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MiR-181b targets *Six2* and inhibits the proliferation of metanephric mesenchymal cells in vitro

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that down-regulate gene expression by binding to target mRNA for cleavage or translational repression, and play important regulatory roles in renal development. Despite increasing genes have been predicted to be miRNA targets by bioinformatic analysis during kidney development, few of them have been verified by experiment. The objective of our study is to identify the miRNAs targeting *Six2*, a critical transcription factor that maintains the mesenchymal progenitor pool via self-renewal (proliferation) during renal development. We initially analyzed the 3′UTR of *Six2* and found 37 binding sites targeted by 50 putative miRNAs in the 3′UTR of *Six2*. Among the 50 miRNAs, miR-181b is the miRNAs predicted by the three used websites. In our study, the results of luciferase reporter assay, realtime-PCR and Western blot demonstrated that miR-181b directly targeted on the 3′UTR of *Six2* and down-regulate the expression of *Six2* at mRNA and protein levels. Furthermore, EdU proliferation assay along with the *Six2* rescue strategy showed that miR-181b suppresses the proliferation of metanephric mesenchymal by targeting *Six2* in part. In our research, we concluded that by targeting the transcription factor gene *Six2*, miR-181b inhibits the proliferation of metanephric mesenchymal cells in vitro and might play an important role in the formation of nephrons.

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1. Introduction

MicroRNAs (miRNAs) refer to a huge category of small non-coding RNA molecules (18–25 nucleotides in length) that down-regulate gene expression mostly through mRNA degradation or translation repression [1]. One strand of the mature miRNA is assembled into the RNA-induced silencing complex (RISC), then imperfectly base pairing binds to the 3′untranslated region (UTR) of targeted mRNAs by which reduces the expression of the target proteins [2]. Decrease of the targets abundance is widely believed to be modulated by numerous mechanisms containing reduction of the target mRNA stability, augmenting the rate of mRNA degradation, inhibition of translational initiation and elongation as well as induction of deadenylation [2]. It is estimated that one individual miRNA is able to target numerous mRNAs, meanwhile a single mRNA could also be targeted by multiple miRNAs [3,4], thus complicated altering gene expression regulatory networks have been well-established. The conservative estimates imply that about no less than 30%, even 60% approximately, protein-coding genes expressed in the genome can be targeted and down-regulated by more than 1000 miRNAs [5].

With the progression of in-depth study and characterization of miRNAs, their functions are thought to be involved in a large number of biological processes, including development, homeostasis, and diseases [2]. The roles of miRNAs in the regulation of renal development, physiology, and pathology have been considered as an important and potentially fruitful area of research. The miRNAs, such as miR-192, -194, -204, -215, and -216 are higher abundant in kidney compared to other organs, suggesting that these miRNAs may be involved in kidney [6]. Overwhelming experimental evidences demonstrated that miRNAs are essential for kidney development. Conditional depletion of Dicer1 in developing ureteric bud epithelium results in parenchymal cysts and hydronephrosis [7]. Podocyte-specific Dicer deprived mice presents proteinuria by 3 weeks along with segmental foot process effacement, as well as glomerulonephritis [8,9]. However, the researches in miRNAs

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regulated kidney development are still in their infancy. The identification and characterization of the miRNAs in various processes of kidney development may lead to breakthroughs in the molecular mechanisms of kidney development.

During kidney development, nephrons originate from a population of self-renewing *Six2* positive nephron progenitor cells (a mesenchymal progenitor pool) which are a subset of metanephric mesenchyme (MM) cells [10]. Formation of the full complement of nephrons and nephron endowment are determined by the balance between self-renewal (proliferation) and consumption of nephron progenitors [10,11]. The former is dependent on *Six2*, a crucial transcriptional regulator, which is required for the maintenance of the progenitor pool [10]. Deletion of *Six2* leads to ectopic differentiation, depletion of cap mesenchyme progenitors, and kidney hypoplasia and dysplasia [11,12]. However, little is known that whether *Six2* can be regulated by miRNAs or not.

In present study, we for the first time cloned and analyzed the 3′UTR of *Six2*. It is shown that the 915 bp 3′UTR of *Six2* is evolutionarily highly conserved across the mammal species. We found that there exist 50 miRNAs and 37 binding sites in the 3'UTR of *Six2*. MiR-181b is the common predicted miRNA by the three bioinformatic websites used and particular conserved across different species among 50 miRNAs. Moreover, it was first to identify that miR-181b can target the 3′UTR of *Six2*, resulting in down-regulation of the *Six2* expression both at mRNA and protein level and miR-181b can inhibit the mK3 cell proliferation at least partially through *Six2*.

2. Methods and materials

2.1. Bioinformatic analysis

The evolutional conservation of *Six2* 3′UTR (NCBI Reference Sequence: NM_011380.2) was retrieved from Genebank database (NCBI). Each putative microRNA that is able to target *Six2* was predicted by commonly cited prediction programs such as miRawalk (http://www.microwalk.org), miRanda (http://microrna.org) and TargetScan release 6.2 (http://www.targetscan.org). MicroRNA sequence used for analysis are acquired from online accessible miRbase (http://www.mirbase.org).

2.2. Plasmid construction

pCDNA3.1-luciferase-m*Six2*–3′UTR-WT, pCDNA3.1-luciferase-m*Six2*–3′UTR-MUT, the miR-181b over-expression vector, pRL-SV40 were used in Dual-luciferase assays. The 3′UTR of murine *Six2*, including the predicted miR-181b target sites was cloned from C57BL/6 mouse genomic DNA by PCR with the forward primer: **5′-cttggtaccgagctc tcctagagctctgttcgcct-3′** and the reverse primer: **5′-tgctggatatctgca cgaacattcacatgagggcg-3′**. The PCR fragments were inserted into the *Eco*RI/*Bam*HI site(down-stream of fly luciferase gene) of the pCDNA3.1-luciferase vector to create pCDNA3.1-luciferase-m*Six2*–3′UTR-WT using the same method (ligation-independent cloning) as reported before [13]. As a negative control, pCDNA3.1-luciferase-m*Six2*–3′UTR-MUT was acquired by introducing eight mutations into the seed region of miR-181 family target sites of pCDNA3.1-luciferase-m*Six2*–3′UTR with the forward primer: 5′-**tccattttacgccctcatg GTTTGTAA cgtgcagatatccag-**3′ (mut) and the reverse primer: 5′-**tgtgctggatatctgcacg TTACAAAC catgagggcgtaaaa-**3′. The mutation method is largely the same as reported previously [14]. A schematic diagram of the luciferase reporter constructs is shown in Fig. 2A. The plasmid pcDNA3.1-luciferase was recombined with the insertion of firefly luciferase gene amplified from pGL3-Basic vector (Promega, Madison, WI, USA) into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) [15]. pRL-SV40 was purchased from Promega. pcDNA3.1(+)-*Six2* CDS, which is *Six2* over-expression vector, was constructed by inserting the DNA fragments containing CDS of *Six2* amplified from the cDNA of mK3 cells with the forward primer:5′-**cttggtaccgagctccaccatgtccatgctgcccacctt-3′** and reverse primer: **5′-tgctggatatctgcaggcagatcgtcagtcagtca-3′**)into pcDNA3.1(+) in the *Bam*HI/*ECO*RI sites. With the intention of using the fluorescence labeling, the fragments of pri-mir-181b were amplified from C57BL/6 mouse genomic DNA with the forward primer: 5′-**tttcaggtcccggataccatcatctactccatggc**-3′ and the reverse primer: 5′-**TTGCACCACCACCGG cctgaaagttcagacagcag**-3′. Each of those fragments were inserted into pdsAAV-CB-EGFP in BamHI sites to construct pdsAAV-CB-EGFP-miR-181b, which is the over-expression vector of miR-181b. All these recombined vector were sequenced.

2.3. Cell culture

HEK 293T cells and mK3 cells (an mouse clonal cell line who representing the uninduced differentiation stage of metanephric mesenchyme [16,17]) were cultured in DMEM (Gibico, Carls-bad, CA, USA), added 10% FBS (Gibico, Carlsbad, CA, USA) and penicillin (1000 units/ml) and streptomycin (1000 μg/ml) in 37 °C with 5% CO_2, 100% humidity.

2.4. Transfection

The vector pCDNA3.1-luciferase-m*Six2*-3′UTR-WT and pCDNA3.1-luciferase-m*Six2*-3′UTR-MUT were co-transfected with pdsAAV-CB-EGFP-miR-181b and pRL-SV40 with calcium phosphate cell transfection method. miR-181b RNA (mimic) (**AACAUUCAUUGCUGUCGGUGGGU**) were synthesized by GuangZhou RiboBio company in China and stored at −80 °C. The miR-181b mimic (50 nM) or scrambled miRNA control was transfected into mK3 cells via Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions.

2.5. Realtime-PCR

MiR-181b mimic (50 nM) or control mimic (50 nM) was transfected into mK3 cells. The total RNA was isolated from mK3 cells 48 h post-transfection using Trizol (Invitrogen, Carlsbad, CA, USA). The mRNA level of *Six2* was detected by SYBR-Green qRT-PCR kit (ComWin Biotech, China) with the sense primer: 5′-**GCCAAGGAAAGGGAGAACAGC**-3′ and anti-sense:primer: 5′-**GAGCAACA GAGCGGGACTGG**-3′ according to the manufacturer's instructions (Promega, Madison, WI, USA). The level of *Six2* mRNA expression was normalized to those of internal control (18 s). The relative gene expression was analyzed by comparative threshold cycle (CT) method ($2-\Delta\Delta CT$) [18].

2.6. Dual-luciferase assays

For luciferase assays, HEK293T cells were seeded onto 24-well plate (0.1 million each well) the day before transfection. 500 ng of pCDNA3.1-luciferase-m*Six2*-3′UTR-WT or pCDNA3.1-luciferase-m*Six2*-3′UTR-MUT and 500 ng of pdsAAV-CB-miR-181b-EGFP or pdsAAV-CB-EGFP were co-transfected into HEK293T cells together with pdsAAV-CB-miR-181b-EGFP. 10 ng of pRL-SV40 was included to serve as internal control. 48 h later, luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The activity of pdsAAV-CB-EGFP-miR-181b was defined as the ratio of firefly luciferase/renilla luciferase, normalized to control vector transfection. All the experiments were performed in triplicate.

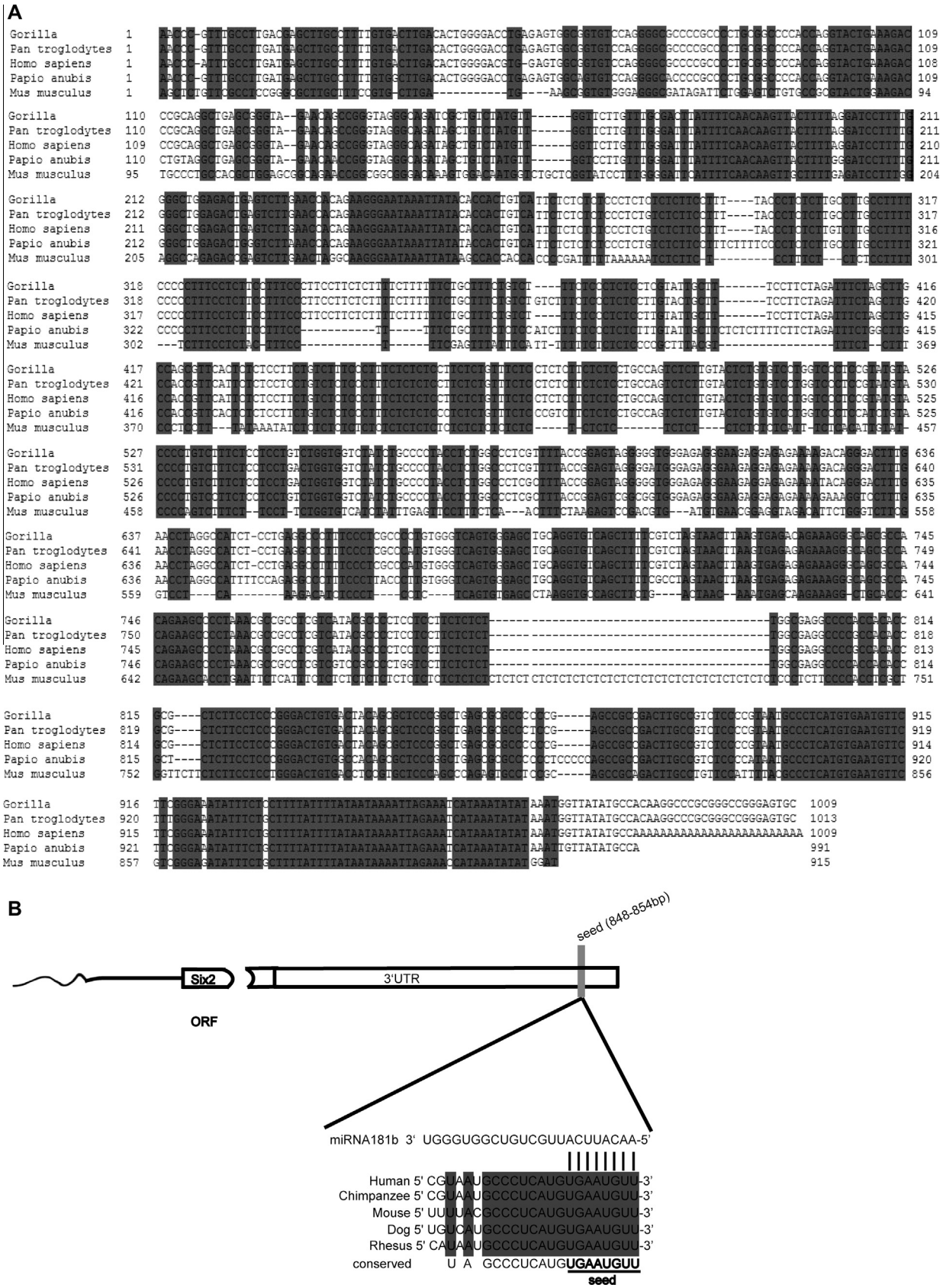

Fig. 1. Bioinformatic analysis of 3′UTR of mouse *Six2* gene and putative binding sites for miR-181b. Genebank was used to analyze the 3′UTR of *Six2*. (A) Several tracks for entire sequence of different 3′UTR of *Six2* across different mammalian. The figure shows that the entire 3′UTR of mouse *Six2* gene is about 915 bp in length and highly conserved in evolution. In the display tracks, conservation is shown in gray. (B) Sequence alignment of miR-181b predicted bind sites in the 3′UTR of *Six2* gene. Binding sites conserved in multiple species. Absolutely conserved nucleotides are shown in gray shadow.

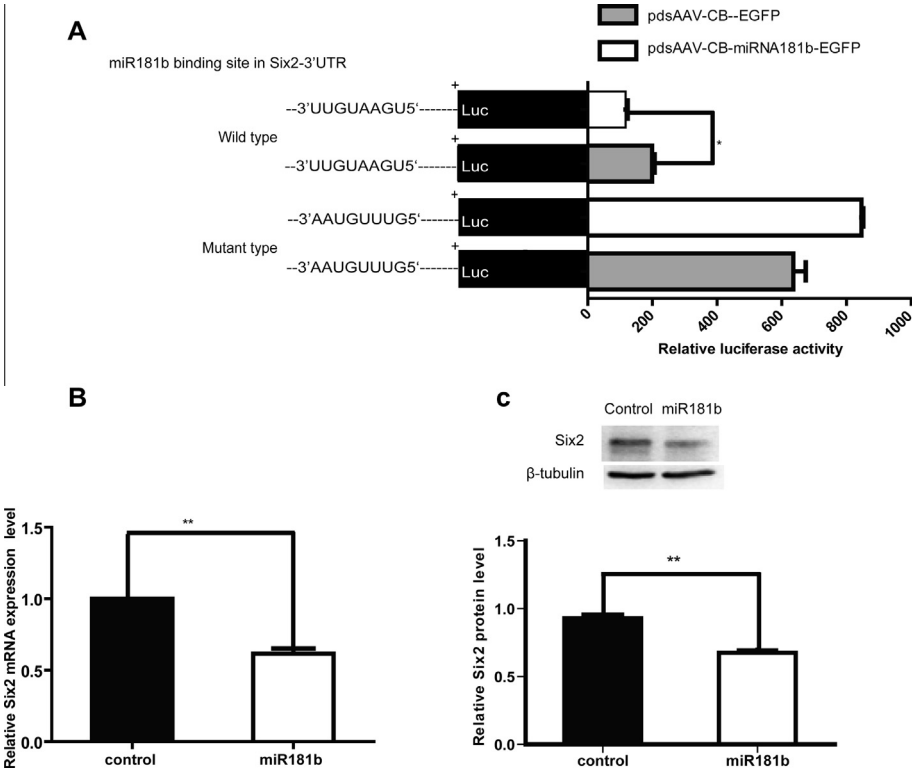

Fig. 2. miR-181b binds to the 3′UTR of *Six2* to suppress mRNA and protein expression level. (A) HEK 293Tcells were co-transfected with pGL-SV40 (renilla control), firefly luciferase reporter containing either wild-type (pcDNA3.1-LUC-*Six2*-3′UTR) or mutant type (pcDNA3.1-LUC-*Six2*-mut-3′UTR, obtained by site-directed mutagenesis of eight base pairs of the miR-181b seed-region in 3′UTR of *Six2*), and either the miRNA expression plasmid pdsAAV-CB-miR-181b-EGFP (indicated as miR-181b) or pdsAAV-CB-EGFP (indicated as control). Luciferase activity was assayed 48 h after transfection using dual luciferase reporter assay, normalized to Renilla control. *p* Values were calculated using Student *t* test. Values represents mean values ± SEM from triplicate experiments. The error bars represent SD. (B) Effect of miR-181b on *Six2* mRNA level. Real-time PCR was performed to analyzed the expression of *Six2* gene 48 h after transfection of either miR-181b or Control mimic (indicated as miR-181b or control on *x*-axis). $^{*}p < 0.05$, relative to the control. *p* Values were calculated using Student *t* test. Values represent mean values ± SEM from three independent experiments. The error bars represent SD. (C) Effect of miR-181b on *Six2* protein level. mK3 Cells were transfected with either miR-181b mimic (50 nm) or control mimic (indicated as either miR-181b or control), and Western-blot was used to show that expression of *Six2* gene were suppressed 48 h after transfection. Quantification of protein level of *Six2* in mK3 cells overexpressing miR-181b. Data are means ± SEM ($n = 3$); $p < 0.01$ miR versus negative control.

2.7. Western blotting

miR-181b mimic (50 nM) or control mimic (50 nM) was transfected into mK3 cells. The cells were washed three times with ice-cold PBS buffer and lysed with RIPA Lysis Buffer (Boster, China). This products were placed on ice for 30 min, and boiled with 5× SDS loading buffer 10 min, 100 °C to extracted proteins. The proteins was separated by 12% SDS–PAGE, electro-transferred to a PVDF membrane (Millipore, USA), and subsequently was blocked with 5% (w/v) fat-free milk in TBST for 1 h at room temperature. A rabbit polyclonal antibody against *Six2* (1:600; proteintech, China) was used as the primary antibody, and Mouse moloclonal anti-beat-tubulin (1:5000, Transgen, China) were used to recognize *Six2* and beta-tubulin, respectively and horseradish peroxidase(HRP)-conjugated goat anti-rabbit or mouse IgG was used as a secondary antibody. The last step of Western blotting antibody detection was performed via Western blot chemiluminescent HRP substrate reagent (Millipore, USA). The expression level of *Six2* protein was nomalized to those of internal control (β-tubulin).

2.8. 5-Ethynyl-2′-deoxyuridine (EdU) assays

mK3 cells were seeded onto 24-well plate (0.05 million each well). 24 h later mK3 cells were co-transfected with miR-181b mimic (50 nm) or control mimic and blank vector (pcDNA3.1(+)) or co-transfected with miR-181b mimic (50 nm) and *Six2* overexpression vector. 24 h after transfection, the cell proliferation of mK3 cells was determined in vitro via the EdU DNA Proliferation in Detection kit (RiboBio, China) based on manufacturer instructions.

2.9. Statistical analysis

All works were performed in triplicate, and the results are presented as the mean ± standard error of the mean (SEM). The Prism 4 software (GraphPad, San Diego, CA, USA) was applied to calculate the statistical results. Analysis was performed by Student's *t* test and repeated measures analyses of variance by one-way analysis of variance (ANOVA) with Tukey's post hoc comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Bioinformatic analysis of Six2 3′UTR

To determine the relationship of *Six2* and miRNAs, we originally analyzed the 3′UTR of *Six2* gene in present study. The whole 3′UTR of *Six2* that lies on chromosome 17 is 915 bp in length and conserved in evolution across mammal species such as Gorilla, *Pan troglodytes*, *Homo sapiens*, *Papio anubis*, Mouse (as illustrated in Fig. 1A). Afterwards,we endeavored to explore the potential miRNAs associated with *Six2*. Hence, we took advantage of miRwalk (http://www.microwalk.org), miRanda (http://www.microRNA.org), and TargetScan release 6.2 (http://www.targetscan.org). As

displayed in Table 1, there are 37 binding sites targeted by 50 putative miRNAs in the 3′UTR of murine *Six2*. Notably, among the 50 miRNAs, miR-181 family are the only miRNAs predicted by all the three websites above (seed site: 5′AACUUCA3′ target site: 5′UGAAUGUU3′). Compared with the 3′UTR of human, chimpanzee, mouse, dog, and rhesus from the TargetScan (http://www.targetscan.org), the alignments with miR-181b displayed in Fig. 1B revealed the high conservation of miR-181b among these species. These data indicated that the 3′UTR of *Six2* is highly conserved and miR-181b most likely target it.

3.2. MiR-181b binding site in the 3′UTR of Six2 is functional

To clarify whether the 3′UTR of *Six2* contains functional miR-181b targeting sequences, we constructed dual-luciferase reporter plasmids (pcDNA3.1(+)-luciferase-*Six2*-3′UTR, wild type; pcDNA3.1(+)-luciferase-*Six2*-3′UTR-mut, mutant type, as mentioned above) (Fig. 2A). With these plasmids and HEK293T cell line, Dual-luciferase reporter assays were performed. For comparison, the relative activity of miRNA was defined as the ratio of firefly/renilla. As Fig. 2A shows, luciferase reporter relative activity was decreased prominently below that of the negative control in HEK293T co-transfected with the miR-181b & the construct of pcDNA3.1-luciferase-*Six2*-3′UTR. Importantly, the suppression was blocked when miR-181b binding site in 3′UTR of *Six2* was mutated, confirming that miR-181b suppressed the expression of a luciferase reporter protein in HEK293T cell via direct interaction with the mouse 3′UTR of *Six2*. Taken together, these data was consistent with bioinformatic predictions and revealed that *Six2* might be targeted by miR-181b functionally. As report goes, both transcriptional repression and mRNA cleavage can be caused via miRNAs regulation [19]. To further verify the effects of miR-181b on the expression of *Six2* in mK3 cells, RT-real-time PCR and Western were performed. As expected, the mRNA level of *Six2* decreased clearly by miR-181b, compared to the control miRNA (negative control) (Fig. 2B). In addition, the protein level of *Six2* was also reduced by miR-181b as illustrated in Fig. 2C. These results suggested that the expression of *Six2* could be down-regulated by miR-181b both at mRNA and protein level.

3.3. Cell proliferation of metanephric mesenchymal can be suppressed by miR-181b and rescued by Six2 partially

To estimate whether miR-181b could affect cell proliferation via *Six2*, mK3 cells were transfected with miR-181b mimic & pcDNA3.1(+), control mimic & pcDNA3.1(+) or miR-181b mimic & pcDNA3.1(+)-*Six2* CDS followed by EdU assay. As exhibited in Fig. 3A and B, compared with control, the ratio of EdU positive cells to all cells in the mK3 cells transfected with miR-181b was reduced, representing the inhibition of cell proliferation. In contrast, the cells transfected with miR-181b & pcDNA3.1(+)-*Six2* displayed a partially-rescued phenotype. These findings demonstrated that the cell proliferation of mK3 can be inhibited by miR-181b, at least by targeting *Six2* in part.

4. Disscussion

The metanephric mesenchyme (MM) houses a class of *Six2* positive nephron progenitor cells which have the trait of self-renewal. It is crucial to balance self-renewal (proliferation) and differentiation (consumption) of nephron progenitors in order to generate the full complement of nephrons during development. The differing fates of the nephron progenitor cells, involve in a network of multiple transcription factors and signaling pathways, one of the most essential being *Six2* gene. In this paper, we focused on miRNA as it is an acknowledged regulator that can downregulate its target genes [19], which may contribute to the regulation of *Six2* in MM cells. Prior work has documented that miRNAs play a

Table 1
The potential miRNAs occupy the putative site on the 3′UTR of *Six2*.[a]

miRwalk			miRNA.org			TargetScan		
Potential miRNAs	Seed sequences	Located sites	Potential miRNAs	Seed sequences	Located sites	Potential miRNAs	Seed sequences	Located sites
miR-532-3p	CCUCCCACACC	46–56	**miR181a**	ACUUACA	848–854	**miR181a**	ACUUACA	848–854
miR-532-3p	CUCCCACACC	46–55	**miR181b**	ACUUACA	848–854	**miR181b**	ACUUACA	848–854
miR509-5p	ACUCCAGAAU	64–73	**miR181c**	ACUUACA	848–854	**miR181c**	ACUUACA	848–854
miR-92a-2	AGGUGGGGA	739–747	**miR181d**	ACUUACA	848–854	**miR181d**	ACUUACA	848–854
miR-489	AUGACACCA	477–485	miR590-3P	AUAAAAUU	887–894	miR23a	UACACU	845–851
miR-881	AGAGAGAUA	384–392	miR-340-5p	UUUAUA	881–886;375–381	miR23b	UACACU	845–851
miR-882	AGGAGAGAG	289–298	miR-543	GAAUGUU	849–855	miR204	UUCCCU	233–239
miR-590-3p	UAAUUUUAU	887–895	miR-491	UUCCCCAC	738–745	miR211	UUCCCU	233–239
miR-181a	AACAUUCA	848–855	miR-882	CUCUCUCC	727–734;342–349;290–297			
miR-188-3p	CUCCCACA	48–55	miR-185	UCUCUCC	728–734;343–349;291–297			
miR-295	ACUCAAAU	488–495	miR-342-3p	GUGUGAG	586–592			
miR-92a-2	GGUGGGGA	739–746	miR-425	GUGUCAU	479–485			
miR-326	CUCUGGGC	794–801	miR-489	UGGUGUCAU	477–485			
miR-330	CUCUGGGC	794–801	miR-539	AUUUCUC	441–448;661–667			
miR-340	UUAUAAAG	375–382	miR-653	UUUCAACA	175–182			
miR-181a-1	AACAUUCA	848–855	miR-150	UGGGAG	50–55			
miR-181b-1	AACAUUCA	848–855						
miR-181c	AACAUUCA	848–855						
miR-181b-2	AACAUUCA	848–855						
miR-671-3p	CCGGUUCU	117–124						
miR-665	CCAGGAGG	764–771						
miR-762	GGGCUGGG	790–797						
miR-674	CACUGAGA	581–588						
miR-694	UGAAAAUG	172–179						
miR-491	GUGGGGAA	738–745						
miR-181d	AACAUUCA	848–855						
miR-653	UGUUGAAA	175–182						

The bold values indicate the miRNAs predicted by the three used bioinformatics websites.
[a] According to the bioinformatics websites: miRwalk (http://www.microwalk.org), TargetScan release 6.2 (http://www.targetscan.org), miRanda (http://www.microRNA.org).

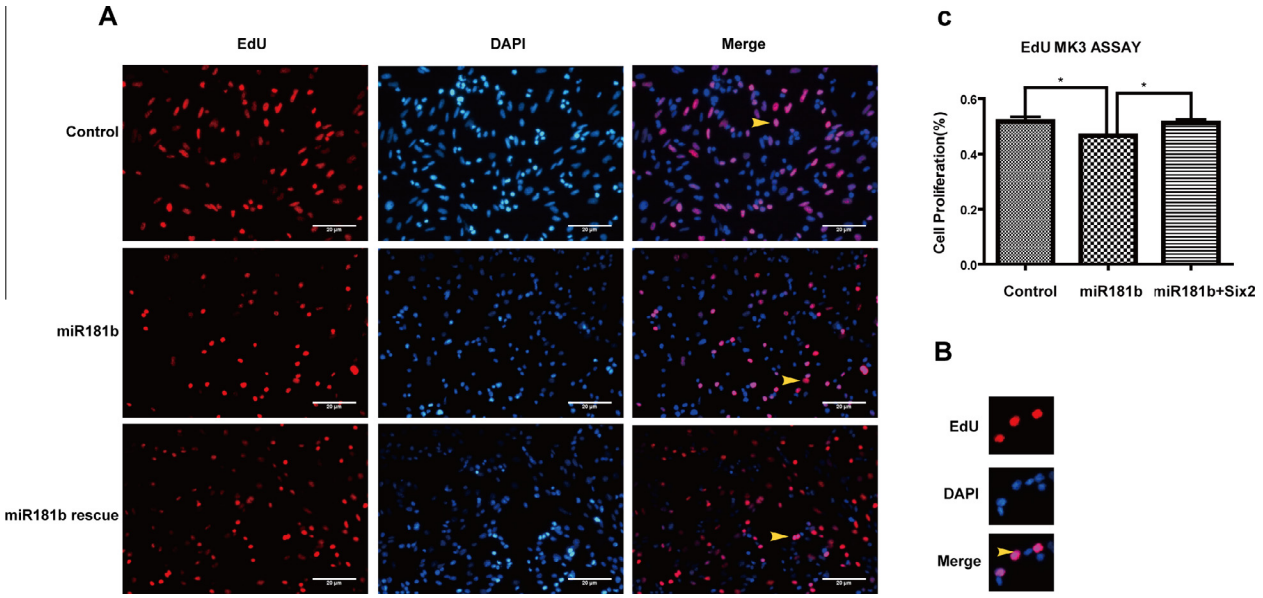

Fig. 3. Overexpression of miR-181b inhibits mK3 cells proliferation. (A) Proliferating mK3 cells were labeled with EdU (red) and cell nucleus were stained with DAPI (blue). The results represented by the pictures, accessed by fluorescent microscopy (200×). The arrowheads indicate cells undergoing proliferation and (B) EdU positive cells percentage (EdU%) were quantified. Results were presented as mean ± SEM ($n = 3$). $p < 0.05$ miR versus negative control.

vital role in the regulation of kidney development by negatively regulating the expression of target genes [20]. These researches, however, have not clarified whether miRNAs could target *Six2* and make the MM cells fate decisions. In this study, we initially utilized bio-informatic websites to analyze the 3′UTR of *Six2*. The results showed that the 3′UTR of murine *Six2* contains 915 nucleotides that provide 37 potential binding sites for 50 miRNAs regulation. MiR-181 family is the most commonly predicted miRNAs by all the three bioimformatic websites metioned above among so many putative miRNAs. This prediction indicates that miR-181 may have the tendency to target *Six2* and regulate the expression of *Six2* during renal development.

The miR-181 family includes four highly conserved mature miRNAs. The four mature products, miR-181a, miR-181b, miR-181c and miR-181d are found to be down-regulated in many different tumors, such as chronic lymphocytic leukemia, breast cancer, acute myeloid leukemia, glioblastoma, glioma, multiple myeloma, papillary thyroid carcinoma, hepatocellular carcinoma [21–28] and appear to act as tumor suppressor genes [28]. When it comes to miR-181b, the miRNA functions as tumor suppressor through inhibition of cell proliferation in glioma cells [21]. Thereupon, we wonder if miR-181b could target *Six2* and affect the fate (proliferation) of metanephric mesenchymal cells?

In this study, we first reported that the 3′UTR of *Six2* contains functional miR-181b targeting sequence(s). Our data suggested that over-expression of miR-181b repressed *Six2* 3′UTR reporter activity in the luciferase assays. Meanwhile, miR-181b down-regulated the expression of *Six2* at both mRNA and protein level, inhibiting the proliferation of mK3 cells. These findings are in good agreement with previous work [21], confirming that miR-181b can downregulate the expression of the target gene and further cause apparent effect on inhibition of proliferation. What' more, our results expand the prior work and point out specifically that miR-181b functionally targets *Six2* and inhibits cell proliferation of metanephric mesenchymal cells by down-regulating *Six2* expression and thus further helps us to understand the intricate molecular regulation during the formation of nephrons.

Although our results provide clear evidence for the vital role of miR-181b towards the central gene *Six2* in kidney mesenchyme, there are some limitations that are worth to be noted. The complete regulatory mechanisms of miR-181b during renal development still needs further investigation. Furthermore, future work should also focus on other target genes and functions of miR-181b in kidney development.

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