Submitted: 18 November, 2022 Accepted: 25 May, 2023 Published: 30 September, 2023 DOI:10.22514/jomh.2023.082

ORIGINAL RESEARCH

JOMH Journal of Men's Health

Testosterone promotes human foreskin fibroblast growth through miR-143-3p targeting IGFBP-3

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Abstract

Testosterone is an important male hormone, which could improve the maintenance and recovery of gonadal function in males as well as the repair of human hypospadias and cell fibrosis. Our study focused on investigating the regulatory effect of testosterone in human foreskin fibroblasts (HFF-1) and regulatory mechanisms involved. In this study, HFF-1 cells were treated with testosterone, and cell viability and migration were assessed by cell counting kit-8 (CCK8) and Transwell assays. The expression levels of androgen receptor (AR), miR-143-3p and insulin-like growth factor binding protein-3 (IGFBP-3) were measured by quantitative real-time PCR (qRT-PCR), Western blotting, and immunofluorescence. In addition, a potential binding site for miR-143-3p on IGFBP-3 was predicted and its direct binding was further confirmed by a dual luciferase reporter assay. These results showed that testosterone increased the viability and migration of HFF-1 cells. Testosterone could down-regulate miR-143-3p and up-regulate IGFBP-3 and AR. Overexpression of miR-143-3p hindered HFF-1 cell viability and negatively regulated IGFBP-3, whereas inhibition of IGFBP-3 impeded cell viability and migration. Furthermore, miR-143-3p was found to directly bind to IGFBP-3. Overexpression of IGFBP-3 countered the regulation of HFF-1 cells by miR-143-3p mimics. In conclusion, this study showed that testosterone promoted the proliferation and migration of HFF-1 cells and AR signaling, at least *via* the miR-143-3p/IGFBP-3 axis. This discovery presents a novel insight for testosterone application in male disorders like hypospadias.

Keywords

Testosterone; miR-143-3p; IGFBP-3; HFF-1 cells

1. Introduction

Hypospadias, a congenital malformation in male newborns, is the second most common with a prevalence of 1 in 200–300 in newborn males [1]. However, the etiology of most patients with hypospadias remains unclear. Hypospadias is thought to be a clinical manifestation of the interaction of hormonal and genetic factors during the development of the external genitalia [2]. Recent epigenetic investigations have identified an imbalance in human foreskin fibroblasts (HFF) as an important aspect of the disease etiology [3]. Previous studies have reported that low doses of bisphenol A can regulate the pathological development of hypospadias and that the progression might be related to dysregulated gene expression in HFF [4]. Therefore, targeting the growth of foreskin fibroblasts may provide potential concepts for disease treatment.

The growth and development of male external genitalia are primarily regulated by androgens. Testosterone, an endogenous androgen, exerts potent androgenic effects and other biological activities. Besides, testosterone is a widely prescribed drug. Several studies have found that testosterone plays a crucial role in male gonadal dysfunction, cardiovascular disease, and type 2 diabetes [5–7]. Research has also demonstrated that testosterone can result in abnormal proliferation of prostatic epithelial and stromal tissues [8, 9]. Additionally, previous evidence highlights the efficacy of testosterone in the repair of human hypospadias and in treating other male diseases [10]. A recent study showed that the local application of testosterone to human foreskin tissue has been found to increase the density of blood vessels and reduce fibrosis [11]. Nevertheless, there is a paucity of studies investigating the effect of testosterone on HFF growth in order to mediate the development of hypospadias.

MicroRNAs (miRNAs) consist of 19–23 nucleotides and are a form of small non-coding RNAs. They help regulate disease progression by inhibiting the expression of target mRNAs by preventing their translation or promoting their degradation [12]. Furthermore, miRNAs also have been demonstrated to be participated in various cellular processes that control disease progression [13]. Testosterone has been found to target miRNAs to influence fat deposition and carcinogenesis in breast tissue [14, 15]. However, the role of testosterone in regulating miRNA levels and its ability to impact hypospadias remains unknown. Previous studies have demonstrated that

miR-143-3p regulates specific target genes to control the onset and progression of various diseases, including pneumonia [16], rheumatoid arthritis [17], and tumors [18, 19]. In addition, miR-143-3p can regulate prostate cell differentiation, proliferation, and apoptosis [20]. A study previously reported the translocation of miR-143-3p in early passaged and senescent HFFs by utilizing a miRNA microarray dataset and verified that miR-143-3p hindered early HFF proliferation and induced cell cycle arrest [21]. Therefore, we hypothesize that miR-143-3p is a potential target of testosterone.

The insulin-like growth factor binding protein (IGFBP) family consists of peptides that share similar structures, each approximately 260 amino acids long [22]. These peptides display different binding sites for miRNAs and are crucial in cell apoptosis and angiogenesis [23, 24]. In addition, IGFBP can combine with IGF to influence cell proliferation, migration, differentiation, angiogenesis and apoptosis [25]. IGFBP-3 is a polypeptide, and the function of IGFBP-3 largely depends on the cellular environment. IGFBP-3 plays a vital role in circulation due to its high affinity with IGF [26, 27]. According to research, IGFBP-3 regulates smooth muscle cell proliferation and apoptosis in the corpus cavernosum and also modulates oxidative stress and fibrosis [28]. IGFBP-3 is involved in regulating the growth and migration of castration-resistant prostate cancer cells [29]. Additionally, previous study showed that testosterone can stimulate the secretion of IGFBP-3 in HFF [30], although the exact regulatory mechanism is yet to be elucidated.

The purpose of this study was to examine the impact of testosterone on HFF-1 cell growth and proliferation and identify the regulatory mechanism involving miR-143-3p. Our findings offer novel evidence and understanding for the potential use of testosterone in hypospadias repair or other urinary system diseases, as well as fibrosis-related diseases.

2. Materials and methods

2.1 Cell acquisition and culture

Human foreskin fibroblasts (HFF-1) cells (CBP60935) were sourced from COBIOER (Nanjing, China) and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were cultivated under an incubation of 37 °C and 5% CO_2, and utilized for experimentation after 3–4 days of growth. Cells in the logarithmic growth period were grown until they were adherent (24 h) and were ready for subsequent treatments in six-well plates (2×10^5 cells/well).

To investigate the role of testosterone, HFF-1 cells were grouped as follows: Normal group consisting of cells in their usual state, and the Testosterone group consisting of cells treated with 10 nM testosterone for 24 h after growing adherently [30]. Testosterone (58-22-0) was ordered from Sigma-Aldrich (St. Louis, MO, USA) to explore the regulatory effect of testosterone in HFF-1 cells.

To study the impact of miR-143-3p effect, HFF-1 cells were grouped as follows: Normal control (NC) mimics group consisting of cells transfected with NC mimics for 48 h after growing adherently, the miR-143-3p mimics group consisting of cells transfected with miR-143-3p mimics for 48 h after growing adherently. Afterwards, both groups were exposed to 10 nM testosterone for 24 h.

To evaluate the function of IGFBP-3, HFF-1 cells were divided into four groups: the si-NC group consisting of cells transfected with si-NC for 48 h after growing adherently, and the si-IGFBP3 group consisting of cells transfected with si-IGFBP3 for 48 h after growing adherently. Afterwards, both groups were exposed to 10 nM testosterone for 24 h.

To determine whether miR-143-3p functions through IGFBP-3, HFF-1 cells were divided into four groups: the NC mimics group consisting of cells transfected with NC mimics for 48 h after growing adherently, the miR-143-3p mimics group consisting of cells transfected with miR-143-3p mimics for 48 h after growing adherently, the miR-143-3p mimics + oe-NC group consisting of cells co-transfected with miR-143-3p mimics and oe-NC for 48 h after growing adherently, and the miR-143-3p mimics + oe-IGFBP3 group consisting of cells co-transfected with miR-143-3p mimics and oe-IGFBP3 for 48 h after growing adherently. Afterwards, all groups were exposed to 10 nM testosterone for 24 h.

2.2 Cell transfection

Plasmids si-NC, si-IGFBP3, oe-NC and oe-IGFBP3, along with lentiviruses (miR-143-3p mimics and NC mimics) were synthesized by GenePharma (Shanghai, China). The transfection of plasmids and/or lentiviruses into HFF-1 cells was carried out using Lipofectamine 2000 reagent (11668019, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and the cells were incubated for 48 h. Plasmids oe-NC and oe-IGFBP3 (HG-HO000598) were acquired from HonorGene (Changsha, China). The sequence of IGFBP-3 was obtained from NCBI, and primers were designed to isolate the target gene. The target fragment was then ligated into the pcDNA3.1(+) vector.

2.3 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by Trizol from HFF-1 cells, and the concentration of RNA was measured by a spectrophotometer. Reverse transcription was then carried out with a reverse transcription kit (CW2569, CWBIO, Taizhou, China) following the manuals. Subsequently, we performed qRT-PCR with UltraSYBR Mixture (CW2601, Taizhou, CWBIO, China) using cDNA as the template and the primer sequences given in Table 1. The qRT-PCR was performed on PIKOREAL96 system from Thermo Fisher Scientific (Waltham, MA, USA). Relative levels were calculated and analyzed by the $2^{-\Delta\Delta CT}$ method with normalizing *β-actin* and *U6* [31].

2.4 Western blotting

Western blot analysis of HFF-1 cells was performed as described previously [32]. Cells were washed with pre-cooled phosphate buffer saline (PBS) and then mixed with radio immunoprecipitation assay (RIPA) lysis solution (AWB0136b, Abiowell, Changsha, China). The samples were then collected and crushed by ultrasound after scraping the cell suspension with a cell scraper. After lysing for 10 min, the supernatant

TABLE 1. Primer sequences.

Name	Sequences
β-actin	Forward ACCCTGAAGTACCCCATCGAG Reverse AGCACAGCCTGGATAGCAAC
U6	Forward CTCGCTTCGGCAGCACA Reverse AACGCTTCACGAATTTGCGT
IGFBP-3	Forward AGTAATGGCACAATTCTTCGGAT Reverse CTATGTGCTCCCAGTGTCC
miR-143-3p	Forward TGAGATGAAGCACTGTAGCTC Reverse GCTGTCAACGATACGCTACGTA

IGFBP: insulin-like growth factor binding protein.

was collected into 1.5 mL tubes by centrifugation. Protein concentrations were then determined with a bicinchoninic acid (BCA) protein quantitative kit (AWB0104, Abiowell, Changsha, China), and 5× loading buffer was replenished to each sample before boiling them in water for 5 min. Next, protein samples were separated by electrophoresis and transferred to nitrocellulose (NC) membranes with a constant current through a DYCZ-40D mini transfer electrophoresis apparatus (LIUYI, Beijing, China). Next, the membranes were incubated in 5% skimmed milk powder (Yancheng Saibao, Yancheng, China) at normal temperature for 1 h and sealed at 4 ℃ overnight. Then, we added primary antibodies, including IGFBP-3 (10189-2-AP, 1:500, Proteintech, Chicago, IL, USA), androgen receptor (AR, 22089-1-AP, 1:5000, Chicago, IL, Proteintech, USA), and *β-actin* (66009-1-Ig, 1:5000, Chicago, IL, Proteintech, USA). The following day, secondary antibodies, including HRP goat anti-mouse IgG (SA00001-1, 1:5000, Chicago, IL, Proteintech, USA), HRP goat anti-rabbit IgG (SA00001-2, 1:6000, Chicago, IL, Proteintech, USA) were added to the membranes after three washes with PBST (PBS containing 0.05% Tween 20) and incubated at room temperature for 1.5 h. The electrochemiluminescence (ECL) was used for development, and the images were acquired with a gel imaging system. Lastly, protein band densities were quantified by Quantity one 4.6 software (Bio-Rad, Hercules, CA, USA) and presented as histograms.

2.5 Measure of cell viability by cell counting kit-8 (CCK8) assay

HFF-1 cell viability evaluated by CCK8 assays as previously reported [33]. In brief, HFF-1 cells were incubated in a 96-well plate (0030730119, Eppendorf, Germany) with 5×10^3 cells per well at a volume of 100 μL (5 replicate wells for each group). The cells were incubated in the dark at 37 ℃ for 24 h. After 24 h of incubation, 10 μL of CCK8 reagent (NU679, Dojindo, Kumamoto Ken, Japan) was added to each well, and the cells were further incubated at 37 ℃ with 5% CO_2 for 4 h. The absorbance of each sample was measured using a Bio-Tek microplate analyzer (MB-530, Heales, Shenzhen, China). The growth inhibition rate was calculated based on the mean value of the absorbance readings.

2.6 Immunofluorescence (IF)

AR expression was determined by IF as described previously [34]. To prepare the histological sections, they were initially baked at 60 ℃ for 12 h and then cleared in xylene for 20 min. Subsequently, they were subjected to a series of ethanol, including 75%, 85%, 95% and 100% concentrations (5 min at each grade), followed by a pure water wash. Sections were immersed into ethylene diamine tetraacetic acid (EDTA) buffer and exposed to continuous microwave heating for 24 min. After cooling, the cells were rinsed with 0.01 M PBS (Abiowell, Changsha, China), followed by immersion in a sodium borohydride solution. Cells were soaked in 75% ethanol solution for 1 min, after which Sudan Black dye was applied. The sections were sealed in 5% bovine serum albumin (BSA) for 1 h before being incubated with AR antibody (Ab133273, 1:50, Abcam, Cambs, UK) at 4 ℃ overnight. The following morning, sections were incubated with conjugated Affinipure Goat Anti-Rabbit IgG (H+L) secondary antibody (SA00013-2, proteintech, Chicago, IL, USA) at 37 ℃ for 60–90 min. DAPI (4,6-diamidine-2-phenylindole dihydrochloride) was applied for staining nuclear at 37 ℃. Finally, the sections were sealed with glycerol buffer before being observed under a fluorescence microscope.

2.7 Transwell assays

Cell migration was assessed by Transwell chambers as previously described [35]. The lower layer of the transwell chamber (3428, Corning Incorporated, Corning, NY, USA) was filled with 500 μL of DMEM containing 10% FBS. HFF-1 cells were trypsinized and suspended in serum-free DMEM at a density of 2×10^6/mL. Subsequently, 100 μL of the cells were added to each well and the chamber was incubated at 37 ℃ for 48 h. After incubation, the upper chamber was removed and the cells placed in new wells containing PBS (SH30256.01, Hyclone, South Logan, UT, USA). The cells were then fixed using 4% paraformaldehyde and stained using 0.1% crystal violet. The upper surface cells were visualized using an inverted microscope. After that, the chamber was taken out and 500 μL of 10% acetic acid was added for depigmentation. The optical density (OD) value was determined three times using a microplate analyzer at 550 nm.

2.8 Dual-luciferase reporter assays

First, we predicted that the binding sites of miR-143-3p on IGFBP-3 by applying the "miRNA and Targets" function at https://starbase.sysu.edu.cn/ [36]. As previously described [37], the regulatory elements of the target gene were artificially mutated according to the predicted binding sites. Then, the wild-type (WT) and mutated (MUT) sequences were inserted into expression vectors with firefly luciferase to control the transcriptional expression of luciferase. HFF-1 cells were divided into four groups: the NC mimics + IGFBP-3 WT group (HFF-1 cells were co-transfected with pHG-miRTarget-IGFBP-3 WT-3U plasmid and NC mimics at a final concentration of 50 nM), the miR-143-3p mimics + IGFBP-3 WT group (HFF-1 cells were co-transfected with pHG-miRTarget-IGFBP-3 WT-3U plasmid and miR-143-3p mimics at a final

concentration of 50 nM), the NC mimics + IGFBP-3 MUT group (HFF-1 cells were co-transfected with pHG-miRTarget-IGFBP-3 MUT-3U plasmid and NC mimics at a final concentration of 50 nM), and the miR-143-3p + IGFBP-3 MUT group (HFF-1 cells were co-transfected with pHG-miRTarget-IGFBP-3 MUT-3U plasmid and miR-143-3p mimics at a final concentration of 50 nM). A luciferase detection kit (E1910, Promega, Madison, WI, USA) was applied to measure the activities of firefly and renilla luciferases in the four groups in GloMax 20/20 (Promega, Madison, WI, USA) according to the product instructions. Renilla luciferase was used as an internal reference.

2.9 Data analysis

GraphPad Prism 8.0 statistical software (Graphpad, La Jolla, CA, USA) was applied for data processing [38]. The Student's *t*-test and one-way analysis of variance (ANOVA) were used to analyze and calculate the significance of the difference between the different groups. $p < 0.05$ was statistically significant.

3. Results

3.1 Testosterone promoted migration and proliferation of HFF-1 cells

Recent evidence suggests that an imbalance in human foreskin fibroblasts is an important part of the hypospadias etiology [3, 4]. Testosterone is an endogenous androgen that contributes to hypospadias repair in humans [10]. Therefore, we applied testosterone reagent to HFF-1 cells for 24 h to assess the impact of testosterone on the proliferation and migration of these cells. The CCK8 assays revealed that the Testosterone group had significantly higher cell viability than the Normal group (Fig. 1A). The Testosterone group also showed higher levels of cell migration compared to the Normal group (Fig. 1B). Our observations suggested that testosterone contributed to promoting the proliferation and migration of HFF-1 cells.

Androgen receptor (AR) can bind to testosterone and regulate cell differentiation and proliferation, thus mediating prostate organogenesis and development [39]. AR is closely associated with the development of hypospadias, and testosterone increases AR expression in fibroblasts [40, 41]. Consequently, we examined the expression of AR in each group to investigate whether testosterone affects AR signaling. The fluorescence intensity of AR in the Testosterone group were elevated compared to the Normal group (Fig. 1C). Western blotting further confirmed that the abundance of AR in the Testosterone group was greater (Fig. 1D). These findings indicated that testosterone promoted the expression of AR.

3.2 Testosterone regulated the expression of miR-143-3p and IGFBP-3 in HFF-1 cells

Next, we attempted to explore the underlying mechanism. Testosterone can target miRNA to regulate disease development [14, 15]. In a prior study, it was found that miR-143-3p hindered early HFF proliferation [21]. Evidence has indicated that miR-143-3p/IGFBP was shown to be involved in the osteogenic differentiation of dental pulp stem cells [42]. Additionally, testosterone could stimulate the secretion of IGFBP-3 in HFF [30]. This prompted us to investigate if miR-143-3p/IGFBP-3 was a potential target of testosterone.

To determine whether testosterone-mediated HFF-1 cell growth involves miR-143-3p and IGFBP-3, we measured the expression levels of these molecules using qRT-PCR in HFF-1 cells. We observed a significant down-regulation of *miR-143-3p* expression in the Testosterone group when compared to the Normal group, whereas the relative levels of *IGFBP-3* were significantly higher in the Testosterone group (Fig. 2A). The western blotting data for IGFBP-3 expression were in agreement with the qRT-PCR results (Fig. 2B), which confirms that testosterone has a regulatory impact on miR-143-3p and IGFBP-3 in HFF-1 cells. Accordingly, these findings indicated that miR-143-3p/IGFBP3 could act as a target of testosterone.

3.3 miR-143-3p targeted IGFBP-3

Having established that testosterone impacts the expression of both miR-143-3p and IGFBP-3, we sought to investigate whether miR-143-3p targeted IGFBP-3. The prediction results from specialist software suggested that miR-143-3p and IGFBP-3 had a binding site (Fig. 3A), thus indicating miR-143-3p could target IGFBP-3. To confirm this, we carried out a dual-luciferase reporter assay to assess the binding between miR-143-3p and IGFBP-3. Our data demonstrated that compared to the NC mimics + IGFBP-3 WT group, the luciferase intensity of the miR-143-3p mimics + IGFBP-3 WT group was lower. In contrast, the luciferase intensity of the miR-143-3p mimics + IGFBP-3 MUT group was similar to that of the NC mimics + IGFBP-3 MUT group (Fig. 3B), which confirmed that miR-143-3p can bind to IGFBP-3.

3.4 Overexpression of miR-143-3p inhibited the growth-promoting effect of testosterone on HFF-1 cells

Next, we wondered whether the regulation of testosterone on HFF-1 cells was through miR-143-3p/IGFBP3. To delve deeper into the effect of miR-143-3p on HFF-1 cells, we treated testosterone-exposed HFF-1 cells with miR-143-3p mimics. As a result, miR-143-3p mimics elevated the level of *miR-143-3p* compared to the NC mimics group (Fig. 4A). We found that cell viability in the miR-143-3p mimics group was notably inhibited (Fig. 4B), indicating that overexpression of miR-143-3p hindered HFF-1 cell viability. In addition, the abundance of IGFBP-3 exhibited a downward trend in the miR-143-3p mimics group (Fig. 4C). Our results indicated that miR-143-3p interfered with the proliferation of testosterone-exposed HFF-1 cells and limited the expression of IGFBP-3.

3.5 IGFBP-3 silencing attenuated the growth-promoting effect of testosterone on HFF-1 cells

Next, we transfected IGFBP-3 silencing sequences into testosterone-induced HFF-1 cells to assess the function of

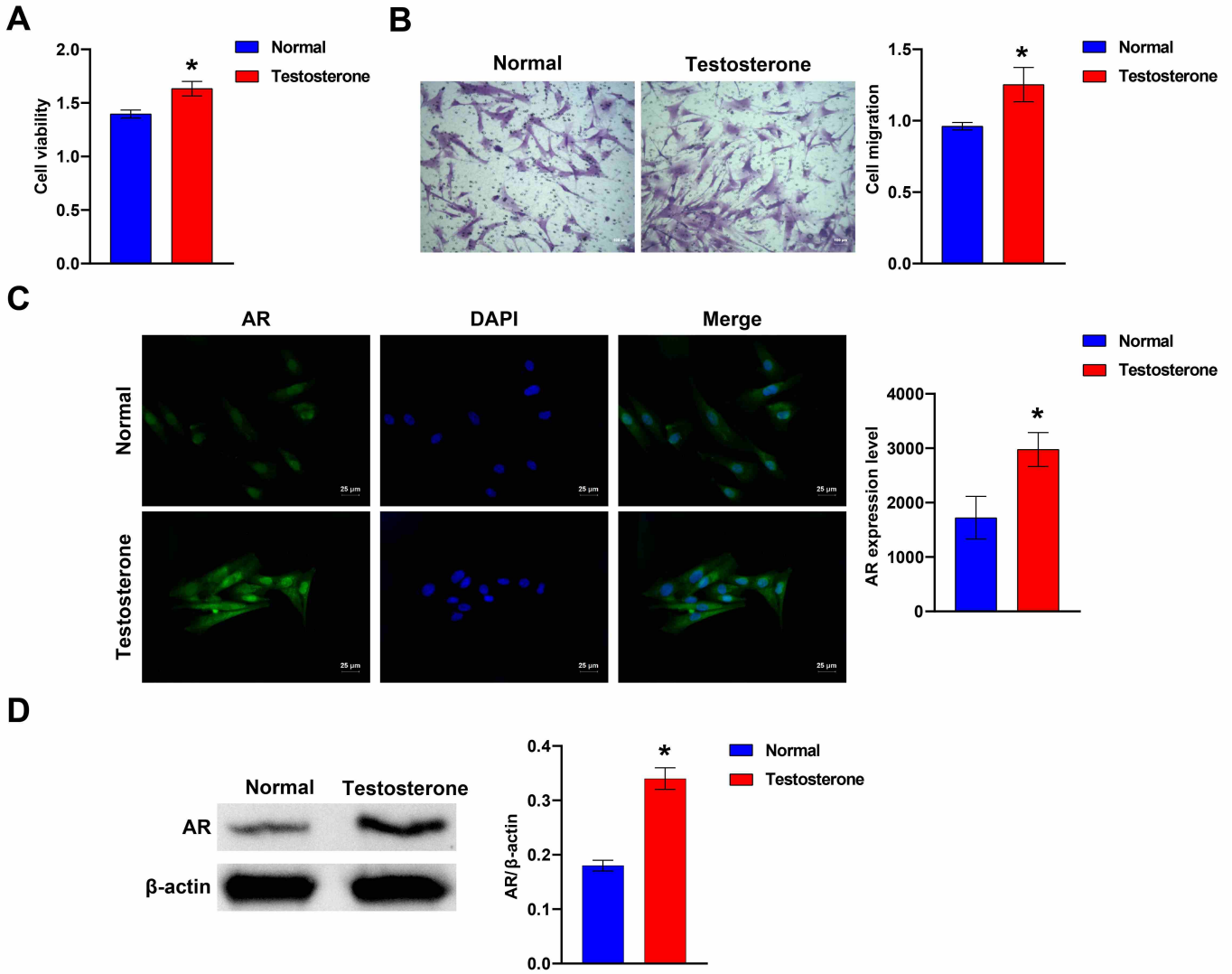

FIGURE 1. Detection of HFF-1 cell proliferation and migration. (A) CCK8 assays were used to measure cell viability. (B) Transwell assay for cell migration. Scale bar = 100 μm. (C) The expression of AR was determined by immunofluorescence. Scale bar = 25 μm. (D) The protein abundance of AR was analyzed by western blotting. The student's *t*-test was used to analyze statistical significance. *$p < 0.05$ *vs.* Normal group. AR: androgen receptor; DAPI: 4,6-diamidine-2-phenylindole dihydrochloride.

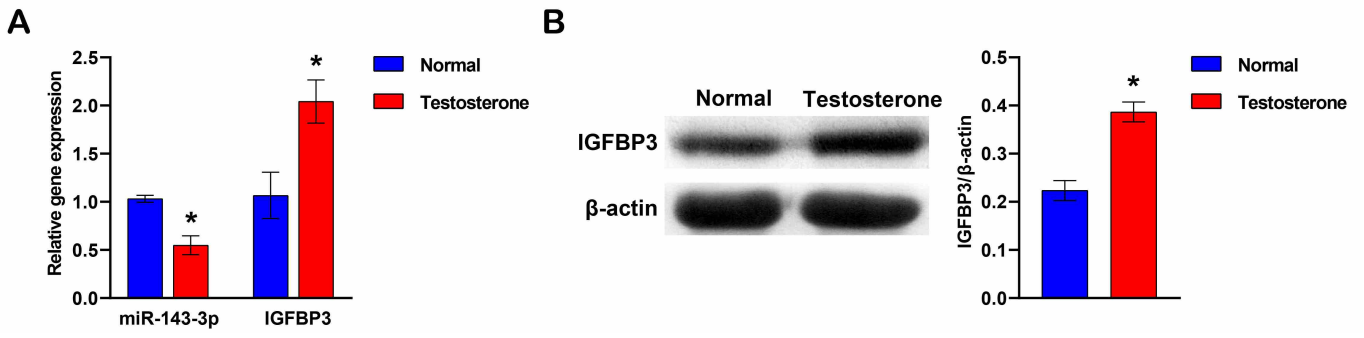

FIGURE 2. Down-regulation of miR-143-3p and up-regulation IGFBP-3 in HFF-1 cells. (A) Expression levels of *miR-143-3p* and *IGFBP-3* by qRT-PCR. (B) Protein abundance of IGFBP-3 by western blotting. The student's *t*-test was used to analyze statistical significance. *$p < 0.05$ *vs.* Normal group. *IGFBP-3*: insulin-like growth factor binding protein-3.

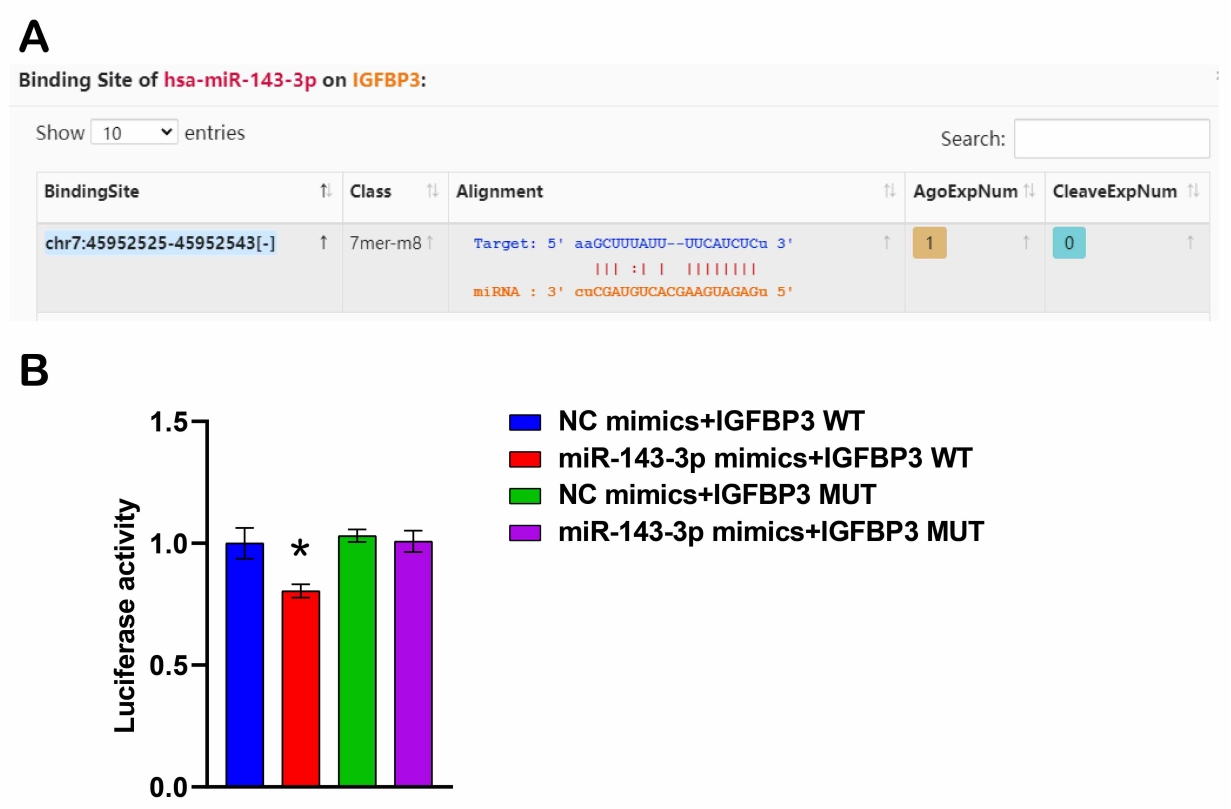

FIGURE 3. Prediction and validation of miR-143-3p targeting IGFBP-3. (A) Bioinformatics prediction of the target relationship between miR-143-3p and IGFBP-3. (B) Dual-luciferase reporter assays were used for the validation of miR-143-3p targeting IGFBP-3. One-way ANOVA was used to analyze statistical significance. *$p < 0.05$ *vs.* NC mimics + IGFBP3 WT group. WT: wild-type; MUT: mutated; *IGFBP-3*: insulin-like growth factor binding protein-3.

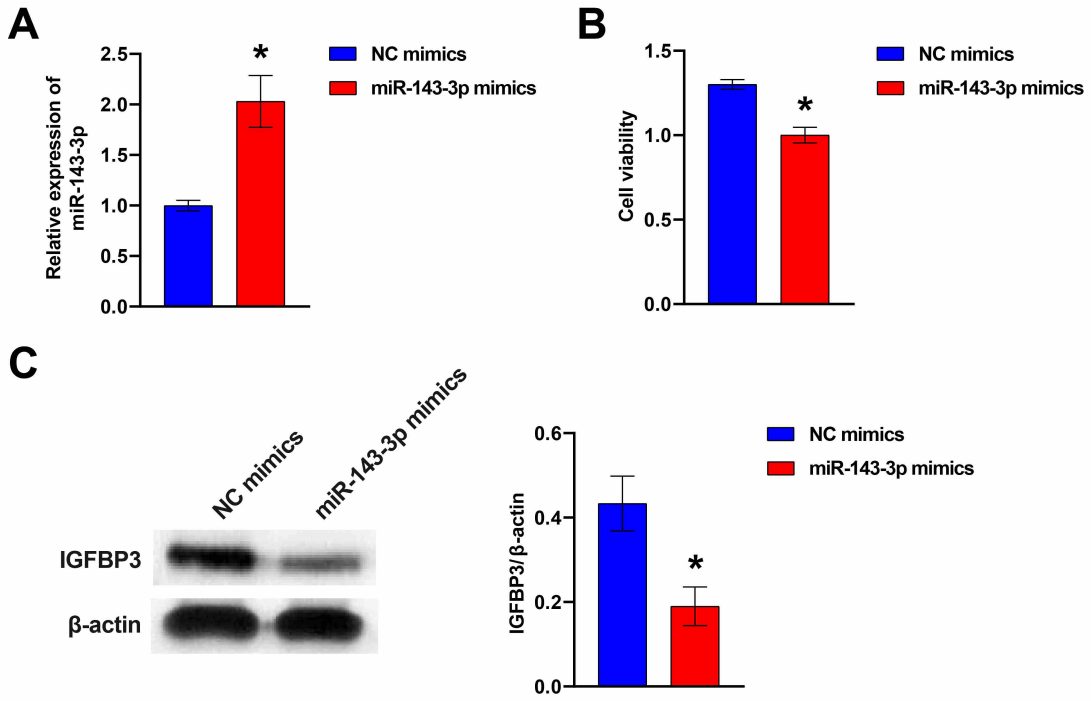

FIGURE 4. miR-143-3p could limit HFF-1 cell growth and IGFBP-3 expression. (A) qRT-PCR for measurement of *miR-143-3p* expression. (B) CCK8 assays for HFF-1 cell viability. (C) Western blotting for analysis of the abundance of IGFBP-3. The student's *t*-test was used to analyze statistical significance. *$p < 0.05$ *vs.* NC mimics group. NC: nitrocellulose; *IGFBP-3*: insulin-like growth factor binding protein-3.

IGFBP-3. The relative mRNA level of *IGFBP3* was reduced by si-IGBP3 compared to the si-NC group (Fig. 5A). The protein abundance of IGFBP-3 was reduced in the si-IGFBP3 group (Fig. 5B). In addition, si-IGFBP3 also restricted HFF-1 cell proliferation and migration (Fig. 5C,D). In a word, our findings indicated that IGFBP-3 silencing impeded testosterone-induced cell proliferation and migration and limited the expression of IGFBP-3 in HFF-1 cells.

3.6 miR-143-3p regulated testosterone-induced HFF-1 cell growth and AR expression *via* IGFBP-3

We wondered whether the regulation of miR-143-3p on testosterone-exposed HFF-1 cells was through IGFBP-3, given that IGFBP-3 is a target of miR-143-3p. To explore the relationship between miR-143-3p and IGFBP-3 in testosterone-induced HFF-1 cells, we introduced miR-143-3p mimics and the IGFBP-3 overexpression vector jointly. Additionally, *miR-143-3p* level exhibited an upward trend and *IGFBP-3* level exhibited a downward trend in the miR-143-3p mimics group. Compared to the miR-143-3p mimics + oe-NC group, oe-IGFBP3 decreased *miR-143-3p* levels and increased *IGFBP-3* levels (Fig. 6A). Additionally, miR-143-3p mimics reduced the protein abundances of IGFBP-3 and AR. However, oe-IGFBP3 elevated the protein levels of IGFBP-3 and AR compared to the miR-143-3p mimics + oe-NC group (Fig. 6B). Moreover, miR-143-3p mimics hindered HFF-1 cell proliferation. However, miR-143-3p mimics + oe-IGFBP3 resulted in stronger cell viability compared to the miR-143-3p mimics + oe-NC group (Fig. 6C). Similarly, the migration of HFF-1 cells was limited in the miR-143-3p mimics group. However, oe-IGFBP3 promoted HFF-1 cell migration compared to the miR-143-3p mimics + oe-NC group (Fig. 6D). Our observations suggested that miR-143-3p could inhibit IGFBP-3 to impede the proliferation, migration, and AR expression of testosterone-exposed HFF-1 cells.

4. Discussion

Hypospadias is a congenital malformation affecting the external male genitalia, which can lead to hypogonadism and increase the risk of cardiovascular disease in adulthood [38]. Given these phenomena, it is of great significance to explore the pathogenesis of hypospadias. Targeting proliferation, apoptosis, and the cell cycle of urethral plate fibroblasts may affect the progression of hypospadias [43]. Studies have shown that abnormal foreskin development affects the progression of hypospadias, and that HFF cells are critical for foreskin development [3]. Therefore, this study used HFF-1 cells in an attempt to investigate possible regulatory mechanisms. Our data showed that testosterone contributed to the proliferation and migration of HFF-1 cells and promoted AR expression, in part *via* miR-143-3p/IGFBP-3.

Testosterone, as an androgen, is essential to human health. The efficacy of testosterone replacement therapy for gonadal recovery and treating male disorders has already been proven [44, 45]. The reduction of testosterone levels often triggers hypogonadism and a physical decline in men, often manifested clinically as easy fatigue, loss of libido, erectile dysfunction, muscle and weight loss [46–48]. Notably, testosterone replacement therapy is considered the current standard practice for patients with gonadal dysfunction [49]. Preoperative androgen stimulation contributes to hypospadias repair [50, 51]. Abnormal androgen signaling is strongly associated with the development of hypospadias [52, 53], and testosterone has also been found to affect the outcome of hypospadias surgery [54]. From this, we hypothesized that testosterone might play a role in the growth of HFF-1 cells and, therefore, in the development of hypospadias. Our study findings showed that testosterone promoted the proliferation and migration of HFF-1 cells, and also promoted the accumulation of AR. Studies have shown that AR binds to testosterone, thereby regulating cell differentiation and proliferation to mediate prostate organogenesis and development [39]. AR has found to be closely associated with the development of hypospadias, and testosterone could increase AR expression in fibroblasts [40, 41]. These results suggested that testosterone could be participating in the pathogenesis of hypospadias by regulating HFF cell proliferation and migration, as well as AR signaling.

Recent reports indicate that miR-181a mediates H_2O_2-induced oxidative stress and HFF cell senescence [55]. Many reports have proven that miR-143-3p could be effective for different diseases [16]. miR-143-3p could regulate specific target genes in cancer, cardiovascular disease and other diseases, as well as affect the disease process. Previous studies have demonstrated that both testosterone and miR-143-3p can affect cell proliferation and differentiation, hinting at a possible regulatory relationship between them [56, 57]. Therefore, we investigated whether testosterone could regulate miR-143-3p. Our validation showed that testosterone could facilitate the migration and proliferation ability of HFF-1 cells, while overexpression of miR-143-3p could significantly restrain HFF-1 cell migration and proliferation. This information indicated that both testosterone and miR-143-3p could affect HFF-1 cell migration and proliferation. Testosterone has been shown to exert its effects by targeting miRNAs [58], leading us to hypothesize that testosterone and miR-143-3p might be connected. Further analysis revealed that testosterone downregulated the expression of miR-143-3p, which indicated that testosterone could negatively regulate miR-143-3p. It could be inferred that testosterone promoted the proliferation of HFF-1 cells by negatively regulating miR-143-3p, but specific regulatory mechanism involved remains unclear.

According to previous studies, testosterone has been shown to have a regulatory effect on the expression level of IGFBP-3 in humans [59], confirming that testosterone can regulate IGFBP-3. Our research has also supported this finding, as testosterone was found to positively regulate the expression of IGFBP-3. Furthermore, we observed that IGFBP-3 silencing reduced testosterone-induced proliferation and migration of HFF-1 cells, indicating that testosterone may increase the level of IGFBP-3 to promote the proliferation and migration of HFF-1 cells. It has been reported that IGFBP-3 can affect the progression of many cancers by inhibiting the binding of receptors and blocking their anti-apoptotic activity [60–62]. It has also been reported that IGFBP-3 inhibits angiogenesis

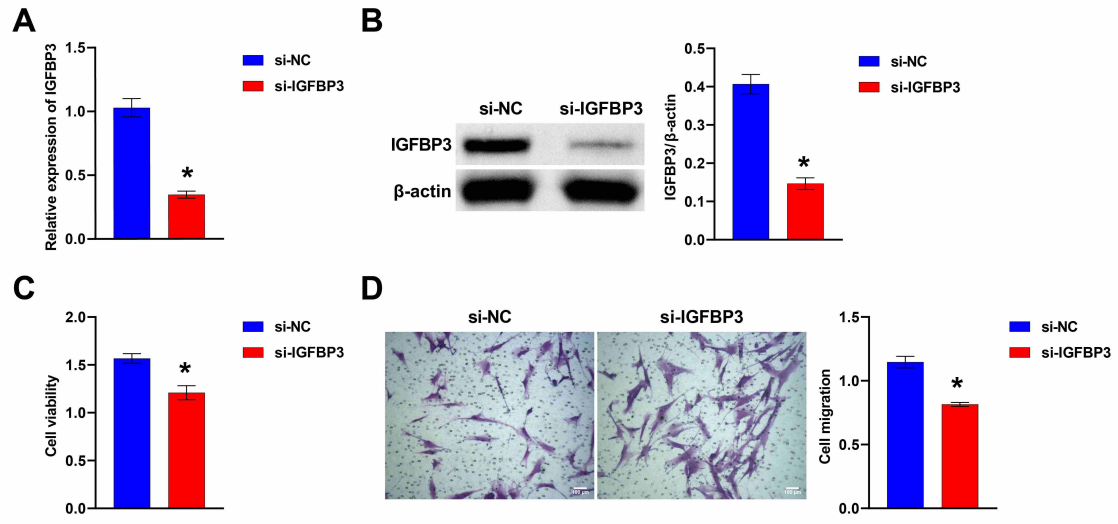

FIGURE 5. IGFBP-3 silencing inhibited the growth of HFF-1 cells. (A) qRT-PCR for measurement of *IGFBP-3* expression. (B) The abundance of IGFBP-3 was examined by western blotting. (C) The viability of HFF-1 cells was detected by CCK8 assays. (D) Transwell assay for cell migration. Scale bar = 100 μm. The student's *t*-test was used to analyze statistical significance. *$p < 0.05$ *vs.* si-NC group. NC: nitrocellulose; *IGFBP-3*: insulin-like growth factor binding protein-3.

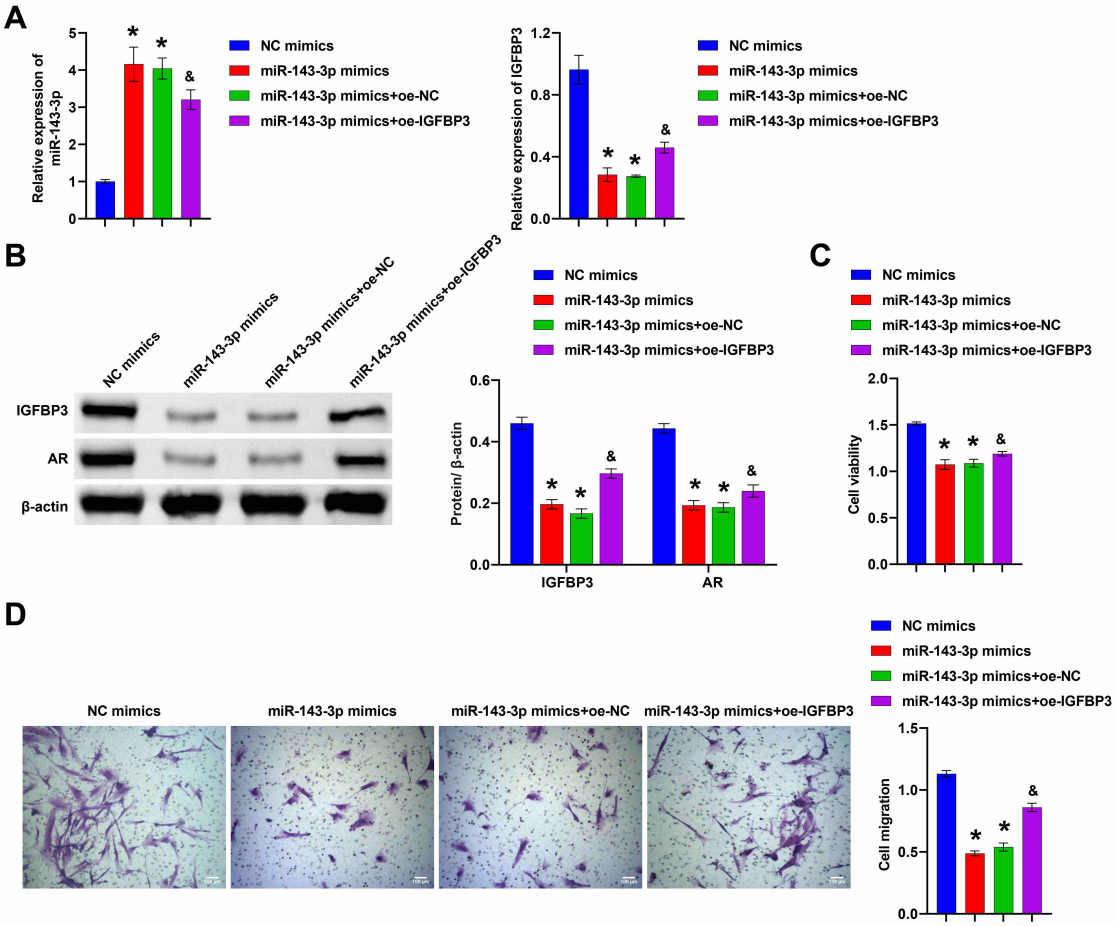

FIGURE 6. miR-143-3p targeted IGFBP-3 to regulate testosterone-induced HFF-1 cell growth and AR expression. (A) qRT-PCR for measurement of *miR-143-3p* and *IGFBP-3* expression. (B) Western blotting for detection of IGFBP-3 and AR. (C) HFF-1 cell viability was detected by CCK8 assays. (D) Transwell assay for cell migration. Scale bar = 100 μm. One-way ANOVA was used to analyze statistical significance. *$p < 0.05$ *vs.* NC mimics group; & $p < 0.05$ *vs.* miR-143-3p mimics + oe-NC group. NC: nitrocellulose; AR: androgen receptor; *IGFBP-3*: insulin-like growth factor binding protein-3.

and affects apoptosis [63, 64]. Accumulating reports indicate that IGFBP-3 acts as a target gene for some miRNAs [65]. For example, miR-449a plays a role in non-obstructive azoospermia [66]. Below physiological levels, corticosterone could target the miR-124-3p/HDAC5 axis to block testosterone synthesis [67]. These miRNAs are thought to target IGFBP-3, and their functions are closely related to testosterone levels, thus indicating the need for further research on IGFBP-3. Based on previous results, we considered whether IGFBP-3 is a target of miR-143-3p, thereby affecting testosterone-mediated regulation pathway. We found a miR-143-3p binding site on IGFBP-3 gene through bioinformatics prediction, thus providing an initial suggestion of a targeting relationship between the two factors. Subsequently, we verified that miR-143-3p directly targeted IGFBP-3 and regulated its expression by a dual-luciferase reporter assay. We found that miR-143-3p was negatively correlated with IGFBP-3, thus indicating that miR-143-3p could negatively regulate IGFBP-3. Based on these findings, we co-transfected miR-143-3p mimics and IGFBP-3 overexpression vector into testosterone-induced HFF-1 cells to further analyze that miR-143-3p was acting through IGFBP-3. We found that IGFBP-3 overexpression countered the inhibitory effect of miR-143-3p on HFF-1 cell proliferation and migration and AR expression. This result suggests that testosterone inhibits miR-143-3p to increase IGFBP-3, which ultimately promotes HFF-1 cell proliferation and migration and AR signaling.

Nevertheless, this study has some limitations that need to be taken into account. This study predominantly explored the effects of testosterone at the cellular level, and whether these findings can be validated in human and animal samples needs further study. Due to the limitation of funds and time, our mechanistic study did not include a blank group, that is, a group that was not treated with testosterone. Moreover, this study had limited exploration of phenotypes and mechanisms, such as the regulation of HFF-1 cell apoptosis, invasion, and cycle by testosterone; the relationship between AR signaling and HFF-1 cell growth; and the effect of miRNA on testosterone levels. Additionally, there is still insufficient conclusive evidence to establish a direct link between dysregulated HFF-1 cell growth and hypospadias. Further research is required to thoroughly investigate the intricate mechanisms and regulatory pathways of testosterone. Our future work aims at exploring this direction and achieving this objective.

5. Conclusions

This study was designed to examine the function of testosterone in the growth of HFF-1 cells and to confirm the underlying regulatory mechanisms involved. We propose that testosterone can regulate miR-143-3p to target IGFBP-3, thereby promoting the growth of HFF-1 cells and activating AR signaling. These findings could have significant implications for the treatment of male diseases such as hypospadias, possibly providing a novel idea for the therapeutic application of testosterone.

AVAILABILITY OF DATA AND MATERIALS

The data used to support the findings of this study are available from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

QLP, YWZ and WT—designed the study, performed the research, analysed data, and wrote the paper. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This study was supported by Hunan Provincial Health Commission Scientific Research Project (20200748).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Qian-Long Peng, Yao-Wang Zhao, Wen Tian. Testosterone promotes human foreskin fibroblast growth through miR-143-3p targeting IGFBP-3. Journal of Men's Health. 2023; 19(9): 15-25. doi: 10.22514/jomh.2023.082.