



MicroRNA profiling reveals the role of miR-133b-3p in promoting apoptosis and inhibiting cell proliferation and testosterone synthesis in mouse TM3 cells

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Abstract

Late-onset hypogonadism (LOH) is an age-related clinical and biological syndrome in which serum testosterone deficiency is an important characteristic and diagnostic indicator. In this study, we firstly analyzed the difference in the expression level of three miR-133 s (including miR-133a-3p, miR-133a-5p, and miR-133b-3p) in rat testis samples, blood samples from mice before and 1 wk after testis removal, and mouse TM3 cells. Secondly, the mimics and inhibitors corresponding to the three miR-133 s of mouse were transfected into TM3 cells separately to determine the correlation between the three miRNAs. Finally, using mouse TM3 cells to analyze the effect of miR-133b overexpression or inhibition on the proliferation and apoptosis of mouse testicular Leydig cells, the effect on genes related to testosterone synthesis, and the effect on the level of testosterone in the culture medium. We found that, compared with the testis tissue of newborn rats, miR-133a-5p was increased in adult rats, and miR-133a-3p and miR-133b-3p were decreased. In addition, 1 wk after the testis was removed, the expression levels of these three miRNAs in the blood of adult mice decreased. The correlation of the three miRNAs was summarized, and it was found that miR-133b-3p played an important role in it. In TM3 cells, overexpression of miR-133b-3p suppressed the proliferation and promotes apoptosis of cells, suppressed the expression level of most genes related to cell proliferation and testosterone synthesis, and the concentration of testosterone in the culture medium decreased while these phenomena can be reversed by the inhibition of miR-133b-3p expression. It was found that miR-133b-3p can regulate testosterone production in TM3 cells at least by targeting FSCN1. The above results suggest that miR-133b-3p plays an important role in regulating testosterone synthesis. These findings also provide new candidate diagnostic indicators for late-onset hypogonadism in men and provide new clues for the further study of pathogenesis.

Keywords Leydig cells · TM3 cells · Testosterone synthesis · MicroRNA · miR-133

Introduction

Late-onset hypogonadism (LOH) is an age-related clinical and biological syndrome characterized by deficient serum testosterone levels and diffuse symptoms including sexual dysfunction, decreased muscle mass and strength, osteoporosis, hot flashes, insomnia, fatigue, depression, irritability, and anxiety (Gray *et al.* 1991; Feldman *et al.* 2002; Wang *et al.* 2009; Huhtaniemi 2020). The prevalence of LOH in men ages 40 to 79 years averages approximately 2.1%, and the prevalence increases gradually from 0.1% in men ages 40 to 49 to 5.1% in men ages 70 to 79 (Huhtaniemi 2020). Testosterone supplementation can improve clinical symptoms caused by testosterone deficiency, but it does not apply to similar clinical symptoms without hormone deficiency (Wang *et al.* 2009; Bassil 2011; Huhtaniemi 2020).

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The pathogenesis of LOH remains to be further studied, but at least it involves the level of sex hormones and its complex influencing factors in the body. For middle-aged and older men, it's reported that serum total testosterone decreased by 1.6% per year, free testosterone decreased by 1.2% per year, and albumin-bound testosterone decreased by 1.0% per year, and then calculated bioavailable testosterone decreased 2 to 3% per year (Gray *et al.* 1991; Feldman *et al.* 2002). Because hypothalamic GnRH secretion declines during aging, there is a compensatory increase in FSH and LH concentrations in peripheral blood, and changes in the circadian rhythm of LH secretion from the pituitary portal system: the daily pulse frequency increases and the pulse amplitude decreases (Takahashi *et al.* 2005). However, pure age-related changes in the gonadal axis are rare, and in more cases these older adults have concurrent obesity or chronic disease (Wu *et al.* 2010; Grossmann *et al.* 2020). Decreased testosterone secretion associated with obesity and chronic disease is not associated with a compensatory increase in gonadotropin levels, suggesting that pituitary function is disturbed (Huhtaniemi 2020). The researchers used estradiol to stimulate the hypothalamic-pituitary-gonadal axis and found that negative feedback regulation was weakened, suggesting a decline in neuroendocrine function in the elderly. Additionally, addition of LH in an in vitro cell culture system does not help senescent Leydig cells restore their ability to synthesize testosterone, suggesting that Leydig cells themselves may be dysfunctional (Chen *et al.* 2002). Electron microscope analysis and histological observation of testicular tissue in aged men showed that the mitochondria of Leydig cells and the smooth endoplasmic reticulum were vacuolated, and the testicular tissue was insufficient and degenerated. In the testis, some researchers have reported that the amount of Leydig cells and Sertoli cells decreased significantly during aging (Neaves *et al.* 1984; Rebourcet *et al.* 2017), while others reported no significant decrease of Leydig cells (Petersen *et al.* 2015). Testosterone synthesis in the testes declines during aging, but whether this is due to intrinsic aging of Leydig cells or due to impaired function or the number of Leydig cells in an aging tissue environment requires further study. Elucidation of these is important for understanding the pathogenesis of LOH and for rational application of therapeutic measures.

Chen and colleagues found the significantly altered expression of miR-133a-3p, miR-125a-5p, and miR-361-5p in LOH patients' plasma (Chen *et al.* 2016). The researchers found that the level of miR-133a-3p in peripheral blood was negatively correlated with the severity of symptoms in elderly men, and believed that miR-133a-3p was suitable for the diagnosis of LOH (Chen *et al.* 2016). Using miRNA's target gene prediction database (miRanda), bioinformatics

analysis predicts miR-133a's potential target genes, a very complex target gene network is presented, many genes related to metastasis, inflammation, cell cycle, apoptosis, and endocrine (EGFR, FSCN1, SRF, Akt, IGF1R, LASP1, cyclin D2, etc.) and many signaling pathways (Wnt, PI3K/Akt, ERK, MAPK, Sox4-EMT, RFFL-p53/p21, TGF- β /Smad, EGFR/Akt, Jak-STAT signaling pathway, etc.) are all related to it (Chen *et al.* 2016). EGFR is a cell surface receptor that binds to epidermal growth factor (EGF), which induces receptor dimerization and tyrosine autophosphorylation, promoting cell growth, proliferation, and survival. Phosphorylated EGFR recruit adaptor proteins like GRB2, which in turn activate at least four major downstream signaling cascades, including STAT, PLCgamma-PKC, PI3 kinase-AKT, and RAS-RAF-MEK-ERK modules (Runkle *et al.* 2016). The expression product of FSCN1 is a member of the fascin family of actin-binding proteins that play key roles in cell migration, motility, adhesion, and cellular interactions (<https://www.ncbi.nlm.nih.gov/gene/6624>). The SRF encoded a ubiquitous nuclear protein that regulates the activity of many genes involved in cell cycle regulation, differentiation, growth, and apoptosis (<https://www.genecards.org/cgi-bin/carddisp.pl?gene=SRF>). In many pathways, such as MAPK pathway, the SRF is regulated as a downstream target (Liang *et al.* 2011). The Akt-encoded product is one member of the human AKT serine-threonine protein kinase family, which is phosphorylated by phosphoinositide 3-kinase (PI3K) (Fayard *et al.* 2010). These AKT proteins regulate cellular metabolism, survival, proliferation, and angiogenesis in normal and malignant cells. The insulin-like growth factor I receptor, encoded by IGF1R, has a key role in transformational events (<https://www.ncbi.nlm.nih.gov/gene/3480>). High-level expression of IGF1R is found in most malignant tissues as an anti-apoptotic factor to enhance cell survival. The downstream signaling pathways of IGF1R include PI3K/Akt and MAPK/ERK (Zhang *et al.* 2011). LASP1-encoded LIM and SH3 protein 1 can induce lung epithelial-mesenchymal transition through the TGF- β 1/Smad/Snail pathway, and the LASP1 and TGF- β /Smad3 signaling pathways can be regulated by miR-133a to inhibit lung cancer progression (Shen *et al.* 2020; Xue *et al.* 2021). CCND2 encoded cyclin D2 is required for the G1/S transition of the cell cycle, and high-level expression of CCND2 has been found in testicular tumors. CCND2 has multiple downstream pathways, including STAT5, Wnt, and PI3K/Akt signaling (Pirozzi *et al.* 2021).

miR-133 was first identified in mice, and its homologues also exist in other species, including humans and rats. Associated genes in each species often encode multiple miRNAs with the same or similar mature sequences. miR-133a-1, miR-133a-2, and miR-133b were found on human chromosomes 18, 20, and 6, respectively, with corresponding mature microRNA products as miR-133a-3p, miR-133a-5p,

and miR-133b-3p (Kozomara and Griffiths-Jones 2011). In this study, firstly, the expression levels of miR-133 in rat testis samples, blood samples from mice before and 1 wk after testis removal, and mouse TM3 cells were detected. Secondly, the mimics and inhibitors corresponding to the three miRNAs of mouse were transfected into TM3 cells separately to determine the correlation between the three miRNAs. Finally, using mouse TM3 cells to analyze the effect of miR-133b overexpression or inhibition on the proliferation and apoptosis of mouse testicular Leydig cells, the effect on genes related to testosterone synthesis, and the effect on the level of testosterone in the culture medium.

Materials and methods

Predict target genes of miR-133b that may be closely related to testosterone synthesis For humans and mice, the genes expressed in testes and that may be regulated by miR-133b were predicted by two databases (<https://rgd.mcw.edu/rgd-web/report/gene>, <https://www.ncbi.nlm.nih.gov/gene>). Targetscan (https://www.targetscan.org/vert_80/) and miRDB (<http://www.mirdb.org/>) are used to predict the target gene regulated by miR-133b. The expression amount of FSCN1 in different tissues is searched in the SRA database (<https://www.ncbi.nlm.nih.gov/sra/?term=>).

Quantitative real-time PCR Total RNA of TM3 cells and testis samples was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and examined with a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). Using a reverse transcription reagent kit (TOYOBO, FSQ-101, Osaka Japan), the first strand cDNA was synthesized following manufacturer's protocol. Using the Applied Biosystems SYBR Green mix kit (TOYOBO, FSQ-201, Osaka Japan) and the SLAN-48P fluorescence quantitative PCR instrument (Shanghai Hongshi Medical Technology, Shanghai, China), PCR was performed following manufacturer's protocol. The target gene expression levels were normalized against the expression levels of mouse β -actin or U6 in the test samples, and the $2^{-\Delta\Delta Ct}$ method was adapted for quantitative analysis. The primer sequences used in this study are shown in Table 1.

Plasmid construction and cell transfection The mimics/inhibitor and negative control of the three miRNAs were purchased from Wuhan GeneCreate Biological Engineering Co., Ltd (GeneCreate, Shanghai, China). Each transfection was performed according to the manufacturer's instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Animal The SD rats and Balb/C mice used in this experiment were all purchased from Beijing Vital River Laboratory

Animal Technology Co., Ltd (VRL, Beijing, China). The experimental protocols were approved by the animal experimental ethical inspection of the Laboratory Animal Centre, Wenzhou Medical University (wydw299-0510), and meet the guidelines of the responsible governmental agency.

Cell culture The mouse TM3 cell line was introduced from Procell (Procell, Wuhan, China, CL-0234). TM3 cells were grown in DMEM (Hyclone, Logan, SH30022.01B), 10% FBS (Gibco, Maitland, Australia, 10,099-133), and 1% Penicillin–Streptomycin Solution Cell proliferation analysis (Cytiva, Logan, SV30010) incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Cell proliferation analysis The proliferation of TM3 cells was measured by CCK-8 cell viability test kit (Cell Counting Kit-8, Solarbio, Beijing, China). Cells were seeded in 96-well plates with a concentration of 5×10^3 cells per well. Twenty-four hours after transfection, the cells were stained with 10 μ L of CCK-8 reagent for 2 h. According to the absorbance at 450 nm, cell viability was detected using a spectrophotometer (Xpspectrum, Shanghai, China).

Cell cycle analysis The cell cycle of processed TM3 cells was also measured by propidium iodide (PI) cell cycle staining following the steps below. (1) Harvesting cells: Aspirate and discard the culture medium, wash the cells with 2 ml PBS, add 1 ml trypsin, digest for 1 min in a 37°C incubator, add culture medium to stop the digestion, collect the cell suspension, centrifuge at 1000 rpm for 5 min, and discard the supernatant. (2) Cell fixation: Add 5 ml of cold 70% ethanol, mix well while adding, and fix overnight at 4°C. (3) Cell staining: Collect the cells by centrifugation, wash the cells twice with 5 ml of PBS, centrifuge at 1000 rpm for 5 min, and discard the supernatant. Add 500 μ L permeabilization solution (100 μ g/ml RNase A, 0.2% Triton X-100) and incubate at 37°C for 30 min. Wash the cells twice with 5 ml of PBS, and stain with 50 μ g/ml PI (Solarbio, CA1510, Beijing, China) for 10 min. (4) Preparation for flow analysis: Pass through a 300-mesh screen, centrifuge at 1000 rpm for 5 min, and discard the supernatant. Add 5 ml PBS to wash away excess PI, resuspend the cells with 200 μ L PBS, and detect by Beckman Coulter's CytoFlex flow cytometry platform (Beckman Cytotflex, Brea, CA).

Cell apoptosis analysis Cell apoptosis was measured using the Annexin V-FITC/Propidium Iodide staining kit (Beyotime Institute of Biotechnology, Jiangsu, China). TM3 cells were seeded in 6-well plates with a concentration of 10^6 cells/ml. Cells were labeled with Annexin V-FITC for 5 min in the dark, followed by the addition of 5 mg propidium iodide to each sample for 30 min. Finally, over 20,000 total cells were measured per sample using Beckman Coulter's CytoFlex flow cytometry platform (Beckman Cytotflex, California, USA).

Table 1. RT-PCR primers and siRNA

mmu-miR-133a-3p	F: ACACTCCAGCTGGGTTTGGTCCCCTTCAAC, R: TGGTGTCTGGAGTCG Stem loop RT primer: CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCAGCTGGT
mmu-miR-133a-5p	F: ACACTCCAGCTGGGCTGGTAAATGGAA, R: TGGTGTCTGGAGTCG Stem loop RT primer: CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGATTGGTT
mmu-miR-133b-3p	F: ACACTCCAGCTGGGTTTGGTCCCCTTCAAC, R: TGGTGTCTGGAGTCG Stem loop RT primer: CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTAGCTGGT
Egfr	F: GGACAAACAGAACTGGGCTTAGG R: AATCGCACAGCACCAATCAGG
Fscn1	F: ACCAGCAGCAGCGACACC R: CCACCTTGAGAGCCACCTTATTG
Srf	F: TGCCTCAACTCGCCAGACTC R: CTTCACTGTGTCCTTGGTTTCCC
Akt1	F: GCTACTTCCTCCTCAAGAACGATG R: GCAGGCAGCGGATGATAAAGG
Igflr	F: TCCTCCACATCCTGCTCATCTC R: GACCAGTGCGTAGTTGTAGAAGAG
Lasp1	F: AATCTCCGCCTCAAGCAACAG R: CTGCCACCACGCTGAAACC
Cnd2	F: GGAACCTGGTAGTGTGGGTAAGC R: TGCGAAGGATGTGCTCAATGAAG
Klf15	F: TGTTGCCCTCGCCATCTC R: TCCACTGCCACCACCACTG
Ccn2	F: AGGACCGCACAGCAGTTGG R: GGCAGTTGGCTCGCATCATAG
Slc2a4	F: GCCAGCCTACGCCACCATAG R: TACTAAGAGCACCGAGACCAACG
U6	F: CTCGCTTCGGCAGCACATATAC R: CGAATTTGCGTGTATCCTTGC
mbeta-actin	F: CCGCTGCCTCTTCCTCCTC R: AACGCTCATTGCCGATGGTG
Si-NC	Guide: UUCUCCGAACGUGUCACGUTT Passenger: ACGUGACACGUUCGGAGAATT
Si-FSCN1	Guide: ACUGAUUUGGUCAUUUUUCUG Passenger: GAAAAAUGACCAAAUCAGUAAU
Si-FSCN2	Guide: UUAUUUCCAGUUCAAAGCUU Passenger: GCUUUGAACUGAAAAUAAAG

Western blotting WB detected the related proteins Wnt3a, PI3K, Akt, ERK, MAPK, TGF- β , Smad2, EGFR, Jak, and STAT1. Total protein was isolated from TM3 cells by RIPA buffer (Beyotime, P0013D, Jiangsu, China) with 1% complete EDTA-free and 1 mM PMSF protease inhibitor cocktail (Beyotime, P1006-1). The protein concentration of the samples was then measured using the BCA protein detection kit (CW BIO, Beijing, China, CW2011S). A total of 50 μ g of protein per sample was electrophoresed on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) gel, and transferred to a nitrocellulose membrane (Millipore, Shanghai, China, IPVH00010). The membrane was then blocked with 25 mM Tris and 140 mM NaCl with 0.05% Tween-20 containing 5% nonfat milk and immunoblotted with antibody against Wnt3a (Proteintech, Wuhan, China,

26,744–1-AP), PI3K (Proteintech, Wuhan, China, 20,584–1-AP), Akt (Affinity, Cincinnati, OH, AF6261), ERK (Abcam, ab32537), MAPK (Proteintech, Wuhan, China, 14,064–1-AP), TGF- β (Proteintech, Wuhan, China, 21,898–1-AP), Smad2 (Proteintech, Wuhan, China, 12,570–1-AP), EGFR (Proteintech, Wuhan, China, 66,455–1-Ig), Jak (Proteintech, Wuhan, China, 66,466–1-Ig), STAT1 (Proteintech, Wuhan, China, 10,144–2-AP), and GAPDH (Proteintech, Wuhan, China, 60,004–1-Ig). The target proteins on the membrane were detected with ECL Plus kit (Beyotime Biotechnology, Jiangsu, China) according to the product manual.

Testosterone concentration detection in cell culture supernates Human Testosterone (T) ELISA Kit (CUSABIO, Wuhan, China, CSB-E05099h) was used for the quantitative

determination of testosterone concentrations in cell culture supernates according to the kit's instructions.

Statistical analysis Each experiment was performed at least 3 times and data are represented as the means $\pm$ standard deviations (SD). The data analysis was performed using SPSS 20.0 (SPSS, Inc., Chicago, IL). By one-way analysis of variance (ANOVA), the differences were assessed, and $P < 0.05$ was considered statistically significant.

Results

Target genes of miR-133b that may be closely related to testosterone synthesis For humans and mice, there are three target genes expressed in testes and may be regulated by miR-133b: Klf15 (Kruppel-like factor 15), Ccn2 (Cellular communication network factor 2), and Slc2a4 (Solute carrier family 2 member 4). The expression product of Klf15 is a zinc finger protein, which can bind to the promoter of the 17 β -hydroxysteroid dehydrogenase type 5 gene to upregulate gene expression and promote testosterone synthesis. The Ccn2 expression product can be combined with fibronectin, has growth factor activity, and participates in the aging process. The expression product of Slc2a4 is a protein with glucose transmembrane transporter and D-glucose transmembrane transporter activities; it can improve the tolerance of cells to hypoxia, and promote the cell's response to insulin stimulation and glucose uptake. It should be emphasized that Klf15 and Slc2a4 are both regulated by miR-133b-3p. Based on the above information, we believe that the miR-133b is very likely to be expressed in the testis and has a role in the pathogenesis of LOH.

The expression profiles of miR-133 in rat testis samples and TM3 cells The expression levels of miR-133a-3p, miR-133a-5p, and miR-133b-3p in testis samples of newborn rats and 6-mo-old rats were detected (Fig. 1a). Compared with the testis tissue of newborn rats, miR-133a-5p was increased in adult rats, and miR-133a-3p and miR-133b-3p were decreased. In addition, the testes of 3 adult male Balb/C mice at the age of 8 wks were removed. Take blood samples from mice before and 1 wk after removal, and extract RNA to detect the expression of these three miRNAs (Fig. 1b). One week after the testis was removed, the expression levels of these three miRNAs in the blood of adult mice decreased. We also looked at the expression levels of these three miRNAs in TM3 cells, and found miR-133a-5p's expression level is relatively higher than that of miR-133b-3p (Fig. 1c).

Correlation of expression levels between 3 miRNAs The mimics and inhibitors corresponding to miR-133 s of mice were transfected into TM3 cells and tested to determine the correlation between the three miRNAs (Fig. 2).

After the overexpression of miR-133a-3p, miR-133a-5p's expression level was significantly upregulated, and after the expression of miR-133a-3p was inhibited, miR-133a-5p's expression was significantly downregulated (Fig. 2a, b). There was a positive correlation between the expression levels of these two RNAs. After the overexpression of miR-133a-3p, miR-133b-3p's expression was significantly downregulated, and miR-133b-3p's expression remained unchanged after the expression of miR-133a-3p was inhibited.

Overexpression or inhibition of miR-133a-5p has no effect on miR-133a-3p's expression (Fig. 2c, d). Overexpression of miR-133a-5p has no significant effect on miR-133b-3p's expression, but the inhibition of 133a-5p expression has a significant promotion effect on miR-133b-3p's expression.

After the overexpression of miR-133b-3p, the expression level of miR-133a-3p increased significantly (Fig. 2e). After the inhibition of miR-133b-3p's expression level, miR-133a-3p's expression was significantly downregulated (Fig. 2f). A positive correlation between these two RNAs' expression levels was found. After overexpression of miR-133b-3p, miR-133a-5p's expression level decreased, and after inhibition of miR-133b-3p's expression, miR-133a-5p's expression level increased, showing a negative correlation between these two RNAs.

From the above experimental results, the correlation of miR-133 s' expression levels in TM3 cells was summarized, and miR-133b-3p has an important role in it (Fig. 2g).

Overexpression or inhibition of miR-133b-3p affects the proliferation and apoptosis of TM3 cells After transfection of miR-133b-3p's mimics or inhibitor into TM3 cells, the proliferation of TM3 cells was measured by CCK-8 cell viability test kit and PI Cell Cycle Staining, and TM3 cells' apoptosis was measured using the Annexin V-FITC/Propidium Iodide staining kit.

After transfection of miR-133b-3p mimics into TM3 cells, compared with the negative control groups (miR-133b-3p mimics NC and TM3), the proliferation ability of the cells was significantly weakened (Fig. 3a). After miR-133b-3p mimics transfected TM3 cells, compared with miR-133b-3p mimics NC group cells, cells in G1 phase increased, cells in S phase remained unchanged, and cells in G2 phase decreased (Fig. 3b), and the number of apoptotic cells increased significantly (Fig. 3c).

After miR-133b-3p inhibitor transfected into TM3 cells, compared with the negative control groups (miR-133b-3p inhibitor NC and TM3), the proliferation ability of the cells showed an upward trend (Fig. 3a). After miR-133b-3p

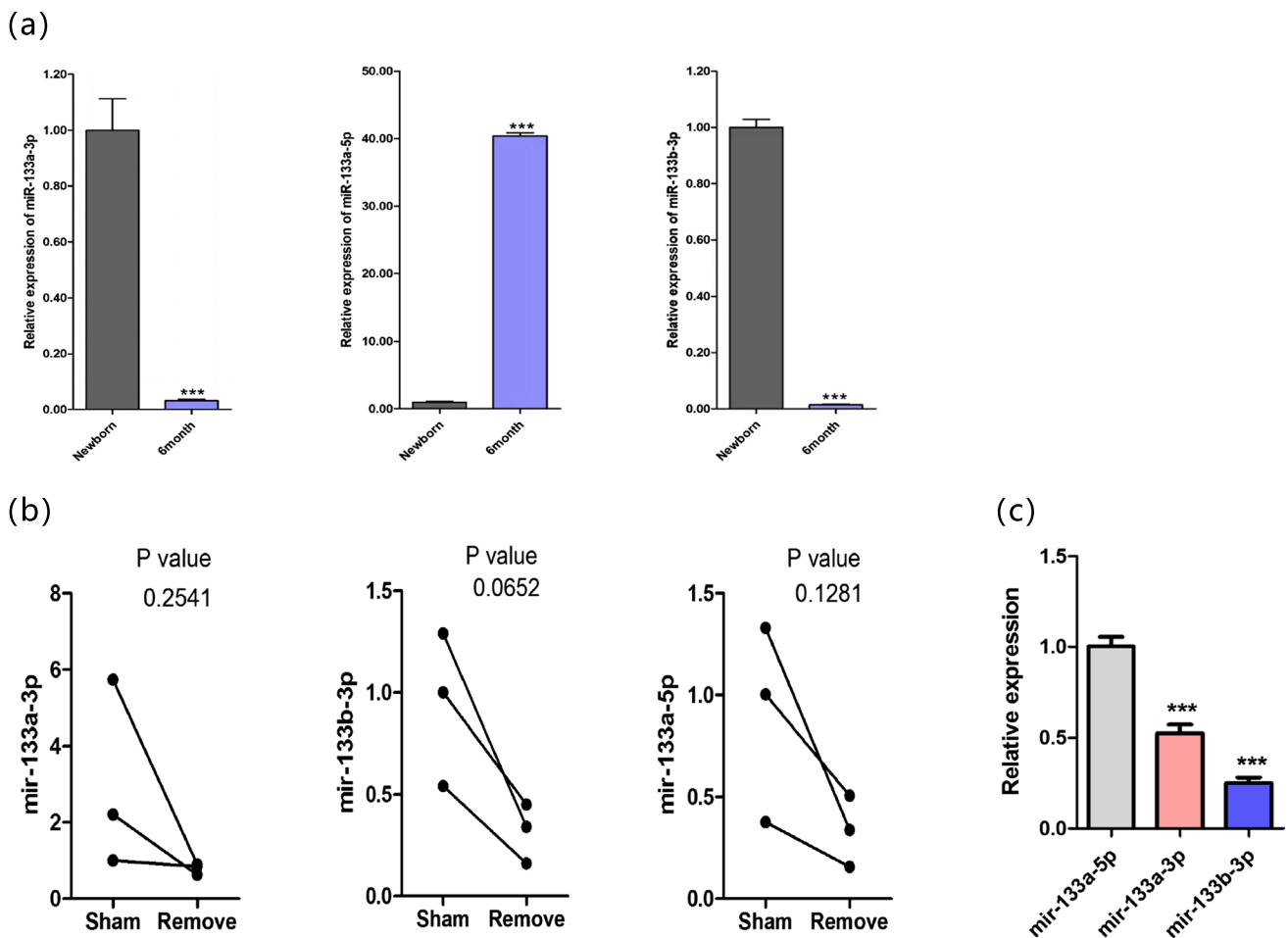


Figure 1. The expression profiles of miR-133 in mouse testis samples and TM3 cells. (a) The relative expressions of three miR-133 s in testis samples of newborn rats ($n=3$) and 6-mo-old adult rats ($n=3$) separately. (b) The relative expressions of these three miRNAs in the

blood of adult mice ($n=3$) before and 1 wk after testicle removal. (c) The expression levels of these three miRNAs in TM3 cells. miRNA Results were normalized to the expression of mbeta-actin. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

inhibitor transfected TM3 cells, compared with mmu-miR-133b-3p inhibitor NC group cells, cells in G1 phase decreased, cells in S phase remained unchanged, and cells in G2 phase increased (Fig. 3b), and the number of apoptotic cells decreased significantly (Fig. 3c).

The effect of overexpression or inhibition of miR-133b-3p on the expression level of important genes related to proliferation, apoptosis, and testosterone synthesis in TM3 cells The effects of overexpression or inhibition of miR-133b-3p on the expression level of important genes related to the proliferation, apoptosis, and testosterone synthesis in TM3 cells were analyzed. These genes including EGFR, FSCN1, SRF, Akt, IGF1R, LASP1, CCD2, Klf15, Ccn2, and Slc2a4 and genes involved in the signaling pathways (Wnt, PI3K/Akt, ERK, MAPK, Sox4-EMT, RFFL-p53/p21, TGF- β /Smad, EGFR/Akt, Jak-STAT signaling pathway).

After miR-133b-3p was overexpressed in TM3 cells, the expression level of most genes related to miR-133a decreased significantly, and the expression level of lasp1 did not change significantly (Fig. 4a). At the same time, the expression levels of Ccn2, Klf15, and Slc2a4 did not change significantly (Fig. 4a).

After inhibition of miR-133b-3p's expression in TM3 cells, the expression levels of genes related to miR-133a have an upward trend, and the expression levels of SRF and cyclin D2 have increased significantly (Fig. 4a). At the same time, the expression level of Slc2a4 decreased significantly, while the expression levels of Ccn2 and Klf15 did not change significantly (Fig. 4a).

After overexpressing or inhibiting the expression of miR-133b-3p in TM3 cells, we detected the content of proteins of corresponding genes in the cells (Fig. 4b). On the whole, the synthesis of proteins decreased after the overexpression, while the synthesis of proteins increased after the inhibition.

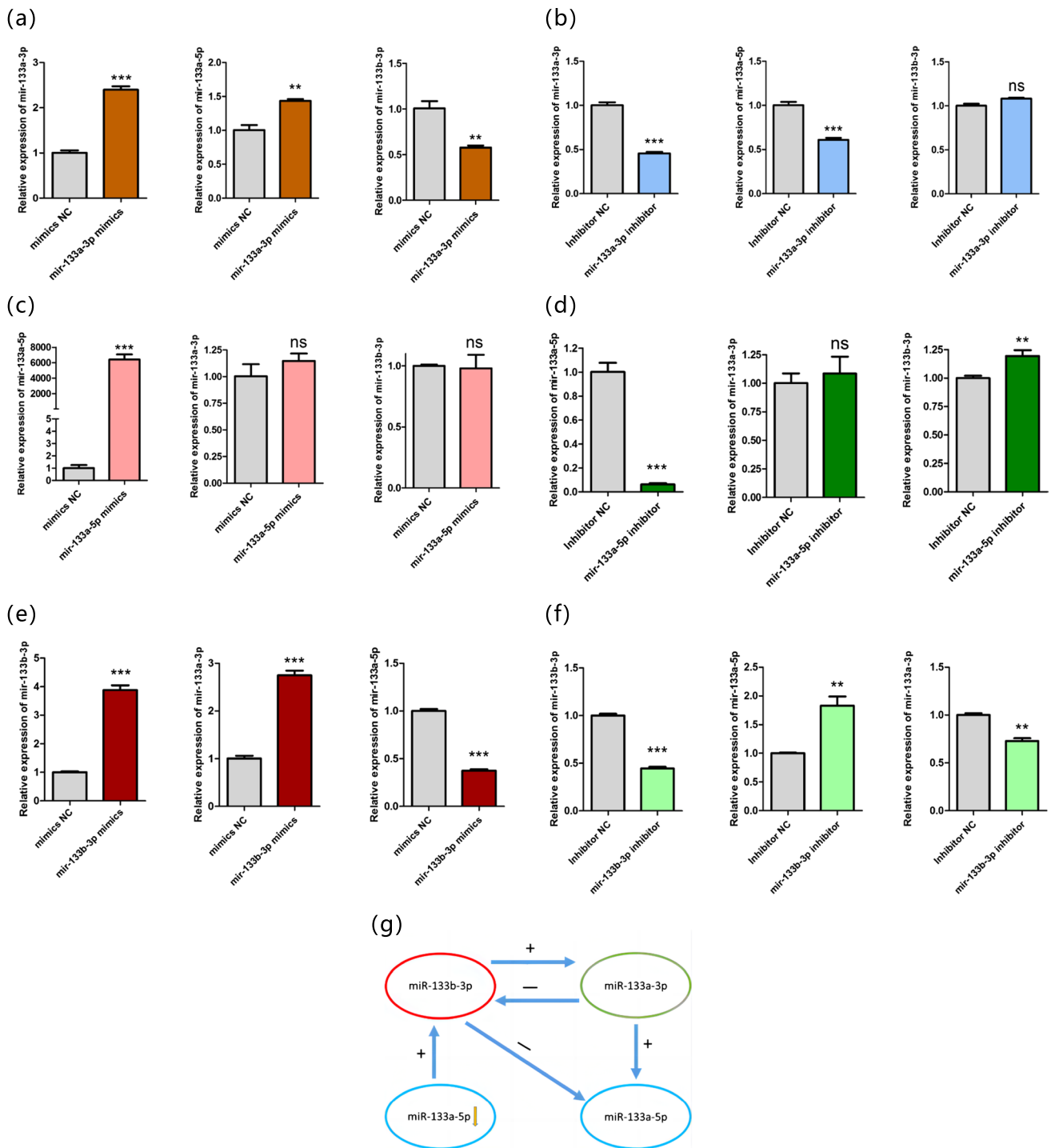


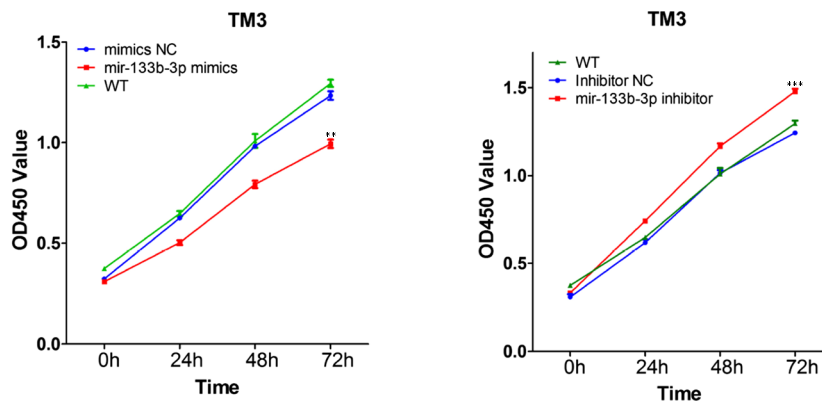
Figure 2. Correlation of expression levels of miR-133 s in TM3 cells. (a, b) The effect of overexpression or inhibition of miR-133a-3p on the expression levels of miR-133a-5p and miR-133b-3p. (c, d) The effect of overexpression or inhibition of miR-133a-5p on the expression levels of miR-133a-3p and miR-133b-3p. (e, f) The effect of

overexpression or inhibition of miR-133b-3p on the expression levels of miR-133a-3p and 133a-5p. (g) Correlation of expression levels of miR-133 s in TM3 cells. miRNA results were normalized to the expression of mbeta-actin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

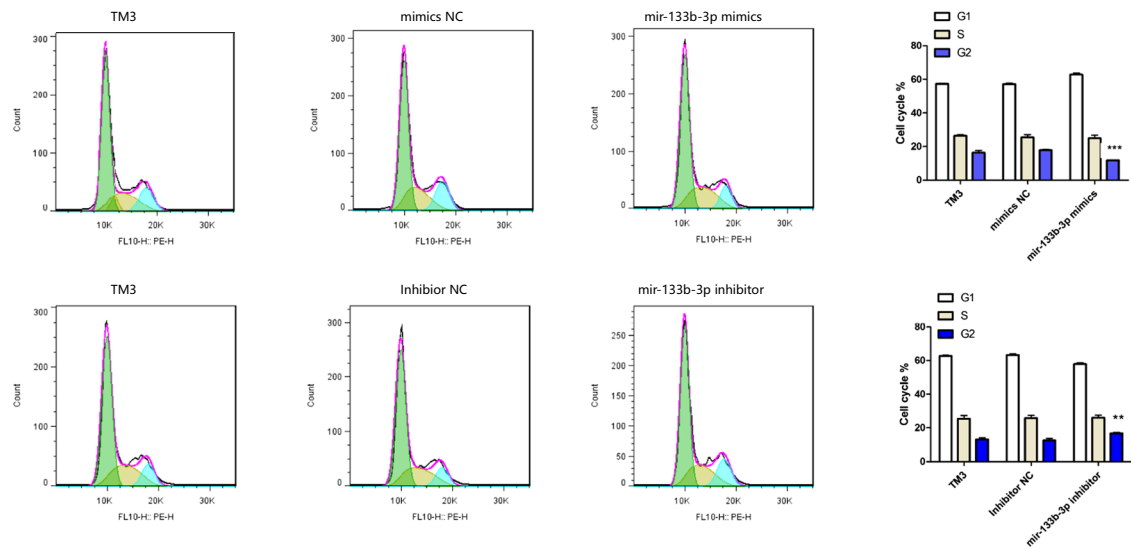
The effects of overexpression or inhibition of miR-133b-3p on testosterone production in TM3 cell culture medium After mmu-miR-133b-3p mimics transfected TM3 cells, compared

with the negative control groups (mmu-miR-133b-3p mimics NC group and TM3 group), the concentration of testosterone in the culture medium decreased (Fig. 5a). After

(a)



(b)



(c)

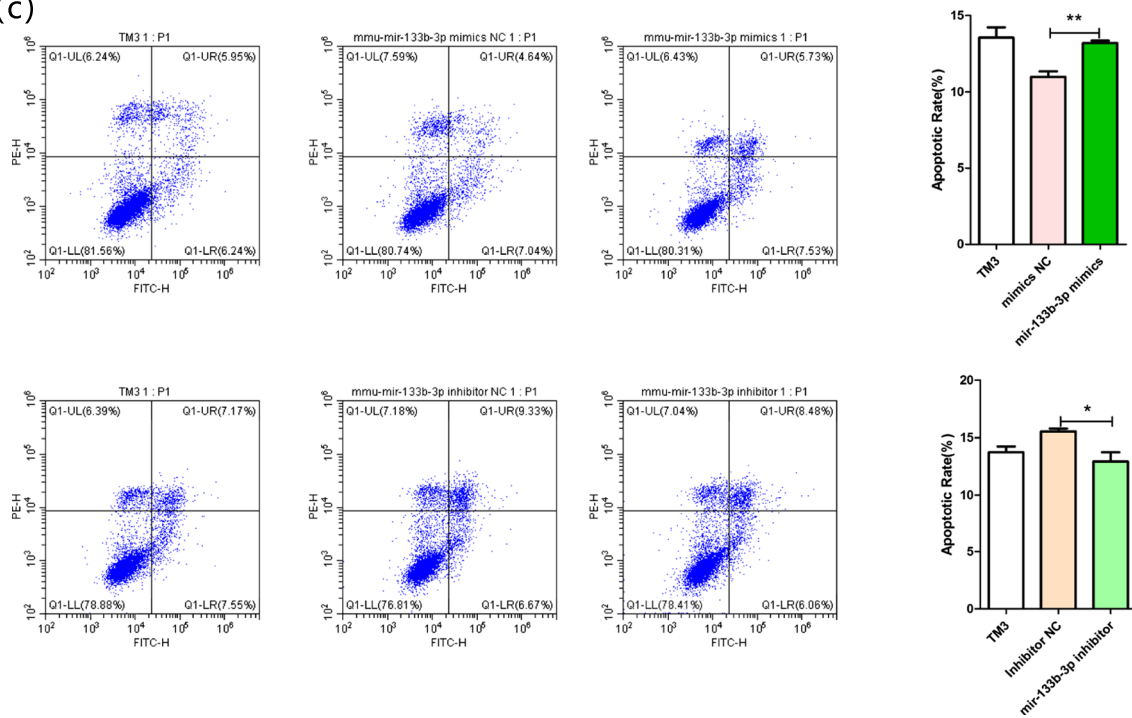


Figure 3. The effect of miR-133b-3p overexpression or inhibition in TM3 cells on cell proliferation and apoptosis. (a) The proliferation of TM3 cells measured by CCK-8 cell viability test kit. (b) The proliferation of TM3 cells measured by PI Cell Cycle Staining. (c) The TM3 cells' apoptosis measured using the Annexin V-FITC/Propidium Iodide staining kit. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

mmu-miR-133b-3p inhibitor transfected TM3 cells, compared with the negative control groups (mmu-miR-133b-3p inhibitor NC group and TM3 group), the concentration of testosterone

in the culture medium increased (Fig. 5b). After FSCN1 silencing, the concentration of testosterone was lower than that of the control group, indicating that FSCN1 plays a role in the production of testosterone in TM3 cells (Fig. 5c).

miR-133b-3p can regulate testosterone production in TM3 cells by targeting FSCN1 In order to find the target genes regulated by mouse mmiR-133b-3b, we first predicted through Targetscan and miRDB online database, selected

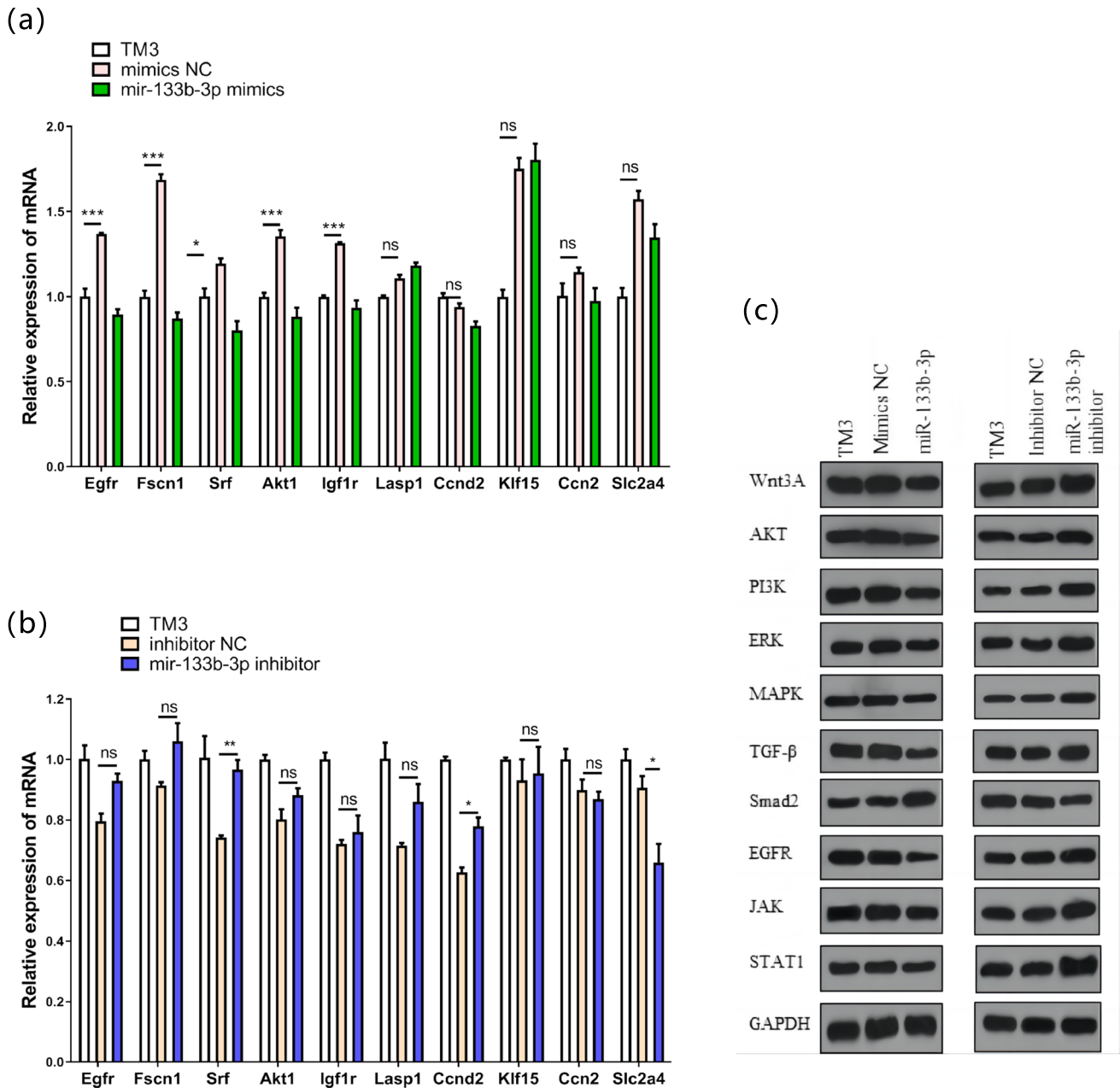
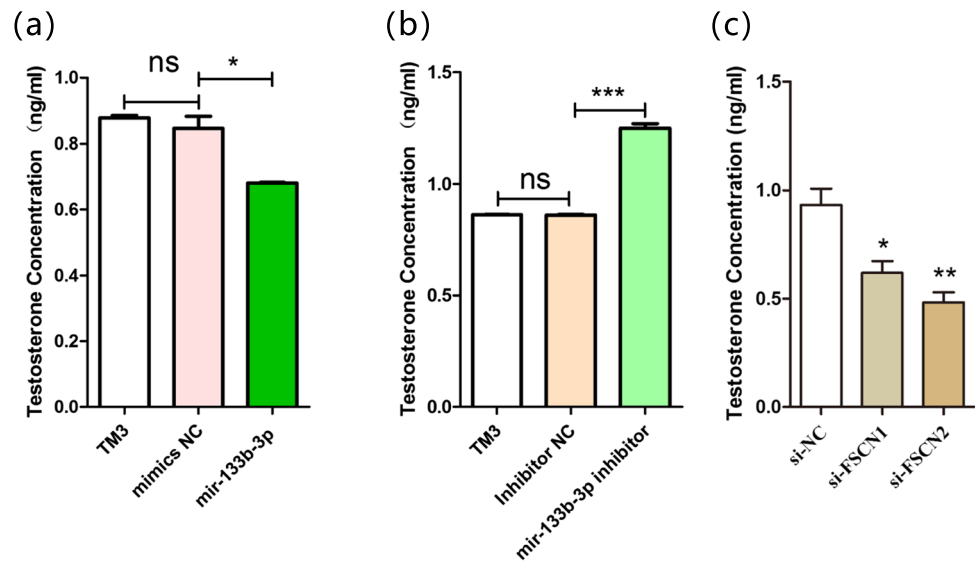


Figure 4. The effect of overexpression or inhibition of miR-133b-3p on the expression of important genes related to proliferation, apoptosis, and testosterone synthesis in TM3 cells. (a) After miR-133b-3p was overexpressed or inhibition in TM3 cells, the expression levels

of genes were detected by RT-PCR. (b) After overexpression or inhibition of miR-133b-3p in TM3 cells, the contents of proteins related to corresponding genes were detected by western blotting. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 5. The effect of over-expression or inhibition of miR-133b-3p on testosterone concentration in the TM3 cells' culture medium. (a) After over-expression of miR-133b-3p in TM3 cells, the testosterone concentration of culture medium was detected using ELISA kit. (b) After inhibition of miR-133b-3p in TM3 cells, the testosterone concentration of the culture medium was detected using ELISA kit. (c) Transfect two siRNAs silencing FSCN1 and control (si- NC) into TM3 cells, and detect testosterone concentration through the kit 24 h later. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



the intersection, and found 309 predicted common genes (Fig. 6a). We then performed Venn analysis between the predicted 309 genes and the genes detected by qPCR in Fig. 4a (EGFR, FSCN1, SRF, Akt, IGF1R, LASP1, CCD2, Klf15, Ccn2, Slc2a4), and found 2 shared protein genes (EGFR and FSCN1) (Fig. 6b). These two genes are related to proliferation, apoptosis, and testosterone synthesis. The FSCN1 expression level was searched from the SRA DATA database of NCBI, and the results showed that FSCN1 was abundantly expressed in testis tissue (Fig. 6c). This suggests that FSCN1 may be involved in the physiological function of testis, so it was finally selected for follow-up verification (Fig. 6d).

The region in the 3'UTR sequence of FSCN1 that binds to mmu-miR-133b-3b was selected and constructed into a pmir-GLO vector (named FSCN1-WT), and a vector with the mutated binding site (named FSCN1-MUT) was constructed. The interaction between FSCN1 mRNA and mmu-miR-133b-3b was verified by dual luciferase assay (Fig. 6d).

Discussion

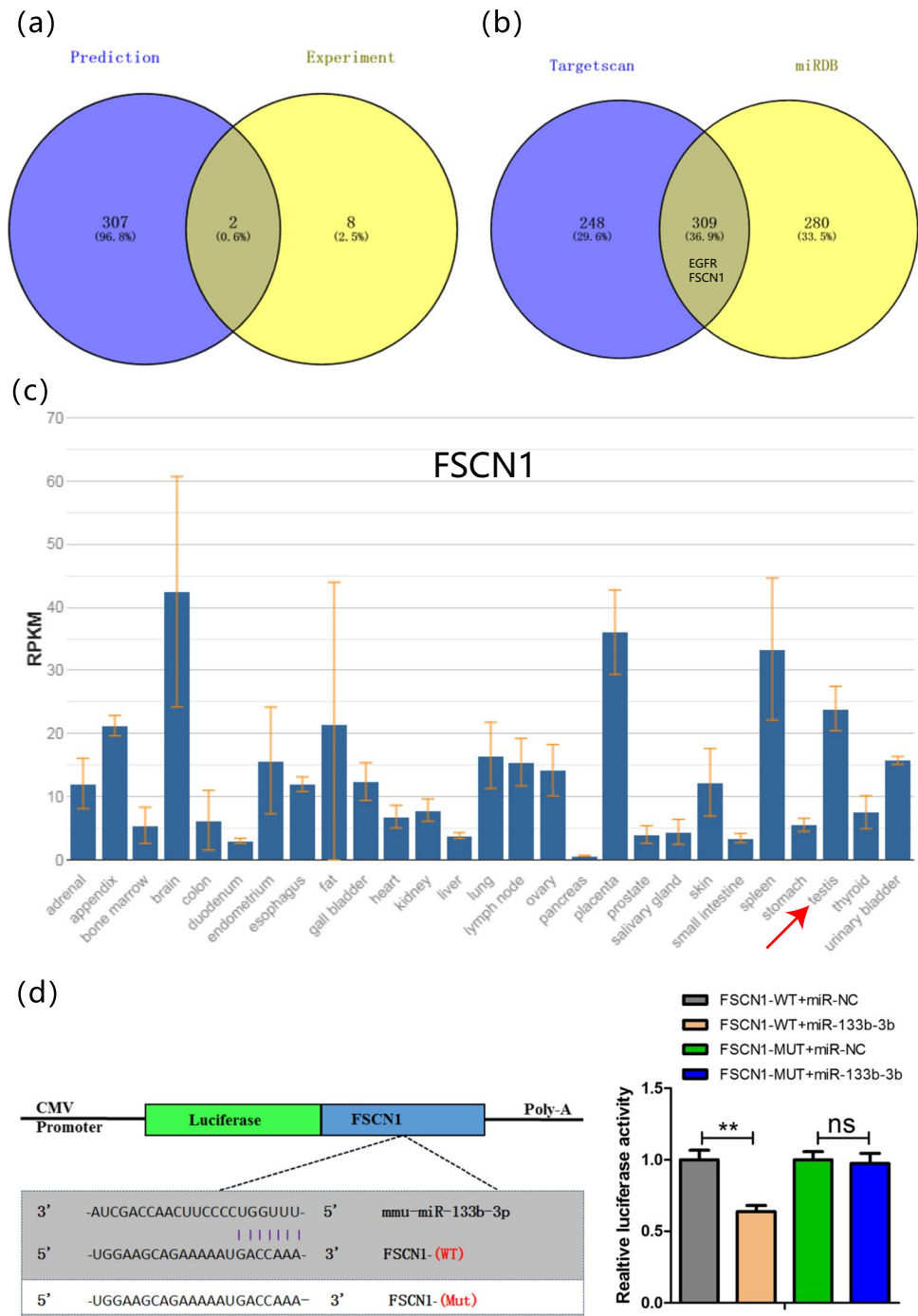
Kraus and colleagues found that the androgen-regulated microRNA miR-375 may have an important role in testosterone deficiency-induced increases in visceral fat mass and associated insulin resistance (Kraus *et al.* 2015). In their further study, it is reported that miR-130a is also regulated by androgen and downregulated in early stages of adipogenesis and regulates the translation of androgen receptor (AR) and adiponectin (ADIPOQ) (Greither *et al.* 2020). It has also been reported that peripheral blood miR-133a-3p level has the diagnostic value for LOH and is

negatively correlated with symptom severity in older men (Chen *et al.* 2016). We speculate from the above research reports that miR-133 may be involved in the regulation of testosterone synthesis.

In this study, we predicted that there are three target genes regulated by miR-133b-3p in testis tissue: Klf15, Cnn2, and Slc2a4 (<https://rgd.mcw.edu/rgdweb/report/gene>). The Klf15 expression product can bind to the promoter of 17 β -hydroxysteroid dehydrogenase type 5 gene, upregulate gene expression, and promote testosterone synthesis. However, although overexpression or inhibition of miR-133b-3p could affect testosterone synthesis, it had no effect on Klf15 expression, indicating that miR-133b-3p may regulate testosterone synthesis through other pathways. The expression product of Cnn2 has growth factor activity and participates in the aging process. In this study, overexpression of miR-133b-3p tends to inhibit the expression of Cnn2, and can inhibit Leydig cells' proliferation. This result suggests that 133b-3p may inhibit Leydig cell proliferation at least by inhibiting Cnn2 expression. In fact, Cao and his colleagues have found that miR-133b exerts targeted inhibition of Cnn2 expression, thereby reducing TGF- β 1-induced epithelial-mesenchymal transition in HK2 cells and renal interstitial fibrosis in aged mice with unilateral ureteral obstruction (Cao *et al.* 2021). Compared with the control group, the inhibition of miR-133b-3p expression could not affect the expression of Cnn2, but could promote the proliferation of Leydig cells. This result suggests that inhibition of 133b-3p expression promotes Leydig cell proliferation through other pathways.

In this study, it is found that miR-133a-3p expression has a negative feedback effect on miR-133b-3p expression. On the other hand, increased expression of miR-133b-3p

Figure 6. MiR-133b-3p targets FSCN1 to regulate the level of testosterone. (a) Predict target genes regulated by mmu-miR-133b-3b through Targetscan and miRDB databases, and conduct Venn analysis. (b) Through Venn analysis of 309 predicted target genes and 10 genes verified by qPCR experiment, two common target genes EGFR and FSCN1 were found. (c) The expression level of FSCN1 in tissues was searched through SRA DATA database. (d) The interaction between mmu-miR-133b-3b and FSCN1 was verified by double luciferase experiment. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



could inhibit the miR-133a-5p expression, while inhibition of miR-133a-5p expression would further promote the expression of miR-133b-3p. It is suggested that miR-133b-3p has an important regulatory role in the number of Leydig cells and testosterone synthesis in the testis. Li and his colleagues found that miR-133a promotes primary myoblasts proliferation and inhibits its apoptosis in cattle (Li *et al.* 2018). As a muscle-specific lncRNA, muscle differentiation-related lncRNA (MDNCR) can promote

myoblast differentiation and apoptosis by adsorbing miR-133a, thereby enhancing the expression of miR-133a's target gene GosB (Li *et al.* 2018). Bioinformatics analysis using miRNA target gene prediction database (miRanda) revealed that potential target genes of miR-133a are involved in metastasis, inflammation, cell cycle, apoptosis, and endocrine (e.g., EGFR, FSCN1, SRF, Akt, IGF1R, LASP1, cyclin D2, etc.) and various signaling pathways (Wnt, PI3K/Akt, ERK, MAPK, Sox4-EMT, RFFL-p53/

p21, TGF- β /Smad, EGFR/Akt, Jak-STAT signaling pathway, etc.). After overexpression of miR-133b-3p, the expression levels of most genes related to testosterone synthesis and cell proliferation decreased, and the overall effect was that TM3 cell proliferation and testosterone synthesis decreased. After inhibition of miR-133b-3p expression, most genes related to testosterone synthesis and cell proliferation increased, and the overall effect was the increase of TM3 cell proliferation and testosterone synthesis. FSCN1 is an F-actin-bundling protein and thus may be critical for normal sperm morphology and sperm motility (Cheng *et al.* 2007). In this study, it was found that *miR-133b-3p can regulate testosterone production in TM3 cells at least by targeting FSCN1*. We speculate that miR-133b-3p may also regulate TM3 cell proliferation and testosterone synthesis via miR-133a, which needs to be confirmed by future studies. Interestingly, Yao *et al.* found that miR-133b can directly inhibit the transcriptional activator GLI3 and then indirectly promote the expression of Cyclin B1 and Cyclin D1 to activate human Sertoli cell proliferation (Yao *et al.* 2016). In a previous multicenter study, we analyzed the epidemiological characteristics of LOH in China, including associations between smoking, sex hormone levels, and late-onset hypogonadism in men differ depending on age (Liu *et al.* 2021). In this study, it was found that the expression level of miR-133b-3p was decreased in adult rat testis compared with neonatal rat testis. miR-133b-3p inhibits Leydig cell proliferation but promotes Sertoli cell proliferation; these phenomena suggest that the role of miR-133b-3p in the regulation of testicular physiology is more complex than we know and require further investigation.

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Author contribution Chengshuang Pan wrote the original draft. Didi Wu and Honggang Li assisted in bioinformatic analysis and technical guidance. Qianjin Fei and Chengshuang Pan and Jianyuan Jin and Jiujia Zheng performed the research. Xuefeng Huang edited the original draft. Xiangbin Kong designed the research study and edited the original draft. All the authors read and approved the final manuscript.

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Data availability Data supporting the findings of this study are available from the respective authors upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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