The effect and mechanism of Grim19 on mouse sperm quality and testosterone synthesis

Yue Zhao, Haoran Liu, Yang Yang, Wenqian Huang and Lan Chao

Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, People’s Republic of China

Correspondence should be addressed to L Chao; Email: qlszcl@163.com

Abstract

Abnormal sperm parameters such as oligospermia, asthenospermia, and teratozoospermia result in male factor infertility. Previous studies have shown that mitochondria play an important role in human spermatozoa motility. But the related pathogenesis is far from elucidated. The aim of this study was to investigate the association between gene associated with retinoid-interferon-induced mortality 19 (GRIM19) and asthenospermia. In this study, Grim19 knockout model (Grim19+/− mouse) was created through genome engineering. We showed that compared with WT mice, the sperm count and motility of Grim19+/− mice were significantly reduced. Grim19 may contribute to sperm count and vitality by influencing the mitochondrial membrane potential, intracellular reactive oxygen species production, and increasing cell apoptosis. The spermatogenic cells of all levels in the lumen of the seminiferous tubules were sparsely arranged, and the intercellular space became larger in the testis tissue of Grim19+/− mice. The serum testosterone concentration is significantly reduced in Grim19+/− mice. The expression of steroid synthesis-related proteins STAR, CYP11A1, and HSD3B was decreased in Grim19+/− mice. To further confirm whether changes in testosterone biosynthesis were due to Grim19 downregulation, we validated this result using Leydig cells and TM3 cells. We also found that Notch signaling pathway was involved in Grim19-mediated testosterone synthesis to some extent. In conclusion, we revealed a mechanism underlying Grim19 mediated spermatozoa motility and suggested that Grim19 affected the synthesis of testosterone and steroid hormones in male mouse partly through regulating Notch signal pathways.

Reproduction (2022) 163 365–377

Introduction

The incidence of male infertility is increasing annually and has become a global health problem. Abnormal sperm parameters such as oligospermia, asthenospermia, and teratozoospermia result in male factor infertility. The pathogenesis of male infertility is complex, studies have shown that the interaction between genes and the environment is an important cause of damage to male reproductive function, especially spermatogenesis (Lafferty et al. 2020). Previous studies have shown that mitochondria play an important role in human spermatozoa motility (Ferramosca et al. 2012). But the related pathogenesis is far from elucidated.

Gene associated with retinoid-interferon-induced mortality 19 (GRIM19) was originally identified as a critical regulatory protein for interferon-beta and retinoic acid-induced cell death. It has been positioned at human chromosome 19p13.2, and coded for a novel 16 kDa protein (Angell et al. 2000). GRIM19 is a functional subunit of mitochondrial respiratory chain complex I, which plays a role in cellular energy metabolism (Fearnley et al. 2001), and maintenance of mitochondrial membrane potential (MMP) (Lu & Cao 2008). GRIM19 is distributed in many tissues of normal human bodies (Kapoor 2013). Mitochondria’s role as an energy provider is surely fundamental for sperm motility. Therefore, GRIM19 might play a dual protein function involved in cell death and mitochondrial metabolism. GRIM19 might modulate sperm functions through mitochondria-dependent pathways. But so far, there are not many studies on the occurrence and mechanism of GRIM19 in male reproductive diseases, and we need further research to find out.

The primary function of the testes is to produce sperm and testosterone. The Leydig cells of the testis distributed between the seminiferous tubules are the main field of sex hormone synthesis in males and more than 95% of testosterone is synthesized and secreted by this cell (Tremblay 2015). Testosterone is the body’s major androgen. It is necessary for the completion of spermatogenesis and maintenance of secondary sexual characteristics (Chen et al. 2014). Disrupting testosterone production may affect male reproductive functionality.

Previous studies have shown that GRIM19 defects can cause the abnormality of the mitochondrion structure, morphology, and cellular distribution (Huang et al. 2004, Chao et al. 2015) and GRIM19 plays an important
role in human spermatozoa motility by affecting the mitochondrial function (Yang et al. 2017). Therefore, the aim of this study is to investigate the correlations with an association between low levels of GRIM19 and asthenospermia and to explain its mechanism.

Materials and methods

Animals

The Grim19 knockout model (Grim19+/- mouse, C57 BL/6) was created by CRISPR/Cas9-mediated genome engineering from Nanjing Institute of Biomedical Research, Nanjing University. Transcript ENSMUST00000110167.4 has five exons, with the ATG start codon at exon 2 and TAG stop codon at exon 5. Specific gRNAs in introns 2–3 and 3–4 was designed, which directed Cas9 endonuclease cleavage of the Grim19 gene and created a double-strand break. The breaks were repaired by nonhomologous end joining, and the Grim19 gene was disrupted by deletion of exon 3 (cagagaactccctcaggagctcagagaaagagagggagtggtgagc aggggttctgtgaggatgagggaactcctac). Grim19 homogeneous knockout mice (Grim19+/-) died in the early stage of embryonic development. The DNA samples extracted from the tails of all Grim19+/- mice participating in the experiment were subjected to agarose gel electrophoresis for genotype identification, the following primer sequences were used: forward: 5'-CCACCCCCAAGTGTAAAACTATC-3' and reverse: 5'-GCACAGGCGAGGAACTAC-3'.

Eight to ten-week-old Grim19+/- male mice and littermate male wildtype C57BL/6 mice (WT mice) were selected as the experimental group and the control group, respectively. All mice were raised under the same specific pathogen-free (SPF) conditions. The cage and feed were changed weekly to maintain a stable environment. All procedures were approved by the Animal Care and Use Committee of Shandong University (Shandong, China).

Sperm motility statistics and morphological observations

The testicular organ coefficient calculation: after the mice had been sacrificed by dislocating the cervical spine, mice were weighed, and the right and left sides of testicular tissues were collected to gently remove fat pads around the tissues before weighing. The testicular organ coefficient was calculated by using the following calculation: the testis weight (g)/body weight (g) of animal × 100 (g).

Sperm count: after the animals were sacrificed, the epididymides were immediately separated and placed in a warm culture dish containing 1 mL Hank’s balanced salt solution free of calcium and magnesium. The tissue was minced and placed in an incubator at 37°C for 15 min. The tissue was blown to make the sperm fully swim out, and after the sperm was evenly dispersed, 10 µL sperm was taken for the blood count board and counted the number of sperm in five squares to calculate sperm density.

Sperm motility measurement: the collected sperm was dropped on the glass slide of the wet preparation, after the sperm stopped being suspended (within 1 min), the following types of sperm (at least 200) are observed under a 200x phase-contrast microscope. We divided sperm into progressive motility (PR), non-progressive motility (NP), immotility (IM). The formula for sperm motility is as follows: progressive sperm%=PR/(PR+NP+IM) × 100% and total sperm motility%=(PR+NP)/(PR+NP+IM) × 100%.

Sperm morphology: the sperm morphology was observed by sperm morphology fast staining solution (Diff-Quik method) under a 1000x phase-contrast microscope.

Tissue collection and histological analysis

Testis and epididymis were dissected from Grim19-deficient and control mice immediately after death by dislocating the cervical spine, fixed in 4% (v/v) paraformaldehyde for up to 24 h, and embedded in paraffin. Five-micrometer-thick sections were cut and mounted on glass slides. After deparaffinization, the sections were stained with hematoxylin–eosin for histological analysis.

Detection of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was estimated by JC-1 kit (Beyotime, Haimen, China). Briefly, sperm were resuspended to a final cell concentration of 5 × 10⁶/mL and then incubated with an equal volume of JC-1 staining working solution (5 µmg/mL) at 37°C for 20 min. The stained samples were then analyzed by flow cytometry (CytoFLEX, Beckman, CA, USA). The JC-1 was excited by a 490 nm argon laser, and the results were recorded using green fluorescence (GRN-HLog) and red fluorescence (RED-HLog) channels. Forward and side scatter measurements were taken to generate a scatter plot, which was used to gate sperm cells and did not include dead cells and debris. At least 10,000 sperm were analyzed for each sample by CytExpert Flow Cytometry Software.

Detection of reactive oxygen species production

The level of reactive oxygen species (ROS) in the spermatozoa was quantified by Reactive Oxygen Species Assay Kit (DCFH-DA) (Beyotime) at a concentration of 10 µmol/L. Sperm at a density of 5 × 10⁶/mL were suspended in DCFH-DA and incubated in the dark for 30 min at 37°C. After washing three times with PBS, the stained samples were then analyzed by flow cytometry (CytoFLEX, Beckman). Forward and side scatter measurements were taken to generate a scatter plot, which was used to gate sperm cells and did not include dead cells and debris. At least 10,000 sperm were analyzed for each sample by FlowJo and CytExpert Flow Cytometry Software.

Detection of apoptosis

An Annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China) was used to detect the apoptosis of the spermatozoa samples. After rinsing with PBS, 5 × 10⁵ spermatozoa were suspended in 500 mL binding buffer. Then, 5 mL Annexin V-FITC was mixed with the suspension, and 5 mL propidium iodide was added. The samples were incubated

Reproduction (2022) 163 365–377

https://rep.bioscientifica.com
for 15 min at room temperature in the dark and then analyzed by flow cytometry (CytoFLEX, Beckman) equipped with an argon ion laser tuned at 488 nm wavelength. The physical parameters of forward and side light scattering were used to set the main gate to exclude dead cells and debris by CytExpert Flow Cytometry Software.

**Immunohistochemistry analysis**

Immunohistochemistry (IHC) analysis of tissues from at least four mice for each group was performed. The slides were deparaffinized in xylene for 15 min and repeated, dehydrated sequentially in graded alcohol from 100 to 75% (v/v) (5 min). The tissues were then immersed in sodium citrate antigen retrieval solution to retrieve (15 min boiling in a microwave at the medium power setting), and endogenous peroxidase activity was purged twice using a peroxidase inhibitor (sp9000, Zsbio) for 10 min at room temperature. The sections were washed with PBS and blocked with normal goat serum (sp9000, Zsbio) for 15 min at room temperature. Next, the tissues were incubated with anti-GRIM19 (Abcam, 1:200), polyclonal anti-CYP11A1 (Abcam, 1:200), and the polyclonal anti-HSD3B (Abcam, 1:200), overnight (about 12 h) at 4°C. The polyclonal antibody was replaced with PBS in the negative control. Then they were incubated at room temperature with biotin-labeled goat anti-mouse/rabbit IgG polymer (sp9000, Zsbio). After removing the secondary antibody, the sections were stained with DAB kit (ZLI-9018, Zsbio). All slides were counterstained with hematoxylin (Solarbio), dehydrated, and mounted in glycerol gelatine (Solarbio). Finally, observe the sample with an inverted microscope. One randomly selected section per mice was used for each analysis, and the proportion of positive cells in each area was counted in three randomly selected fields. The average of the three fields was used in the data analysis.

**Isolation and cultivation of Leydig cell**

Leydig cells were isolated from the testes of four 10-week-old male WT mice and four Grim19+/− mice. The testis was immediately separated, the tunica albuginea was removed, and collagenase digestion was then combined with Percoll (Solarbio, Beijing, China) density centrifugation to isolate Leydig cells (Hedger & Eddy 1986). Firstly, 8 mL 0.5 mg/mL type 1 collagenase (Gibco) was used to enzymatically digest Leydig cells at 37°C for 15 min. The collagenase activity was terminated by adding in DMEM-F12 (Gibco) containing 10% (v/v) fetal bovine serum (FBS; Gibco). The mixture was then filtered through a 75-µm filter and centrifuged at 400 g for 5 min to exclude seminiferous tubules. Next, the dispersed cells were washed with DMEM-F12 and layered over a Percoll gradient (70, 58, 30, and 15% (v/v)). The gradient was centrifuged for 30 min at 3000 g. Four concentration gradients of Percoll separation testicular cells can be centrifuged to produce three Layer cells, viewed from top to bottom, layer 1 (15–30%, 1.035 g/mL) was mainly composed of small round cells and cell debris, and layer 2 (30–58%, 1.076 g/mL) was composed of sperm and small fragments of interstitial cells, the third layer (58–70%, 1.085 g/mL) was mainly composed of Leydig cells. The Leydig cells were cultured in DMEM-F12 (Gibco) supplemented with 5% (v/v) fetal equine serum (Solarbio Life Sciences, Beijing, China), 2.5% (v/v) FBS (Gibco), and 1% (v/v) penicillin–streptomycin (Solarbio Life Sciences) at 37°C in a humidified atmosphere containing 5% CO2. The purity of the Leydig cells obtained was >80% as evaluated by the HSD3B staining method (Sharpe & Fraser 1983). T25 cell culture flasks are used for cell culture. When Leydig cells reached 85–90% confluency, the cells were digested with trypsin-EDTA solution (Solarbio, Beijing, China) and passaged with a new growth medium. During cell growth, the cells were replaced with a new growth medium every 48–60 h. Leydig cells were cultured for approximately 10 days and passaged two times before being used for subsequent experiments.

**TM3 cells culture and transfection**

Mouse testis Leydig cell line TM3 (ZQ-0100) was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. TM3 were cultured in DMEM-F12 (Gibco) supplemented with 5% (v/v) fetal equine serum (Solarbio Life Sciences), 2.5% (v/v) FBS (Gibco) and 1% (v/v) penicillin–streptomycin (Solarbio Life Sciences) at 37°C in a humidified atmosphere containing 5% CO2. T25 cell culture flask was used for routine cell culture. When the TM3 cells reached 85–90% confluency, we digested the cells and passaged them with a new growth medium at 48–60 h intervals.

Six-well plates were used when cells needed to be treated with reagent; 3 × 105 cells were seeded per well and were allowed to reach 70% confluence and were then incubated in serum-free DMEM-F12 for 12 h. Lipofectamine 2000 (Invitrogen) was used to knock down Grim19 mixed with siRNA (siRNA) (GenePharma, Shanghai, China), in accordance with the manufacturer’s instructions. Six microliter of lipofectamine 2000 was mixed with 119 µL DMEM-F12 concentrations and in parallel 2500 ng Grim19 siRNA was mixed gently with 125 µL DMEM-F12 incubated for 3 min at RT. Then we added siRNAs to diluted Lipofectamine 2000 and incubated the mixture for 20 min at RT to allow siRNA–lipid complexes to form. Later, the cells were transfected with Grim19 siRNA–lipid complexes as the Grim19 siRNA group and with negative control siRNA–lipid complexes as the negative control group. Similarly, in subsequent experiments, Notch-related pathway ligands siRNA were transfected, 6 µL lipofectamine 2000 was mixed with 119 µL DMEM-F12 concentrations, and in parallel 2500 ng Grim19 siRNA was mixed gently with 125 µL DMEM-F12 incubated for 5 min at RT. Then we added siRNAs to diluted Lipofectamine 2000 and incubated the mixture for 20 min at RT to allow siRNA–lipid complexes to form. Later, the cells were transfected with Grim19 siRNA–lipid complexes as the Grim19 siRNA group and with negative control siRNA–lipid complexes as the negative control group. Similarly, in subsequent experiments, Notch-related pathway ligands siRNA were transfected, 6 µL lipofectamine 2000 was mixed with 119 µL DMEM-F12 concentrations, while 2500 ng siRNA was mixed gently with 125 µL DMEM-F12. After 48 h of incubation, cells were used for RNA extraction, and after 72 h of incubation, they were used for western blotting. The following sequences were used in Table 1.

**ELISA detection of hormone levels**

The mouse serum testosterone was measured with a mouse testosterone ELISA kit (BlueGene, Shanghai). The blood of the mouse was taken out of the eyeball and centrifuged at 1600 g for 20 min to obtain the serum. In addition, the supernatant of cells transfected with Grim19 for 48 h was collected as the sample and the testosterone hormone level in the cell supernatant was analyzed. Ten microliter balance solution was dispensed into 100 µL samples and mixed well. One
Table 1 Primers sequences used for siRNA.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>5’-UUUCUCCGAGCGUGACGUTT-3’</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5’-CACUCAGAULUGUUGCGAATTT-3’</td>
</tr>
<tr>
<td>Grim19</td>
<td>5’-GGAAUGGACCGAUCGUATT-3’</td>
</tr>
<tr>
<td>Notch4</td>
<td>5’-GGCCACUCUGUGAGAAGAATT-3’</td>
</tr>
<tr>
<td>Jagged1</td>
<td>5’-GCCAGACCGAUAUGCGAATT-3’</td>
</tr>
<tr>
<td>Jagged2</td>
<td>5’-GCCGCGGAGGGAGAUCUATT-3’</td>
</tr>
<tr>
<td>Delta3</td>
<td>5’-GCCGCGGAGGGAGAUCUATT-3’</td>
</tr>
<tr>
<td>Delta4</td>
<td>5’-GCCGACAGUUGCGAUAUGCGAATTT-3’</td>
</tr>
</tbody>
</table>

hundred microliter standards and 110 µL samples were added to the wells and 100 µL of PBS (pH 7.0-7.2) was added in the blank control well. After which 100 µL horseradish peroxidase (HRP)-labeled detection antibody was added separately in each well (not blank control well), the contents of the wells were thoroughly mixed by swirling gently and incubated at 37°C for 60 min. After washing the microtiter plate with 1x cleaning solution and repeating this process five times, 50 µL substrate A and 50 µL substrate B were added to each well including blank control well and incubated for 15 min at 37°C in the dark. The reactions were terminated by adding 50 µL stop solution to each well including blank control well. The optical density (OD) was determined at 450 nm using a microplate reader immediately. The standard curve was used to determine the concentration of samples. Averaged the duplicate readings for each standard and sample and all OD values were subtracted by the mean value of blank control before result interpretation. A standard curve was constructed by plotting the concentration on the horizontal (x) axis against the average OD for each standard on the vertical (y) axis and a best fit curve was drawn using statistical software to generate a four parameter logistic (4-PL) curve-fit or logit-log linear regression curve. The concentration of samples corresponding to the mean absorbance from the standard curve was calculated.

**RNA extraction and quantitative RT-PCR**

Twenty milligrams of testicular tissue were ground in a mortar with a pestle in liquid nitrogen and then 200 µL RIPA lysis buffer (Beyotime, Shanghai, China) was added and incubated on ice for 30 min. Similarly, every 2.5 x 10⁶ cells were added 200 µL RIPA lysis buffer. The mixture was centrifuged at 13,858 g for 25 min at 4°C. The supernatant was obtained to quantify protein concentration with BCA Protein Assay Kit (Solarbio, Beijing, China). Samples were adjusted to the same protein concentration by adding 5x SDS-PAGE protein sample buffer and RIPA, and then incubated in a 98°C metal bath for 10 min. Then, the proteins were separated in 12% SDS-PAGE gels in 1x Tris-Glycine running buffer (T1070, Solabio, Beijing) at a constant pressure of 80 V for 1 h and 120 V for 1 h. The proteins were transferred onto PVDF membranes in 1x rapid transfer buffer (WB4600, NcmBlot, Suzhou, China) at a constant current of 400 mA for 25 min. The membrane was blocked with fast blocking buffer (P30500, NcmBlot, Suzhou, China) for 10 min at room temperature, and subsequently incubated with primary antibodies against the following proteins: anti-GRIM19 (Abcam, 1:1000), anti-STAR (Cell Signaling Technology, 1:1000), anti-CYP11A1 (Abcam, 1:1000), anti-HSD3B (Abcam, 1:1000), anti-GAPDH (Abcam, 1:1000) overnight at 4°C. After washing with Tris-buffered saline Tween-20 (TBST), the membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 h (Zhongshan Golden Bridge Biotechnology Co., Ltd, China). Immunoreactivity was determined using an ECL system. ImageJ software was used to detect the grayscale value of bands for calculating the relative expression.

**Protein extraction and western blotting**

Twenty milligrams of testicular tissue were ground in a mortar with a pestle in liquid nitrogen and then 200 µL RIPA lysis buffer (Beyotime, Shanghai, China) was added and incubated on ice for 30 min. Similarly, every 2.5 x 10⁶ cells were added 200 µL RIPA lysis buffer. The mixture was centrifuged at 13,858 g for 25 min at 4°C. The supernatant was obtained to quantify protein concentration with BCA Protein Assay Kit (Solarbio, Beijing, China). Samples were adjusted to the same protein concentration by adding 5x SDS-PAGE protein sample buffer and RIPA, and then incubated in a 98°C metal bath for 10 min. Then, the proteins were separated in 12% SDS-PAGE gels in 1x Tris-Glycine running buffer (T1070, Solabio, Beijing) at a constant pressure of 80 V for 1 h and 120 V for 1 h. The proteins were transferred onto PVDF membranes in 1x rapid transfer buffer (WB4600, NcmBlot, Suzhou, China) at a constant current of 400 mA for 25 min. The membrane was blocked with fast blocking buffer (P30500, NcmBlot, Suzhou, China) for 10 min at room temperature, and subsequently incubated with primary antibodies against the following proteins: anti-GRIM19 (Abcam, 1:1000), anti-STAR (Cell Signaling Technology, 1:1000), anti-CYP11A1 (Abcam, 1:1000), anti-HSD3B (Abcam, 1:1000), anti-GAPDH (Abcam, 1:1000) overnight at 4°C. After washing with Tris-buffered saline Tween-20 (TBST), the membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 h (Zhongshan Golden Bridge Biotechnology Co., Ltd, China). Immunoreactivity was determined using an ECL system. ImageJ software was used to detect the grayscale value of bands for calculating the relative expression.

**Statistical analysis**

The experimental data were statistically analyzed using the Student's t-test in GraphPad Prism software (ver. 9.1.0). Data were presented as mean ± s.d. A P-value <0.05 was considered statistically significant (NS, no significance).
Table 2 Primers sequences used for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>5’-CATGTAAGTTGCTATCCAGAC-3’</td>
<td>5’-CTCTTTATGCAAGCCGAT-3’</td>
</tr>
<tr>
<td>Grim19</td>
<td>5’-GAACCCCTTGACATCAGGGTAC-3’</td>
<td>5’-ATCTGAAAGGTTCCCTCCTGG-3’</td>
</tr>
<tr>
<td>Star</td>
<td>5’-AGAGGTGTGCTGCTGGAGA-3’</td>
<td>5’-TCTTGGACCTCTGATGAC-3’</td>
</tr>
<tr>
<td>Cyp11a1</td>
<td>5’-TACATATTGACCCACACAA-3’</td>
<td>5’-TACTGAGGATACATGAC-3’</td>
</tr>
<tr>
<td>Hsd17b</td>
<td>5’-TTTCGCTCAGATCCGTTGG-3’</td>
<td>5’-TTGCTACAAACAGCAGGT-3’</td>
</tr>
<tr>
<td>Hsd3b</td>
<td>5’-CTCTACTGCTGCTCCCTTCT-3’</td>
<td>5’-TCCCTCCCAGGTACATCC-3’</td>
</tr>
<tr>
<td>Notch1</td>
<td>5’-TGTTGCCTCTTCTACTGCG-3’</td>
<td>5’-TGTTGCCTTCCAGACCATCAG-3’</td>
</tr>
<tr>
<td>Notch2</td>
<td>5’-TGGAGGACTGTGCTGAGGA-3’</td>
<td>5’-GGAGACCCCTGAACTTACG-3’</td>
</tr>
<tr>
<td>Notch3</td>
<td>5’-TTCCTCCCAGATCCGTTGG-3’</td>
<td>5’-TCCCTCCCAGTACACATCAG-3’</td>
</tr>
<tr>
<td>Notch4</td>
<td>5’-CATACCCCAGATCAGTGAC-3’</td>
<td>5’-GAACCCCTGACATCAGG-3’</td>
</tr>
<tr>
<td>Jagged1</td>
<td>5’-GGAGATTAGGAGACGGACTC-3’</td>
<td>5’-GGAGACCCCTGAACTTACG-3’</td>
</tr>
<tr>
<td>Jagged2</td>
<td>5’-TGTTGAGTGTGCTGAGGA-3’</td>
<td>5’-GGAGACCCCTGAACTTACG-3’</td>
</tr>
<tr>
<td>Delta1</td>
<td>5’-GTAGAGGACTGTGCTGAGGA-3’</td>
<td>5’-GGAGACCCCTGAACTTACG-3’</td>
</tr>
<tr>
<td>Delta2</td>
<td>5’-GCAGGACTGTGCTGAGGA-3’</td>
<td>5’-GGAGACCCCTGAACTTACG-3’</td>
</tr>
<tr>
<td>Delta3</td>
<td>5’-GGGACTGTGCTGAGGA-3’</td>
<td>5’-GGAGACCCCTGAACTTACG-3’</td>
</tr>
<tr>
<td>Delta4</td>
<td>5’-GGGACTGTGCTGAGGA-3’</td>
<td>5’-GGAGACCCCTGAACTTACG-3’</td>
</tr>
</tbody>
</table>

Figure 1 The effect of Grim19 expression level on mouse sperm quantity and testicular structure. (A) The difference in testicular shape and weight between WT mice (n = 4) and the Grim19+/− mice (n = 4). (B) The difference in sperm count, progressive sperm motility, and total sperm motility between WT mice (n = 4) and the Grim19+/− mice (n = 4). (C) Observation of sperm morphology between WT mice (n = 4) and the Grim19+/− mice (n = 4). Scale bar = 25 μm. (D) Tests tissue structure changes between WT mice (n = 4) and Grim19+/− mice (n = 4) using HE staining method. Scale bar = 50 μm. (E) Epididymal structure changes between WT mice (n = 4) and Grim19+/− mice (n = 4) using HE staining method. Scale bar = 50 μm. (F) The MMP was estimated by JC-1 kit. The MMP was estimated by JC-1 kit. In the dot plot, the cells with a high MMP were observed in the upper right quadrant (Q1), and the cells with a low MMP were identified in the lower right quadrant (Q4). The percentage of sperm relative membrane potential in Grim19+/− mice (n = 4) were significantly lower than that in WT mice (n = 4). (G) The level of ROS in the spermatozoa was quantified by DCFH-DA. Flow cytometric analysis showed that there was a considerable increase in the intracellular ROS production in Grim19+/− mice (n = 4) compared with WT mice (n = 4). (H) Annexin V-FITC was used to detect the apoptosis of the spermatozoa samples. The flow cytograms are shown that the numbers in the upper right quadrant (Q1), upper left quadrant (Q2), lower left quadrant (Q3) and lower right quadrant (Q4) represent the relative number of late apoptosis, damage, survival, early apoptotic cells. Grim19+/− mice (n = 4) had significantly more apoptotic cells (Q1+Q4) than in WT mice (n = 4). MMP, mitochondrial membrane potential; NS, no significance; ROS, reactive oxygen species.
Results

The sperm count and motility of Grim19+/- mice was reduced, and the testicular structure was abnormal

Compared with WT mice, there was no difference in relative testicular shape and weight in Grim19+/- mice (Fig. 1A). To assess the effect of Grim19 expression on sperm quality, sperm was collected from WT and Grim19+/- mice for sperm counting and viability analysis. Sperm count of Grim19+/- mice was significantly reduced compared with WT mice (P = 0.0191). The progressive motility of sperm (P < 0.0001) and total motility (P = 0.0397) in the Grim19+/- mice were lower than those of WT mice (Fig. 1B). Using Diff-Quik method to detect mouse sperm morphology, the morphology of sperm exhibited no clear differences between the Grim19+/- and WT mice (Fig. 1C). Further tests histology analysis showed that the structure of the seminiferous tubules in the testis tissue of the Grim19+/- mice was complete, the spermatogenic cells of all levels in the lumens of the seminiferous tubules were sparsely arranged, and the intercellular space became larger (Fig. 1D). The lumens of the mouse epididymis tissue were arranged tightly, and the cell morphology was normal (Fig. 1E). The above studies indicate that Grim19 deficiency may affect testis structure and sperm quality, and its mechanism needs to be further explored.

In Grim19+/- mice sperm, intracellular MMP were decreased, ROS production and apoptosis were increased

Lack of GRIM19 may lead to the collapse of MMP and increased apoptosis, which was found in previous studies (Chen et al. 2015). Therefore, the levels of MMP, ROS, and apoptosis of mouse sperm were evaluated. JC-1 was used to estimate the MMP of the spermatozoa. Flow cytometric analysis showed that the percentage of sperm with ratio of red to green in Grim19+/- mice group were significantly lower than that in WT mice, suggesting that the spermatozoa in Grim19+/- mice had a lower MMP compared with WT mice (P = 0.0015) (Fig. 1F). Mitochondria are the main source of ROS produced by sperm, especially through the formation of superoxide in the electron transfer chain. Compared with WT mice, Grim19+/- mice had increased reactive oxygen content as shown in Fig. 1G (P = 0.0047). We tested the effect of Grim19 gene knockout on mouse sperm apoptosis. Flow cytometric analyses of Annexin V/PI in Grim19+/- mice and the controls were presented in Fig. 1H. Significantly more apoptotic cells were found in Grim19+/- mice than in WT mice (P = 0.0258). These results indicate that Grim19 may participate in the impact on sperm count and vitality by influencing the MMP, intracellular ROS production, and increasing cell apoptosis.

The expression of testosteronde and steroid synthesis-related proteins was decreased in Grim19+/- mice

The expression and localization of GRIM19 in the testis was detected, the results of immunohistochemical staining showed that GRIM19 was mainly distributed in the testicular seminiferous tubule-interstitium (Fig. 2A). To explore whether the testosterone biosynthesis in Grim19-deficient mice was defective, the expression of key steroidogenic enzymes was examined by IHC. HSD3B and CYP11A1 protein was detected in the interstitium of the testis. The levels of CYP11A1 (P < 0.0001) and HSD3B (P = 0.0007) were reduced in the testis of Grim19+/- mice (Fig. 2B and C). The level of testosterone hormone in mice serum was detected by ELISA. Compared with the WT mice, the level of testosterone hormone in mice serum of the Grim19+/- mice was reduced (P = 0.0104) (Fig. 2D). The batch variation coefficient (CV) of ELISA between the Grim19+/- mice and the WT mice are 1.74 and 2.85%. Then, we verified this idea by quantitative RT-PCR and western blot. As shown in Fig. 2E, the expression of Grim19 (P = 0.0929), Star (P = 0.0006), Cyp11a1 (P = 0.0131), and Hsd3b (P = 0.0002) mRNA was reduced in Grim19 deficient testes compared with the control group and Hsd17b expression was not statistically significant. The protein expression of key steroidogenic proteins such as STAR (P = 0.0009), CYP11A1 (P = 0.0246), and HSD3B (P = 0.0128) was reduced in Grim19-deficient testes (P = 0.0003) (Fig. 2F and G).

The expression of steroid synthesis-related proteins was decreased in Grim19+/- mice Leydig cells

It has been verified that the expression of steroid synthesis-related proteins in the testes of Grim19+/- mice is reduced. To further confirm the effect of the loss of Grim19 expression on the main endocrine functions of Leydig cells, we isolated and cultured Leydig cells in vitro. The cells were identified by HSD3B staining, and Leydig cells were stained blue-black. The purity of the obtained Leydig cells >80% (Fig. 3A). To further determine the expression of steroid synthesis-related proteins in mice Leydig cells. The quantitative RT-PCR results were shown in Fig. 3B, compared with the WT mice, the mRNA expression of Grim19 (P = 0.0023), Star (P = 0.0313), Cyp11a1 (P = 0.0194), and Hsd3b (P = 0.0742) in the Grim19+/- mice was reduced and Hsd17b expression was not statistically significant. The results of western blot detection showed that the expression of testicular biosynthesis proteins such as STAR (P = 0.0023), CYP11A1 (P = 0.0087), and HSD3B (P = 0.0073) was lower in the Grim19-deficient Leydig cells (P = 0.0097) than that of the control group (Fig. 3C and D).
Figure 2 The localization of GRIM19 in testis and the expression of testosterone and steroid synthesis-related proteins in mice testis. (A) IHC staining showed that GRIM19 was mainly distributed in the interstitium of the seminiferous tubules of the testis. Scale bar = 200 μm (50 μm). (B) (C) The expression of CYP11A1 and HSD3B in testis tissue of WT mice (n = 4) and Grim19+/− mice (n = 4) by IHC staining. Scale bar = 120 μm (50 μm). (D) The level of testosterone hormone in mice serum in the Grim19+/− mice (n = 4) and the WT mice (n = 4) by ELISA. (E) The expression of Grim19, Star, Cyp11a1, Hsd3b, and Hsd17b mRNA using RT-RCR in testis of WT mice (n = 4) and Grim19+/− mice (n = 4). (F) (G) The protein expression of GRIM19, STAR, CYP11A1, and HSD3B in testis of WT mice (n = 4) and the Grim19+/− mice (n = 4) by western blotting. All experiments were repeated three times. IHC, immunohistochemistry; NS, no significance.
Downregulation of Grim19 expression in TM3 cells resulted in decreased expression of testosterone and steroid synthesis-related proteins

To further verify the in vivo experimental results that Grim19 affects the reduction of testosterone synthesis in mouse testes, we used TM3 mouse testicular stromal cells to construct an in vitro model of Grim19 differentially expressed cells. The results confirmed that Grim19 influences testosterone synthesis in TM3 cells. The regulation was consistent with the observations in vivo. The expression of Grim19 was successively downregulated by transfection of siRNA in TM3 cells (P = 0.0065) (Fig. 3E). The level of testosterone hormone in the transfected cell supernatant was detected by ELISA. Compared with the control group, the level of testosterone hormone in the cell supernatant of the experimental group was reduced (P = 0.0197) (Fig. 3F).
The ELISA batch variation coefficients (CV) of the Grim19 siRNA group and the control group were 1.93 and 2.52%. It may be due to a small number of cells and too much medium, resulting in low testosterone concentration in the cell supernatant and small differences between groups. As shown in Fig. 3G compared with the control group, the mRNA expression of Star (P = 0.0189), Cyp11a1 (P = 0.0011), and Hsd3b (P = 0.0004) in the Grim19 siRNA group was reduced and Hsd17b expression was not statistically significant. Detected by western blot, the expression of such as STAR (P = 0.0421), CYP11A1 (P = 0.0092), and HSD3B (P = 0.0482) was lower in the Grim19 siRNA group than that of the control group (Fig. 3H and I).

**NOTCH signaling pathway was involved in Grim19-mediated testosterone synthesis in TM3**

Notch signaling in Leydig cells is indirectly related to gonocyte fate and germ cell development. In this study, we detected the effect of downregulation of Grim19 expression on Notch signaling pathway-related ligands and receptors. First, we found that while downregulating the expression of Grim19, the expression of Notch signaling related molecular ligands mRNA such as Notch4 (P < 0.0001), Delta3 (P = 0.0029), Delta4 (P < 0.0001), Jagged1 (P = 0.0006), and Jagged2 (P = 0.0057) was reduced in Fig. 4A. To further explore which Notch receptor regulates the expression of key enzymes for steroid synthesis, we transfected Notch4, Delta3, Delta4, Jagged1, and Jagged2 siRNA to TM3 cells respectively. The quantitative RT-PCR results were shown in Fig. 4B, compared with the control group, the mRNA expression of Cyp11a1 (P = 0.0017) and Hsd3b (P < 0.0001) in the Delta3 siRNA group (P = 0.0396) was reduced and the mRNA expression of Star was not statistically significant. The results of western blot detection showed that the expression of testicular biosynthesis proteins such as CYP11A1 (P = 0.0002) and HSD3B (P < 0.0001) was lower in the Delta3 siRNA group than that of the control group while there was no statistical difference in the expression of STAR (Fig. 4C). As shown in the Fig. 4D, the mRNA expression of Star (P = 0.0002), Cyp11a1 (P = 0.0011), and Hsd3b (P = 0.0458) in the Delta4 siRNA group (P = 0.0086) was reduced compared with the control group. Detected by western blot, the expression of such as STAR (P = 0.0039), CYP11A1 (P = 0.0495), and HSD3B (P = 0.005) was lower in the Delta4 siRNA group than those in the control group (Fig. 4E). After Notch4, Jagged1, and Jagged2 siRNA were transfected into TM3 cells, the detection results were not statistically significant. The above results indicate that Delta3 and Delta4 ligands of the Notch pathway are involved in the process of Grim19 regulating testosterone synthesis at the transcription and translation levels, respectively, but the specific mechanism needs further study.

**Discussion**

Mitochondria is an organelle that participates in the process of apoptosis and is the control center of cell death. As the energy supply center of sperm, mitochondria are closely related to sperm motility. The decrease of mitochondrial metabolism is also one of the main mechanisms of asthenospermia. The mitochondria in sperm are mainly located in the middle of the sperm flagella, and the ATP produced by them provides energy support for sperm activities. The specific factors that can lead to the weakening of mitochondrial function are very complicated, mainly including the decrease of respiratory chain complex activity, the decrease of MMP, the change of mtDNA, etc., and the three can influence each other. The normal MMP is a prerequisite for maintaining oxidative phosphorylation to generate ATP (Durairajanayagam et al. 2021). MMP has been used as an indicator to evaluate male reproductive function (Aitken & Drevet 2020). Mitochondria are also the main source of oxygen free radicals in the body. ROS are by-products of oxidative phosphorylation and are necessary for sperm to maintain normal function. Essentially, ROS are involved in mediating several key processes, such as sperm tyrosine phosphorylation, cholesterol exclusion, hyperactivation, and sperm-egg interactions (Alamo et al. 2020). High concentration of ROS is very easy to oxidize the mitochondrial inner membrane, DNA, and other ultrastructures, resulting in loss of mitochondrial function, and ultimately cell necrosis or apoptosis.

GRIM19 has a complicated mechanism of action. It not only participates in the function of the respiratory chain of cell mitochondria but also has the effect of initiating apoptosis (Sun et al. 2009, Sun et al. 2010, Tripathy et al. 2010, Tammineni et al. 2013). GRIM19 is essential for the maintenance of MMP. Lu and Cao (2008) believed that the C-terminal region of GRIM19 is necessary to maintain the transmembrane potential, and the complete transmembrane potential can protect cells from apoptosis. Impaired mitochondrial function and decreased sperm motility should be the result of multiple factors and pointed out the direction for the diagnosis and treatment of asthenospermia.

Previous research confirmed that the expression of GRIM19 was significantly lower in the spermatozoa of men with asthenozoospermia (Yang et al. 2017), we used animal experiments to explored its mechanism, which further proves the correlation between the level of Grim19 and an occurrence of asthenozoospermia. Therefore, we concluded that Grim19 may participate in the impact on sperm count and vitality by influencing the MMP, intracellular ROS production, and increasing cell apoptosis.

In testis, the synthesis of steroid hormones by Leydig cells is synthesized through the synergistic action of a carrier protein and a steroid-producing
enzyme cascade. The abnormal expression of any of these steroidogenic enzymes may lead to testosterone synthesis disorders (Lin et al. 1995, Colvin et al. 2001, Aghazadeh et al. 2015). Cholesterol is transported by STAR protein into the inner mitochondria where it is converted to pregnenolone by Cytochrome P450 Cholesterol Side Chain Lyase (P450scc, also called CYP11A1). Pregnenolone is further catalyzed to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B) and then to 17α-hydroxyprogesterone and androstenedione. Finally, 17α-hydroxyprogesterone is converted to testosterone by 17β-hydroxysteroid dehydrogenase (HSD17B) (Payne & Hales 2004, Miller & Auchus 2011). In addition to the classical pathway Cyclic AMP (c-AMP)/Protein Kinase A (PKA) pathway (Ho et al. 2016), some signaling pathways (Tai & Ascoli 2011, Li et al. 2020), cytokines (Dubre & Tremblay 2007, Lai et al. 2014), transcription factors (Martin & Tremblay 2010, Tremblay 2015) and ncRNA (Liu et al. 2014) can also be regulated synthesis of testosterone. But so far, the targets of various signaling pathways, the target genes of transcription factors, the interaction between transcription factors, and their post-translational modification mechanisms are still unclear and need further study.

In the present study, we found that the expression of key steroidogenic proteins such as STAR, CYP11A1, and HSD3B in Leydig cells was reduced in Grim19-deficient testes. This directly leads to a significant drop in serum testosterone levels. However, the expression level of...
GRIM-19 affects sperm and testosterone

Hsd17b mRNA is not significantly different from that of the control group, indicating that Grim19 cannot change the synthesis of testosterone by affecting the expression level of Hsd17b mRNA.

Notch is a highly evolutionarily conserved signaling pathway that consists of four Notch receptors (Notch 1–4) and five types of Notch ligands (Delta1, Delta3, Delta4, Jagged1, and Jagged2) in mammals (Grandbarbe et al. 2007, Maier 2019). When binding occurs between the transmembrane Notch receptor and the relative ligand expressed on neighboring cells, the Notch intracellular domain is cleaved by a γ-secretase, and Notch signaling is activated (Yuan et al. 2015). Notch signaling in Leydig cells is indirectly related to gonocyte fate and germ cell development (Tang et al. 2008). In this study, we used TM3 cells, a mice adult Leydig cell line, to study the effect of downregulation of Grim 19 expression on Notch signaling pathway-related ligands and receptors. In the experiment, we found that while downregulating the expression of Grim 19, the expression of steroid synthesis protein decreased, and the related ligands Notch4, Delta3, Delta4, Jagged1, and Jagged 2 of the Notch signaling pathway also changed. We guessed that the related ligands of the Notch pathway were mediated the process of Grim 19’s influence on steroid synthesis protein. To further explore which Notch receptor regulates the expression of key steroidogenic enzymes, we transfected the above-mentioned ligand siRNA and found that Delta3 and Delta4 influenced the expression of steroid synthesis protein. Although the results are not statistically significant, the expression of STAR is slightly increased at the transcription level. But at the translation level, the expression of STAR is slightly downregulated, which is the opposite of the expression at the transcription level. Studies have found that post-transcriptional regulatory events (such as micro RNA, alternative splicing, and polyadenylation) and post-translational modifications (phosphorylation, ubiquitination, and acetylation) involved in regulating gene expression in mammalian played an important role in testicular development and sperm maturity (Licatalosi 2016, Liao et al. 2018, Santos et al. 2020). Therefore, this difference in the expression patterns of testosterone synthesis genes at the transcription and translation levels may be caused by strict regulation of post-transcriptional and post-translational events. However, there were limitations to this study. No experiments were conducted to dissect the upstream signaling pathways that mediated the Grim 19 downregulation of the Notch signaling pathway. Moreover, the downstream effectors mediating the action of Notch on steroidogenic gene expression remain unclear. Thus, further study is warranted to resolve these issues.

In the seminiferous epithelium, spermatogonial stem cells will constantly renew themselves to maintain the number of spermatogonial stem cells throughout the life process, and they will further differentiate to produce many spermatogenic cells. Studies have found that Notch signals in Sertoli cells regulate the proliferation and differentiation of spermatogenic cells by regulating the expression of glial cell line-derived neurotrophic factor and cytochrome P450 family 26, subfamily b, polypeptide 1 (cytochrome P450, family 26, subfamily b, polypeptide 1, and CYP26B1), thereby maintaining the homeostasis of spermatogonial stem cells (Marei et al. 2014). In addition, a research reported that Notch signaling controls germ cell fate, identity, meiosis, and differentiation of spermatids (Murta et al. 2014). The impact of Grim19+/- mouse sperm quality and quantity may be related to the decrease in testosterone synthesis caused by impaired Notch signaling in Leydig cells and it may also control the number of spermatogenic cells by affecting the Notch pathway in testicular supporting cells. It may involve the balance between self-renewal and differentiation of spermatogonial stem cells, but this requires further experiments to explore.

Conclusion

In conclusion, Grim19 can affect sperm count and motility through sperm MMP, ROS, and apoptosis level. Grim19 may regulate the synthesis of testosterone and steroid hormones through the Notch signaling pathway to a certain extent.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Natural Science Foundation of China (grant numbers 82071620, 81571511 and 81701528), the Shandong Provincial Key Research and Development Project (grant number 2019GSF107004).

Author contribution statement

Y Z and L C conceived and wrote the paper. Y Z performed the experiments and analyzed the data. Y Z, H R L, W Q H, Y Y and L C reviewed and approved the draft. L C obtained ethical approval. Y Z and L C approved the final draft.

Acknowledgements

The authors thank the Key Laboratory of Gynecologic Oncology (Shandong Province, Jinan, China) for providing laboratory equipment. The authors are sincerely grateful to all the patients participating in this study, and we thank all mice sacrificed in our study.
References


Aitken RJ & Drevet JR 2020 The importance of oxidative stress in determining the functionality of mammalian spermatogonia: a two-edged sword. Antioxidants 9 111. (https://doi.org/10.3390/antiox9020111)


Licitatoros DD 2016 Roles of RNA-binding proteins and post-transcriptional regulation in driving male germ cell development in the mouse. Advances in Experimental Medicine and Biology 907 123–151. (https://doi.org/10.1007/978-3-319-29073-7_6)


Sharpe RM & Fraser HM 1983 The role of LH in regulation of Leydig cell responsiveness to an LHRR agonist. Molecular and Cellular Endocrinology 33 131–146. (https://doi.org/10.1016/0303-7207(83)90162-4)


Tai P & Ascoli M 2011 Reactive oxygen species (ROS) play a critical role in the cAMP-induced activation of Ras and the phosphorylation of ERK1/2 in Leydig cells. Molecular Endocrinology 25 885–893. (https://doi.org/10.1210/me.2010-0049)


https://rep.bioscientifica.com


Received 1 October 2021
First decision 28 October 2021
Revised manuscript received 28 February 2022
Accepted 21 March 2022