Research Article

Eif2s3y Promotes the Proliferation of Spermatogonial Stem Cells by Activating ERK Signaling

Mengfei Zhang,1 Na Li,1 Wenqing Liu,1 Xiaomin Du,1 Yudong Wei,1 Donghui Yang,1 Zhe Zhou,1 Fanglin Ma,1 Sha Peng,1 Shiqiang Zhang,1 Xin He,1 Chunling Bai,2 Guangpeng Li,2 and Jinlian Hua1

1College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering & Technology, Northwest A&F University, Yangling, Shaanxi 712100, China
2State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, School of Life Sciences, Inner Mongolia University, Hohhot 010070, China

Correspondence should be addressed to Jinlian Hua; jinlianhua@nwsuaf.edu.cn

Received 8 October 2020; Revised 9 January 2021; Accepted 12 January 2021; Published 30 January 2021

1. Introduction

Spermatogenesis is essential for the continuation of most species. The reduction of spermatogonial stem cells (SSCs) can destroy spermatogenesis and lead to male infertility [1, 2]. In addition to maintaining stable spermatogenesis, studies in mice have shown that a small fraction of undifferentiated spermatogonia can regenerate spermatogenic lineage after being isolated from donor tissues and transplanted into the testis of recipient males lacking endogenous reproductive lines [3]. These regenerated spermatogonia are often referred to as spermatogonial stem cells. SSCs are located on the basement membrane of seminiferous tubules, and the delicate control of SSC self-renewal and differentiation critically determines sperm production in male animals [2, 4]. Therefore, a defect in SSC proliferation usually results in reduced germ cell number or even male infertility [5].

Chemotherapeutic drugs, such as busulfan and cisplatin, cause male reproductive damage and long-term infertility by damaging SSCs [6, 7]. In human reproductive medicine, SSCs can be used to solve infertility caused by spermatogenesis and maturation disorders [8]. Spermatogonial stem cell transplantation (SSCT) has many potential applications and may have a significant impact on society. Successful spermatogenesis has not been achieved following the transplantation of human testis tissue. However, there have been
successful cases of animal SSCT, such as mice, dogs, and non-human primates [9, 10]. Thus, improving the proliferation ability of SSCs is critical for the rapid restoration of male reproductive capacity.

Eukaryotic translation initiation factor 2 subunit 3 and structural gene Y-linked (Eif2s3y) is located on the Y chromosome of male animals and is traditionally considered to be involved in the formation of the eIF2 polymer to mediate translation initiation [11, 12]. In recent years, several studies have shown that Eif2s3y is essential for mouse spermatogenesis [13, 14]. In 2014, Yamauchi et al. reported that mouse progeny could be generated by male germ cells with the Y chromosome contribution limited to only two genes, Sry and Eif2s3y [15, 16]. Importantly, Eif2s3y may be the only Y chromosome gene required to drive mouse spermatogenesis. In our previous studies, increased efficiency of haploid cell induction has been detected in Eif2s3y-overexpressing (oeEif2s3y) embryonic stem cells (ESCs) [17]. However, how Eif2s3y improves the efficiency of spermatogenesis is still unclear.

In the present study, we wanted to explore the role and regulatory mechanism of Eif2s3y in dairy goats. We obtained the Eif2s3y gene fragment of dairy goats and found that the expression level of Eif2s3y in the testis was significantly higher than that in other tissues. In addition, we found that Eif2s3y promoted goat SSC proliferation dependent on the extracellular regulated protein kinases (ERK) signaling pathway. The SSC experiment showed that Eif2s3y could increase the number of SSCs transplanted into busulfan-treated mice. Our study may provide an efficient approach for the repair of male spermatogenic cells in large animals and improve the efficiency of livestock genetic breeding in the future.

2. Materials and Methods

2.1. Animal Experiments. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People’s Republic of China, Policy No. 2006 398) and were approved by the Animal Care and Use Center of the Northwest A&F University.

Different tissues and testes at different ages (1, 3, 6, 9, 12, 18, and 24 months) of Guanzhong dairy goats were supplied by Yaoan slaughterhouse in the Yangling Agricultural High-tech Industrial Demonstration Zone. Three male goats from different ages were used in the testis collection. These tissues were then used to extract RNA by using RNAiso Plus (#9109, Takara Bio Inc., Japan).

The male ICR mice used for the infertile mouse model were purchased from Dashuo Laboratory Animal Limited Company in Chengdu, China. Twenty 7-week-old male mice were treated with busulfan (B2635-25G, Sigma-Aldrich by Merck) at a dose of 30 mg/kg for 2 weeks to be rendered infertile. These busulfan-treated mice were used for spermatogonial transplantation [1, 18].

2.2. Cell Culture and Preparation of Dairy Goat SSCs. The procedures for isolating and purifying SSCs were in accordance with a previous study, and the morphology and functionality of SSCs we used have been verified [19–21]. The procedures for isolating and purifying SSCs are as follows. Testes from dairy goats of 3 months were aseptically collected. After washing five times with phosphate-buffered saline (PBS) containing 100 U/mL penicillin and 100 mg/mL streptomycin, testes were cut into small pieces by using sterile scissors. Seminiferous epithelial cells were incubated with an enzyme cocktail containing 0.1% collagenase IV (Invitrogen) and 10 μg/mL DNase I (Sigma-Aldrich by Merck) at 37°C for 30 min, and the cell suspension was blended every 10 min at the same time. The dissociated fragments were then digested with 0.25% trypsin (Invitrogen) for 15 min, followed by neutralization with Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Gibco, MA, USA). The cell suspension was then filtered by 40 μm copper meshes to exclude the seminiferous tubules. Then, the cell suspension was plated in culture dishes and incubated in an atmosphere composed of 5% CO2 at 37°C for 2 hours.

Nonadherent SSCs were obtained and removed to a new dish when the Leydig cells attached to the culture dish. Then, these cells were purified by the MASC technique to obtain Thy1-positive cells. Dairy goat SSCs were cultured in a medium containing DMEM/F12 (Invitrogen) with 1% FBS, 10% KSR (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich by Merck), 1% nonessential amino acids (Invitrogen), 1% L-glutamine (Invitrogen), 10 ng/mL basic fibroblast growth factor (bFGF, Millipore), 10 ng/mL GDNF (Reproach), 50 ng/mL Gfra1 (Sino Biological, Inc., Beijing, China), and 20 ng/mL epidermal growth factor (EGF, Sino Biological, Inc.) [22, 23]. These cells were cultured for 12 hours at 37°C, supplemented with 5% CO2 in the air. The medium was refreshed every day. The dairy goat SSCs were passaged by TrypLE (Invitrogen).

2.3. Seminiferous Tubule Transplantation. For SSCT, approximately 100 μL of a single cell suspension or medium was injected through the efferent duct into the left testis or right testis of busulfan-treated mice, respectively. The testis which was injected with the medium was the control group. The seminiferous tubule injection protocol was conducted as previously reported [24, 25]. These testes were collected for analysis 4 weeks after injection [26].

2.4. Construction of Recombination Plasmid. The primer sequences for the dairy goat Eif2s3y CDS clone were designed according to the published Mus musculus Eif2s3y mRNA sequence (XM_006531609) were as follows: forward: 5′-AGAATTCTTCCGGCAAGATGGCG-3′, reverse: 5′-AGGGCCCGCTTCCATGCAATG-3′.

Eif2s3y was amplified from the dairy goat testicular cDNA by a reverse transcription-polymerase chain reaction. Then, the specific fragments were cloned into the pCDH-CMV-MCS-EF1 vector. Nucleotide fragments for knocking down experiments which were sent to biological companies for synthesis were as follows: 5′-CCGGGAACAGATACTTGCAATTTCGACTGAGTACAAATGGAATGATCTGTTCTTTTTGTG-3′. The specific nucleotide fragments were
cloned into the CD513B-U6-shEif2s3y vector. The recombinant plasmid pCDH-CMV-Eif2s3y-EF1-puro (oeEif2s3y), CD513B-U6-shEif2s3y (shEif2s3y), and assistant plasmids PAX2 and VSVG were stored in Shaanxi Centre of Stem Cells Engineering & Technology, Northwest A&F University [27].

2.5. Lentivirus Preparation and Infection. Lentivirus production was described previously [28]. Assistant plasmids PAX2 and VSVG were cotransfected with pCDH-CMV-Eif2s3y-EF1-puro or CD513B-U6-shEif2s3y in HEK293T cells. The oeEif2s3y or shEif2s3y lentivirus was collected 48 hours later after substituted. The primary SSCs were infected with lentivirus oeEif2s3y or shEif2s3y when the density reached 80% complementing with polybrene (Sigma-Aldrich by Merck) to increase transfection efficiency. The infected SSCs were then cultured with a medium containing 500 ng/mL puromycin (Sigma-Aldrich by Merck) for 1 week in order to increase the proportion of positive cells.

2.6. Ethynyl-Deoxyuridine (EdU) Incorporation Assay. EdU incorporation assay was performed as per the manufacturer’s instructions (C10310-1, RiboBio, Guangzhou, China). SSCs planted in a 48-well plate were incubated with the 50 μM EdU medium for 2 h. Then, the EdU medium was discarded. Cells were fixed with 4% paraformaldehyde at room temperature for 15 min and decolorized in 2 mg/mL glycine for 10 min. After washing with PBS, cells were permeated by 0.5% Triton X-100 for 10 min. The staining buffer was added and incubated in the dark at room temperature for 30 min. After washing with PBS, the nuclei were visualized by Hoechst 33342 (Sigma-Aldrich by Merck). The cells were washed three times and observed under a fluorescence microscope.

Three culture wells were used in each group. At least three cell images and 300 cells per well were taken randomly. The ratio of the number of red fluorescent cells to the number of blue fluorescent cells is the ratio of positive cells [28]. The proportion of positive cells is positively correlated with the cell proliferation rate.

2.7. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis. The qRT-PCR analysis was in accordance with a previous report [17]. Tissues and cells were harvested at the proper time, and total RNAs were extracted using the TRIzol reagent (RNAiso Plus, #9109, Takara Bio Inc., Japan). RNA integrity was analyzed by agarose gel electrophoresis, and the concentration was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Lot 00887496, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Aliquots of undiluted cDNA were stored at -20°C and used for RT-PCR and real-time PCR. RT-qPCR was conducted on a CFX Connect Real-Time System (Bio-Rad, California, USA) using the SYBR Premix Real-Time PCR Kit (FP215-01, Tiangen Biotech, Beijing, China) in accordance with the manufacturer’s instructions. The expression levels of mRNAs were normalized to GAPDH and β-actin. The qRT-PCR primers used in this article are listed in Supplemental Table 1. All primer sequences were determined through established GenBank sequences. The PCR efficiency was evaluated and analyzed by agarose gel electrophoresis.

2.8. Immunofluorescence (IF) Staining. Immunofluorescence staining of testes and SSCs was conducted as previously reported [27]. The primary antibodies used in this study are listed as follows: rabbit anti-eIF2y (1:200; PA5-31177, Thermo Fisher Scientific, MA, USA), rabbit anti-DDX4 (1:200; ab13840, Abcam, Cambridge, UK), mouse anti-ZBTB16 (1:200; sc-28319, Santa Cruz Biotechnology, CA, USA), rabbit anti-STR8A1 (1:200; ab49602, Abcam, Cambridge, UK), mouse anti-GFRA1 (1:200; sc-271546, Santa Cruz Biotechnology, CA, USA), rabbit anti-SOX9 (1:200; ab185230, Abcam, Cambridge, UK), and rabbit anti-SATB2 (1:100; bs-20388R, Bioss, Beijing, China). Secondary antibodies are as follows: Alexa Fluor 488-goat anti-rabbit IgG (1:400; ZF-0511, ZSGB-BIO, Beijing, China) and Alexa Fluor 568-goat anti-mouse (1:400; ZF-0513, ZSGB-BIO, Beijing, China).

2.9. Population Doubling Time (PDT) Determination. The PDT of dairy goat SSCs was estimated according to the protocol described previously [29]. Briefly, cells were serially subcultured; the initial seeding cell number and the total cell number cultured 24 h later were all counted, respectively. PDT was calculated according to the formula $PDT = \left(\log_{10}(N_t/N_0)\right) \times t$, where $N_0$ means the number of seeded cells, $N_t$ indicates the number of cells after $t$ (h) of culturing, and $t$ means the duration of cell culturing hours.

2.10. Western Blotting. Western blotting (WB) was estimated according to a previous article [27]. The antibodies used in this study are listed as follows: anti-eIF2y (1:500; PA5-31177, Thermo Fisher Scientific, MA, USA), anti-PCNA (1:500; BM0104, Boster, Wuhan, China), anti-Cyclin D (1:500; WL01435a, Boster, Wuhan, China), anti-Cyclin D (1:500; BM0104, Boster, Wuhan, China), anti-ERK (1:200; #9194, CST, Boston, USA). The results were detected using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and quantified using ImageJ (V1.48d).

2.11. Bioinformatics Analysis. The dairy goat Eif2s3y CDS was sequenced by Sangon, China. Multiple sequence alignment among different species was performed by DNAMAN software, and the phylogenetic tree was depicted with MEGA 4.1. The amino acid sequences of Eif2s3y proteins in different species were also analyzed by DNAMAN software. The protein secondary structure was predicted by DNAStar software. The domains contained in Eif2s3y protein were predicted by the SWISS-MODEL Workspace website and RasMol software [30].

2.12. ERK Pathway Inhibitor and Activator. To confirm the function of ERK signaling in Eif2s3y regulation, SSCs were treated with 1 μM ERK pathway inhibitor PD0325901 (APEX BIO Technology LLC, A3013, Houston, USA) or
Relative mRNA expression

(a)

(b)

Figure 1: Continued.
10 μM ERK pathway activator 12-O-tetradecanoylphorbol-13-acetate (TPA, APExBIO Technology LLC, N2060, Houston, USA) for 24h, respectively [31]. PD0325901 effectively inhibits the phosphorylation of ERK1/2 in multiple cell lines, while TPA activates the phosphorylation of ERK1/2 [32]. There were three replicates in each group of cells. The diluent of the reagent is DMSO.

### 2.1.3. Statistical Analysis

Relative gene expression was analyzed by the comparative Ct method (2^(-ΔΔCt) method). To compare significant differences, a two-tailed Student’s t-test was used. The results were represented as mean ± SD. All results were replicated at least 3 times. Statistical analyses were analyzed by SPSS 20.0 software and GraphPad Prism software (La Jolla, CA). P values < 0.05 were considered statistically significant (*P < 0.05, **P < 0.01, and ***P < 0.001).

### 3. Results

#### 3.1. The Expression Pattern of Eif2s3y in Dairy Goats

First, we performed qRT-PCR to clarify the expression pattern of Eif2s3y in dairy goats. The results showed that Eif2s3y was widely expressed in different tissues including the brain, kidney, heart, liver, ovary, spleen, lung, and testis (Figure 1(a)). Of note, the testis showed the highest expression level of Eif2s3y (P < 0.01) and this level tended to increase gradually over time (P < 0.05), and it sustained high levels after sexual maturity than before (Figure 1(b)). Then, immunofluorescence staining was performed to analyze the expression pattern of eIF2γ in the testes of 3- and 24-month-old goats. The result showed that Eif2s3y could be expressed in SSCs, Sertoli cells, and Leydig cells (Figure 1(c); Supplemental Fig. 2A and B). Of note, lots of sperms could be observed in the testes of 24-month-old goats while not in those of 3-month-old goats (Figure 1(d); Supplemental Fig. 2C). We could see from these results that Eif2s3y was highly expressed in testes and eIF2γ protein mainly existed in the cytoplasm. The high expression of Eif2s3y in spermatogonia made us want to study their function in these cells.

#### 3.2. Structure and Bioinformatics Analysis of Eif2s3y in Dairy Goats

A pair of specific cloning primers for the CDS region of the dairy goat Eif2s3y gene was designed as described in Materials and Methods. We further cloned the Eif2s3y gene of dairy goats by PCR, and three repetitions were made (Figure 2(a)). The fragments whose sizes were between 1000 bp and 2000 bp were considered to be the goat Eif2s3y gene. Next, we inserted the gene into the pMD18-T vector for sequencing analysis, which showed that the size of the CDS region of the dairy goat Eif2s3y gene was 1413 bp. We uploaded the sequence information to the National Center for Biotechnology Information (NCBI) and obtained a formal gene serial number (GenBank: KP326346.1).

Eif2s3y gene sequences of Homo sapiens, Microcebus murinus, Capra hircus, Bos taurus, Rattus norvegicus, Mus musculus, Tokudaia osimensis, Loxodonta africana, and Xenopus tropicalis obtained from NCBI indicated that this gene was widely expressed in different species (Figure 2(b)). We analyzed the phylogenetic tree of Eif2s3y and compared their nucleotide and amino acid sequences (Figure 2(b)). The results showed a 97.98% similarity for amino acid among different species and suggested that Eif2s3y was highly conserved among different species (Figure 2(c)). Then, we predicted the protein structure of Eif2s3y through SWISS-
MODEL Workspace and found an important binding region comprising four tandem zinc finger domains (Figure 2(e)). Another prediction gave a schematic map of protein domain analysis of Eif2γ by NCBI CD-Search (Figures 2(d) and 2(e)). These results indicated that Eif23γ was conserved and might have similar functions in different species.
**Figure 3: Continued.**
3.3. Overexpression of Eif2s3y Promotes the Proliferation of Dairy Goat SSCs. The Eif2s3y fragment was inserted into the pCDH-CMV-MCS-EF1-puro vector, and a recombinant plasmid pCDH-CMV-Eif2s3y-EF1-puro was successfully constructed (Figures 3(a) and 3(b)). The pCDH-Eif2s3y and pCDH lentivirus were collected as described in Materials and Methods. The morphology and function of primary SSCs that we used to verify the direct effects of Eif2s3y have been verified in the past experimental studies [19, 21]. The primary cells and pure spermatogonia are shown in Supplementary Figure 1A. We examined the expression of several marker genes of SSCs by qRT-PCR and IF staining. The expression of SSC marker genes Zbtb16, GFRa1, and Stra8 was significantly higher in the pure spermatogonia (Supplemental Figure 1B). The same conclusion was obtained by immunofluorescence staining (Supplemental Figure 1C). We successfully enriched SSCs.

The SSCs were infected with lentivirus pCDH-Eif2s3y or pCDH. After screening by 500 ng/mL puromycin (Sigma) for one week, oeEif2s3y cells and control cells were established (Figure 3(c)). Interestingly, the morphology of oeEif2s3y cells changed and the edge of colonies became unsmooth, showing a certain extent of differentiation. According to a previous report, the Eif2s3y defect would block the production of spermatogonia and result in infertility in mice [13]. Our results showed that Eif2s3y promoted the proliferation of goat SSCs,
Figure 4: Continued.
as reflected by the higher proliferation rate of oeEif2s3y cells in cell number counting (Figure 3(d)). The population doubling time (PDT) of oeEif2s3y cells was significantly reduced from 34.6 hours to 28.9 hours. The results were further strengthened by EdU incorporation assay (Figures 3(e) and 3(f)). In accordance with these findings, we found that the expression levels of proliferation-associated genes (Pcna, Cyclin D) and self-renewal-associated gene (Zbtb16) were increased in oeEif2s3y cells (Figure 3(g)). The proportion of red positive cells was consistent with the cell proliferation rate. Western blotting was applied to examine the effect of transgenic Eif2s3y (Figure 3(h)). In oeEif2s3y SSCs, statistical analysis showed that the expression of ZBTB16, eIF2γ, Pcna, Cyclin D was higher than in Control SSCs (Figure 3(i)). Collectively, these data demonstrated that overexpression of Eif2s3y promoted the proliferation of goat SSCs.

3.4. Eif2s3y Deficiency Reversed the Goat SSC Growth Rate. Since overexpression of Eif2s3y could promote SSC proliferation, we wondered whether knockdown of Eif2s3y expression would inhibit this proliferation. Recombinant plasmid CD513B-U6-shEif2s3y was successfully constructed (Figures 4(a) and 4(b)). Seven days after infection with lentivirus, RT-PCR analysis confirmed the successful knocking down of Eif2s3y expression (shEif2s3y) in goat SSCs. The efficiency of the two interfering fragments was 60% or 90%, respectively (Figure 4(g)). We chose the more efficient U6-Vector2 for future experiments (Figures 4(c) and 4(e)).

The population doubling time of shControl and shEif2s3y SSCs was 35.9 or 44.8 hours, respectively (Figure 4(d)). Then, we evaluated the proliferation rate by EdU staining; the percentage of EdU-positive shEif2s3y cells was lower than that of shControl (Figures 4(e) and 4(f)). Compared with the shControl group, the expression levels of proliferation-associated genes Pcna and Cyclin D and self-renewal-associated gene Zbtb16 in the shEif2s3y group were significantly decreased (Figures 4(g) and 4(h)). Western blotting analysis got the same results (Figure 4(i)). These experiments showed that Eif2s3y deficiency reversed goat SSC proliferation.

![Figure 4: Depletion of Eif2s3y resulted in proliferation abnormality in goat SSCs. (a, b) The schematic of lentivirus plasmid CD513B-U6-MCS and CD513B-U6-shEif2s3y. (c) Typical images of shControl (left) and shEif2s3y (right) SSCs. Scale bar, 200 μm. (d) Proliferation curve of shControl and shEif2s3y SSCs and the results of population doubling time (PDT) determination. (e) EdU incorporation assay of shControl (up) and shEif2s3y (down) SSCs. (f) Scale bar, 400 μm. (f) The ratio of EdU-positive cells to total cells. Data are presented as mean ± SD and are represented by three independent repetitions. (g) RT-PCR analysis of the expression levels of Eif2s3y, Pcna, Cyclin D, and Zbtb16 in shControl and shEif2s3y SSCs in vitro. (h) Western blotting detected the protein expression of ZBTB16, eIF2γ, PCNA, and Cyclin D in shControl and shEif2s3y SSCs. GAPDH was used as a loading control. (i) Gray intensity analysis of WB results normalized to GAPDH in (h). Data are presented as mean ± SD and are represented by three independent repetitions; *P < 0.05, **P < 0.01, and ***P < 0.001.]
Figure 5: Continued.
Figure 5: Eif2s3y promotes the colonization of goat SSCs in SSCT. (a) OeEif2s3y or Control SSCs were injected into the seminiferous tubules of busulfan-treated mice. The figure here shows two typical pictures of the testis during injection. Scale bars, 1 mm and 5 mm as indicated. (b) Morphology of testes and epididymides transfected with oeEif2s3y or Control SSCs. (c) The testicular (left) and epididymis (right) weight/body mass ratio in two groups. (d) H&E staining of the mouse testis 30 days after injection. Scale bars, 50 μm (left) and 20 μm (right). (e) Statistical plots of the diameter of seminiferous tubules (left) and the thickness of seminiferous epithelia (right) from oeEif2s3y and Control SSC-transplanted mice. Each group counted at least 30 round seminiferous tubules from 10 mice. (f) H&E staining of the mouse epididymis 30 days after injection. Scale bars, 50 μm (left) and 20 μm (right). (g) Statistical plots of the diameter of epididymis tubules from oeEif2s3y and Control SSC-transplanted mice. Each group counted at least 30 epididymis tubules from 10 mice. (h) RT-PCR analysis of the expression of Eif2s3y, Pcna, Zbtb16, and Cyclin D in Testis-Control and Testis-oeEif2s3y. (i) Immunofluorescence staining of DDX4 (green) in SSC-transplanted testes. The nuclei were stained with Hoechst 33342 (blue). Scale bar, 100 μm. These white arrows represented typical DDX4-positive germ cells. DDX4 is a representative marker for pan-germ cells. (j) Immunofluorescence staining of ZBTB16 (green) in SSC-transplanted testes. The nuclei were stained with Hoechst 33342 (blue). Scale bar, 100 μm. These white arrows represented typical ZBTB16-positive spermatogonial stem cells. ZBTB16 is a representative marker for undifferentiated spermatogonia. Data are presented as mean ± SD and are represented by three independent repetitions; *P < 0.05, **P < 0.01, and ***P < 0.001; N.S. means P ≥ 0.05.
3.5. *Eif2s3y* Could Increase the Colonization Rate of Goat SSCs in SSCT. Spermatogonial stem cell transplantation technology has been an effective method to study SSCs since 1994 [1, 26, 33]. Some previous research had proved the reliability of our transplantation technique [17]. To investigate the contribution of *Eif2s3y* in SSCs, oe*Eif2s3y* SSCs and Control SSCs were transferred into the seminiferous tubules of twenty infertile mice treated with busulfan (Figures 5(a) and 6(a)).
Figure 7: Continued.
Testes transplanted with oeEif2s3y SSCs were heavier than those in the control group \( (P = 0.014) \), while the weight of the epididymis did not change significantly \( (P = 0.43) \) (Figures 5(b) and 5(c)).

H&E staining of the transplanted testes showed that more germ cells were observed in the Eif2s3y group (Figure 5(d)). Additionally, the diameter of seminiferous tubules \( (P < 0.01) \) and the thickness \( (P < 0.001) \) of seminiferous epithelia were significantly increased in the Eif2s3y group (Figure 5(e)). However, the diameter of epididymis tubules was not significant between these two groups (Figures 5(f) and 5(g)). In addition, RT-PCR analysis showed that Eif2s3y, Pcna, Zbtb16, and Cyclin D were overexpressed in the oeEif2s3y group (Figure 5(h)). Importantly, immunofluorescence staining showed that the Eif2s3y group had more DDX4-positive germ cells and more ZBTB16-positive SSCs in the testes (Figures 5(i) and 5(j)). Thus, overexpression of Eif2s3y might contribute to improving the survival rate and proliferation of goat SSCs in SSCT. However, no sperm was found in either group, which might be caused by different species (Figure 5(d)).

3.6. Eif2s3y Promotes the Proliferation of SSCs by Activating the ERK Signaling Pathway. To confirm the proliferation mechanism of Eif2s3y in goat SSCs, we investigated the proliferation-related signaling pathways through western
blotting in oeEif2s3y and shEif2s3y SSCs. Our previous study found that the expression of Eif2s3y increased the phosphorylation level of ERK. Then, SSCs were treated by 1 μM ERK pathway inhibitor PD0325901 [31] or 10 μM ERK pathway activator TPA [32] for 24 hours, respectively. DMSO was used for the control group. As expected, PD0325901 treatment significantly inhibited the proliferation of oeEif2s3y SSCs, while TPA treatment efficiently restored the proliferation of SSCs transplanted with shEif2s3y, as analyzed by EdU incorporation assay (Figures 7(a)–7(d)).

Western blotting analysis was further conducted to explore the underlying mechanisms, and we found that the MEK-ERK signal could directly and indirectly participate in the proliferation of SSCs. When ERK signaling was activated by Eif2s3y overexpression or by TPA treatment, the expression levels of PCNA and Cyclin D were both increased. The results showed that the ERK signaling blockade, either by Eif2s3y interference or by PD0325901 treatment, significantly inhibited the expression levels of PCNA and Cyclin D (Figures 7(e) and 7(f)). In comparison, the results showed that the ERK signaling blocked, either by Eif2s3y interference or by PD0325901 treatment, significantly inhibited the expression levels of PCNA and Cyclin D, both of which were essentially needed during cell proliferation (Figures 7(g) and 7(h)). According to the above experimental results, we hypothesized that Eif2s3y activated the downstream ERK signaling pathway to regulate proliferation genes.

According to the above experimental results, we proposed a mechanism diagram of how Eif2s3y activated the downstream ERK signaling pathway to promote SSC proliferation and restoration of male spermatogenic cells in goat testes (Figure 6(b)).

4. Discussion

Eif2s3y is widely expressed in different male animals and recognized as a translation initiation factor [11, 34]. In recent studies, we found that Eif2s3y could regulate the proliferation of goat SSCs. The expression levels of proliferation- and self-renewal-related genes Cyclin D, Cyclin A, Pena, and Plzf were upregulated in oeEif2s3y SSCs and downregulated in shEif2s3y SSCs (Figures 3(g) and 4(g)). Our results showed that Eif2s3y played an important role in male reproduction of dairy goats, and these results were consistent with previous studies in mice that Eif2s3y played a critical role in male spermatogenesis [14, 15, 35]. Importantly, we found a regulatory pathway of Eif2s3y in male reproduction with ERK signaling involved. This finding was in line with previous studies reported by us and other groups that the MEK/ERK signaling pathway played an important role in cell proliferation, differentiation, and cell cycle progression [36, 37]. However, how Eif2s3y regulates ERK signaling remains to be further studied.

SSCT technology is an effective method to identify the characteristics of SSCs cultured in vitro [1]. We transferred oeEif2s3y SSCs into the seminiferous tubules of infertile mice and found that Eif2s3y could enhance the colonization of germ cells (Figure 5(d)). Moreover, no mature sperm was observed in the epididymis (Figure 5(e)). The blood-testis barrier (BTB) made goat SSCs transplanted into mouse testes survive [5]. However, the species relationship between goats and mice was so far that mice could not produce goat sperms. As a contrast, we injected CDS13B-U6-shEif2s3y lentivirus into the seminiferous tubules of wild-type mice and found that spermatogenesis was blocked and the germ cells in seminiferous tubules were very loose. Another research group directly knocked out the mouse Eif2s3y gene with TALEN technology, which led to testicular hypoplasia and male infertility [13]. More generally, all these results of animal experiments in vivo indicated that Eif2s3y played an important role in spermatogenesis.

Our bioinformatics analysis showed that Eif2s3y was highly conserved among different species (Figure 2(c)). Therefore, the interference experiment of mice might also be applicable to goats. Dairy goat Eif2s3y was located on the Y chromosome and encoded a 471 amino acid protein which contained a compact zinc finger domain and an N-terminal GTP binding domain (Figures 2(d) and 2(e)). In mice, Eif2s3x was a homologous gene of Eif2s3y and shared 98% of amino acid sequence identity and almost all of the RNA binding domains with Eif2s3y [35]. However, recent studies have found that these two genes might not completely replace each other. Eif2s3x has been found to play an irreplaceable role in the early development of organs such as the brain and pancreas [38–40]. Meanwhile, a recent study showed that Eif2s3y was more effective in masculinizing mice during sex growth at 12.5 days of mouse embryonic development [41]. However, in dairy goats, it was still unclear whether a homologous gene of Eif2s3y exists and how they worked together. Thus, future work is essentially needed to answer these questions.

In conclusion, our study found a novel role of Eif2s3y in the male reproduction of dairy goats. This finding might provide an important basis for the repair of male infertility and spermatogonial stem cell transplantation toward realizing the regeneration of germplasm in large animals.

Data Availability

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

Conflicts of Interest

The authors declare that no conflict of interest exists.

Authors’ Contributions

The contributions of the authors involved in this study are as follows: J LH: experimental design and financial support; MFZ, NL, and WQL: performance of cellular and molecular experiments, data analysis, and manuscript preparation; XMD, YDW, DHY, and ZZ: preparation of lentivirus injection, mouse model, and primary SSCs; and FLM, NL, SP, SQZ, XH, CLB, and GPL: assistance of experiments and revision and discussion of the manuscript. Mengfei Zhang and Na Li contributed equally to this work. The author order was determined by negotiation.
Supplementary Materials

Supplementary 1. Supplemental Figure 1: isolation and enrichment of dairy goat spermatogonia. (A) The primary cells and pure spermatogonia isolated from healthy dairy goat testes; scale bar = 200 μm. (B) RT-PCR analysis of the expression of Zbtb16, GFRa1, and Stra8 in primary cells and pure spermatogonia. Data are presented as mean ± SD and are represented by three independent repetitions; *P < 0.05, **P < 0.01. (C) Immunofluorescence staining of ZBTB16 (up), STRA8 (middle), and GFRa1 (down) in pure spermatogonia. The nuclei were stained with Hoechst 33342 (blue). Scale bar, 200 μm. ZBTB16, STRA8, and GFRa1 are representative markers for SSCs.

Supplementary 2. Supplemental Figure 2: some additional pictures. (A) Immunofluorescence staining of mouse (up) and goat (down) testes. Only IgG (1:200) was used for the fluorescence experiments as the parallel negative antigen control to prove that there was no false positive in our immunofluorescence staining. (B) Immunofluorescence staining of SOX9 (up) and StAR (down) in goat testes. The nuclei were stained with Hoechst 33342 (blue). Scale bar, 200 μm. Species-specific IgG antibody for immunofluorescence experiments as the parallel negative antigen control to prove that there was no false positive in our immunofluorescence staining. (B) Immunofluorescence staining of SOX9 (up) and StAR (down) in goat testes. The nuclei were stained with Hoechst 33342 (blue). Scale bar, 100 μm. SOX9 is a representative marker for Sertoli cells, and StAR is a representative marker for Leydig cells. (C) H&E staining of 3-month-old and 24-month-old goat testes. Scale bars, 50 μm (left) and 20 μm (right).

Supplementary 3. Supplemental Figure 3: full unedited images. (A) Full unedited PCR images of Figure 2(a) data. (B) Full unedited western blotting images of Figure 7(c) data. (C) Full unedited western blotting images of Figure 7(g) data.

Supplementary 4. Supplemental Table 1: the sequence and length of primers used in qRT-PCR amplification.

Supplementary 5. Supplemental File 1: normal distribution test for experimental data.

Supplementary 6. Supplemental File 2: experimental information for qRT-PCR.

References

[1] R. L. Brinster and M. R. Avarbock, "Germ cell transmission of donor haplotype following spermatogonial transplantation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 24, pp. 11303–11307, 1994.

[2] H. Nishimura and S. W. L’Hernault, "Spermatogenesis," *Current Biology*, vol. 27, no. 18, pp. R988–R994, 2017.

[3] M. C. Nagano, "Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice," *Biology of Reproduction*, vol. 69, no. 2, pp. 701–707, 2003.

[4] K. Guan, K. Nayernia, L. S. Maier et al., "Pluripotency of spermatogonial stem cells from adult mouse testis," *Nature*, vol. 440, no. 7088, pp. 1199–1203, 2006.

[5] M. Kanatsu-Shinohara, N. Ogonuki, S. Matoba, A. Ogura, and T. Shinohara, "Autologous transplantation of spermatogonial stem cells restores fertility in congenitally infertile mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 117, no. 14, pp. 7837–7844, 2020.

[6] A. P. Grigg, R. McLachlan, J. Zajac, and J. Szer, "Reproductive status in long-term bone marrow transplant survivors receiving busulfan-cyclophosphamide (120 mg/kg)," *Bone Marrow Transplantation*, vol. 26, no. 10, pp. 1089–1095, 2000.

[7] M. Delessard, J. Saulnier, A. Rives, L. Dumont, C. Rondanino, and N. Rives, "Exposure to chemotherapy during childhood or adulthood and consequences on spermatogenesis and male fertility," *International Journal of Molecular Sciences*, vol. 21, no. 4, p. 1454, 2020.

[8] R. L. Brinster, "Male germline stem cells: from mice to men," *Science*, vol. 316, no. 5823, pp. 404–405, 2007.

[9] M. Hutka, L. B. Smith, and R. T. Mitchell, "Xenotransplantation as a model for human testicular development," *Differentiation*, vol. 97, pp. 44–53, 2017.

[10] E. Ntemou, P. Kadam, D. van Saen et al., "Complete spermatogenesis in intratesticular testis tissue xenotransplants from immature non-human primate," *Human Reproduction*, vol. 34, no. 3, pp. 403–413, 2019.

[11] W. C. Merrick, "Mechanism and regulation of eukaryotic protein synthesis," *Microbiological Reviews*, vol. 56, no. 2, pp. 291–315, 1992.

[12] B. S. Shin, J. R. Kim, S. E. Walker, J. Dong, J. R. Lorsch, and T. E. Dever, "Initiation factor eIF2γ promotes eIF2-GTP-Met-tRNAiMet ternary complex binding to the 40S ribosome," *Nature Structural & Molecular Biology*, vol. 18, no. 11, pp. 1227–1234, 2011.

[13] Y. Matsubara, T. Kato, K. Kashimada et al., "TALEN-mediated gene disruption on Y chromosome reveals critical role of EIF2S3Y in mouse spermatogenesis," *Stem Cells and Development*, vol. 24, no. 10, pp. 1164–1170, 2015.

[14] S. Mazeyrat, N. Saut, V. Grigoriev et al., "A Y-encoded subunit of the translation initiation factor Eif2 is essential for mouse spermatogenesis," *Nature Genetics*, vol. 29, no. 1, pp. 49–53, 2001.

[15] Y. Yamauchi, J. M. Riel, Z. Stoytcheva, and M. A. Ward, "Two Y genes can replace the entire Y chromosome for assisted reproduction in the mouse," *Science*, vol. 343, no. 6166, pp. 69–72, 2014.

[16] Y. Yamauchi, J. M. Riel, V. A. Ruthig, E. A. Ortega, M. J. Mitchell, and M. A. Ward, "Two genes substitute for the mouse Y chromosome for spermatogenesis and reproduction," *Science*, vol. 351, no. 6272, pp. 514–516, 2016.

[17] N. Li, W. Ma, Q. Shen et al., "Reconstitution of mammalian male germ cell specification from mouse embryonic stem cells using defined factors in vitro," *Cell Death and Differentiation*, vol. 26, no. 10, pp. 2115–2124, 2019.

[18] M. Kanatsu-Shinohara, H. Morimoto, and T. Shinohara, "Fertility of male germline stem cells following spermatogonial transplantation in infertile mouse models," *Biology of Reproduction*, vol. 94, no. 5, p. 112, 2016.
H. Zhu, C. Liu, M. Li, J. Sun, W. Song, and J. Hua, "Optimization of the conditions of isolation and culture of dairy goat male germline stem cells (mGSCs)," *Animal Reproduction Science*, vol. 137, no. 1-2, pp. 45–52, 2013.

S. Takashima and T. Shinohara, "Culture and transplantation of spermatogonial stem cells," *Stem Cell Research*, vol. 29, pp. 46–55, 2018.

H. Zhu, J. Ma, R. du et al., "Characterization of immortalized dairy goat male germline stem cells (mGSCs)," *Journal of Cellular Biochemistry*, vol. 115, no. 9, pp. 1549–1560, 2014.

H. Kubota, M. R. Avarbock, and R. L. Brinster, "Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 47, pp. 16489–16494, 2004.

Y. Zhang, S. Wang, X. Wang, S. Liao, Y. Wu, and C. Han, "Endogenously produced FGF2 is essential for the survival and proliferation of cultured mouse spermatogonial stem cells," *Cell Research*, vol. 22, no. 4, pp. 773–776, 2012.

T. Ogawa, J. M. Aréchaga, M. R. Avarbock, and R. L. Brinster, "Transplantation of testis germinal cells into mouse seminiferous tubules," *The International Journal of Developmental Biology*, vol. 41, no. 1, pp. 111–122, 1997.

M. Kanatsu-Shinohara, N. Ogonuki, K. Inoue, A. Ogura, S. Toyokuni, and T. Shinohara, " Restoration of fertility in infertile mice by transplantation of cryopreserved male germ-line stem cells," *Human Reproduction*, vol. 18, no. 12, pp. 2660–2667, 2003.

S. Goodyear and R. Brinster, "Spermatogonial stem cell transplantation to the testis," *Cold Spring Harbor Protocols*, vol. 2017, no. 4, 2017.

Z. du, S. Xu, S. Hu et al., "Melatonin attenuates detrimental effects of diabetes on the niche of mouse spermatogonial stem cells by maintaining Leydig cells," *Cell Death & Disease*, vol. 9, no. 10, p. ???, 2018.

Q. Lei, Q. Pan, N. Li et al., "H19 regulates the proliferation of bovine male germline stem cells via IGF-1 signaling pathway," *Journal of Cellular Physiology*, vol. 234, no. 1, pp. 915–926, 2018.

J. Fang, Y. Wei, X. Teng, S. Zhao, and J. Hua, "Immortalization of canine adipose-derived mesenchymal stem cells and their seminiferous tubule transplantation," *Journal of Cellular Biochemistry*, vol. 119, no. 4, pp. 3663–3670, 2018.

M. Li, C. Liu, H. Zhu et al., "Expression pattern of boule in dairy goat testis and its function in promoting the meiosis in male germline stem cells (mGSCs)," *Journal of Cellular Biochemistry*, vol. 114, no. 2, pp. 294–302, 2013.

S. M. White, M. L. Avantaggiati, I. Nemazanyy et al., "YAP/-

[TAZ] inhibition induces metabolic and signaling rewiring resulting in targetable vulnerabilities in NF2- deficient tumor cells," *Developmental Cell*, vol. 49, no. 3, pp. 425–443.e9, 2019, e9.

L. Li, C. Ge, D. Wang, L. Yu, J. Zhao, and H. Ma, "Dehydroepiandrosterone reduces accumulation of lipid droplets in primary chicken hepatocytes by biotransformation mediated via the cAMP/PKA-ERK1/2 signaling pathway," *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, vol. 1863, no. 6, pp. 625–638, 2018.

Y. Qin, L. Liu, Y. N. He et al., "Testicular busulfan injection in mice to prepare recipients for spermatogonial stem cell trans-plantation is safe and non-toxic," *PLoS One*, vol. 11, no. 2, article e0148388, 2016.

G. Borck, B. S. Shin, B. Stillier et al., "eIF2y mutation that disrupts eIF2 complex integrity links intellectual disability to impaired translation initiation," *Molecular Cell*, vol. 48, no. 4, pp. 641–646, 2012.

C. Murata, Y. Kuroki, I. Imoto, and A. Kuroiwa, "Ancestral Y-linked genes were maintained by translocation to the X and Y chromosomes fused to an autosomal pair in the Okinawa spiny rat Tokudaia muenninki," *Chromosome Research*, vol. 24, no. 3, pp. 407–419, 2016.

Z. Niu, L. Zheng, S. Wu et al., "Ras/ERK1/2 pathway regulates the self-renewal of dairy goat spermatogonial stem cells," *Reproduction*, vol. 149, no. 5, pp. 445–452, 2015.

K. Hasegawa, S. H. Namekawa, and Y. Saga, "MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells," *Stem Cells*, vol. 31, no. 11, pp. 2517–2527, 2013.

S. Moortgat, J. Desir, V. Benoit et al., "Two novel EIF2S3 mutations associated with syndromic intellectual disability with severe microcephaly, growth retardation, and epilepsy," *American Journal of Medical Genetics. Part A*, vol. 170, no. 11, pp. 2927–2933, 2016.

M. Skopkova, F. Hennig, B. S. Shin et al., "EIF2S3 mutations associated with severe X-linked intellectual disability syndrome MEHMO," *Human Mutation*, vol. 38, no. 4, pp. 409–425, 2017.

S. K. Young-Baird, B. S. Shin, and T. E. Dever, "MEHMO syndrome mutation EIF2S3-I259M impairs initiator Met-tRNAiMet binding to eukaryotic translation initiation factor eIF2," *Nucleic Acids Research*, vol. 47, no. 2, pp. 855–867, 2019.

E. A. Ortega, Q. Salvador, M. Fernandez, and M. A. Ward, "Alterations of sex determination pathways in the genital ridges of males with limited Y chromosome genes," *Biology of Reproduction*, vol. 100, no. 3, pp. 810–823, 2019.