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miR-544 Regulates Dairy Goat Male Germline Stem Cell Self-Renewal via Targeting PLZF

Wencong Song,[1] Hailong Mu,[1] Jiang Wu,[2] Mingzhi Liao,[3] Haijing Zhu,[4] Liming Zheng,[1] Xin He,[1] Bowen Niu,[1] Yuanxin Zhai,[1] Chunling Bai,[5] Anmin Lei,[1] Guangpeng Li,[5]* and Jinlian Hua[1]*

[1]*College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering & Technology, Key Lab for Animal Biotechnology of Agriculture Ministry of China, Northwest A&F University, Yangling, Shaanxi 712100, China*

[2]*College of Agriculture, Guangdong Ocean University, Zhanjiang 524088, China*

[3]*College of Life Science, Northwest A&F University, Yangling, Shaanxi 712100, China*

[4]*College of Life Science, Yulin College, Yulin University, 719000, China*

[5]*Key Laboratory for Mammalian Reproductive Biology and Biotechnology, Ministry of Education, Inner Mongolia University, Hohhot 010021, China*

ABSTRACT

The balance between the self-renewal and differentiation of male germline stem cells (mGSCs) is critical for the initiation and maintenance of mammalian spermatogenesis. The promyelocytic leukemia zinc finger (PLZF), a zinc finger protein, is a critical factor for maintaining the self-renewal of mGSCs, so, evaluation of the PLZF pathway in mGSCs may provide a deeper insight into mammalian spermatogenesis. miRNA was also an important regulating factor for the self-renewal and differentiation of mGSCs; however, there is currently no data indicating that which miRNA regulate the self-renewal and differentiation of mGSCs via PLZF. Here, we predicted the prospective miRNA targeting to PLZF using the online Bioinformatics database-Targetscan, and performed an analysis of the dual-luciferase recombinant vector, psiCHCEKTM-2-PLZF-3′UTR. miR-544 mimics (miR-544m), miR-544 inhibitors (miR-544i), Control (NC, scrambled oligonucleotides transfection), pPLZF-IRES2-EGFP or PLZF siRNA were transfected into mGSCs; the cells proliferation was evaluated by BRDU incorporation assay and flow cytometry, and the mGSC marker, GFRa1, PLZF, KIT, DAZL, and VASA expression were analyzed by RT-qPCR, immunofluorescence and Western blot. The results showed that miR-544 regulates dairy goat male germline stem cell self-renewal via targeting PLZF. Our study identifies a new regulatory pathway for PLZF and expands upon the PLZF regulatory network in mGSCs. J. Cell. Biochem. 116: 2155–2165, 2015.

KEY WORDS: PLZF; miR-544; MALE GERMLINE STEM CELLS (mGSCs); DAIRY GOAT

Spermatogonial stem cells (SSCs), also named male germline stem cells (mGSCs), are progenitor cells that maintain spermatogenesis, produce spermatozoa, and transmit genetic information from parents to descendants [Brinster, 2002; Lee and Shinohara, 2011]. Spermatogonia are the foundation of spermatogenesis and valuable for the study of spermatogenesis. Spermatogenesis is governed by orchestrated, phase-specific gene expression patterns that are tightly controlled at the transcriptional and post-transcriptional levels. Some transcription factors (TFs) and cytokines play critical roles in the fate of mGSCs [Mullaney and Skinner, 1992]. Those TFs varies, for example, Glial cell line-derived neurotrophic factor (GDNF) is an important factor for promoting spermatogonial self-renewal [Buageaw et al., 2005], and GDNF family receptor α-1 (GFRa1) is one of the receptor of GDNF. VASA gene (also named

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*Correspondence to: Dr. Jinlian Hua and Dr. Guangpeng Li, College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering & Technology, Northwest A&F University, Yangling, Shaanxi 712100, China.
E-mail: jinlianhua@nwsuaf.edu.cn; gpengli@immu.edu.cn

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DDX4) encodes another RNA-binding protein that is highly conserved across species and expressed specifically in the germline in several model organisms such as flies, mice, and humans [Castrillon et al., 2000; Medrano et al., 2012]. Deleted in AZoospermia-Like (DAZL) is essential for the germ cells development at meiosis [Saunders, 2003], and the stem cell factor receptor (KIT) also is required for the germ cell development [Busada et al., 2015]. However, promyelocytic leukemia zinc finger protein (PLZF), GFRa1, and VASA are associated with the self-renewal and pluripotency of mGSCs [Hobbs et al., 2010]. As a TF, PLZF is indispensable for murine SSC maintenance [Hobbs et al., 2010], which regulates many developmental processes during embryonic and postnatal life [Yang and Oatley, 2014], and the proliferation and self-renewal of male germline cells through mammalian target of rapamycin (MTOR) [Hobbs et al., 2010].

miRNAs are important regulators of gene expression that primarily act post-transcriptionally to control the expression of target mRNAs [Wu et al., 2014]. Increasing evidence has shown that several classes of miRNAs are expressed in male germ cells and play critical roles in small-RNA-mediated regulation of spermatogenesis [Yadav and Kotaja, 2014]. Several TFs are also regulated by miRNAs, such as ets variant 5 (ETV5) regulated by miR-21, which was important for the self-renewal of male germline cells [Chen et al., 2005]. However, there remains a lack of information as to whether some miRNAs regulated the PLZF in the self-renewal and differentiation of male germline stem cells.

miR-544, as one of the 18 miRNAs in the 14q32 miRNA-clusters, regulates both cell proliferation and the cell cycle in various cancers and may be a diagnostic marker of both gastric cancer and glioblastoma [Ma et al., 2012; Zhi et al., 2013]. However, there is little information on miR-544 regulation of TFs in SSCs and stem cells.

Dairy goats are economically important animals and provide milk, meat, fur, and other valuable products. They are also used as animal models for biomedical research and mGSC could be a very good model to study dairy goat mGSCs [Wu et al., 2014]. Therefore, we predicted the prospective miRNAs that target to PLZF and checked them by the dual-luciferase assay on dairy goat mGSCs. Then the expression profiles of miR-544, a candidate miRNA targeted PLZF, were explored in the different tissues of dairy goat. When the miR-544 mimics were transfected in dairy goat mGSCs, the expression of genes related to the self-renewal and differentiation of dairy goat mGSCs was determined, especially PLZF. Finally, we found that miR-544 promoted the differentiation of mGSCs by targeting PLZF. As a result, our finding provides a new regulation mechanism for the PLZF regulatory network in mGSCs and this study increases the understanding of mammalian spermatogenesis.

MATERIALS AND METHODS

mGSC CULTURE

The mGSCs derived from the dairy goat testis was maintained in our laboratory and cultured under the following culture conditions: DMEM/F12 (Invitrogen, Carlsbad, CA), supplemented with 10% bovine fetal serum (FBS, Hyclone, Logan, UT), 4 mM L-glutamine (Invitrogen), and 1% non-essential amino acids (Invitrogen) [Zhu et al., 2014].

BIOINFORMATICS PREDICTION

To predict the miRNAs targeting PLZF during spermatogenesis, the targetscan (http://www.targetscan.org/vert_61/) database was used [Dweep et al., 2011; Lewis et al., 2005].

CELL TRANSFECTION

miR-544 mimics (miR-544m), inhibitors (miR-544i), and PLZF siRNA were purchased from Genepharma Co. (Shanghai, China). The mGSCs were transfected in a 48-well plate, and scrambled oligonucleotides transfection (NC) was used as a control. The miR-544 mimics/inhibitors, and PLZF siRNA were diluted to 0.2 ng in 50 μl of Opti-MEM (Invitrogen) reduced serum medium. The protocol is referred as Yu et al. (2014). The cells' self-renewal and differentiation features were detected by real time quantitative PCR (RT qPCR) and immunofluorescence methods after those cells being transfected with miRNA for 48 hours.

The pPLZF-IRES2-EGFP recombinant plasmid was transfected into the mGSCs-I-SB cells [Zhu et al., 2014] in 48-well plates. The transfection protocol was performed as Song et al. (2013). The cells were examined by RT-qPCR and immunofluorescence after 48 h.

REAL-TIME QUANTITATIVE PCR (RT-qPCR) ANALYSIS

The total RNA from dairy goat testis tissues and cells transfected with pPLZF-IRES2-EGFP, miRNA-, and PLZF siRNA-treated cells were reverse transcribed into cDNA using the M-MuLV Reverse Transcriptase reagent kit according to the manufacturer's instructions (Thermo Scientific, New Haven, CT). RT-qPCR was performed on a CFX96 RT-qPCR detection system (Bio-Rad, CA 94547) according to the instructions for the BioEasy SYBR Green I RT-qPCR kit (Bioer Co. Ltd., Hangzhou, China). The RT-qPCR was performed as described previously by Wu et al. (2014). The relative expression levels of the target genes and differentially expressed miRNAs were normalized to *Gapdh* and *5S* RNA expression for each sample, respectively. The relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ [Wu et al., 2014]. The primers for the validated mRNAs and miRNAs are listed in Table S1.

IMMUNOFLUORESCENCE STAINING

The immunofluorescence staining was performed as described previously [Yu et al., 2014]. Briefly, cells transfected with miR-544m, miR-544i, or NC were cultured in 48-well plates and fixed with 4% formaldehyde for 10 min at room temperature. The cells were then washed with Phosphate Buffered Saline (PBS) for two times, and 5 min each. The cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature and blocked with 1% BSA for 30 min at room temperature. Then, the cells were incubated with primary antibodies, including Goat anti-Rabbit IgG plonclonal antibody: GFRa1 (1:100, Santa Cruz), PLZF (1:200, Santa Cruz Biotechnology, CA), VASA (1:200, Abcam), DAZL (1:500, Abcam), and mouse monoclonal antibody, KIT (1:200, Biolegend, San Diego, CA) overnight at 4°C. The Donkey anti-Rabbit IgG Secondary Antibody, Alexa Fluor® 488 conjugate (1:500, Molecular Probes) was used according to the manufacturer's instructions. Concurrently, the

negative controls were stained with conjugated secondary antibodies, goat anti-rabbit IgG, and goat anti-mouse IgG alone. The cell nuclei were stained with Hoechst 33342. Images were captured with an Evos F1 fluorescence microscope (AMG).

FACS ANALYSIS

For FACS-mediated cell-cycle and apoptosis analysis, $2*10^6$ mGSCs transfected with miR-544m, miR-544i, NC, pPLZF-IRES2-EGFP, or PLZF siRNA were treated by trypsinization and washed with pre-chilled PBS. Then, the cells were treated with 1 ml of reagent A and 10 μl of reagent B for 30 min at 37°C in the dark according to the manufacturer's instructions (Affinity BioReagents, China). Cell-cycle and apoptosis analysis were performed on an EPICS ELITE apparatus (Beckmann–Coulter) using the MultiCycle software (Phoenix Flow Systems, Inc.) [Wu et al., 2014].

LUCIFERASE REPORTER ASSAY

The dual-luciferase reporter vectors were generated by cloning the entire 3′UTR or mutant 3′UTR of PLZF into the psiCHCEKTM-2 vector (Promega, WI) at the site digested with NotI and XhoI. PLZF 3′UTR fragment cloning was performed using PCR (Table S2). According to the predicted interaction sites, five seed bases of miR-544 were mutated by PCR and enzyme digestion methods to construct the Mut-PLZF-3′UTR. The firefly luciferase vector was used as an internal reference, and the mutated 3′UTR of PLZF (Mut-PLZF) was used as the control. A total of 50 ng of psiCHCEKTM-2-PLZF-3′UTR (PLZF 3′UTR) or psiCHCEKTM-2 -Mut-PLZF-3′UTR (Mut-PLZF-3′UTR) and miR-544m were co-transfected into HeLa cells in a 48-well plate using Lipofectamine 2000 (Invitrogen). After 48 h, all target validation assays were performed with the dual-luciferase reporter system (Vigorous Biotechnology, Beijing) according to the manufacturer's instructions and our previous studies [Li et al., 2013; Yu et al., 2014]. The activities were measured with a BHP9504 optical analysis reader (Hamamatsu, Japan).

WESTERN BLOT

Total cell extracts were prepared from mGSCs transfected with pPLZF-IRES2-EGFP, miRNAs, or PLZF siRNA, and the proteins were extracted in 1 × SDS–PAGE sample loading buffer. The total cell proteins were resolved by SDS–PAGE, transferred to a 0.22 μm PVDF membrane at approximately 200 mA for 90 min, and probed with β-ACTIN (1:1000, Beyotime, China), C-MYC (1:500, Chemicon

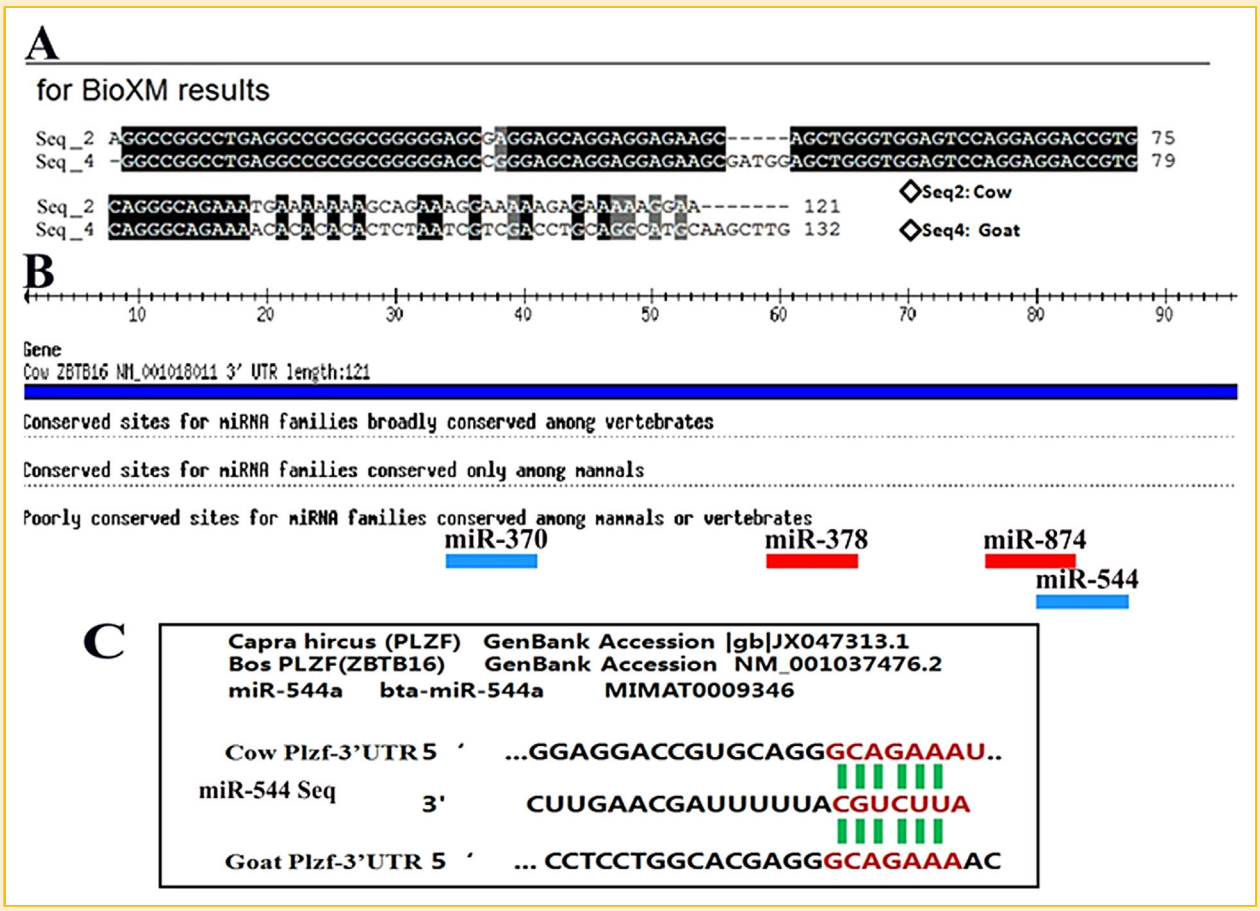

Fig. 1. The candidate miRNAs that targeting to PLZF were analysis by bioinformatics tool. (A) The 3′UTR of PLZF in cow was compared to that of dairy goat by BioXM software. (B) The putative miRNAs targeting to PLZF were analyzed by targetscan. (C) The predicted targeted sites for both cow and dairy goat.

Temecula), CXCR4 (1:300, Santa Cruz), PLZF (1:300, Santa Cruz), and VASA (1:500, Abcam). Horseradish peroxidase-conjugated anti-rabbit was used as a secondary antibody (1:1000, Beyotime). Detection was performed using the Thermo Scientific Pierce ECL Western blot substrate (Thermo Scientific). The results were analyzed with a Tanon-410 automatic gel imaging system (Tanon Corporation, China) [Yu et al., 2014].

BRDU INCORPORATION

After transfections with pPLZF-IRES2-EGFP, miRNAs, or PLZF siRNA, mGSCs proliferation was determined with a BRDU incorporation assay as described previously [Song et al., 2013]. BRDU-positive cells were detected by incubation of the cells in TRITC-conjugated secondary antibody (1:500, Millipore) for 1 h at room temperature. After three washes in PBS, the cells were visualized by fluorescence microscopy and analyzed for BRDU incorporation.

STATISTICAL ANALYSIS

The data are presented as the mean ± SEM. Differences in the expression of specific markers were evaluated using Student's *t*-test (Excel, Microsoft Corporation). The results of the different treatments were considered statistically significant at $P < 0.05$ and were highly significant at $P < 0.01$.

RESULTS

PROSPECTIVE miRNAs TARGET PLZF

The dairy goat PLZF-3′UTR was obtained by RT-PCR and was approximately 120 bp in length. The PLZF-3′UTR (1–90 bp) was highly homologous between dairy goat and bovine (Fig. 1A). Because the online bioinformatics software, targetscan, does not contain the dairy goat database, we used the highly homologous fragment of the bovine PLZF-3′UTR to identify prospective miRNAs. We found the following four candidate miRNAs: miR-370, miR-378, miR-544, and miR-874 (Fig. 1B and C), which have the seeding sequences that bind the PLZF-3′UTR.

To evaluate the effects of the miRNAs on PLZF, the four predicted miRNAs were screened and transfected into primary mGSCs. The results showed that compared to the NC-treated group, PLZF expression in the miR-370 and miR-874 transfected group was up-regulated in primary mGSCs, whereas it was downregulated in mGSCs-I-SB. By contrast, PLZF expression levels in both the miR-378 and miR-544-transfected groups were specifically downregulated in primary mGSCs and mGSC line (mGSCs-I-SB) (Fig. 2A and B). Furthermore, we verified the miRNAs screening by co-transfection of miR-378 or miR-544 with the PLZF-3′UTR luciferase vector, and the results showed that miR-544 is the optimal candidate miRNA that targets PLZF (Fig. 2C).

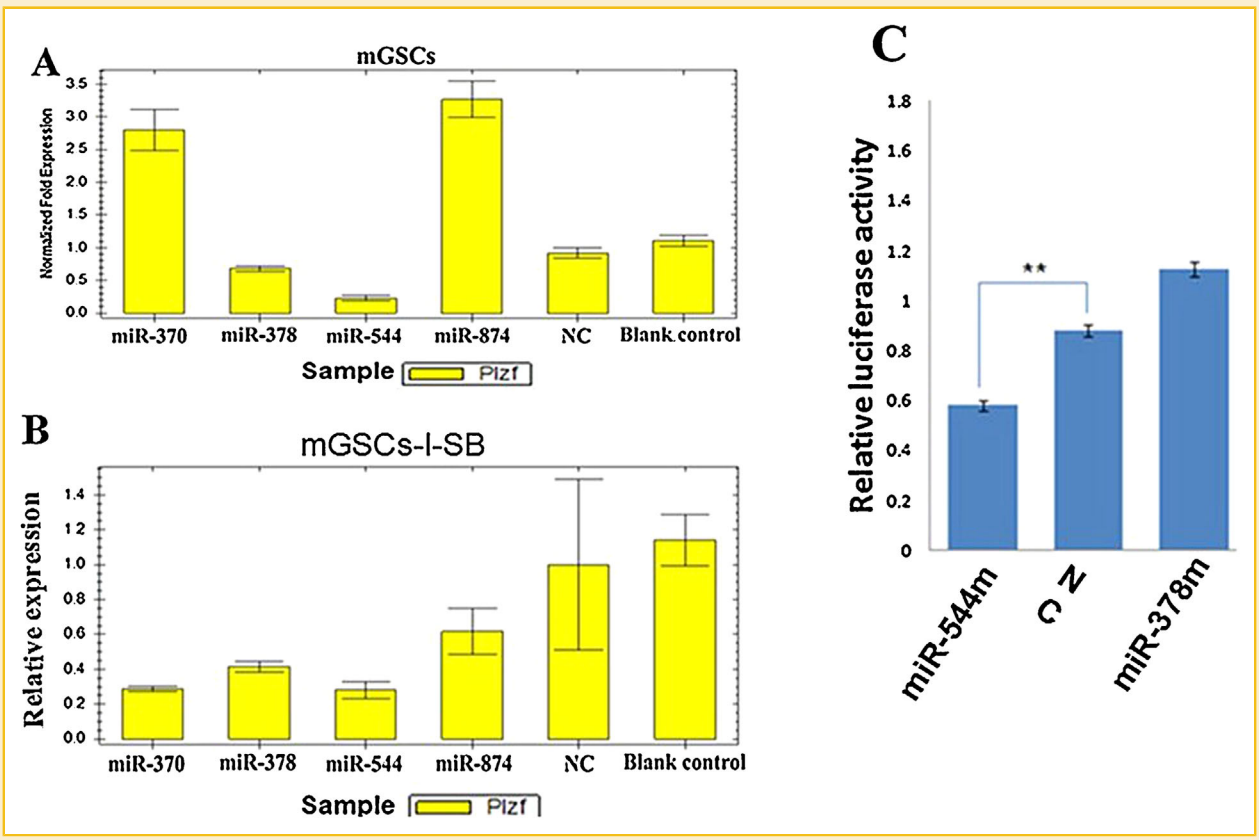

Fig. 2. Screening for miRNAs. (A) PLZF expression was detected after primary mGSCs were transfected with four selected predicted miRNAs. (B) PLZF expression was detected after primary mGSCs-I-SB cell line were transfected with four selected predicted miRNAs. (C) The luminescence intensity analysis for miR-544m was performed after the cells were transfected with psiCHCEKTM-2-PLZF-3′UTR. $^{**}P < 0.01$.

miR-544 EXPRESSION PROFILES IN DAIRY GOAT TISSUES

The expression of miR-544 in dairy goat tissues was detected by RT-qPCR. The results showed that miR-544 expression in the brain, heart, liver, and kidney was much higher than in the spleen, testis, and lung. In testis, the expression of miR-544 was highest at 12 month (M), followed by 1.5 year (Y), and 6.5 M, and the lowest expression was at 3 M (Supplemental Fig. S1A). Intriguingly, the expression levels of both PLZF and miR-544 in dairy goat tissues are completely opposite (Supplemental Fig. S1A), which suggests that miR-544 inhibits PLZF expression.

To test whether miR-544 regulates PLZF, we analyzed the expression of miR-544 and PLZF in mGSCs transfected pPLZF-IRES2-GFP, miR-544m, miR-544i, miR-544m in combination with pPLZF-IRES2-GFP and NC. The results of in vitro miRNA transfection experiment showed that overexpressed miR-544m specifically inhibited the expression of PLZF (Supplemental Fig. S1B).

CONSTRUCTION OF PLZF-3′UTR AND Mut-PLZF-3′UTR DUAL LUCIFERASE REPORTING VECTOR

PLZF-3′UTR and Mut-PLZF-3′UTR were constructed with the psiCHCEKTM-2 dual luciferase reporter vector (Supplemental Fig. S2) by PCR. The recombinant dual luciferase reporter vector of psiCHCEKTM-2-PLZF-3′UTR and psiCHCEKTM-2 -Mut-PLZF-3′UTR were verified by a restriction enzyme digestion with XhoI and NotI. The results showed that there were two amplification band lengths. The bands of PLZF-3′UTR or Mut-PLZF-3′UTR were approximately 100 bp, and the dual luciferase reporter vector was approximately 6000 bp (Supplemental Fig. S2A). The Mut-PLZF-3′UTR was designed using five site mutations of PLZF-3′UTR (Supplemental Fig. S2B and C).

miR-544 TARGETS DIRECTLY TO PLZF

The PLZF-3′UTR or Mut-PLZF-3′UTR dual luciferase reporter vector was co-transfected with miR-544 into HeLa cells, and we detected the luciferase activities. We found that compared to the NC-treated group, the luciferase activity in the miR-544m and PLZF-3′UTR co-transfection group decreased significantly. The luciferase activity in the miR-544m and Mut-PLZF-3′UTR co-transfection group also decreased; however, the level remains higher than in the former group (Fig. 3A).

Western blot was performed to determine the PLZF expression in the miR-544m or pPLZF-IRES2-EGFP transfected mGSCs. The results showed that PLZF expression in the miR-544m-treated group was lower than the NC and miR-544i-treated groups. The pPLZF-IRES2-EGFP-treated group had higher PLZF expression than the NC group, whereas the level in PLZF siRNA-transfected group was lower than the NC-treated group (Fig. 3B and C; Supplemental Fig. S1B). All of the results indicate that miR-544 inhibits PLZF expression by targeting its 3′UTR directly.

THE EFFECTS OF miR-544 ON THE PROLIFERATION OF DAIRY GOAT mGSCs

To determine whether miR-544 effects the proliferation of dairy goat mGSCs, the proliferation of mGSCs was determined by BRDU incorporation assay after treatment with miR-544m, miR-544i, NC,

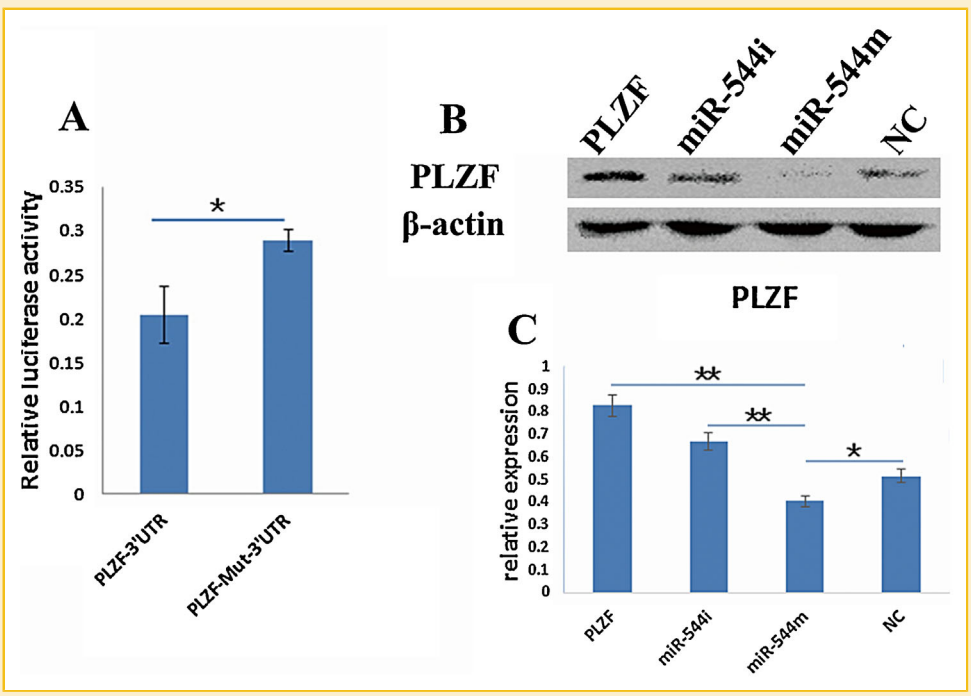

Fig. 3. PLZF is the direct target of miR-544. (A) The luminescence intensity analysis of the psiCHCEKTM-2 -Mut-PLZF-3′UTR and psiCHCEKTM-2 -PLZF-3′UTR vectors ($^{**}P < 0.01$). (B) Western blot detection for PLZF after mGSCs were transfected with miR-544. (C) Quantification of B. $^{**}P < 0.01$, $^{*}P < 0.05$.

siRNA, and pPLZF-IRES2-EGFP at 48 h. The results showed that the percentage of BRDU positive cells was significantly higher in both the miR-544i and pPLZF-IRES2-EGFP transfection groups than the NC transfected group (Fig. 4); however, the BRDU positive cell rate was significantly decreased in the miR-544m and PLZF siRNA-treated groups. The results showed that the number of cells and BRDU positive rate in the miR-544m transfection group were significantly lower than in the miR-544i or NC transfected group (Fig. 4; Supplemental Fig. S3B).

THE EFFECT OF MIR-544 ON MGSC CELL CYCLE

The cell cycle was determined by flow cytometry analysis after mGSCs-I-SB treatment with miR-544m, miR-544i, NC, siRNA, and pPLZF-IRES2-EGFP after 48 h. The results showed that the percentage of cells at the G2/M phase is lower in the miR-544m-treated group than the NC-treated group, whereas there was no significant difference between the miR-544i- and NC-treated groups (Fig. 5A). Furthermore, the cell rate was higher at S phase and lower at G1 phase in the pPLZF-IRES2-EGFP-treated group than the NC-treated group, whereas the PLZF siRNA-treated group is opposite the pPLZF-IRES2-EGFP-treated group (Fig. 5B). Taken together, our results suggest that PLZF promotes mGSC cell proliferation, whereas miR-544m specifically downregulates PLZF and inhibits mGSC proliferation (Figs. 1–5).

Simultaneously, we used FACS to analyze whether miR-544 affect the apoptosis of mGSCs, the results showed that the percentage of apoptotic cells was increased in miR-544m transfection compared to the miR-544i or NC transfected group (Fig. 6). Moreover, the rate of bigger cells in the miR-544m transfection was higher than the NC group, and the miR-544i treated group was opposite to the miR-544m-treated (Supplemental Fig. S3A). Because the larger size cell and apoptotic rate may be upregulated by MTORC1 [Hobbs et al., 2010; Ji et al., 2010], we detected the expression of MTORC1 by RT-qPCR. The results showed that the MTORC1 expression was significantly higher in miR-544m transfected mGSCs than in both the miR-544i and NC treated group (Supplemental Fig. S3B).

miR-544 REGULATES THE SELF-RENEWAL OF mGSCs VIA TARGETING PLZF

From above results, we found miR-544 specifically downregulates PLZF, and suppresses the proliferation, and increases the apoptosis rate in apoptosis. We further evaluate whether miR-544 affects the other mGSC specific genes. RT-qPCR and immunofluorescence were used to detect the expression of PLZF, GFRa1, DAZL, VASA, and KIT after miR-544 transfection in mGSCs (Fig. 7; Supplemental Fig. S4–6). The results showed that the expression levels of the self-renewal markers of mGSCs, PLZF, GFRa1, and VASA were lower in the miR-544m treated group compared to NC-treated group;

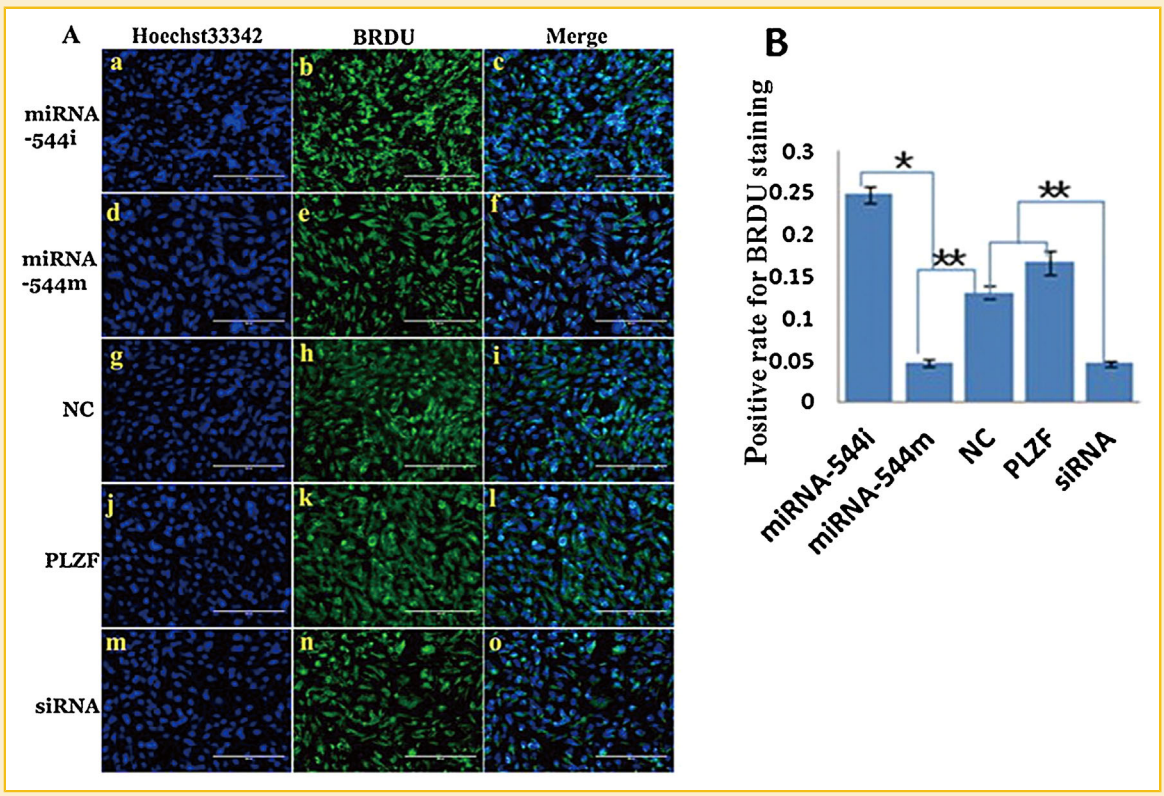

Fig. 4. BRDU incorporation assay in mGSCs transfected with miR-544. (A) Immunofluorescence analysis of the BRDU incorporation assay in mGSCs transfected with miR-544m, miR-544i, NC (scrambled oligonucleotides transfection), siRNA, and PLZF. (B) The rate of BRDU positive cells in mGSCs transfected with miR-544m, miR-544i, NC, siRNA, and PLZF, which is repetitive for labeling of y axis. $^{**}P < 0.01$; $^{*}P < 0.05$.

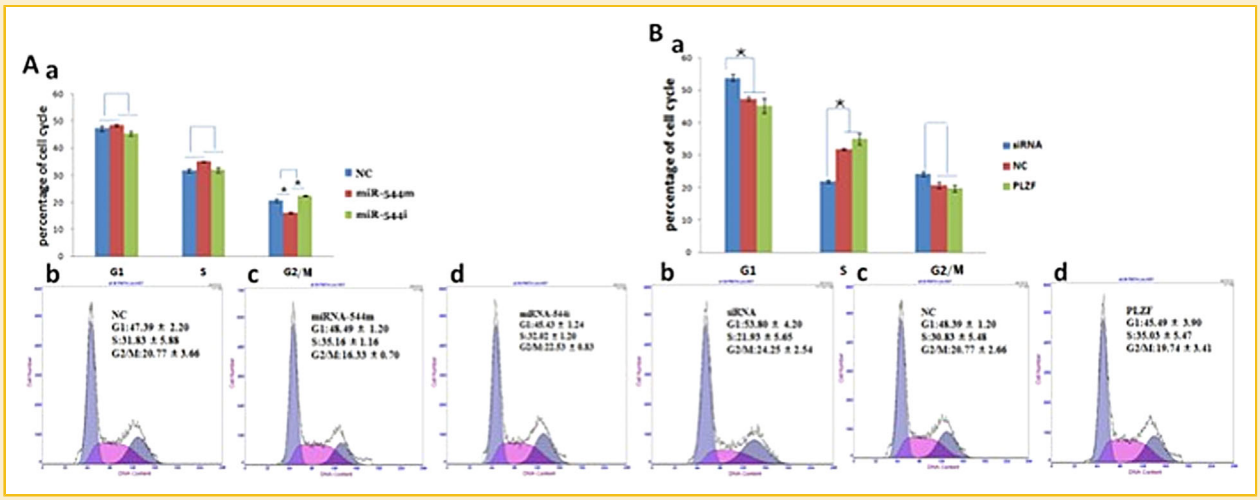

Fig. 5. The effect of miR-544 and PLZF on mGSC cell cycle. (A) The percentage of cells at the G2/M, S, and G1 phase in mGSCs transfected with miR-544m, miR-544i-, and NC. (B) The percentage of cells at the G2/M, S, and G1 phase in mGSCs transfected with pPLZF-IRES2-EGFP, PLZF siRNA, and control.

however, expression of the differentiation markers, including DAZL and KIT, were higher in the miR-544m-treated group and lower in the miR-544i-treated group (Supplemental Fig. S4–6).

Moreover, the expression of VASA (germ cell specific gene), PLZF, C-MYC (a TF that regulates cell cycle progression, apoptosis, and cell transformation.) and CXCR4 (an α-chemokine receptor specific to SDF-1) was detected by Western blot after mGSCs transfection with miR-544m, miR-544i, PLZF siRNA, and pPLZF-IRES2-EGFP. The results showed that PLZF expression was the lowest in the miR-544m-treated group and highest in the pPLZF-IRES2-EGFP group, whereas, there was significant difference between the miR-544i-treated group and miR-544m-treated, or between pPLZF-IRES2-EGFP group and PLZF siRNA-treated group (Fig. 8). The VASA expression trends were similar to PLZF, and the VASA expression was not significantly different between the miR-544i and PLZF siRNA treatment group. CXCR4 expression was highest in the pPLZF-IRES2-GFP-treated group, which was significantly higher than that in miR-544m, miR-544i, and PLZF siRNA treatment

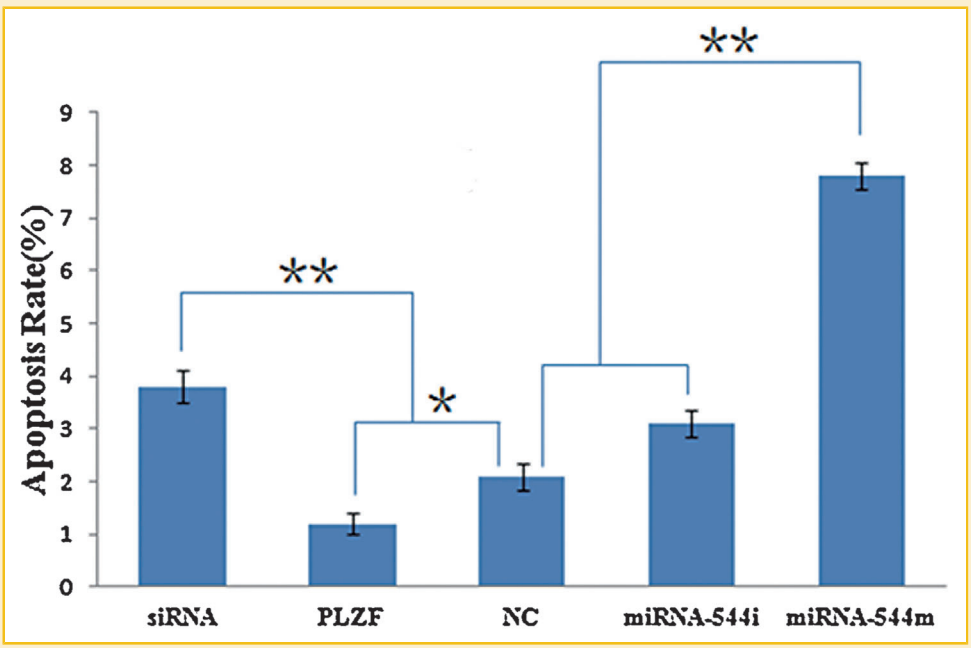

Fig. 6. Apoptosis cell rate analyzed by flow cytometry, the apoptosis cell rate of mGSCs transfected with miR-544m, miR-544i, NC, siRNA, and PLZF. $^{**}P < 0.01$; $^{*}P < 0.05$.

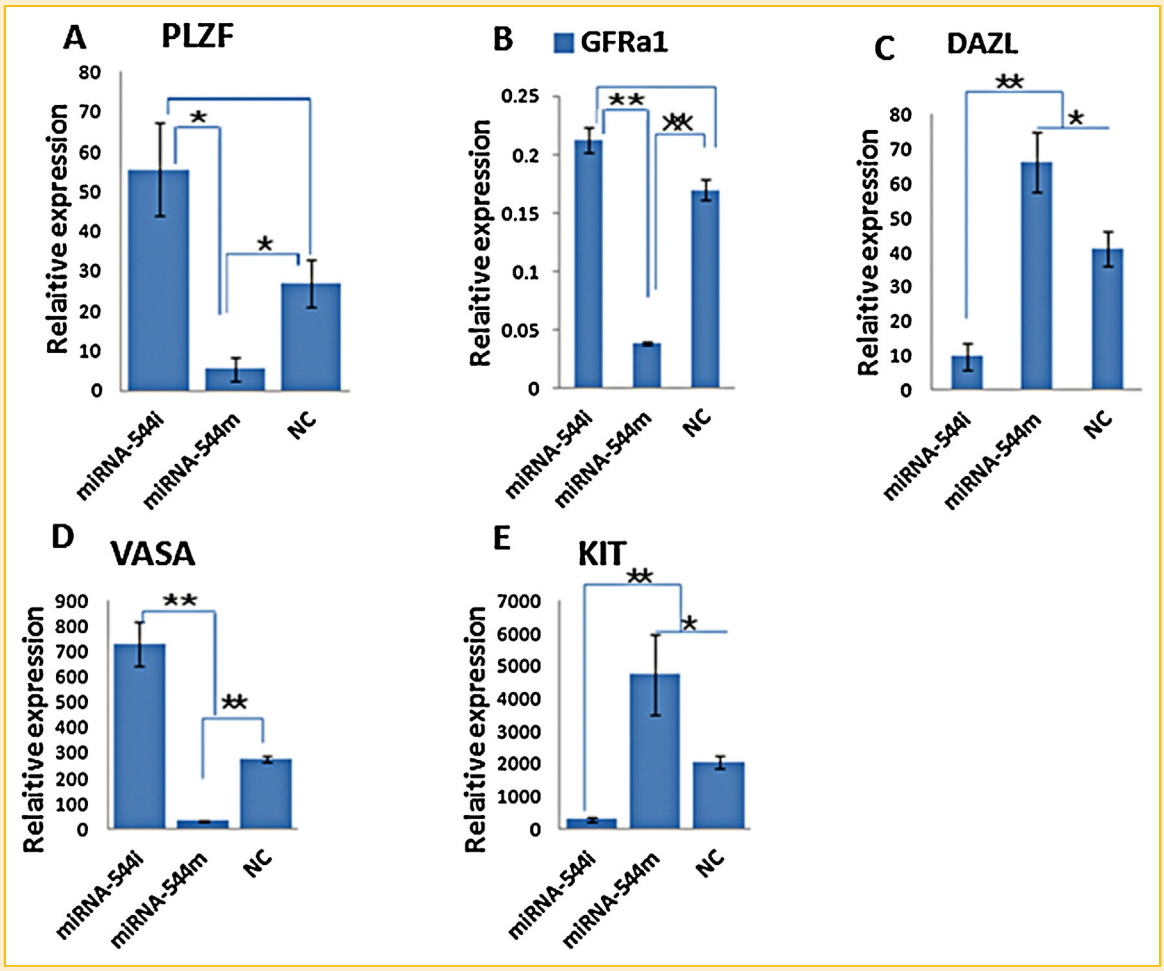

Fig. 7. RT-qPCR detection of PLZF, GFRa1, DAZL, VASA, and KIT expression after transfection of miR-544. $^{**}P < 0.01$; $^{*}P < 0.05$.

groups. By contrast, the highest C-MYC expression was found in the miR-544i-transfected group, whereas the lowest expression was in the miR-544m-treated group, and there was no significant difference between the PLZF siRNA-treated group and pPLZF-IRES2-EGFP-treated. Thus, these results support our hypothesis that miR-544 might upregulate the germ cells' differentiation markers while hinder the self-renewal and pluripotency marker to control the fate of mGSCs, and the effects depend on the target-PLZF (Fig. 9).

DISCUSSION

This study shows that miR-544 regulates PLZF in mammalian mGSCs. PLZF, as a TF, is essential for goat testis development and spermatogenesis [Song et al., 2013]. A previous study showed that PLZF is a spermatogonia-specific transcription factor in the testis that is required to regulate mGSC self-renewal and maintenance of the SSCs in humans and mice [Buaas et al., 2004; Costoya et al., 2004], and that it cooperates with other differentiation associated regulators to interact with a network to regulate the mGSC self-renewal and differentiation. Our previous study showed that PLZF promotes self-renewal and maintenance of SSCs in dairy goat [Song et al., 2013]. Proliferation, apoptosis, pluripotency, and spermatogenesis associated proteins including OCT4 and C-MYC were upregulated by PLZF over-expression, PLZF may also function in dairy goats similar to other species to maintain the self-renewal of mGSCs [Song et al., 2013].

miRNAs, a type of small RNA, play critical roles in mammalian spermatogenesis. However, the majority of miRNA studies have focused on the miRNAs present in meiotic and post-meiotic germ cells, although several studies describe their expression, by microarray or sequence analysis of the miRNAs present in the mammalian testis at different stages of development [Ro et al., 2007; Buchold et al., 2010; Jung et al., 2010; Niu et al., 2011; Shin et al., 2011; Tong et al., 2012]. There are a few studies on miRNA regulation of SSCs or cultured mGSCs. miRNAs participate in SSC self-renewal and spermatogenesis, including miR-221, miR-21, the miR-449 clusters, and miR-34b/c. miR-221 plays a crucial role in maintaining the undifferentiated state of mammalian spermatogonia through repression of KIT expression [Yang et al., 2013a]. miR-21 is important in maintaining the spermatogonia population and for spermatogonia self-renewal [Niu et al., 2011]. miR-34c regulates the differentiation of mouse

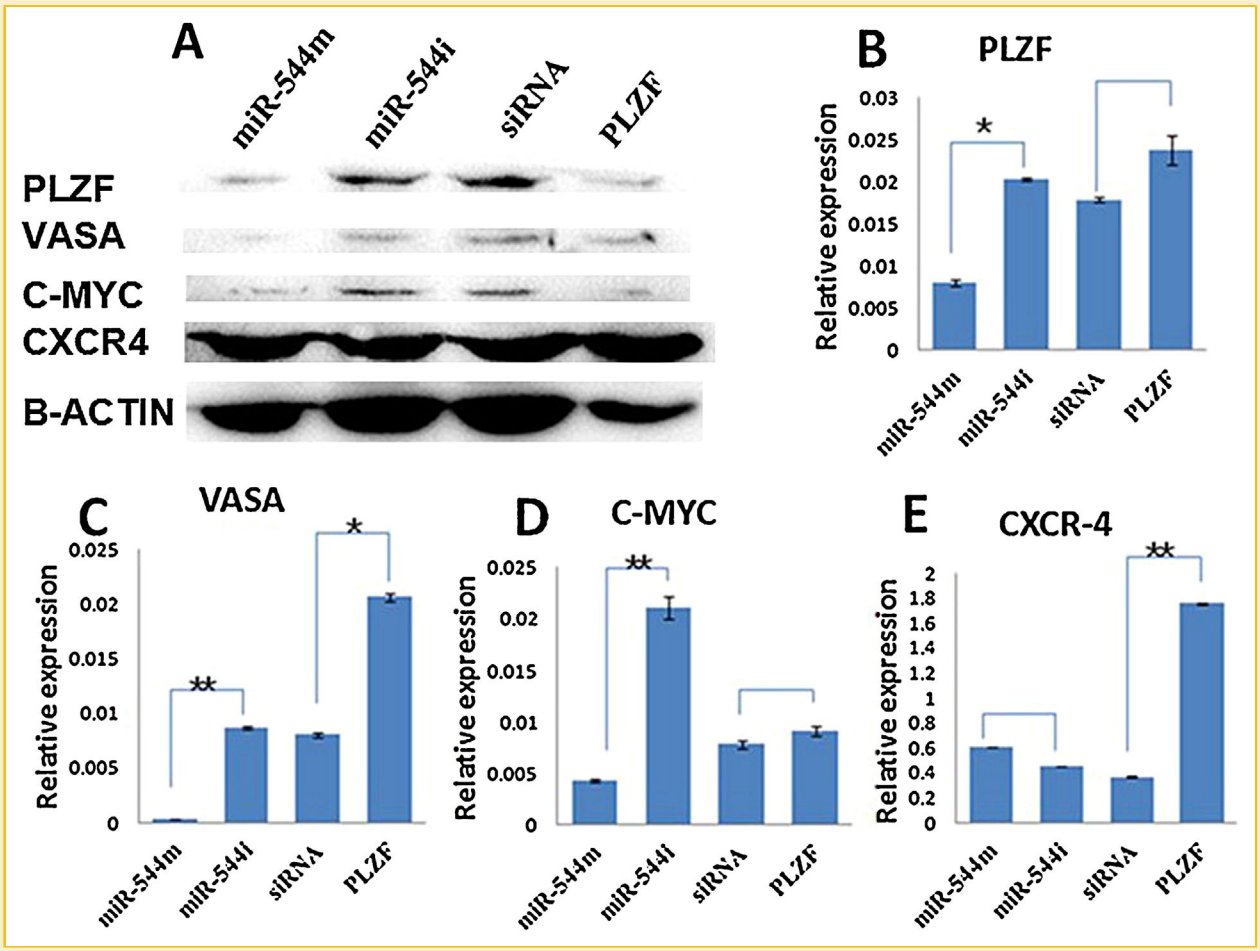

Fig. 8. Western blot analysis of PLZF, VASA, C-MYC, and CXCR4 expression after transfection of miR-544. (A) The regulation of miR-544 to the mGSCs' self-renewal markers were detected by Western blotting. (B–E) Quantification of (A). $^{**}P < 0.01$; $^{*}P < 0.05$.

embryonic stem cells into male germ-like cells through RARg and promotes SSC differentiation by targeting Nanos2 [Yu et al., 2014; Zhang et al., 2012]. The expression pattern of the miR-449 cluster resembles that of miR-34b/c during spermatogenesis, and the miR-449 cluster may have the same roles in the regulation of male germ cell development as miR-34b/c [Bao et al., 2012]. miR-544, as one of the 18 miRNAs in the 14q32 miRNA-clusters, regulates both cell proliferation and the cell cycle in various cancers and may be a diagnostic marker of both gastric cancer and glioblastoma [Ma et al., 2012; Zhi et al., 2013]. However, there is little information on miR-544 regulation of TFs in SSCs. Our results showed that miR-544 is the optimal candidate miRNA that targets PLZF through online bioinformatics software-targetscan analysis, and in vitro miRNAs screening by co-transfection of miRNAs with the PLZF-3′UTR luciferase vector. Results demonstrated that miR-544 participates in regulation of the self-renewal of SSCs and spermatogenesis in dairy goats. The role of miR-544 in dairy goat mGSCs shares a similar function with the miRNAs discussed above that are specific for SSCs and premeiotic cells in either mice or humans. The novelty of our results is that we identified a new miRNA- miR-544 that regulates the mGSC self-renewal in mammals via targeting PLZF. Moreover, the expression profiles of miR-544 and PLZF in the different tissues and testis at different ages were complete opposite to support that miR-544 might inhibit the target-PLZF.

MiRNAs play a critical role in many biological events, including cell proliferation, differentiation, and cell apoptosis [Bueno et al. 2008]. For example, miR-544 plays different roles in different types of tissues and cells. Previous studies identified miRNAs that are testis-enriched compared to other tissues [Ro et al., 2007; Buchold et al., 2010; Jung et al., 2010; Niu et al., 2011; Shin et al., 2011; McIver et al., 2012; Tong et al., 2012]. Here, our results showed that miR-544 plays an important role in mGSC self-renewal, although it has lower expression in the testis than other tissues. MiR-544 was confirmed to target PLZF directly using the dual-luciferase and Western blot assays. We further explored the effects of miR-544 on mGSC self-renewal; the expression of self-renewal related genes, such as GFRa1, PLZF, CXCR4, and C-MYC; the differentiation markers, including KIT and DAZL; and the cell-cycle after transfection of miR-544. CXCL12–CXCR4 signaling in mouse SSCs was found to be important for establishing stem–cell niches and self-

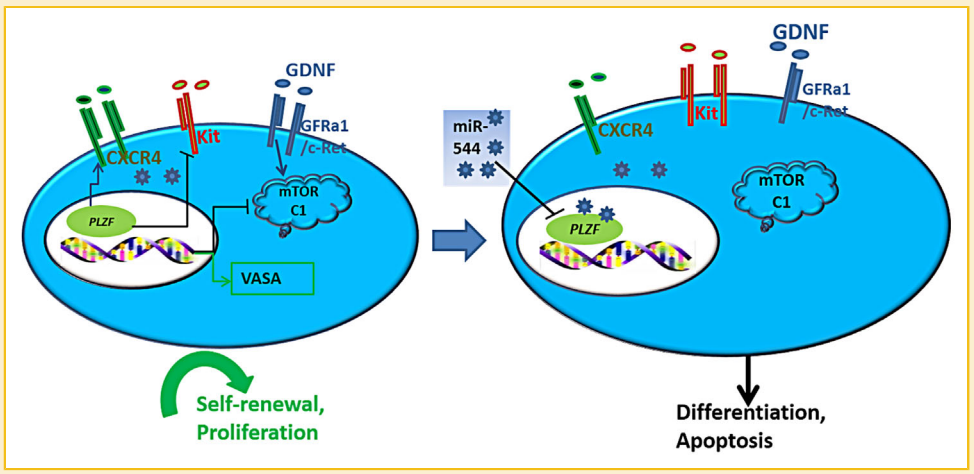

Fig. 9. Model for the function of miR-544 in the regulation of dairy goat mGSC self-renewal and differentiation.

renewal [Yang et al., 2013b]. PLZF, one of the critical self-renewal marker of SSC; Redd1, a negative regulator of mammalian TOR complex 1 (mTORC1), is crucial for maintaining the undifferentiated state of SPCs [Hobbs et al., 2010; Liao et al., 2014]. In PLZF knockout spermatogonial stem/progenitor cells (SPCs), the downregulation of Redd1 was increased, and results in the increase of mTORC1 activity, the expression of ERK-stimulated molecules (Gfra1 and Ret) were decreased, and AKT activity was decreased [Hobbs et al., 2010; Liao et al., 2014]. KIT is bound by KIT ligand (KITL) and is also essential during differentiation of a number of stem cell populations including SSC and embryonic stem cells (ESCs) [Edling and Hallberg, 2007; Busada et al., 2015]. VASA, a germ cell marker, is the highest expression in spermatogonia, then reduced in spermatocytes, low expression in spermatids, and absent in sperm.

Our results demonstrated that the PLZF was specifically downregulated by miR-544, and the expression of GFRa1, CXCR4, and C-MYC also were decreased dependent on PLZF decreasing, simultaneously, the percentage of BRDU positive cells and the S stage cells in the miR-544 overexpression group was reduced; however, the differentiation marker of SSCs, KIT, and DAZL were increased. In combination with the previous studies, we summarize the function of miR-544 for regulation mGSC self-renewal in dairy goat (Fig. 9). Specifically, at the normal stage, the expression level of miR-544 is relatively low, whereas PLZF was normally expressed, and mGSC maintain undifferentiation state. At this stage, PLZF promotes the expression of GFRa1, CXCR4, C-MYC, and VASA, and inhibits C-KIT and MTORC1 and DAZL [Hobbs et al., 2010; Song et al., 2013]. The mGSCs may take advantage of GDNF for remaining their self-renewal ability [Buageaw et al., 2005]. When exogenous miR-544 was added, PLZF expression was suppressed, which might lead to upregulation of the expression of DAZL, KIT, and MTORC1, whereas CXCR4, MYC, GFRa1, and VASA were downregulated. Therefore, GDNF was not used effectively for mGSCs; and the mGSCs will differentiate [Hobbs et al., 2010; Song et al., 2013; Liao et al., 2014].

In conclusion, our study identifies a new miRNA-miR-544 that targets to PLZF, and our data further show that miR-544 regulates the self-renewal and differentiation of mGSCs via targeting PLZF in dairy goat. Our findings may provide more information for miRNAs studies in mammals' mGSCs, and help us further understand mammalian spermatogenesis.

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