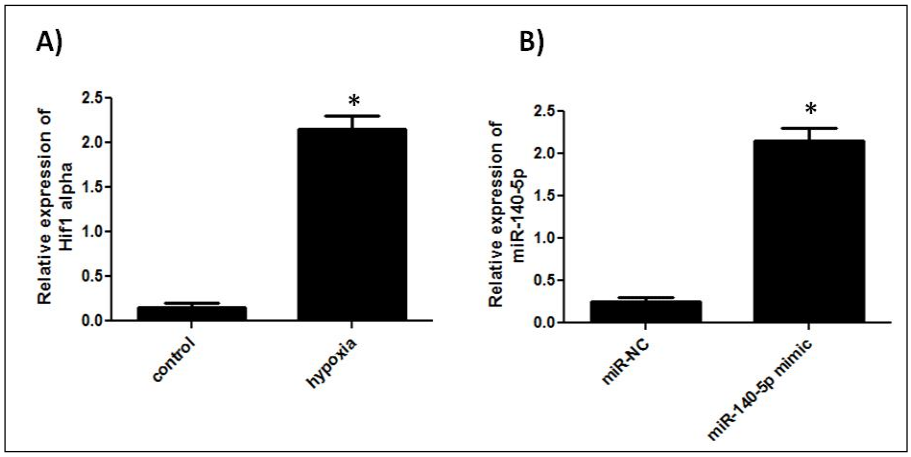

Fig 1: Levels of HIF-1 alpha and miR-140-5p in hypoxia stimulated HK-2 cells. (A) The expression of HIF-1 alpha and (B) miR-140-5p was detected using qRT-PCR in HK-2 cells in hypoxia induced HK-2 cells and compared with control. *p<0.05 compared to control.

with $CoCl_2$ and expression levels of miR-140-5p in hypoxia stimulated HK-2 cells is depicted in Fig 1A and Fig 1B, respectively. Tissue/cell-type specific expression of miR-140-5p has been reported earlier (Zhang *et al*. 2015). MiR-140-5p is frequently upregulated in mesenchymal tissues (Hwang *et al*. 2014). MiR-140-5p expression has been reported to decrease in primary colorectal carcinomas tissues and liver metastatic tissues (Zhai *et al*. 2015b). In addition, downregulation of miR-140-5p has been observed in rat cerebral tissues following middle cerebral artery occlusion (Sun *et al*. 2016).

Effects of miR-140-5p on hypoxia-stimulated cell viability and apoptosis

Considering miR-140-5p down-regulation in hypoxia-induced HK-2 cells, we performed transfection assays to determine its role in hypoxia-stimulated HK-2 cell toxicity and fibrogenesis. We transfected miR-140-5p mimic and then stimulated with hypoxia for 12 h. miR-140-5p overexpression was successfully constructed in HK-2 cells by miR-140-5p mimic transfection, as demonstrated by quantitative real-time PCR (Fig 2A). CCK-8 assay showed miR-140-5 pup-regulation decreases cell viability in hypoxia-treated cells (Fig 2B).

Similarly, miR-140-5p up-regulation increased apoptosis in hypoxia treated cells (Fig 2C). Moreover, we analyzed the impact of miR-140-5p on key mediators of apoptosis. We found that hypoxia-stimulation significantly elevated mRNA (Fig 2D) and protein (Fig 2E) levels of pro-apoptotic (Bad, Bax) and reduced anti-apoptotic Bcl-2 were notably attenuated by miR-140-5p overexpression.

Effects of miR-140-5p on hypoxia-stimulated fibrogenesis

Similarly, we analyzed miR-140-5p impact on the key mediators of fibrosis. Expression levels of pro-fibrosis, including CTGF, Fibronectin and Collagen I were all elevated after hypoxia treatment, which were partially mitigated by miR-140-5p mimic transfection. Hypoxia-stimulation significantly elevated pro-fibrosis related mRNA (Fig 3A) and protein (Fig 3B) levels and was notably attenuated by miR-140-5p overexpression. It has been shown that CTGF, Fibronectin and collagen I are substantially expressed in various forms of tissue fibrosis, including renal fibrotic diseases (Cabello-Verrugio *et al*. 2012).

MiR-140-5p directly targeted TLR4 in HK-2 cells

TargetScan was used to predict binding sites of miR-140-5p in TLR4 32 -UTR (Fig 4A). MiR-140-5p mimic decreased the luciferase activity mediated by WT TLR4 3′-UTR, while no apparent effects were found in the miR-NC or MUT TLR4 groups (Fig 4B). Furthermore, expression levels of TLR4 were analyzed in hypoxia-stimulated HK-2 cells. MiR-140-5p suppressed the elevated protein expression of TLR4 induced by hypoxia treatment (Fig 4C). Furthermore, TLR4 mRNA was significantly upregulated in hypoxia-stimulated HK-2 cells (Fig 4D). Indeed, TLR4 mediates a range of diverse signaling pathways under apoptosis. Chen *et al*. (2018) found that patrinia represses apoptosis by inhibiting TLR4 associated signaling pathways in BRL-3A cells. There have been suggestions that angiotension II (Ang II) could induce mesangial cells apoptosis by activating TLR4/MyD88 signaling pathway (Lv *et al*. 2009). Many molecules have

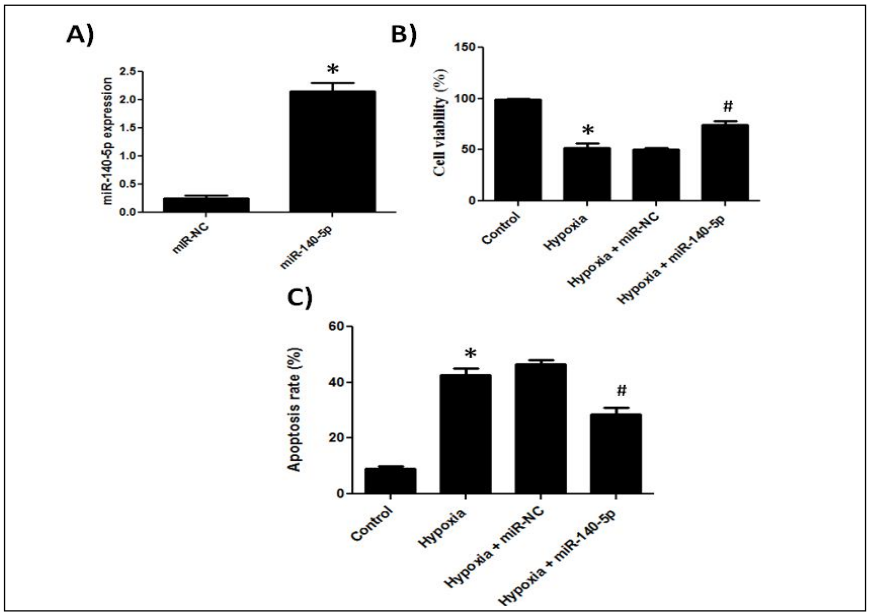

Fig 2: MiR-140-5p overexpression counters the hypoxia-caused HK-2 cell viability loss. (A) The expression level of miR-140-5p was determined in HK-2 cells after miR-140-5p mimic or miR-NC transfection, followed by hypoxia treatment using qRT-PCR. (B) Cell viability of HK-2 cells was assessed by CCK-8 assay. (C) Representative images of apoptotic cell. *p<0.05 compared to control while as # p<0.05 compared with hypoxia + miR-NC.

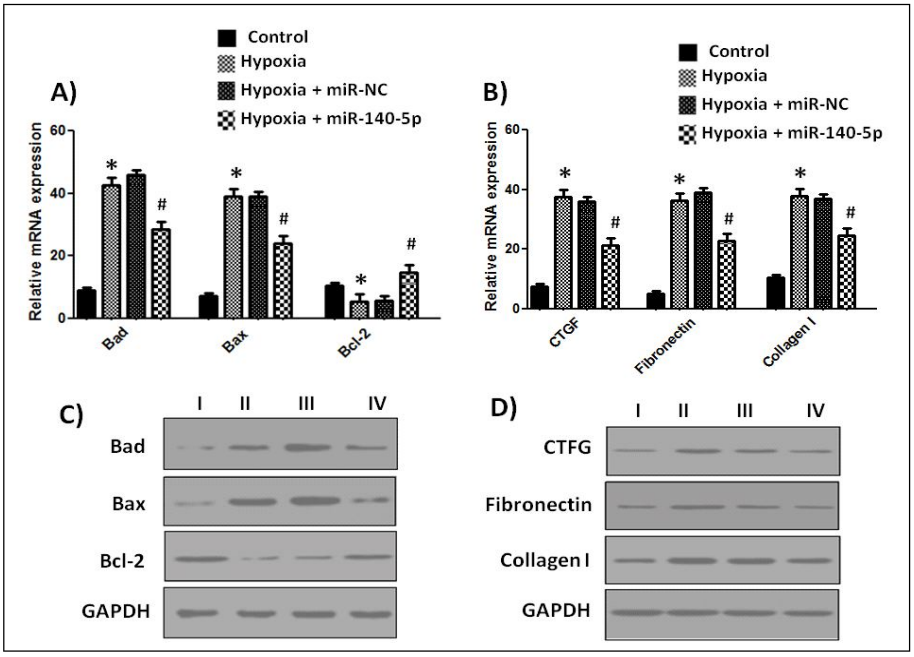

Fig 3: MiR-140-5p overexpression inhibited hypoxia-induced apoptosis and renal fibrogenesis related factors in HK-2 cells. HK-2 cells were treated with hypoxia for 12 h after transfection with miR-140-5p mimic or miR-NC. (A) Shows mRNA expression levels of Bad, Bax and Bcl-2. (B) Shows mRNA expression levels of CTGF, Fibronectin and Collagen I. (C) Shows protein levels of Bad, Bax and Bcl-2 proteins, while as (D) Shows protein levels of CTGF, Fibronectin and Collagen I. Lane (I) = Control, lane II = Hypoxia, Lane (III) = Hypoxia + miR-NC and Lane (IV) = Hypoxia + miR-140-5p. *p<0.05 compared to control while as # p<0.05 compared with hypoxia + miR-NC.

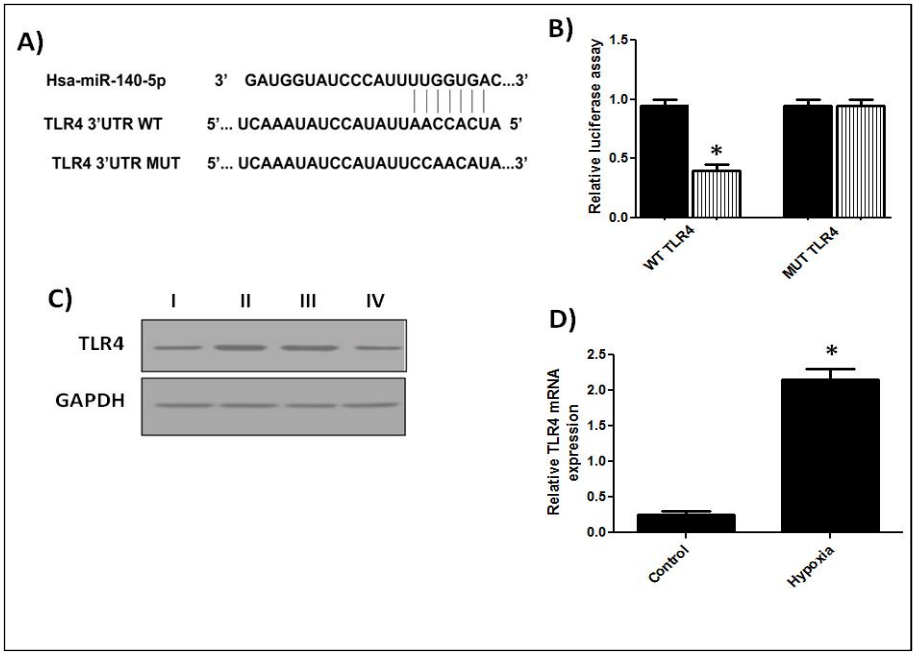

Fig 4: TLR4 was a direct target of miR-140-5p. (A) Shows predicted binding sites of miR-140-5p in TLR4 32 -UTR. (B) Shows luciferase assay (C) Shows protein expression level of TLR4 protein expression. Lane (I) = Control, lane II = Hypoxia, Lane (III) = Hypoxia + miR-NC and Lane (IV) = Hypoxia + miR-140-5p. (D) Shows expression ofTLR4 mRNA in hypoxia-caused HK-2 cells. *p<0.05 compared to control.

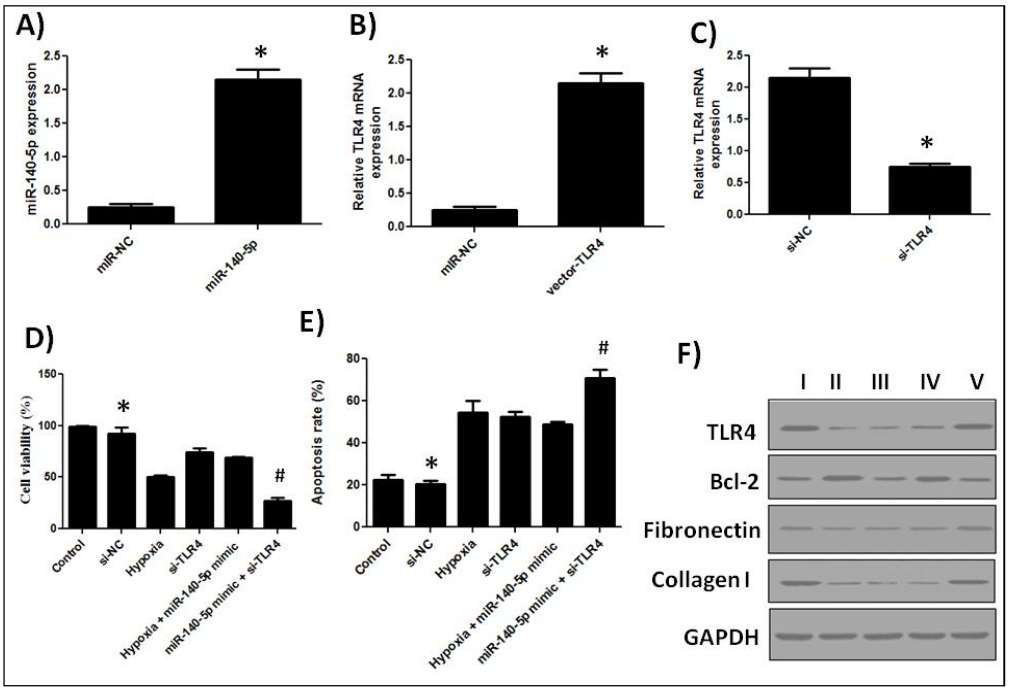

Fig 5: TLR4 was involved in the effects of miR-140-5p on hypoxia-induced apoptosis and renal fibrogenesis of HK-2 cells. HK-2 cells were transfected with si-TLR4, si-NC, miR-140-5p mimic together with TLR4 or empty vector, respectively and then treated with HG for 24 h. (A and B) Shows expression levels of miR-140-5p and TLR4 after use of mimic and si-RNA respectively. (C) Expression levels of TLR4 in cells tranfected with TLR4-vector compared to control. (D) Cell viability of HK-2 cells was assessed by CCK-8 assay. (E) Shows apoptotic assay. (F) Western blotting analysis was performed to detect the protein expression levels of TLR4, Bcl-2, Fibronectin and Collagen I. Lane (I) = Control, Lane (II) = si-NC, Lane (III) = Hypoxia, Lane (IV) = Hypoxia + miR-140-5p mimic and Lane (V) = miR-140-5p mimic + si-TLR4. *p<0.05 compared to control, while as # p<0.05 compared with hypoxia + miR-NC.

been shown to promote mitochondria-mediated apoptosis *via* upregulation of Bad/Bax and downregulation of Bcl-2 (Zhang *et al*. 2013).

MiR-140-5p attenuated hypoxia-stimulated toxicity and fibrogenesis of HK-2 cells by down-regulating TLR4

The above data indicated TLR4 was up-regulated in hypoxia-stimulated cells and miR-140-5p directly target TLR4. We thus speculated that TLR4 might be a downstream functional regulator involved in miR-140-5p regulating hypoxia-stimulated toxicity and fibrogenesis. To validate our hypothesis, transfection of si-TLR4, si-NC, miR-140-5p mimic together with TLR4 or empty vector was carried out in hypoxia-stimulated HK-2 cells (Fig 5 A, B and C). Then, we examined the effects of TLR4 knockdown or overexpression on cell viability, apoptosis and fibrogenesis. As shown in Fig 5D, TLR4 knockdown significantly increased cell viability, while TLR4 overexpression remarkably countered miR-140-5p up-regulation effects on cell viability in hypoxia-induced HK-2 cells. In line with this, TLR4 knockdown mimics, while overexpression attenuated miR-140-5p effects on hypoxia-stimulated cell apoptosis in HK-2 cells (Fig 5E). Moreover, miR-140-5p up-regulation effects on TLR-4, Bcl-2, Fibronectin and Collagen I was mimicked by TLR4 knockdown, but reversed by TLR4 overexpression (Fig 5F). The multiple lines of evidence indicate that TLR4 is also crucial for organ fibrosis (Pulskens *et al*., 2010b). Seki and colleagues have described that TLR4 could promote TGF-β signaling to contribute to hepatic fibrosis (Seki *et al*. 2007). TGF-β can induce the secretion of proteins like Collagen I and Fibronectin to promote fibrosis initiation and progression (Schnaper *et al*. 2003).

CONCLUSION

In conclusion, miR-140-5p inhibits hypoxia-associated cell toxicity and renal fibrogenesis by targeting TLR4. The detailed mechanisms underlying these processes need further investigation; however our study will deepen the understanding of hypoxia-induced nephrotoxicity and renal fibrogenesis, and open new clues into novel therapeutic treatments.

Conflict of interest

All the authors declare no competing financial interests.

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