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RESEARCH ARTICLE

MicroRNA-10b regulates the renewal of spermatogonial stem cells through Kruppel-like factor 4

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MicroRNAs (miRs) are functionally important in spermatogenesis, which is the self-renewal or differentiation of spermatogonial stem cells (SSCs). Here, we report a novel role for miR-10b in regulating the self-renewal of mouse SSCs. We showed that miR-10b was highly expressed in mouse SSCs in vitro and enhanced SSC proliferation. Knockdown of miR-10b significantly increased the apoptosis of SSCs compared with controls. Kruppel-like factor 4 was found to be a target gene of miR-10b in the enhancement of SSC proliferation. These findings further our understanding of the self-renewal and differentiation of SSCs and provide a basis for the diagnosis, treatment, and prevention of male infertility.

KEYWORDS

KLF4, miR10b, self-renewal, spermatogonial stem cells

1 | INTRODUCTION

Tens of millions to hundreds of billions of mature sperms are produced daily in the testicular tube. Sperms differentiate from spermatogonial cells, and mature sperm production is dependent on the continuous activity of germline stem cells. The renewal of spermatogonial stem cells (SSCs) maintains the stem cell pool in testicular tubes, so is crucial for spermatogenesis.

SSCs reside on the basement membrane of the seminiferous tubule in the testis. They are almost completely surrounded by Sertoli cells, which form a microenvironment or niche. Growth factors and extracellular signals within this niche control whether SSCs undergo the self-renewal or differentiation process of spermatogenesis.[1,2]

Many studies have suggested that RNA-induced silencing complex components and a number of microRNAs (miRs) are associated with the mechanisms of spermatogenesis.[3,4] For example, deletion of the RNA-induced silencing complex component Dicer in germ cells or Sertoli cells led to the disruption of spermatogenesis and male mouse infertility.[5,6] Additionally, miR-383, miR-184, miR-21, miR-106a, and miR-224 were reported to regulate spermatogenesis,[7-11]playing an important role in the regulation of SSC growth and differentiation. Because around 1000 miRs are known to exist in mouse genomes, it is highly likely that other miRs will also regulate the fate of SSCs.[8]

In our previous study, we found that miR-10b was expressed in different age of mice testis.[12] As the testicular development, the expression of miR-10b presents the gradient descending. It may be involved in the process of spermatogenesis. There were little information on miR-10b effect on mouse SSCs and its mechanism. Here, we report a novel role for miR-10b in regulating the self-renewal of SSCs.

2 | MATERIALS AND METHODS

2.1 | Animals

CD-1 mice were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd. All animal procedures were approved by the Institutional

Jiang Li, Xiang Liu and Xiaopeng Hu contributed equally to this work.

Animal Care and Use Committee of Shanghai. The procedures were also performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

2.2 | Isolation and culture of mouse SSCs

Testes from 6-day-old mice were collected, washed in phosphate-buffered saline (PBS), and then cut into small pieces. A 2-step enzymatic isolation, purification, and culture were performed as described in previous studies.[13] SSCs were cultured in medium(minimum essential medium α medium, MEM-α) containing 10% foetal bovine serum (Life Technologies), 30 mg/ml pyruvate (Amresco), 2 mM L-glutamine (Amresco), 50 mM β-mercaptoethanol (Biotech), 6 mg/ml penicillin (Amresco), 1 mM nonessential amino acids (Invitrogen), 20 ng/ml mouse epidermal growth factor (PeproTech), 10 ng/ml human basic fibroblast growth factor (PeproTech), 10 ng/ml mouse glial cell line-derived neurotrophic factor (Peprotech), 10 ng/mL mouse leukaemia inhibitory factor (Santa Cruz Biotechnology), and 100 μg/ml transferrin (Sigma).

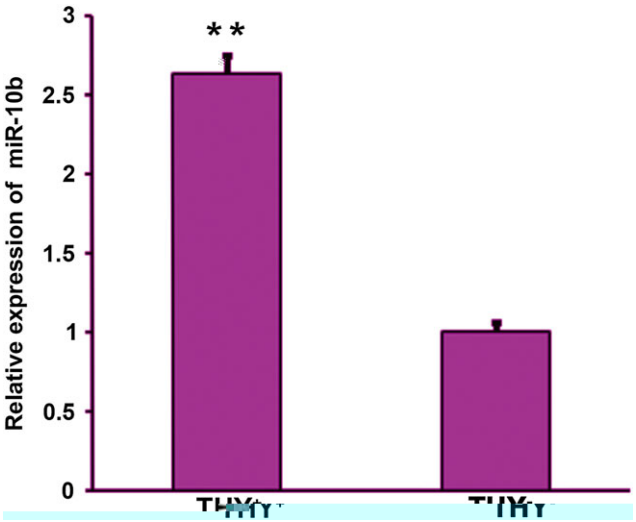

FIGURE 1 **miR-10b is highly expressed in SSCs in vitro**. Relative expression of miR-10b in Thy1$^+$-enriched cells was compared to that in Thy1$^-$-cells (mainly somatic cells of the testis). Bars indicate means ± SD from 3 independent experiments; **, $P < .01$

2.3 | Reverse transcription PCR and quantitative real-time PCR

Total RNA was extracted from SSCs and testes using TRIzol reagent according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using moloney murine leukaemia virus reverse transcriptase in a 20 μl volume containing reverse transcription primer, 25 μM oligo(dT) for mRNA, 10 μM random primers for U6 rRNA, and 2 μM miR-specific primers. PCR analysis was performed with Taq DNA polymerase. Primers are listed in supplementary Table S1.

Quantitative real-time (qRT)-PCR was carried out with SYBR® Premix Ex Taq (Takara, Shanghai, China) in a 20 μl reaction volume on a 7500 Real-Time PCR System using the following protocol: 95°C for 30s, followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds, then 95°C for 15 seconds, 60°C for 60 seconds, and

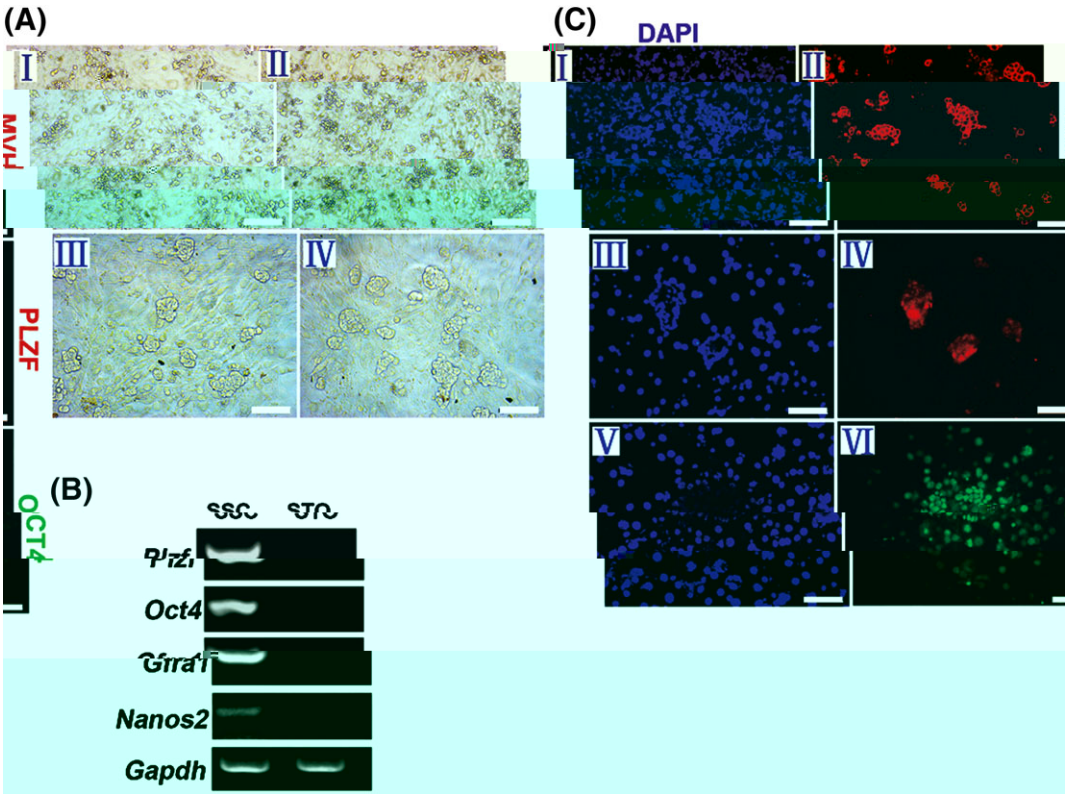

FIGURE 2 **Characteristics of cultured SSCs. A,** SSCs formed colonies after 4 serial passages. I, the first passage; II, the second passage; III, the third passage; IV, the fourth passage. Scale bar, 100 μm. **B,** Reverse transcription PCR detection of *Plzf*, *Oct4*, *Gfra1*, and *Nanos2* mRNA expression in SSCs. STO cells were negative controls. **C,** II, IV, and VI: immunofluorescence staining of MVH, OCT4, and PLZF expression in SSCs. I, III, V: DAPI staining of nuclei. Scale bar, 100 μm.

95°C for 15 seconds. Data analysis was performed using the $2^{-\Delta\Delta}$Ct method. qRT-PCR primers from Generay Biotech Co., Ltd. (Shanghai, China)are listed in supplementary Table S1.

2.4 | Knockdown of Kruppel-like factor 4 (*K 4*), miR-10b overexpression, and miR-10b-knockdown in SSCs

The *Klf4*-knockdown plasmid, miR-10b overexpression plasmid, and miR-10b-knockdown plasmid were designed and packaged in lentiviruses by HANBIO (Shanghai, China). The *Klf4* small interfering RNA sequences were: top strand,5′-GATCCGGTCATCAGTGTTAGCAAA TTCAAGAGATTTGCTAACACTG. ATGACCTTTTTTC-3′,and bottom strand, 5′-AATTGAAAAAAGGTCATCAGTGTTAGCAAATCTCTTGAA TTTGCTAACACTGATGACCG-3′. SSCs were infected with lentiviruses containing the miR-10b overexpression plasmid, miR-10b-knockdown plasmid, or control for 24 hours in 48-well plates. After infection, SSC cells positive for the different plasmids were screened by puromycin(3 ng/ul)selection.

2.5 | Apoptosis assay

Cell apoptosis was detected using an Annexin VApoptosis Detection Kit APC (Affymetrix eBioscience) according to the manufacturer's protocol. Cells were then sorted by flow cytometry.

2.6 | Immunofluorescence staining

Cells cultured in 48-well plates were fixed with 4% formaldehyde for 30 minutes at room temperature and washed three times with PBS, for 5 minutes each time. They were permeabilized by 0.5% Triton X-

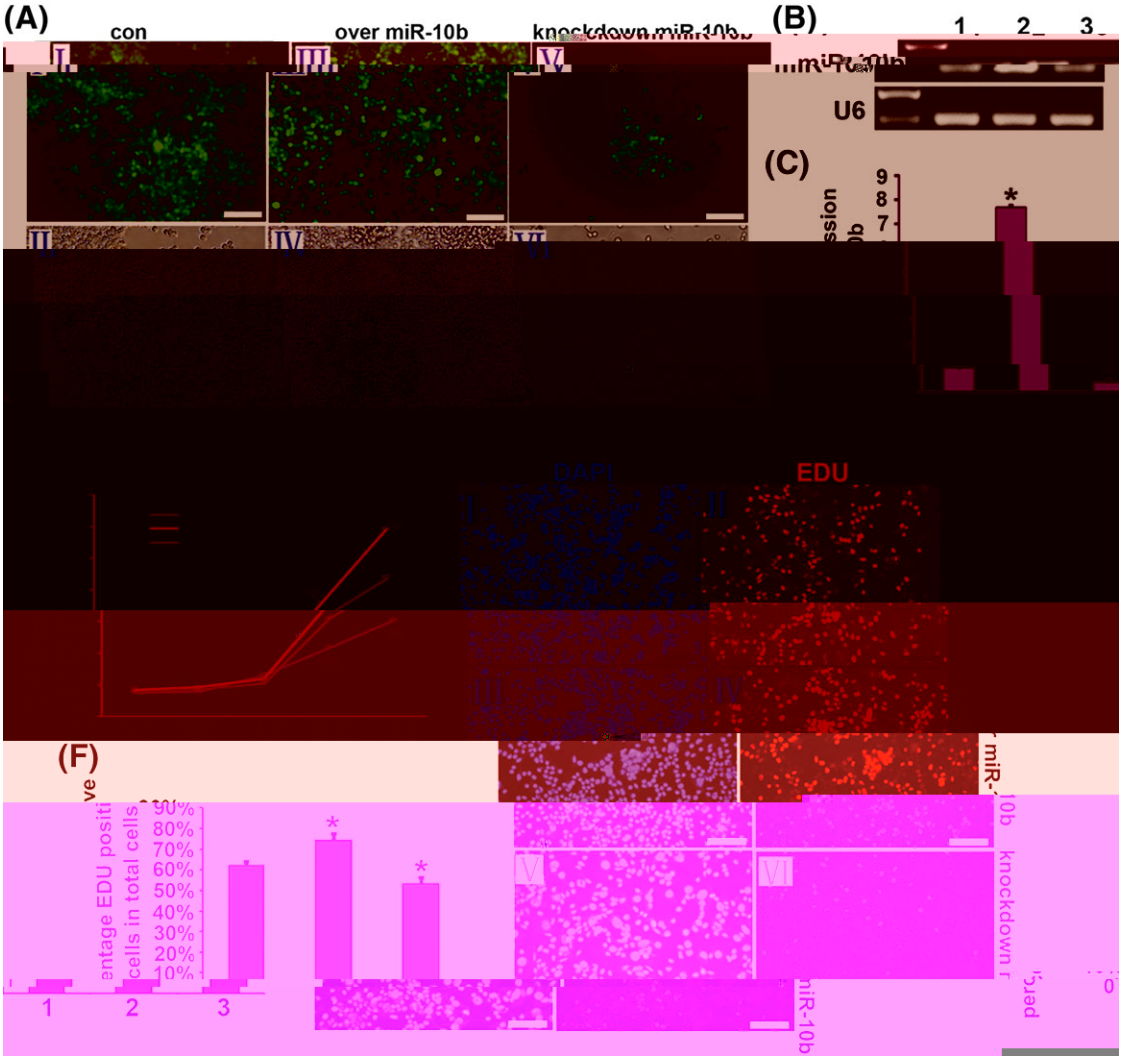

FIGURE 3 **miR-10b enhanced the proliferation of SSCs in vitro.A,** I,III, V: SSCs were infected with control GFP-lentivirus (I), overexpressing miR-10b-lentivirus (III), or knockdown miR-10b-lentivirus (V) for 24 hours.II, IV, VI: Light field. Scale bar, 100 μm. **B,** Reverse transcription PCR detection of miR-10b in SSCs after lentivirus infection. Lane 1, control; lane 2, overexpressing miR-10b; lane 3, miR-10b knockdown. **C,** relative expression of miR-10b in SSCs after lentivirus infection. 1, control; 2, overexpressing miR-10b; 3, miR-10b knockdown. Bars indicate means ± SD from 3 independent experiments; *, $P < .05$. **D,** CCK-8 assay of SSC proliferation. Measurements were taken hourly from 6 to 72 hours after infection with overexpressing miR-10b-lentivirus (red line), knockdown miR-10b-lentivirus (purple line), or control GFP-lentivirus (yellow line). The experiment was performed in triplicate. Bars indicate means ± SD; *, $P < .05$. **E,** EDU assay of SSC proliferation in control, overexpressing miR-10b, or miR-10b knockdown SSCs. I, III, V: DAPI staining of nuclei. II, IV, VI: EDU-positive cells. Scale bar, 100 μm. **F,** EDU assay data were statisticed. 1, control; 2, overexpressing miR-10b; 3, miR-10b knockdown. Bars indicate means ± SD from 3 independent experiments; *, $P < .05$

100 for 15 minutes at room temperature and blocked with 10% goat serum for 30 minutes at room temperature. Cells were then incubated overnight at 4°C with primary antibodies against rabbit-anti-MVH (1:200, Santa Cruz), rabbit-anti-OCT4 (1:180, Santa Cruz), and mouse-anti-PLZF (1:150, Santa Cruz). Appropriate FITC- or TRITC-conjugated secondary antibodies(1:200,Proteintech) were used following the manufacturer's manual.

2.7 | Western blotting

For western blot analysis, SSC proteins were extracted and denatured for separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Approximately 40 μg of protein lysate from each sample was loaded. Nylon membranes were incubated with primary antibodies (rabbit-anti-*KLF4*, 1:200, Santa Cruz; rabbit-anti-GAPDH,1:3000, Cell Signaling Technology) at 4°C overnight, then with horseradish peroxidase-conjugated secondary antibodies(1:5000,Thermo Scientific) at 37°C for 2 hours. The results were visualized with enhanced chemiluminescence.

2.8 | EdU proliferation assay

Five-ethynyl-2′-deoxyuridine (EdU, 50 μM; Cell Light EdU DNA Imaging Kit, Guangzhou RiboBio, Guangzhou, China) was added to SSC cultures for 2 hours at 37°C. The cells were then stained by discarding the EdU medium mixture, fixing the cells in 4% paraformaldehyde at room temperature for 30 minutes, and washing them with 2 mg/ml glycine for 5 minutes on a shaker. Triton X-100 (0.5%) was then added for 10 minutes, after which they were washed with PBS. Then, 1 × Apollo was added for 30 minutes, the cells were protected from light on a shaker, then washed 3 times with PBS containing 0.5% Triton X-100. Finally, 1 × Hoechst33342 was added for 10 minutes, during which the cells were again protected from light on a shaker at room temperature. Images were captured with a Leica fluorescence microscope.

2.9 | CCK8 proliferation assay

Spermatogonial stem cells (30 cells/μl) were plated in 96-well plates and cultured for 24 hours. After this, CVTK solution (Research Science, Shanghai, China) was added to each plate, and the absorbance at 490 nm was measured using a microplate reader.

3 | RESULTS

3.1 | miR-10b is highly expressed in SSCs

We previously showed that miR-10b was expressed in the testis of postnatal mice at different ages, and that its expression gradually reduced

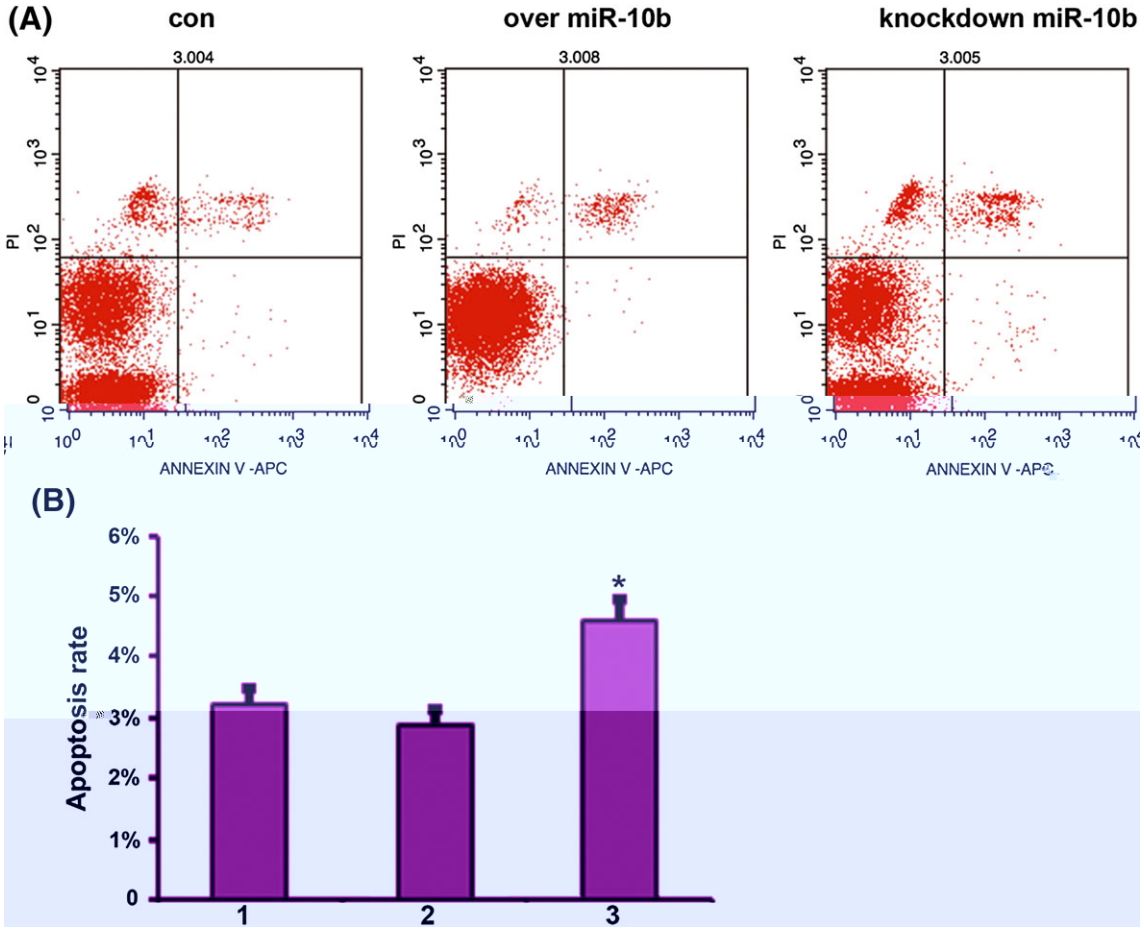

FIGURE 4 **Apoptosis assay. A,** Apoptosis was detected in control, overexpressing miR-10b, or miR-10b knockdown SSCs. **B,** Apoptosis assay data were statisticed. 1, control; 2, overexpressing miR-10b; 3, miR-10b knockdown. Bars indicate means ± SD from 3 independent experiments; *, $P < .05$

with increased age.[12] To study the role of miR-10b in SSC development, we examined the testes of mice at postnatal day 6 because testicular tubes of mice at this age mainly contain SSCs and somatic cells.[14] To confirm that miR-10b is expressed in SSCs or somatic cells, we isolated 2 types of cells: spermatagonial cells ($Thy1^+$) and somatic cells ($Thy1^-$) using anti-Thy1 immune magnetic beads.[13,15] qRT-PCR analysis showed that miR-10b was expressed 2.66-fold higher in $Thy1^+$ cells than in $Thy1^-$ cells (Figure 1). These results suggested that miR-10b is mainly expressed in SSCs.

3.2 | SSC isolation, purification, culture, and characteristics

As previously reported, SSCs derived from neonatal mice can be successfully cultured in vitro.[16] We cultured SSCs isolated from postnatal day 6 CD-1 mice on STO feeder cells, and observed that they formed colonies after 4 serial passages (Figure 2A). To characterize these cells, we determined the mRNA expression of *Plzf*, *Oct4*, *Gfra1*, and *Nanos2* using reverse transcription PCR, and found that the cells showed positive expression for all mRNAs (Figure 2B). Immunofluorescence analysis was also positive for PLZF, OCT4, and MVH proteins (Figure 2C).

3.3 | miR-10b enhanced the proliferation of SSCs in vitro

To determine the biological function of miR-10b in SSCs, we performed experiments to detect cell proliferation after the overexpression and knockdown of miR-10b in SSCs in vitro (Figure 3A, B, C). In CCK8 assays, the optical density values of miR-10b-overexpressing cells were significantly higher than control cells at 36 and 48 hours, while values of miR-10b-knockdown cells were significantly lower than controls (Figure 3D). EDU detection also showed that the overexpression of miR-10b significantly increased the number of proliferating cells, while miR-10b knockdown significantly reduced the number of EDU-positive SSCs (Figure 3E, F). These results suggested that miR-10b enhances the proliferation of SSCs in vitro.

3.4 | Knockdown of miR-10b increased the apoptosis of SSCs in vitro

Annexin V-APC, which binds to phosphatidylserine, was used to detect the early stages of apoptosis. Binding experiments with Annexin V-APC and propidium iodide indicated that there was no significant difference between the number of apoptotic cells in miR-10b-overexpressing cells (apoptosis rate, 2.91%) and control cells(apoptosis rate, 3.23%). However, miR-10b knockdown (apoptosis rate, 4.60%) significantly increased the number of apoptotic cells (Figure 4A,B). These results indicated that miR-10b knockdown enhances SSC apoptosis.

3.5 | *K 4* is a target gene of miR-10b in SSCs

Klf4 is a transcription factor involved in cell cycle regulation, apoptosis, and contact inhibition.[17,18] In the testis, *Klf4* is highly expressed in germ cells and Sertoli cells after meiosis,[19] indicating that it plays an

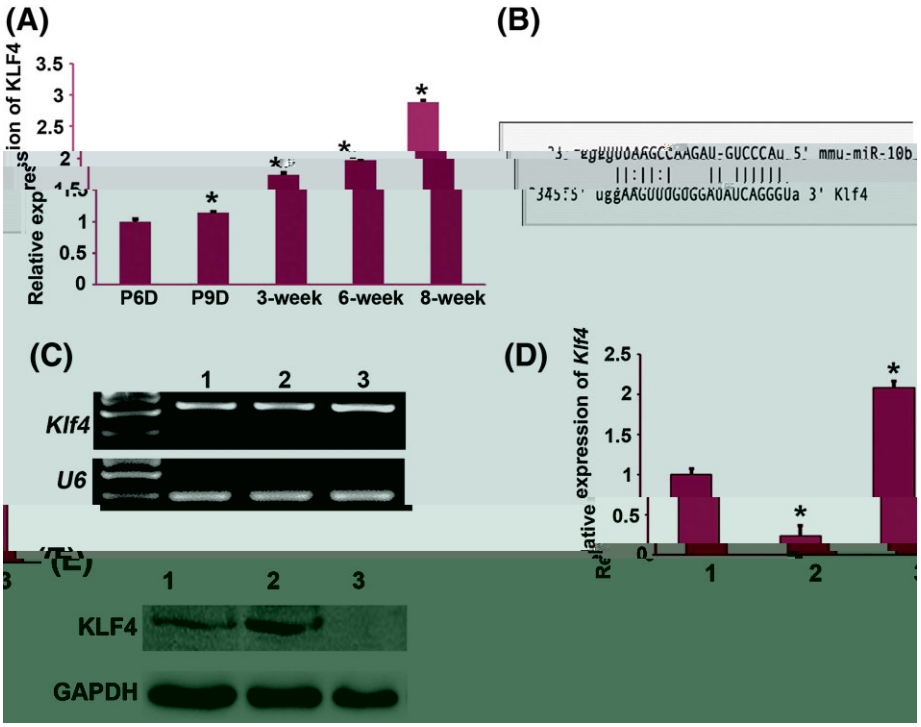

FIGURE 5 ***K 4* is a target gene of miR-10b in SSCs. A,** qRT-PCR showing the relative expression of *Klf4* during testis development (postnatal days 6, 9, and weeks 3, 6, 8). **B,** Bioinformatics software(miRBase) predicted the binding site of miR-10b on the *Klf4* 3′-UTR. **C,** Reverse transcription PCR detected *Klf4* expression in control, miR-10b overexpressing, and miR-10b knockdown SSCs. Lane 1, control; lane 2, overexpressing miR-10b; lane 3, miR-10b knockdown. **D,** qRT-PCR showing *Klf4* mRNA expression in control, miR-10b overexpressing, and miR-10b knockdown SSCs. 1, control; 2, overexpressing miR-10b; 3, miR-10b knockdown. Bars indicate means ± SD from 3 independent experiments; *, $P < .05$. **E,** Western blot detection of *Klf4* protein expression in control, miR-10b overexpressing, and miR-10b knockdown SSCs. Lane 1, control; lane 2, miR-10b knockdown; lane 3, overexpressing miR-10b

important role in cell differentiation in the testis. Using qRT-PCR, we showed that *Klf4* expression increases with increasing age of the mice (Figure 5A).

Bioinformatics software (miRBase) predicted *Klf4* to be a target gene of miR-10b, and suggested that miR-10b can bind to the *Klf4* mRNA 3′ untranslated region (UTR) (Figure 5B). Recently, miR-10b overexpression in KYSE140 and KYSE450 cells led to a reduction of endogenous Klf4 protein expression, whereas silencing of miR-10b in EC9706 cells up-regulated Klf4 protein levels.[20] Here, RT-PCR, qRT-PCR and western blot analysis showed that miR-10b overexpression significantly reduced the expression of Klf4, but that its expression increased in miR-10b-knockdown cells (Figure 5C–E). These results suggested that *Klf4* is a target gene of miR-10b in cultured SSCs.

3.6 | Knockdown of *K 4* enhanced the proliferation of SSCs in vitro

We next tested whether miR-10b enhanced the proliferation of SSCs through *Klf4*. A *Klf4*-shRNA-lentivirus was used to infect SSCs to knock down endogenous *Klf4* expression (Figure 6A). Protein expression of *Klf4* was shown to be significantly reduced after infection compared with control infections (Figure 6B). Furthermore, CCK8 assays revealed that optical density values of *Klf4*-knockdown cells were significantly higher than those of control cells at 24, 36, 48, and 72 hours (Figure 6 C). Additionally, *Klf4* knockdown increased the number of EDU-positive SSC cells in vitro (Figure 6D,E). These results suggested that *Klf4* knockdown enhances the proliferation of SSCs in vitro.

4 | DISCUSSION

miRs play important roles in cell proliferation, differentiation, and apoptosis.[21-23] For example, miR-27a targets the secreted frizzled-related protein 1 gene in human foetal osteoblasts to regulate proliferation, apoptosis, and differentiation.[24]

Spermatogenesis includes 3 main developmental stages, SSC self-renewal, SSC differentiation, and sperm cell deformation, and miR expression has been detected in each. Many miRs participate in regulating developmental stages of the mouse testis and controlling spermatogenesis.[3,4] For example, miR-20 and miR-106 maintain SSC self-

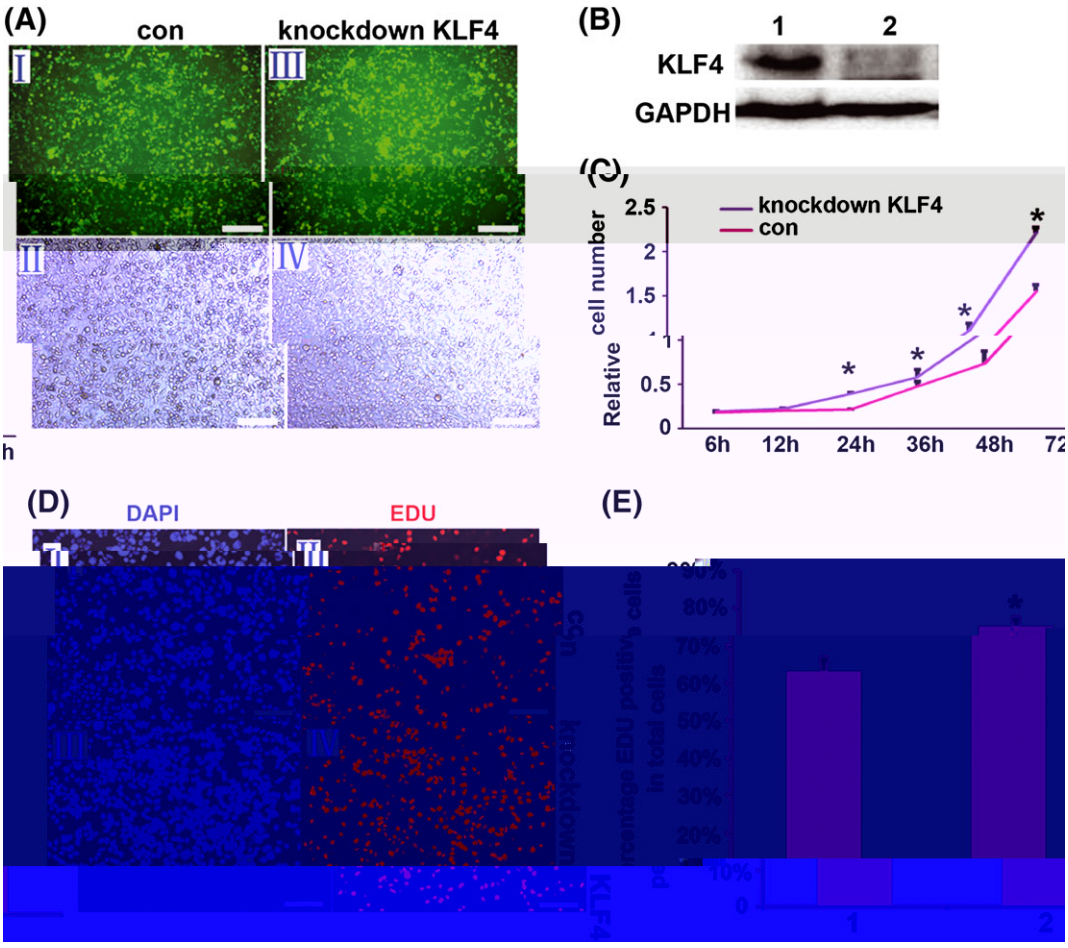

FIGURE 6 **Knockdown of *K 4* enhanced the proliferation of SSCs in vitro. A,** I, III: SSCs were infected with control GFP-lentivirus and knockdown Klf4-lentivirus for 24 hours. II, IV: Light field. **B,** After infection, *Klf4* expression was detected by western blot. Lane 1, control; lane 2, knockdown-Klf4. **C,** CCK-8 assay of SSC proliferation. Measurements were taken hourly from 6 to 72 hours after infection with knockdown Klf4-lentivirus (purple line) and control GFP-lentivirus (red line). The experiment was performed in triplicate. Bars indicate the means ± SD; *, $P < .05$. Scale bar, 100 μm. **D,** EDU assay of SSC proliferation in control and knockdown Klf4 SSCs. I, III: DAPI staining of nuclei. II, IV: EDU-positive cells. Scale bar, 100 μm. **E,** EDU assay data were statisticed. 1, control; 2, knockdown Klf4. Bars indicate means ± SD from 3 independent experiments; *, $P < .05$

renewal through the inhibition of signal transducer and activator of transcription 3 and cyclin-D1 genes, which are specifically expressed in mouse SSCs,[8] miR-184 inhibits SSC differentiation through the inhibition of the nuclear receptor corepressor 2 gene, enabling functional sperms to be expressed in the testes of mice after birth,[11] miR-204 regulates the proliferation of goat SSCs through the target gene sirtuin-1,[25] and miR-21 regulates the transcription factor ETV5 to maintain SSC self-renewal.[10] In the present study, we used qRT-PCR analysis to show that miR-10b was expressed in mouse testis at different ages. During testicular development, miR-10b expression was found to be reduced,[12] suggesting that it may be involved in spermatogenesis. We further showed that miR-10b was mainly expressed in $Thy1^+$ cells, and that it was highly expressed in the testis of mice at postnatal day 6. These results indicate that miR-10b plays a role in the regulation of SSC mitosis. CCK8 and EDU analyses revealed that miR-10b promotes SSC proliferation of SSCs, although the alternative interpretation of the results that miR-10b is involved in regulating SSC differentiation should be tested in a future study.

miRs usually bind to the 3′-UTR sequence of their mRNA targets, but noncanonical targeting of 5′-UTR and coding DNA sequence regions has also been reported.[26-29] In the present study, bioinformatics software(miRBase) predicted *Klf4* as a target gene of miR-10b, and miR-10b was suggested to bind to the *Klf4* mRNA 3′-UTR. qRT-PCR and western blotting provided further evidence that *Klf4* is a target gene of miR-10b in SSCs.

The eukaryotic transcription factor *Klf4* is involved in the regulation of cell proliferation, differentiation, and embryonic development. It is one of the most important induced pluripotent stem cell factors in somatic cell reprogramming, causing stagnation of the cell cycle at G1/S and G2/M phases, thus inhibiting cell proliferation and promoting apoptosis.[17,18] In the testicular tissue, especially after meiosis, *Klf4* is highly expressed in germ cells,[19] indicating its importance in spermatogenesis. Our finding that *Klf4* knockdown enhances the proliferation of SSCs implies that miR-10b binds the 3′ UTR sequence of *Klf4* to regulate its expression and that of SSC proliferation and apoptosis. These findings further our understanding of the self-renewal and differentiation of SSCs and provide a basis for the diagnosis, treatment, and prevention of male infertility.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

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