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Original Article

MicroR-1199-5p targeting SRD5A2 promotes the biological behavior and EMT of hypospadias cells

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Abstract

Hypospadias, an oft-occurring penis anomaly, ranks among neonatal's foremost birth defects. The SRD5A2 can affect male reproductive system development and is abnormally expressed in its epithelial cells. This study exploration aimed at understanding the role of SRD5A2 in the development of hypospadias from a molecular perspective. SRD5A2 levels in hypospadias primary cells were analyzed by Western blot, while targeted interaction with miR-1199-5p was ascertained by dual-luciferase gene reporter assay. In vitro biological experiments were used to confirm the biological function of SRD5A2 in hypospadias. SRD5A2 expression was significantly upregulated, and miR-1199-5p expression was significantly downregulated in hypospadias primary cells. Intervention of SRD5A2 expression can affect cell proliferation, migration, invasion, EMT, and the expression of cell cycle-related proteins. Additionally, we found that SRD5A2 is regulated by upstream miR-1199-5p and can enhance the effect of SRD5A2 on hypospadias cells. Conclusions Silencing SRD5A2 promotes cell proliferation, invasion, and migration blocks the cell cycle at the G1 phase, and simultaneously promotes EMT, cell cycle, and cell proliferation-related protein expression. The biological function of SRD5A2 in hypospadias cells is regulated by miR-1199-5p. SRD5A2 may be an effective therapeutic target for hypospadias.

Keywords: Hypospadias, SRD5A2, EMT, Cell cycle-related, miR-1199-5p.

1. Introduction

Hypospadias is a congenital penile malformation and one of the most common developmental defects of newborns, which involves the abnormal development of the penis and urethra. In recent years, the incidence of hypospadias has increased year by year. A large number of studies show that environmental factors and epigenetic factors play an important role in the etiology of hypospadias. The related factors that increase the risk of hypospadias include maternal hypertension, pre-eclampsia, premature delivery, multiple pregnancies, etc, However, the pathogenesis of hypospadias is still clarified, so it is crucial to explore the key molecular regulatory mechanism of hypospadias to reduce its incidence rate [1, 2]. The urethral suture is formed by the curling and fusion of the urethral plate. The epithelial cells on the urethral plate undergo apoptosis and epithelial-mesenchymal transition (EMT) transformation. Apoptosis and EMT are important processes in the embryogenesis of the urethra, and their activity contributes to the fusion of the urethral suture. Genes linked with an inability to properly fuse urethral folds could potentially contribute to hypospadias [3, 4].

SRD5A belongs to the group of reduced coenzyme II-dependent membrane-bound enzymes, which are mainly found in prostate epithelial cells and have the biological function of converting circulating testosterone into dihydrotestosterone [5-7]. A large number of studies have shown that SRD5A2 affects the development of the male reproductive system, and is up-regulated in diseases such as prostate hyperplasia and prostate cancer[7, 8], and is abnormally expressed in epithelial cells. Some studies have found that the expression of SRD5A2 is targeted by miR-1199-5p, which is located on chromosome 19p13.12 and is a regulatory miRNA of EMT. It can inhibit EMT and tumor cell invasion [5].

Chen [9] conducted whole-genome sequencing on tissue samples from hypospadias patients and found that the expression of SRD5A2 increased in severe hypospadias patients. However, how SRD5A2 affects the pathogenesis of hypospadias patients through regulatory mechanisms has not been clarified. At the same time, how the abnormal expression of SRD5A2 in hypospadias patients is regulated has not been clarified so far.

In this study, we revealed that SRD5A2 has a high expression trend in primary cells of hypospadias, and silencing the expression of SRD5A2 can promote primary cells of hypospadias proliferation, migration, invasion, and promote EMT and cell cycle protein expression levels. In addition, this study demonstrated that the effect of SRD5A2 on primary cells of hypospadias is targeted by miR-1199-

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5p. Therefore, we identified the effect of SRD5A2 on hypospadias and revealed its molecular mechanism.

2. Materials and methods

2.1. Primary cell separation and Cell culture

SD rats were provided by the Guangzhou Experimental Animal Center under SPF conditions. The rats were kept in a 12-hour light/dark cycle, under constant temperature and humidity. Non-pregnant adult female and male rats were paired at a ratio of 1:2 and checked for a vaginal plug on the following afternoon at 4 pm. The day of finding the vaginal plug was designated as gestation day 0 (GD 0). The successfully mated female rats were randomly divided into control and dibutyl phthalate (DBP) exposure groups. Daily weights and separate housing were used. DBP (99.5%, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in peanut oil. Pregnant rats received DBP (750 mg/kg/day) on GD 14-18. Controls received only peanut oil. Normal urethral tissue from control group pups and urethral tissue from pups with hypospadias in the DBP exposure group were collected. Primary cells were extracted from normal rat urethral epithelium and hypospadias rat urethral epithelium by PriCells Biopharmaceutical Co., Ltd. (Wuhan, China).

Primary cells of hypospadias were mixed with complete culture medium (fetal bovine serum with 1% cyanine/streptomycin in DMEM solution (Gibco, Rockville, MD, USA)) and transferred to a culture bottle for cultivation. All these cells were cultured at 37°C with 5% CO2.

2.2. Gene silencing and transduction

shRNAs targeting SRD5A2 and the scramble negative control (sh-NC) were purchased from Suzhou Gemma Genetics (Suzhou, China). PcDNA3.1RNAs targeting SRD5A2 and the scramble negative control (pcDNA3.1-NC) were purchased from Suzhou Gemma Genetics (Suzhou, China). 3.75 μl of diluted Lipofectamine 3000 reagent was added to 250 μl of diluted plasmid vector of each group and incubated for 15 min at room temperature. The viral solution of lentiviral pLKO.1- SRD5A2 shRNA and pLKO.1- SRD5A2 pcDNA-RNA expression vectors were added into primary cells of hypospadias in six-well plates according to the grouping for infection. Primary cells of hypospadias were infected with lentivirus. Infected cells were selected with puromycin (MedChem Express, Monmouth Junction, NJ, USA) (10 μg/mL) for 7-14 days after infection to generate stable primary cells of hypospadias.

2.3. qRT-PCR

TRIzol reagent (Vazyme, Nanjing, China) was utilized for total RNA extraction from cells. A UV spectrophotometer assessed the absorbance of the extracted sample, subsequently calculating its concentration. The cDNA was synthesized using HiScript lll 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). qRT-PCR conditions were as follows: 94°C for 2 minutes, 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes, for 40 cycles. The relative expression of the experimental results was analyzed using the $2^{-\Delta\Delta Ct}$ method compared to GAPDH expression. The primers used in this study were purchased from Sangon Biotech (Shanghai, China) and the primer sequences are shown in Table 1.

2.4. Western blot

The total protein was extracted, which was subsequently transferred membrane and blocked membrane. The membranes were added primary antibody and secondary antibody. The chemiluminescence reagents were mixed in equal volumes of liquid A and liquid B (NCM Biotech, Suzhou. China). The membranes were incubated for 5 min and then detected by JP-K6000 chemiluminescence imager (Jiapeng, Shanghai, China). Protein expression was analyzed using Image J software for optical density values.

2.5. Cell vitality assessment

The cell viability was evaluated by the CCK-8 assay. Initially, this study introduced 2000 cells/well to a 96-well plate. Post adhesion, these cells were kept sans serum-rich media for 12 h. Subsequently, they received fresh, complete medium with CCK-8 diluted 1:10, and were given a one-hour incubation at 37 degrees under 5% CO_2 conditions. Afterwards, this study gauged their OD value at 450 nm, employing our reliable RT-6000 lab instrument from Rayto (Norcross, GA, USA).

2.6. Migration and invasion detection by transwell

For transwell migration and invasion experiments, consider seeding primary cells of hypospadias in the upper chamber, either with or without matrigel (BD Biosciences, Franklin Lakes, NJ, USA), respectively. Following a 24-hour incubation period, gently remove non-migrated or invaded primary cells of hypospadias using a cotton

Table 1. Oligonucleotide sequences used for qRT-PCR.

Primers	Sequence (5'→3')	
SRD5A2	Forward	CACACAATTACGCGCTATGC
	Reverse	TGTGTTTTGCAGAGAAGAGAGC
miR-1199-5p	Forward	GACCCGCGCCGCGCGC
	Reverse	AGTGCAGGGTCCGAGGTATT
CDK2	Forward	CACAATTACGCGCTGATTGACC
	Reverse	TTAGATAACAAGCTCCGTCCG
Cyclin E	Forward	TGATGACGTGTGTCTGAACC
	Reverse	GAACAGGAAGAAAACCTGACC
PCNA	Forward	CACACAATTACGCGCTATGTTTG
	Reverse	GTGTGTTTATGTGTCGAAGCC
GAPDH	Forward	CACACAATTACGCGCTACCA
	Reverse	TATACATTGGGGGTAGGAACAC

swab, then fix the remaining cells on the bottom of the chamber with 4% polyformaldehyde. Apply a 0.2%~0.5% crystal violet stain and randomly capture images from 5 fields (×200 magnification) under an inverted microscope (Shanghai Optical Instrument Factory, Shanghai, China).

2.7. Cell cycle detection by flow cytometry

After an initial 48 h of transfection, our cells (a total of 1×10^7) were prepared gently for cell cycle stage identification. Gentle spinning (at 400 g for 5 minutes) and discarding the supernatant marked this first step towards cell preparation. Cells were then carefully resuspended in 300 µL of PBS before adding 700 µL absolute ethanol. This ethanol preservation process continued in a -20°C fridge for 24 h. Post freezing, gravity aided us again, the remaining solution was spun (at 700 g for 5 minutes), supernatant disposed of, and then PBS wash ensued. This was followed by cell suspension in 0.5 mL of PI/Rnase staining buffer for a brief 15-minute incubation at room temperature. Lastly, flow cytometry was employed to evaluate DNA content and reveal the proportion of cells in each cycle phase. Results were analyzed courtesy of Novo Express software for a clear presentation of cell cycle distribution.

2.9. Dual-luciferase reporter gene assay

The target miRNA of SRD5A2 was predicted using miRWalk (http://mirwalk.umm.uni-heidelberg.de). The 293T cells were seeded in 24-well plates before transfection. Cells firstly received miR let-7a mimics or NC mimics prior to co-transfection with pMIR-USP32-3'UTRw, pMIR-USP32-3'UTRm, or pMIR-REPORT plasmids for 48 h. They were then harvested and analyzed via Double-Luciferase Reporter Assay System (Promega, Madison, WI, USA), following provided guidelines.

2.10. Statistical analysis

In this study, GraphPad Prism 9 statistical software (La Jolla, CA, USA) was used for data analysis. Descriptive statistics for continuous variables are presented as mean ± standard deviation (x ± s). The comparisons between groups were conducted using a T-test and one-way ANOVA. A statistical significance level of $P < 0.05$ was used to determine if there were significant differences.

3. Results

3.1. MiR-1199-5p is low expressed and SRD5A2 is highly expressed in primary cells of hypospadias

To clarify the expression of miR-1199-5p and SRD5A2 in primary cells of hypospadias, this study cultured normal urethral epithelial cells and primary cells of hypospadias and detected the expression of SRD5A2 and miR-1199-5p used Western blot and qRT-PCR, respectively. The results showed that miR-1199-5p was lowly expressed in primary cells of hypospadias, while SRD5A2 was highly expressed in primary cells of hypospadias (Figure 1a-b).

3.2. Silencing SRD5A2 significantly promoted EMT progression and cell cycle-related protein expression, while overexpression of SRD5A2 inhibited EMT progression and cell cycle-related protein expression.

To investigate the effect of SRD5A2 on primary cells of hypospadias, overexpression and silencing expression vectors of SRD5A2 were constructed and transfected into primary cells of hypospadias. qRT-PCR was used to detect SRD5A2 and miR-1199-5p expression. The results showed that SRD5A2 silencing significantly boosted miR-1199-5p expression, while SRD5A2 overexpression rather suppressed it (Figure 2a-b). qRT-PCR and Western blot were used to detect the expression of cell cycle-related proteins. The results showed that the expression of cell cycle proteins (CDK2, cyclinE and PCNA) were up-regulated when SRD5A2 was silenced, and down-regulated when SRD5A2 was overexpressed (Figure 2c-d). Western blot was used to detect N-cadherin, E-cadherin and ZO-1 expression. The results showed that the expression of N-cadherin was up-regulated when SRD5A2 was silenced, and down-regulated when SRD5A2 was overexpressed. The expression of E-cadherin and ZO-1 were down-regulated when SRD5A2 was silenced, and up-regulated when SRD5A2 was overexpressed (Figure 2e). The results indicate that SRD5A2 negatively regulates miR-1199-5p.

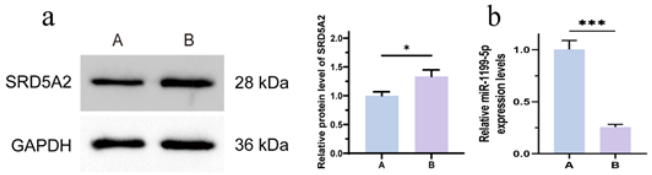

Fig. 1. The expression of miR-1199-5p and SRD5A2 in normal urethral epithelial cells and primary cells of hypospadias. a. Western blot was used to detect the protein expression of SRD5A2 in normal urethral epithelial cells and primary cells of hypospadias. b. qRT-PCR was used to detect the mRNA expression of miR-1199-5p in normal urethral epithelial cells and primary cells of hypospadias. *P<0.05, ***P<0.001. (A: normal urethral epithelial cells; B: primary cells of hypospadias).

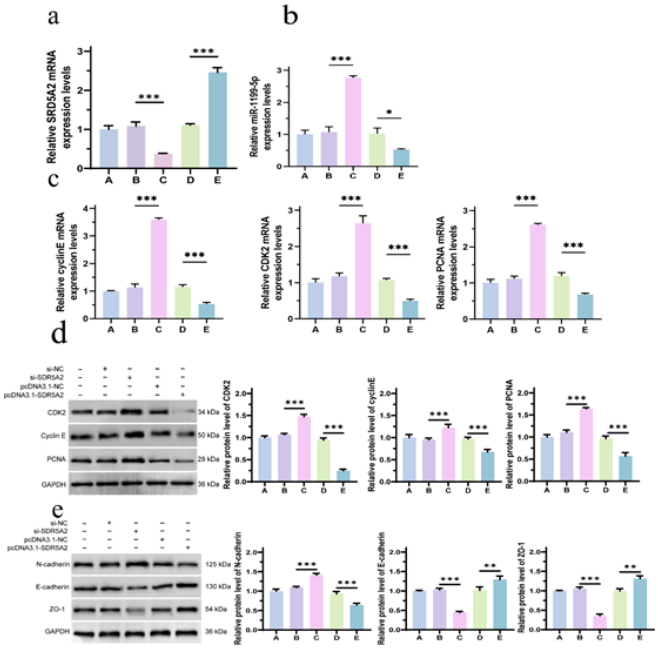

Fig. 2. Silencing or overexpression of SRD5A2 to detect cell cycle and EMT-related gene expression. a. qRT-PCR detection of SRD5A2. b. qRT-PCR detection of miR-1199-5p. c-d. qRT-PCR and Western blot cell cycle genes (CDK2, cyclinE and PCNA). e. Western blot detection of EMT (N-cadherin, E-cadherin as well as ZO-1). *P<0.05, **P<0.01, ***P<0.001. (A: Control; B: si-NC; C: si-SRD5A2; D: pcDNA3.1-NC; E: pcDNA3.1- SRD5A2).

Silencing SRD5A2 significantly promotes EMT process and the expression of cell cycle-related proteins, while overexpression of SRD5A2 inhibits EMT process and the expression of cell cycle-related proteins.

3.3. Silencing SRD5A2 promotes cell proliferation, migration, and invasion, and arrests the cell cycle at the G1 phase. Overexpression of SRD5A2 inhibits cell proliferation, migration, and invasion, and arrests the cell cycle at the G2/M and S phases.

To investigate the effect of SRD5A2 on primary cells of hypospadias, this study constructed SRD5A2 silencing expression or silencing expression vector transfected into primary cells of hypospadias (Figure 2a). CCK-8 determination of cell proliferation showed that silencing expression of SRD5A2 at 24h, 48h, 72h, and 96h significantly promoted cell proliferation (Figure 3a). Flow cytometry determination of transfected cell cycle at 24h showed that when SRD5A2 was silenced, the cell cycle was arrested in the G1 phase, and when SRD5A2 was overexpressed, the cell cycle was arrested in the S phase and G2/M phase (Figure 3b). Transwell migration and invasion and scratch experiments were used to measure transfected cell migration and invasion ability at 24 h.

This study indicated that silent expression of SRD5A2 production facilitated cell migration and invasion, while overexpression of SRD5A2 inhibited hampered it (Figure 3c-e).

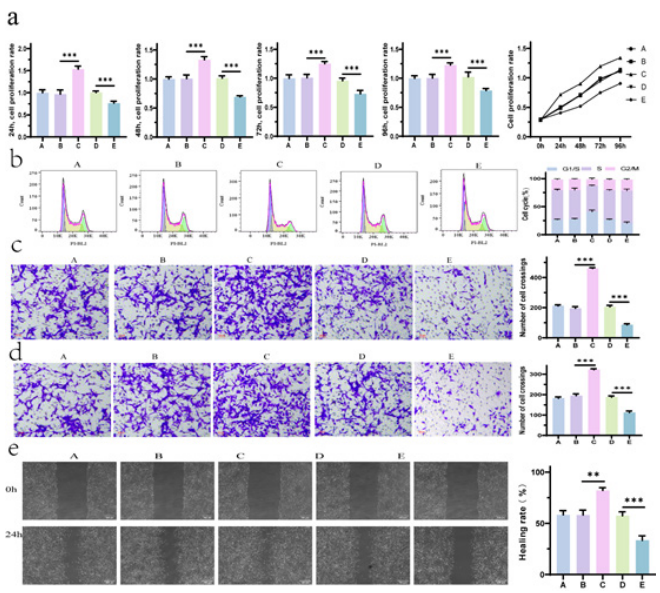

Fig. 3. Silencing or overexpression of SRD5A2 determines cell proliferation, migration, and invasion. a. CCK-8 was used to detect cell proliferation at 24h, 48h, 72h, and 96h. b. Flow cytometry was used to detect the cell cycle. c. Transwell migration assay was used to detect the migration ability of cells. d. Transwell invasion assay was used to detect the migration ability of cells. e. Scratch assay was used to determine the migration ability of cells. **P<0.01, ***P<0.001. (A: Control; B: si-NC; C: si-SRD5A2; D: pcDNA3.1-NC; E: pcDNA3.1-SRD5A2)

3.4. MiR-1199-5p targets SRD5A2 to promote cell proliferation, migration, and invasion and blocks G1 phase of cell cycle

MiRWalk (http://mirwalk.umm.uni-heidelberg.de) was used to predict upstream miRNAs of SRD5A2. MiR-1199-5p was identified as a potential upstream miRNA of SRD5A2. This study dual-luciferase reporter assay showed that miR-1199-5p mimic reduced the luciferase activity of WT-SRD5A2 3'-UTR in 293T cells, but had no significant effect on MUT-SRD5A2 3'-UTR (Figure 4a). Subsequently, we incorporated both miR-1199-5p overexpression and SRD5A2 silence expression vectors transfected into hypospadias primary cells (Figure 4b). CCK-8 assay was used to assess cell activity. Cell migration and invasion were assessed by Transwell migration and invasion assays. Cell cycle was assessed by flow cytometry. These experiments unveiled that SRD5A2 enhanced cell activity, promoted migration and invasion, blocked the cell cycle at G1, and was targeted and regulated by miR-1199-5p (Figure 4c-f).

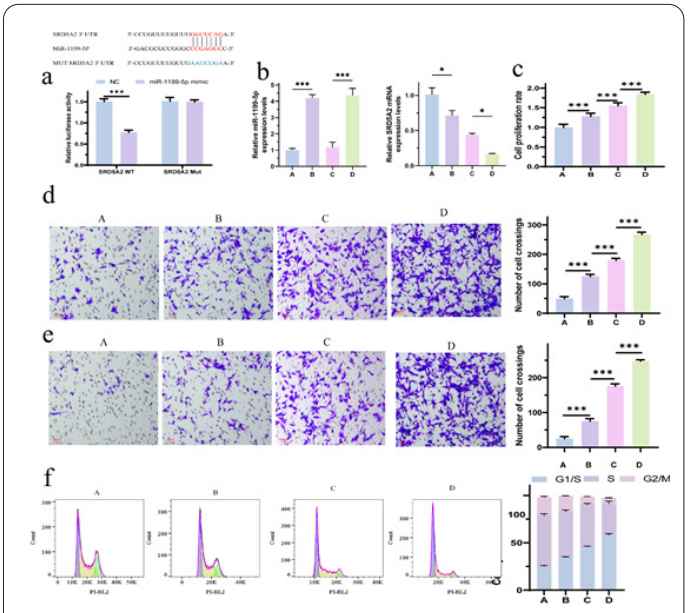

Fig. 4. MiR-1199-5p targets SRD5A2 to promote cell proliferation, migration, and invasion and blocks the cell cycle at the G1 phase. a. Dual-luciferase report detection of the targeting relationship between miR-1199-5p and SRD5A2. b. qRT-PCR was used to detect the transfection efficiency of miR-1199-5p and SRD5A2. c. CCK-8 detection of cell proliferation. d-e. Transwell assay for detecting cell migration ability and cell invasion ability. f. Flow cytometry detected the cell cycle. *P<0.05, ***P<0.001. (A: NC minics; B: miR-1199-5p minics; C: si-SRD5A2; D: miR-1199-5p minics+si-SRD5A2)

3.5. MiR-1199-5p targeting SRD5A2 promotes EMT transformation, and cell proliferation, and increases the expression of cell cycle-related proteins in primary cells of hypospadias

To investigate the effects of miR-1199-5p targeting SRD5A2 on EMT, cell cycle, and cell proliferation-related proteins in hypospadias primary cells. We constructed miR-1199-5p overexpression and SRD5A2 silencing expression vectors and transfected them into hypospadias primary cells (Figure 4b). Western blot was used to detect the expression of E-cadherin, N-cadherin, ZO-1, cell cycle-related proteins (CDK2, cyclinE, PCNA), and proliferation-related genes (PDGF, TGF-β, VEGF). The experimental results showed that SRD5A2 was targeted and regulated by miR-1199-5p to promote EMT (E-cadherin, N-cadherin, ZO-1) (Figure 5a), cell cycle (CDK2, cyclinE, PCNA), and cell proliferation (PDGF, TGF-β, VEGF) protein expression (Figure 5b). These results indicate that

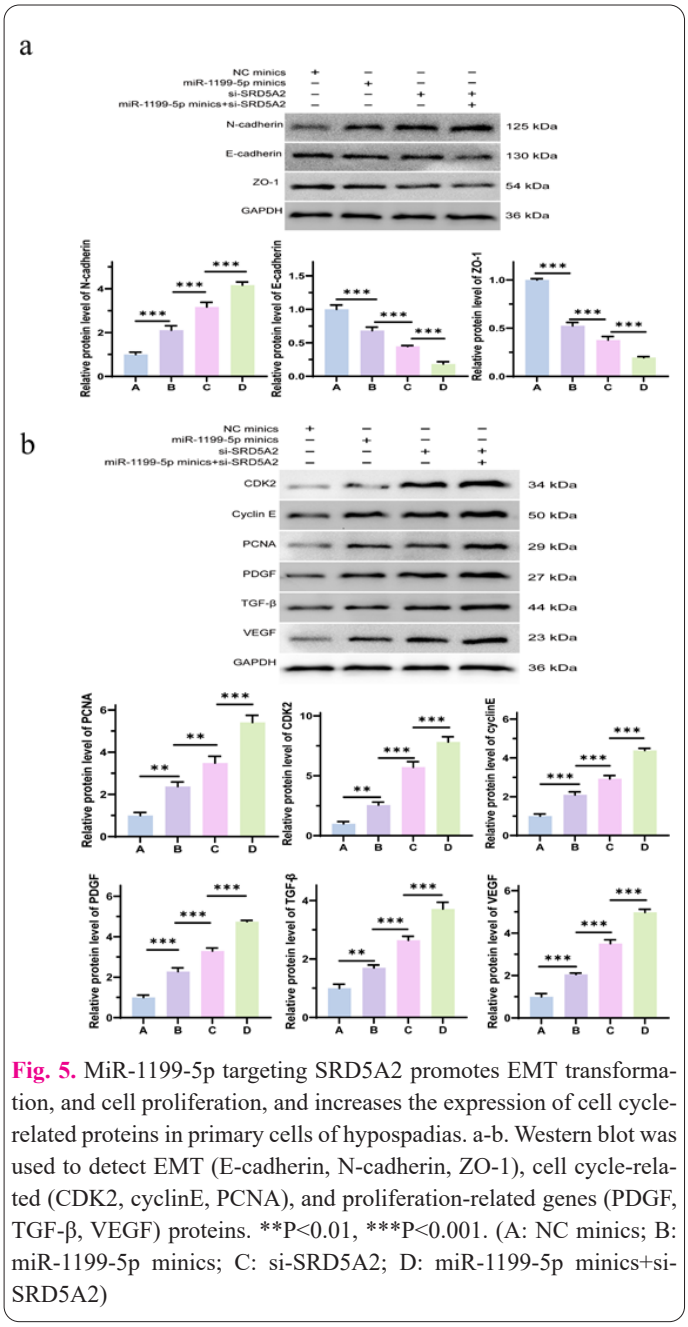

Fig. 5. MiR-1199-5p targeting SRD5A2 promotes EMT transformation, and cell proliferation, and increases the expression of cell cycle-related proteins in primary cells of hypospadias. a-b. Western blot was used to detect EMT (E-cadherin, N-cadherin, ZO-1), cell cycle-related (CDK2, cyclinE, PCNA), and proliferation-related genes (PDGF, TGF-β, VEGF) proteins. **P<0.01, ***P<0.001. (A: NC minics; B: miR-1199-5p minics; C: si-SRD5A2; D: miR-1199-5p minics+si-SRD5A2)

miR-1199-5p is the primary upstream miRNA influencing SRD5A2.

4. Discussion

Hypospadias, an early childhood birth defect, occurs when the forming of the urethral opening becomes distorted during weeks 8-15 of pregnancy. This disruption can lead to unfinished development of the urethra and foreskin, occasionally with a curved penis as well. Yet, the urethral opening typically resides on or near the head (proximal end) of the penis, which can vary in form [10-12]. The etiology of hypospadias involves genetic and environmental factors [1, 13], but the key factors leading to its occurrence have not yet been clarified. SRD5A is a membrane-bound enzyme dependent on NADPH [14]. SRD5A gene polymorphisms are a risk factor for hypospadias [15, 16]. Sequencing studies have found that SRD5A2 is abnormally overexpressed in hypospadias tissue samples [10]. SRD5A mutations lead to complete loss of 5α-RD2 enzyme activity, and 5α-RD2 enzyme deficiency is the most common cause of sexual development disorders [9, 17, 18]. At the same time, SRD5A2 is targeted and regulated by upstream miR-1199-5p [5].

The results of this study showed that SRD5A2 was highly expressed and miR-1199-5p was lowly expressed in primary cells of hypospadias in juvenile rats. EMT, a process where epithelial cells transform into mesenchymal ones, involves the loss of cellular polarity and contact. Such conversion bestows cells with migration and invasion abilities [19]. N-cadherin, E-cadherin as well as ZO-1 are important proteins involved in cell EMT [20, 21]. Reduced N-cadherin and increased E-cadherin as well as ZO-1 usually indicate inhibited EMT [22, 23]. The results of this study showed that silencing SRD5A2 promoted primary cells of hypospadias proliferation, migration and invasion, blocked the cell cycle at the G1 phase, promoted the expression of proteins cyclin E, CDK2, PCNA, and the expression of N-cadherin protein. It also inhibited the expression of ZO-1 and E-cadherin. When SRD5A2 was overexpressed, proliferation, migration and invasion were inhibited, N-cadherin protein expression was suppressed, and E-cadherin and ZO-1 expression was promoted. These results suggest that silencing SRD5A2 can promote proliferation, migration and invasion of primary cells of hypospadias in rats, and promote the EMT process, while overexpression of SRD5A2 can inhibit proliferation, migration and invasion of primary cells of hypospadias in rats, and inhibit the EMT process. In addition, this study also found that miR-1199-5p was highly expressed when SRD5A2 was silenced, and miR-1199-5p was lowly expressed when SRD5A2 was overexpressed.

MiRNA is non-coding RNA that can regulate the transcription or post-transcriptional stability of mRNA, and participate in a series of physiological activities of cells, including metabolism, cell differentiation, etc. [24-26]. MiRNAs play critical roles in guiding changes in your cell's transcripts and subsequently its form and functions during EMT [27-29]. This study aims to further clarify the biological effects of miR-1199-5p targeting SRD5A2 on the regulation of hypospadias cells and the molecular mechanism of EMT transformation. In this study, dual luciferase reporter gene was used to confirm that SRD5A2 is targeted and regulated by upstream miR-1199-5p. MiR-1199-5p has been shown to regulate EMT transformation [5, 27]. Meanwhile, the upregulation of miR-1199-5p is associated with the downregulation of SRD5A2 expression in benign prostatic hyperplasia [5]. The results of this study showed that miR-1199-5p increased when SRD5A2 was silenced in primary cells of hypospadias, and miR-1199-5p decreased when SRD5A2 was overexpressed. At the same time, miR-1199-5p overexpression and SRD5A2 silencing expression vectors were constructed and transfected into primary cells of hypospadias. The results showed that miR-1199-5p overexpression and SRD5A2 silencing expression can promote cell proliferation, migration and invasion, block the cell cycle at the G1 phase, and promote EMT transformation of primary cells of hypospadias. At the same time, it can increase the expression of cell cycle proteins (CDK2, cyclinE, PCNA) and cell proliferation proteins (PDGF, TGF-β, VEGF).

5. Conclusion

In summary, our research results reveal the influence of SRD5A2 on EMT and cell biological behavior of primary cells in hypospadias. Our study shows that the influence of SRD5A2 on primary cells in hypospadias is regulated by

miR-1199-5p. Understanding the role of miR-1199-5p targeting SRD5A2 in EMT of primary cells in hypospadias will help future research and potential treatment methods for hypospadias.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of Hunan Children's Hospital Animal Center.

Informed consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Yifu Chen and Jianjun Hu designed the study and performed the experiments, Liucheng Peng collected the data, Yaowang Zhao analyzed the data, Yifu Chen prepared the manuscript. All authors read and approved the final manuscript.

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