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Stromal interaction molecule 1 is required for neonatal testicular development in mice

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ABSTRACT

Stromal interaction molecule 1 (STIM1) is a transmembrane endoplasmic reticulum protein, and it serves as a Ca^{2+} sensor and activator of store-operated Ca^{2+} entry (SOCE). We have previously identified STIM1 in the proteome profile of mice neonatal testes, revealing STIM1 to be associated with neonatal testicular development. Here, to further explore the location and function of STIM1 in mice testes, we studied the effect of *Stim1* gene knockdown on neonatal testicular development by testicular culture. Our results revealed that STIM1 was primarily located in Sertoli cells. Knockdown of *Stim1* gene using morpholino in neonatal testis caused the mislocation of Sertoli cells and loss of germ cells, which were associated with the aberrant reactive oxygen species (ROS) activation, while inhibition of ROS could partly rescue the phenotypes caused by *Stim1* gene knockdown. In conclusion, our study suggests that STIM1 can maintain neonatal testicular development by inhibiting ROS activation.

1. Introduction

Spermatogenesis involves a complex series of cellular changes leading to the formation of haploid spermatozoa, including mitotic, meiotic and post-meiotic phases [1]. Sertoli cells play a central role in the development of a functional testis. Sertoli cells are the first cells to differentiate recognizably in the indifferent fetal gonad, an event which enables seminiferous cord formation, and, during early postnatal development, Sertoli cells continue to proliferate, differentiate, and mature as nurse-like cells [2]. Successful spermatogenesis relies on these early events in Sertoli cells because abnormal Sertoli cell proliferation or differentiation can disrupt fertility [3,4].

Stromal interaction molecule 1 (STIM1), is a highly conserved type-I membrane, ER-resident protein, and has been well known containing a luminal EF-hand Ca^{2+}-binding domain and several cytosolic protein-protein interaction domains, and it acts as a Ca^{2+} sensor and mediates store-operated Ca^{2+} entry(SOCE) and

Abbreviations: STIM1, Stromal interaction molecule 1; SOCE, store-operated $Ca2^{+}$ entry; ROS, reactive oxygen species; Mo, morpholino oligomer; SSCs, Spermatogonial Stem cells; SOX9, SRY-box 9; GATA4, GATA binding protein 4; DDX4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; LIN28, lin-28 homolog; TUNEL, terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick-end labeling; Wnt, Wingless-related MMTV integration site.

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oxidative stress [5–8]. Global deletion of *Stim1* gene in mice is lethal, indicating that STIM1 is indispensable in the organismal physiology of mammals [9]. In our previous study, we found that STIM1 was predominantly expressed in the cytoplasm of Sertoli cells in fetal mice testis, and knockdown of *Stim1* gene in fetal testis caused severe disruption in testicular cord development, which was associated with the aberrant oxidative stress [10]. We also successfully identified STIM1 in the proteome profile for neonatal mice testis in our previous research [11], and the bioinformatics analysis showed that STIM1 may play a vital role in testicular development. In the present study, we aimed to study the distribution of STIM1 in mice neonatal testis, and study the effects of its knockdown *in vitro*.

2. Materials and methods

2.1. Animals

Pregnant ICR mice were maintained in a controlled environment under a 12/12-h light/dark cycle at 20–22 °C and 50–70% humidity with food and water available *ad libitum*. All experiments on mice were approved by the Animal Ethics Committee of Nanjing Medical University.

2.2. Western blot analysis

Western blot analysis was performed as described previously, with minor modifications [12]. Briefly, testis lysates were separated by electrophoresis, then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, USA). The membranes were then blocked in 5% non-fat milk and incubated overnight with the indicated primary antibodies (Supplementary Table S1), washed, and incubated at room temperature for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (Thermo Scientific, Waltham, USA). The protein signals were then visualized by SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Waltham, USA).

2.3. Immunofluorescence

Mouse testis tissues with 0.5-day, 7.5-day and explants were fixed in 4% (*w/v*) paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA). The tissues were then paraffin embedded and sliced into 5-μm thick sections. Sections were deparaffinized and rehydrated in a graded ethanol series. After antigen retrieval in sodium citrate buffer (pH6.0), the sections were blocked with 5% bovine serum albumin (BSA) (*w/v*) (Sunshine, Nanjing, China) and incubated overnight at 4 °C with primary antibodies (Supplementary Table S1). After washing with phosphate-buffered saline-Tween, the sections were incubated with Alexa-Fluor secondary antibodies (Thermo Scientific, Waltham, USA), and images were captured under a confocal laser microscope (Zeiss LSM710, Carl Zeiss, Oberkochen, Germany).

2.4. Neonatal testis culture

Testes from 3.5-day mice were decapsulated and gently cut into several 1- to 3-mm-diameter pieces. Testis explants were cultured as previously reported [13]. Briefly, 1.5% (*w/v*) agarose gel stands ($10 \times 10 \times 5\ mm^3$ in size and placed in 24-well plates) were prepared one day before the testis culture and incubated with culture medium for more than 24 h. The amount of medium was adjusted so it would come up to half to four-fifths of the height of the agarose gel. Medium was changed every two days. Then, 3–5 testes explants were placed on the medium/air interface of each stand and incubated at 34 °C and 5% CO_2. The culture media contained α-Minimum Essential Medium (α-MEM), 10% KnockOut Serum Replacement (KSR), 1% Non-Essential Amino Acids Solution (NEAA), 0.1%β-Mercaptoethanol and 1% Penicillin-Streptomycin Solution. The explants were cultured for four days with either 20 μM morpholino oligomer (Mo) or its negative control (Ctr). *Stim1* translation-blocking vivo-Mo (oligo sequence: CAAGACGGGCGCACACATCCATGAC) and its control (oligo sequence: CCTCTTACCTCAGTTACAATTTATA) were purchased from Gene Tools (Philomath, Oregon, USA). Coenzyme Q10 (COQ10) was obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and used at a final concentration of 0.1 μM. Reactive oxygen species (ROS) and terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay kits were obtained from Genmed Scientifics (Wilmington, Delaware, USA) and Vazyme (Nanjing, China), respectively.

2.5. Statistical analysis

Experiments were repeated at least three times. The quantitative results are presented as mean ± standard deviation (s.d.). The data were evaluated for statistical differences using Student's *t*-test and one-way ANOVA by Graphpad Software (https://www.graphpad.com/) with * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Results

3.1. STIM1 expression in neonatal mice testes

We used immunostaining to analyze the distribution of STIM1 in neonatal mice testes. The seminiferous epithelium of newborn mouse testis contains two distinct cell types: germ and Sertoli cells. As shown in Fig. 1, in the seminiferous epithelium of 0.5-day testis, gonocytes are evident in the center of the cords with a spherical nucleus, while in the 7.5-day testis, the gonocytes have migrated to the basement membrane of the seminiferous tubule and differentiated into Spermatogonial Stem cells (SSCs). We found that STIM1 was primarily distributed in the cytoplasm of Sertoli cells, while the fluorescence signal was almost undetectable in the gonocytes and SSCs (Fig. 1).

3.2. STIM1 is essential for Sertoli cell orientation and germ cell survival

To explore the function of STIM1 in neonatal testis, we used Mo oligo to knockdown *Stim1* gene, and successfully suppressed STIM1 translation *in vitro*. Western blot analysis showed 52.4% knockdown efficiency of STIM1 by Mo *in vitro* (Fig. 2A and B). As STIM1 is primarily distributed in Sertoli cells, we attempted to compare the orientation and identification of Sertoli cells between the Mo and Ctr groups. We evaluated the expression levels of two Sertoli cell markers, SOX9 [10] and GATA4 [14], in cultured testes. Sertoli cells in the Ctr group were surrounded with the basal membrane consistently (Fig. 2C and E), whereas Sertoli cells in the Mo group departed from the basal membrane and translocated into the adluminal compartment (Fig. 2C and E). The number of Sertoli cells did not appear to differ between the two groups (Fig. 2D and F). In addition, to explore the effect of germ cells after knockdown STIM1, we used DDX4 [15] and LIN28 [16] to label germ cells and SSCs, respectively, and the immunostaining results revealed a significant reduction of DDX4-labeled (Fig. 2G) germ cells and LIN28-labeled SSCs in the Mo group (Fig. 2I); the proportion of reduction was approximately 52.3% (Fig. 2H) and 49.8% (Fig. 2J), respectively. The apoptotic signals were further verified by TUNEL assay, we found that the apoptotic signals showed a 1.9-fold increase in Mo testes,

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