miR-499 promotes immature porcine Sertoli cell growth through the PI3K/AKT pathway by targeting the *PTEN* gene

Hu Gao, Bin Chen, Hui Luo, Bo Weng, Xiangwei Tang, Yao Chen, Anqi Yang and Maoliang Ran

College of Animal Science and Technology, Hunan Provincial Key Laboratory for Genetic Improvement of Domestic Animal, Hunan Agricultural University, Hunan, Changsha, China

Correspondence should be addressed to M Ran or B Chen; Email: ranmaoliang0903@126.com or chenbin7586@126.com

Abstract

Sertoli cells are indispensable for normal spermatogenesis, and increasing evidence has shown that miRNAs participate in the regulation of Sertoli cell growth. However, the functions and regulatory mechanisms of miRNAs in Sertoli cells of domestic animals have not been fully investigated. In the present study, we mainly investigated the regulatory roles of miR-499 in immature porcine Sertoli cells. The results showed that miR-499 was mainly located in the basement section of seminiferous tubules of prepubertal porcine testicular tissue. Overexpression of miR-499 promoted cell proliferation and inhibited apoptosis, whereas miR-499 inhibition resulted in the opposite effect. The *PTEN* gene was directly targeted by miR-499, and the expression of mRNA and protein was also negatively regulated by miR-499 in immature porcine Sertoli cells. siRNA-induced *PTEN* knockdown resulted in a similar effect as an overexpression of miR-499 and abolished the effects of miR-499 inhibition on immature porcine Sertoli cells. Moreover, both miR-499 overexpression and the *PTEN* knockdown activated the PI3K/AKT signaling pathway, whereas inhibition of the PI3K/AKT signaling pathway caused immature porcine Sertoli cell apoptosis and inhibited cell proliferation. Overall, miR-499 promotes proliferation and inhibits apoptosis in immature porcine Sertoli cells through the PI3K/AKT pathway by targeting the *PTEN* gene. This study provides novel insights into the effects of miR-499 in spermatogenesis through the regulation of immature Sertoli cell proliferation and apoptosis.

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Introduction

Spermatogenesis is an intricate process containing three major phases including mitosis, meiosis, and spermiogenesis. This developmental process is precisely regulated to guarantee the generation of highly specialized spermatozoa. Sertoli cells, the sole somatic cells within the seminiferous tubules, play crucial regulatory roles in spermatogenesis. During the embryonic period, Sertoli cells prevent pro-spermatogonia from entering meiosis and promote Leydig cell differentiation. Then, they create a stable micro-environment for spermatozoa and secrete several functional proteins to regulate the multiple biological processes of generative cells during the adult period (Hai *et al.* 2014, Crisostomo *et al.* 2018, Griswold 2018). However, each Sertoli cell has a fixed capacity to support a number of developing germ cells, indicating that sperm production in the testis is governed by the number of Sertoli cells (Johnson *et al.* 2008, Rebourcet *et al.* 2017). In addition, immature Sertoli cells usually exhibit intense proliferation during the fetal to the prepubertal period, which ceases during the mature cell period (Sharpe *et al.* 2003). Hence, immature Sertoli cell proliferation is critical for spermatogenesis and male fertility.

miRNAs, a class of non-coding RNAs, widely participate in regulating multiple biological processes through binding the 3' un-translated region (3'-UTR) of target genes and then causing translational inhibition. Specific deletion of the Sertoli cell-derived Dicer, a key enzyme in miRNA generation, severely impairs Sertoli cell maturation, function, and survival and further leads to infertility. This suggests that miRNAs in Sertoli cells are indispensable for normal spermatogenesis (Papaioannou *et al.* 2009). Recent years have witnessed the elucidation of the functions of several miRNAs in Sertoli cell proliferation and apoptosis, including miR-762 (Ma *et al.* 2016b), miR-638 (Hu *et al.* 2017), miR-1285 (Jiao *et al.* 2015), miR-196a (Zhang *et al.* 2019), miR-34c (Ran *et al.* 2019), miR-10b (Weng *et al.* 2019), and mi-26a (Ran *et al.* 2018a) in pigs; miR-130a (Li *et al.* 2018b), miR-320-3p (Zhang *et al.* 2018), miR-17-92 cluster (Hurtado *et al.* 2018), and miR-202-5p/3p (Wainwright *et al.* 2013) in mice; and miR-133b (Yao *et al.* 2016) and miR-202-3p (Yang *et al.* 2019) in humans, as well as miR-301b-3p/3584-5p (Yin *et al.* 2018) in rats. These abovementioned discoveries indicate that miRNAs are crucial epigenetic regulators in Sertoli cells. However, the functions and mechanisms of most

identified miRNAs have not yet been fully elucidated (Ran *et al.* 2015, Weng *et al.* 2017b).

Our previous study found that miR-499 expression is significantly up-regulated during the embryonic and prepubertal periods compared to that during the adult period (Ran *et al.* 2015). This was further confirmed by another report indicating that miR-499 expression levels in porcine testicular tissues decreased quadratically with increasing age (Zhang *et al.* 2015). In addition, miR-499 participates in regulating multiple cell biological processes, including cell proliferation (Li *et al.* 2016), apoptosis (Zhu *et al.* 2016), and differentiation (Jiang *et al.* 2018, Neshati *et al.* 2018). These clues suggest that miR-499 might play a regulatory role in the growth of immature porcine Sertoli cells, however, which mechanisms are involved remain unknown. The *PTEN* (phosphate and tension homology deleted on chromosome ten) gene, a key negative regulator of the phosphatidylinositol 3 kinases (PI3K)/AKT signaling pathway, has been predicted to be a direct target of miR-499 (Ran *et al.* 2015, Weng *et al.* 2017b). Recent studies have demonstrated that *PTEN* or the *PTEN*-mediated PI3K/AKT signaling pathway are involved in Sertoli cell proliferation or apoptosis in rodents (Niu *et al.* 2015, Wang *et al.* 2015, 2017). We hypothesize that miR-499 participates in the regulation of immature porcine Sertoli cell growth through the PI3K/AKT signaling pathway by targeting the *PTEN* gene. Therefore, the goal of the present study was to investigate the functions and potential molecular mechanisms of miR-499 in cell proliferation and apoptosis of immature porcine Sertoli cells.

Materials and methods

Ethics statement

This study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving animals were approved by the Ethics Committee of the Hunan Agricultural University, China.

Fluorescence in situ hybridization

Fresh testicular tissues were obtained from 1-day-old Shaziling pigs (an indigenous Chinese pig breed) after being given general anesthesia (Zoletil 50, Virbac Co., France). The pig testicular tissues were immediately incubated with 4% paraformaldehyde. The 4- μ m-thick sections were subjected to proteinase K digestion at 37°C for 30 min and then immersed three times in RNase-free water for 3 min each. A cy3-labeled miR-499 probe was designed and synthesized by RiboBio (Guangzhou, China). Hybridization was performed with the cy3-labeled miR-499 probe (final concentration, 25 nM in the cells) using a fluorescent *in situ* hybridization kit (RiboBio, China) following the manufacturer's protocols. After washing three times with PBS, the nuclei were marked by staining with DAPI (50 ng/ml, Sigma) for 5 min. The images were captured using a fluorescence microscope.

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Cell culture and transfection

The commercial swine testis cells (ATCC® CRL-1746™), isolated from swine 80- to 90-day-old fetal testes, have been identified as immature porcine Sertoli cells (Ma *et al.* 2016a). In addition, the marker genes of Sertoli cells including *Sox9*, *Amh*, and *Wt1* are specifically expressed in the commercial swine testis cells (Ran *et al.* 2018b). The immature porcine Sertoli cells were cultured in Dulbecco's modified Eagle medium (HyClone, USA) containing 10% fetal bovine serum (Gibco) at 37°C with 5% CO₂.

To measure the transfection efficiency of immature porcine Sertoli cells, 1.25 μ L of riboTRACER™ Fluorescent Oligo (final concentration, 50 nM in the cells) (Ribobio, China) and 3 μ L of riboFECT TM CP Reagent (Ribobio, China) were diluted in 30 μ L of 1 $\times$ riboFECT TM CP buffer (Ribobio, China) and incubated at room temperature for 15 min. Then, this mixture was added in each well with 465.75 μ L of complete medium for 24 h when the cells clearly reached 80% confluence. We used a GE Healthcare IN Cell Analyzer to observe the cells. At least three independent biological replicates were used in this assay.

For cell transfection, 100 pmol (final concentration, 50 nM in the cells) miR-499 mimic (GenePharma, China), mimic negative control (mimic NC) (GenePharma, China), miR-499 inhibitor (Ribobio, China), inhibitor NC (Ribobio, China), *PTEN* siRNA (Ribobio, China), siRNA NC (Ribobio, China), DMSO (Solarbio, China), LY294002 (PI3K inhibitor, dissolved in 0.5% DMSO, MedChemExpress, USA), inhibitor NC+mimic NC, miR-499 inhibitor+siRNA NC, or miR-499 inhibitor+*PTEN* siRNA was diluted with 250 μ L of serum-free Opti-MEM (Thermo Fisher Scientific Inc.) and incubated at room temperature for 5 min. Then, 5 μ L of Lipofectamine™ 2000 (Invitrogen) was also diluted with 250 μ L of serum-free Opti-MEM and incubated at room temperature for 5 min. These two mixtures were mixed together and incubated at room temperature for 15 min. Finally, the mixtures were added in each well when the cells reached approximately 80% confluence. After cultivation for 6–8 h at 37°C with 5% CO₂, the complete medium was used for cultivation. The sequence information is listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article).

Cell cycle assay

The cell cycle was analyzed using a cell cycle testing kit (Nanjing KeyGen Biotech, China) according to the manufacturer's protocols. After 24 h transfection, cells were washed three times using PBS and harvested in a 1.5-mL centrifuge tube. Then, cells were incubated in 70% (v/v) ethanol overnight at –20°C and then in propidium iodide (PI) solution (50 mg/mL) for 30 min at 4°C. The cell suspension was analyzed on a FACSCanto II flow cytometer (Becton Dickinson, USA). Three independent replicates were conducted for each cell group.

Cell proliferation assay

Cell proliferation was examined using the cell counting kit-8 (CCK-8, Multiscience, China) and 5-ethynyl-2'-deoxyuridine (EdU, Ribobio, China) incorporation assays. Cells were seeded

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in a 96-well culture plate at a density of 1×10^4 cells/well in 100 μ L of culture medium. For the CCK-8 assay, 10 μ L of CCK-8 medium was added in each well at 0 h or 48 h after transfection. Then, cells were incubated for 4 h at 37°C. The absorbance value of each well was detected using an ELISA plate reader (Molecular Devices, USA) at 450 nm. For the EdU assay, 100 μ L of EdU medium (50 μ mol) was added in each well 24 h after transfection, and cells were incubated for 2 h at 37°C. Then, DNA staining solution and EdU staining solution were added in each well to mark living cells (blue) and the proliferating (red) cells according to the manufacturer's protocols, respectively. We used a fluorescence microscope to observe the cells at 20 $\times$ and the ImageJ software to determine cell numbers. At least three independent biological replicates were used in these two assays.

Cell apoptosis assay

Cell apoptosis was detected using an Annexin V-FITC apoptosis detection kit (Nanjing KeyGen Biotech, China) and an ATP assay kit (Beyotime, China). Cells were cultured in 6-well plates with 2 mL medium. After a 24 h transfection, cells were collected in a 1.5-mL centrifuge tube. Before Annexin V-FITC apoptosis analysis, cells were washed three times and double stained with FITC-Annexin V and PI. Then, cell samples were analyzed using a FACSCanto II Flow Cytometer (Becton Dickinson, USA). Percentages of early apoptosis and late apoptosis cells were counted and used as the cell apoptosis rate. The ATP concentration was evaluated using an ATP assay kit (Beyotime, China) according to the manufacturer's protocols. The relative ATP level of experimental groups was normalized with the NC group. In addition, cell survival-related gene (*Bcl2*, *BAX*, and *Caspase-3*) protein expression levels were also measured using the Western blotting method.

Dual-luciferase activity assay

A target site between miR-499 and *PTEN* 3'UTR was predicted using TargetScan and RNAhybrid online software (Fig. 3A). A fragment of the *PTEN* 3'UTR harboring putative binding site of miR-499 was amplified using *PTEN*-3'UTR-W-F and *PTEN*-3'UTR-W-R primers (Supplementary Table 2). Then, a mutant *PTEN* 3'UTR with a six-base mutation at the putative binding site was also amplified using *PTEN*-3'UTR-Mut-F and *PTEN*-3'UTR-Mut-R primers (Supplementary Table 2). The abovementioned two fragments were subcloned into pmirGLO dual-luciferase vectors (Promega). The conducted vectors were co-transfected with miR-499 mimic or mimic NC into immature porcine Sertoli cells. After a 48 h transfection, we measured the luciferase activity of each cell group using the Dual-Glo luciferase assay system (Promega). Renilla luciferase (Promega) activity was used as the internal control.

Real-time qPCR

Real-time quantitative PCR (qPCR) was performed as in our previous studies (Ran et al. 2016, 2017, 2018b, Weng et al. 2017a). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocols. The

primers were designed using Oligo 7.0 software (Supplementary Table 2) and synthesized by Sango Bio. (Shanghai, China). The cDNA of each sample was synthesized using a Primescript first strand cDNA synthesis kit (TaKaRa, China) according to the manufacturer's protocols. The qPCR amplifications were performed on a Thermo Scientific PIKO REAL 96 real-time PCR system using a SYBR Green kit (TaKaRa, China). All qPCR reactions were performed in triplicate. *U6* and *pig-TBP* genes were used as internal controls for miR-499 and protein-coding genes, respectively. The relative expression of each gene was evaluated using the $2^{-\Delta\Delta C_t}$ method.

Western blotting

Total cellular protein was extracted using the radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, China). Then, the protein concentration was measured using a bicinchoninic acid protein assay kit (BCA) (Beyotime, China) according to the manufacturer's protocols. The boiled protein samples were electrophoresed on 10% SDS-polyacrylamide gels and then transferred onto a PVDF membrane (Beyotime, China). The membrane containing protein fractions was blocked with 5% non-fat milk for 2 h and incubated with primary antibodies overnight at 4°C, including PTEN (0.31 μ g/L, Proteintech Group, USA), *Bcl2* (0.3 μ g/L, Proteintech Group, USA), *BAX* (0.34 μ g/L, Proteintech Group, USA), *Caspase-3* (1.2 μ g/L, Abcam, Cambridge, MA), p-PI3K (1 μ g/L, phospho Tyr458, Cell Signaling Technology, USA), p-AKT (1 μ g/L, phospho Ser473, Affinity, USA), and β -actin (1 μ g/L, Proteintech Group, USA). After washing, the membrane was incubated with secondary antibodies (1 μ g/L, Proteintech Group, USA) for 2 h at room temperature. Protein bands were visualized using an ECL advanced Western blotting detection kit (Beyotime, China). β -actin served as the loading control.

Statistical analysis

Data are presented as the mean $\pm$ S.D. Data from the experiment with multiple cell groups were subjected to a one-way ANOVA followed by Duncan's multiple comparison test of significance using SPSS 17.0 software. The *t*-test was used to test the differences in the experiment with only two cell groups. $P < 0.05$ or $P < 0.01$ was considered statistically significant.

Results

Location of miR-499 in prepubertal porcine testicular tissue

To identify the location of miR-499, we conducted a fluorescence *in situ* hybridization assay using fresh pig testicular tissues using 1-day-old samples. As shown in Fig. 1, the miR-499 was mainly located in the basement section of the seminiferous tubules where the Sertoli cells are usually located. This result suggested that miR-499 might play regulation roles in immature Sertoli cell growth because immature Sertoli cells usually exhibit intense proliferation during the prepubertal period.

Figure 1 The location of miR-499 in prepubertal porcine testicular tissue. Testicular tissues of 1-day-old pigs were used in this experiment. Nuclei were marked by staining with DAPI (blue). The miR-499 was marked with red. Scale bar = 100 μ m.

miR-499 promotes immature porcine Sertoli cell proliferation

The transfection efficiency of immature porcine Sertoli cells was measured through transfecting riboTRACER™ Fluorescent Oligo and detected using a GE Healthcare IN Cell Analyzer. The results demonstrated that the green fluorescence signal was evenly detected in cells and that the transfection efficiency could reach 60–70% (Supplementary Fig. 1A). In addition, the relative expression of miR-499 was significantly increased in the miR-499 transfected group (mimic NC, 1.013 ± 0.206 ; miR-499 mimic, 1.862 ± 0.396), whereas significantly decreased in the miR-499 inhibitor group (inhibitor NC, 1.000 ± 0.028 ; miR-499 inhibitor, 0.578 ± 0.029) (Supplementary Fig. 1B).

The cell cycle analysis revealed that miR-499 overexpression increased the percentage of cells in the S phase ($27.550 \pm 0.304\%$) compared with that in the mimic NC transfected cell group ($25.710 \pm 0.898\%$) (Fig. 2A and B). Conversely, fewer miR-499 inhibitor transfected cells were detected in the S phase ($39.283 \pm 0.726\%$), whereas more cells were found in the G1 phase compared to that in the inhibitor NC transfection group ($41.990 \pm 0.443\%$) (Fig. 2A and C). Then, we further examined the relative expression levels of cell cycle-related genes *c-MYC*, *CNNE1*, *CCND1*, and *CDK4* in the abovementioned four cell groups. Expression levels of these four genes were up-regulated in the miR-499 mimic transfected cell group (*c-MYC*, 1.588 ± 0.141 ; *CNNE1*, 1.541 ± 0.138 ; *CCND1*, 1.541 ± 0.139 ; *CDK4*, 2.341 ± 0.201) (Fig. 2E), whereas they decreased in the miR-499 inhibitor transfected cell group (*c-MYC*, 0.558 ± 0.128 ; *CNNE1*, 0.643 ± 0.049 ; *CCND1*, 0.5125 ± 0.173 ; *CDK4*, 0.499 ± 0.037) (Fig. 2F). These results demonstrated that miR-499 promotes cell cycle progression.

Next, we further investigated the role of miR-499 in cell proliferation using the CCK-8 and EdU incorporation assays. The results from the CCK-8 assay revealed that miR-499 overexpression significantly increased the cell proliferation index from 3.571 ± 0.070 to 3.899 ± 0.179

($P < 0.05$), whereas the miR-499 knockdown remarkably inhibited the proliferation index from 3.716 ± 0.061 to 3.462 ± 0.055 ($P < 0.01$) (Fig. 2D). Similarly, the EdU incorporation assay results indicated that miR-499 mimic transfected cells exhibited significantly higher mitotic activity ($49.710 \pm 1.845\%$ of EdU-positive cells) than did the mimic NC transfected cells ($44.040 \pm 2.205\%$ of EdU-positive cells) ($P < 0.01$), whereas the mitotic activity in the miR-499 inhibitor transfected cells was significantly inhibited ($53.270\% \pm 1.338\%$ to $50.420 \pm 1.232\%$ of EdU-positive cells) ($P < 0.01$) (Fig. 2G and H). These results suggest that miR-499 promotes immature porcine Sertoli cell proliferation.

miR-499 inhibits immature porcine Sertoli cell apoptosis

It was shown that miR-499 promotes immature porcine Sertoli cell proliferation; thus, we determined whether miR-499 regulates cell apoptosis. The percentage of apoptosis in immature porcine Sertoli cell decreased from $3.316 \pm 0.210\%$ to $2.653 \pm 0.311\%$ after miR-499 upregulation and increased from $3.083 \pm 0.172\%$ to $3.750 \pm 0.176\%$ when miR-499 was knocked down (Fig. 3A and B). Cell growth is maintained by ATP; therefore, the ATP level can be used to access cell apoptosis (Li et al. 2018a). miR-499 overexpression resulted in an increase in the relative ATP level in immature porcine Sertoli cells (mimic NC, 1.000 ± 0.017 ; miR-499 mimic, 1.130 ± 0.043) ($P < 0.01$), whereas miR-499 knockdown decreased the relative ATP level (inhibitor NC, 1.000 ± 0.032 ; miR-499 inhibitor, 0.880 ± 0.013) ($P < 0.01$) (Fig. 3C). The Bcl2 protein expression, an inhibitor of cell apoptosis, was markedly increased by mimic-induced miR-499 overexpression, whereas it was decreased by the miR-499 knockdown (Fig. 3D). Furthermore, overexpression and knockdown of miR-499, respectively, increased and decreased some apoptosis markers, including Bcl2, BAX, and Caspase-3 protein expression (Fig. 3D). These results indicated that miR-499 inhibits immature porcine Sertoli cell apoptosis.

Figure 2 miR-499 promotes immature porcine Sertoli cell proliferation. The immature porcine Sertoli cells were transfected with mimic NC, miR-499 mimic, inhibitor NC, or miR-499 inhibitor. (A) Cell cycle was analyzed 24 h after transfection using a FACSCanto II flow cytometer. (B and C) The G1, S, and G2 phases of the cell cycle were counted in cells transfected with miR-499 mimic (B) or miR-499 inhibitor (C) ($n=3$). (D) The CCK-8 assay was used to measure the cell proliferation index ($n=3$). (E and F) The mRNA relative expression of cell cycle-related genes *c-MYC*, *CNNE1*, *CCND1*, and *CDK4* were detected in cells transfected with miR-499 mimic (E) or miR-499 inhibitor (F) using a qPCR assay ($n=3$). The *pig-TBP* gene was used as the internal control. (G) Representative images of EdU staining of immature porcine Sertoli cells 24 h after transfection. Scale bar=200 μ m. (H) Results of the analysis of EdU-positive cells ($n=3$). Data are presented as the mean $\pm$ s.d. * $P<0.05$ and ** $P<0.01$.

miR-499 activates the PI3K/AKT signaling pathway via targeting the PTEN gene

To investigate the mechanism involved in the action of miR-499 on immature porcine Sertoli cell proliferation and apoptosis, we predicted the target genes of miR-499 using the TargetScan and RNAhybrid online software and found that the *PTEN* gene was a potential target gene

(Fig. 4A). Then, a fragment of the *PTEN* 3'UTR with the potential target site of miR-499 (wild type, W) or mutant nucleotides (mutant type, Mut) was subcloned into a psi-CHECK-2 dual-luciferase reporter vector. The W or Mut vector was co-transfected with the miR-499 mimic or mimic NC into immature porcine Sertoli cells. The ratio of luciferase activity was significantly suppressed

Figure 3 miR-499 inhibits immature porcine Sertoli cell apoptosis. The immature porcine Sertoli cells were transfected with mimic NC, miR-499 mimic, inhibitor NC, or miR-499 inhibitor. (A and B) Immature porcine Sertoli cell apoptosis phase distributions were checked (A) and counted (B) 24 h after transfection using Annexin V-FITC/PI staining assay ($n=3$). (C) The relative ATP level was measured using an ATP assay kit. (D and E) The Western blot assay was used to detect the protein expression of cell apoptosis marker genes Bcl2, BAX, and Caspase-3 and key proteins in the PI3K/AKT signaling pathway (p-PI3K and p-AKT). The β -actin gene was used as the internal control. Data are presented as the mean $\pm$ s.d. * $P<0.05$ and ** $P<0.01$.

Figure 4 miR-499 directly targets the *PTEN* gene. (A) The *PTEN* gene was predicted as a target of miR-499 using the TargetScan and RNAhybrid online software. (B) Luciferase reporter assay results were quantified after co-transfection of the miR-499 mimic/mimic NC and *PTEN*-3'UTR-W/Mut vector into immature porcine Sertoli cells ($n=3$). Renilla luciferase (Promega) activity was used as an internal control. (C and D) The *PTEN* mRNA (C) and protein expression (D) were measured using qPCR and Western blot assays, respectively ($n=3$). The *Pig-TBP* and β -actin genes were used as internal controls in qPCR and Western blot assays, respectively. Data are presented as the mean $\pm$ s.d. * $P<0.05$ and ** $P<0.01$.

in cells co-transfected with the miR-499 mimic and the *PTEN*-3'UTR-W vector compared with that of other groups ($P < 0.01$) (Fig. 4B). In accordance with luciferase activity results, *PTEN* mRNA (mimic NC, 1.000 ± 0.024 ; miR-499 mimic, 0.569 ± 0.035) and protein expressions were significantly down-regulated by miR-499 overexpression and up-regulated in response to the knockdown of miR-499 (inhibitor NC, 1.000 ± 0.042 ; miR-499 inhibitor, 1.138 ± 0.021) ($P < 0.05$) (Fig. 4C and D). These results demonstrated that miR-499 targets the *PTEN* gene and represses *PTEN* mRNA abundance in immature porcine Sertoli cells.

In previous studies, the *PTEN* gene was found to be a key negative regulator of the PI3K/AKT signaling pathway. Therefore, we further accessed whether the effect of miR-499 on activation of PI3K and AKT was dependent on the *PTEN* gene. Interestingly, overexpression of miR-499 increased p-PI3K and p-AKT protein levels, whereas knockdown of miR-499 decreased these protein levels (Fig. 3E). In addition, the *PTEN* knockdown showed a similar effect with miR-499 overexpression (Fig. 5I). Considered together, we speculate that miR-499 activates the PI3K/AKT signaling pathway by targeting the *PTEN* gene in immature porcine Sertoli cells.

Figure 5 *PTEN* gene deficiency promotes immature porcine Sertoli cell proliferation and inhibits cell apoptosis. *PTEN* siRNA was transfected into immature porcine Sertoli cells to knockdown *PTEN* gene expression. (A and C) The cell cycle was detected (A) and counted (B) using a FACSCanto II Flow Cytometer ($n = 3$). (D) The mRNA relative expression of the cell cycle-related genes *c-MYC*, *CNNE1*, *CCND1*, and *CDK4* were detected using qPCR assay ($n = 3$). The *pig-TBP* gene was used as the internal control. (E and F) The cell proliferation index and mitotic activity were measured using CCK-8 (E) and EdU incorporation assay (F), respectively ($n = 3$). (B and G) Cell apoptosis rate was detected (B) and counted (G) using Annexin V-FITC/PI staining assay ($n = 3$). (H) The relative ATP level was measured using an ATP assay kit. (I) The protein expression of Bcl2, BAX, Caspase-3, p-PI3K, and p-AKT were detected using Western blot assay. The β-actin gene was used as the internal control. Data are presented as the mean $\pm$ S.D. * $P < 0.05$ and ** $P < 0.01$.

PTEN gene deficiency promotes immature porcine Sertoli cell proliferation and inhibits cell apoptosis

We sought to determine whether the *PTEN* gene regulated immature porcine Sertoli cell proliferation and apoptosis. Cell cycle analysis indicated that siRNA-induced *PTEN* knockdown (Supplementary Fig. 1C) significantly decreased the percentage of cells in the G1 phase from $38.480 \pm 0.582\%$ to $42.800 \pm 0.585\%$ ($P < 0.01$) (Fig. 5A and C). In addition, the expression of *c-MYC* (1.654 ± 0.181), *CNNE1* (2.536 ± 0.279), *CCND1* (1.243 ± 0.051), and *CDK4* (4.457 ± 0.514) were significantly up-regulated in the *PTEN* siRNA transfected cell group compared with that of the siRNA NC transfected cell group (designated as 1.0) ($P < 0.05$) (Fig. 5D). The CCK-8 assay results showed that the *PTEN* knockdown promoted the cell proliferation index from 3.833 ± 0.146 to 4.633 ± 0.322 ($P < 0.05$) (Fig. 5E). Similarly, the EdU incorporation assay results demonstrated that cell mitotic activity was significantly increased (from $43.690 \pm 2.18\%$ to $49.990 \pm 3.345\%$ of EdU-positive cells) by the knockdown of the *PTEN* gene ($P < 0.05$) (Fig. 5F).

Next, we clarified the roles of the *PTEN* gene on cell apoptosis. The Annexin V-FITC/PI staining assay revealed that the cell apoptosis rate was significantly repressed from $2.630 \pm 0.104\%$ to $2.067 \pm 0.092\%$ by the silencing of the *PTEN* gene ($P < 0.01$) (Fig. 5B and G). The knockdown of the *PTEN* gene significantly increased the relative ATP level from 1 ± 0.02 to 1.285 ± 0.057 in immature porcine Sertoli cells ($P < 0.05$) (Fig. 5H). Furthermore, the Western blot assay indicated that Bcl2 protein expression was significantly increased, whereas BAX and Caspase-3 protein expression were significantly decreased by knocking down the *PTEN* gene (Fig. 5I). Overall, these data demonstrated that *PTEN* gene deficiency promotes immature porcine Sertoli cell proliferation and inhibits cell apoptosis.

PTEN knockdown offsets the effects of miR-499 inhibition

Our results indicated that the *PTEN* gene knockdown resulted in similar results as those of the overexpression of miR-499 in cell proliferation and apoptosis. We then sought to determine whether the *PTEN* gene knockdown could reverse the roles of miR-499 in immature porcine Sertoli cells. Therefore, we constructed three co-transfection treatments including inhibitor NC+mimic NC, miR-499 inhibitor+siRNA NC, and miR-499 inhibitor+*PTEN* siRNA. Cell cycle results showed that the percentage of cells in the S phase was decreased from $49.840 \pm 2.192\%$ to $45.603 \pm 1.469\%$ in the miR-499 inhibitor+siRNA NC treatment ($P < 0.05$), whereas the miR-499 inhibitor+*PTEN* siRNA treatment increased cell percentage from $14.757 \pm 0.527\%$ to $18.470 \pm 0.774\%$ in the G2 phase ($P < 0.05$) (Fig. 6A).

The relative mRNA expression of *CNNE1*, *CCND1*, and *CDK4* genes were significantly reduced by the miR-499 inhibitor+siRNA NC treatment (0.723 ± 0.011 , 0.512 ± 0.138 , 0.754 ± 0.105) and increased by the miR-499 inhibitor+*PTEN* siRNA treatment (1.435 ± 0.063 , 1.471 ± 0.117 , 1.486 ± 0.183) ($P < 0.05$) compared with that of the inhibitor NC+siRNA NC treatment (designated as 1.0) (Fig. 6B). Correspondingly, the CCK-8 and EdU incorporation assays showed that the *PTEN* knockdown could restrain the miR-499 inhibitor-induced lower proliferation activity of cells (Fig. 6C, D and E). In addition, the Annexin V-FITC/PI staining assay indicated that miR-499 inhibitor+siRNA NC caused a higher cell apoptosis rate ($3.620 \pm 0.600\%$) compared with that of the inhibitor NC+siRNA NC treatment ($2.637 \pm 0.108\%$) ($P < 0.05$), whereas this role was abolished by the *PTEN* knockdown ($2.593 \pm 0.240\%$) ($P < 0.05$) (Fig. 6F). Similarly, the protein expression of Bcl2 decreased significantly ($P < 0.05$), and the protein expression of BAX and Caspase-3 increased in miR-499 inhibitor+siRNA NC co-transfected cells ($P < 0.05$), whereas these effects were attenuated by knocking down the expression of the *PTEN* gene (Fig. 6G). Collectively, these results indicated that the effects of miR-499 inhibition are attenuated by the *PTEN* knockdown.

miR-499 promotes immature porcine Sertoli cell growth through activating the PI3K/AKT signaling pathway

We have demonstrated that both miR-499 overexpression and *PTEN* knockdown activated the PI3K/AKT signaling pathway. Therefore, we sought to determine whether miR-499 regulates immature porcine Sertoli cell proliferation and apoptosis through activating the PI3K/AKT signaling pathway. Inhibition of PI3K/AKT signaling pathway arrested cells in the G1 phase, which was evidenced by more cells in the G1 phase (DMSO, $41.743 \pm 5.147\%$; anti-PI3K/AKT, $54.256 \pm 2.493\%$) and fewer cells in the G2 phase (DMSO, $11.127 \pm 1.182\%$; anti-PI3K/AKT, $4.340 \pm 3.891\%$) (Fig. 7A and C), as well as the down-regulation of the expression of cell cycle-related genes, including *c-MYC* (0.346 ± 0.178), *CNN1* (0.089 ± 0.037), *CCND1* (0.370 ± 0.210), and *CDK4* (0.351 ± 0.164) (Fig. 7D). The CCK8 (DMSO, 3.682 ± 0.103 ; anti-PI3K/AKT, 4.706 ± 0.116) and EdU incorporation assays (from $29.800 \pm 0.008\%$ to $12.910 \pm 0.009\%$ of EdU-positive cells) demonstrated that cell proliferation was inhibited after PI3K/AKT signaling pathway inhibition ($P < 0.05$) (Fig. 7E and F). In addition, PI3K/AKT signaling pathway inhibition caused a higher cell apoptosis rate from $1.923 \pm 0.101\%$ to $2.530 \pm 0.147\%$ and a lower relative ATP level from 39.682 ± 1.488 to 33.509 ± 1.781 in immature porcine Sertoli cells (Fig. 7B, G and H). Correspondingly, the protein expression of Bcl2 decreased, and the protein expression of BAX and Caspase-3 increased in cells

Figure 6 *PTEN* knockdown attenuates the effects of miR-499 inhibition in immature porcine Sertoli cells. Three co-transfection treatments including inhibitor NC + mimic NC, miR-499 inhibitor + siRNA NC, and miR-499 inhibitor + *PTEN* siRNA were conducted in this section. (A) The cell cycle was detected and counted using a FACSCanto II flow cytometer ($n=3$). (B) The mRNA relative expression of cell cycle-related genes *c-MYC*, *CNNE1*, *CCND1*, and *CDK4* were detected using a qPCR assay ($n=3$). The *pig-TBP* gene was used as the internal control. (C) The cell proliferation index was measured using the CCK-8 assay ($n=3$). (D and E) Cell mitotic activity was measured (D) and counted (E) using the EdU incorporation assay ($n=3$). (F) The cell apoptosis rate was detected and counted using the Annexin V-FITC/PI staining assay ($n=3$). (G) The protein expression of Bcl2, BAX, Caspase-3, p-PI3K, and p-AKT were detected using a Western blot assay. The β -actin gene was used as the internal control. Data are presented as the mean $\pm$ s.d. Different letters mean values within each section were significantly different.

with PI3K/AKT signaling pathway inhibition (Fig. 7I). Our results indicated that PI3K/AKT signaling pathway inhibition inhibited immature porcine Sertoli cell growth, which was opposite to that of miR-499 overexpression and *PTEN* knockdown.

Discussion

Sertoli cells are indispensable to guarantee the normal spermatogenesis process. Each Sertoli cell supports a limited number of germ cells, and the Sertoli cells lose proliferation capacity in the pubertal period. Therefore, the proliferation capacity of immature Sertoli cells determines the number of adult Sertoli cells in the pubertal period and further affects spermatogenesis. Most research on Sertoli cell proliferation and maturation focus on protein-coding genes (Griswold 2018), especially in domestic animals. More than 450 mature miRNAs have been identified from pigs, whereas regulatory roles and

mechanisms of most identified miRNAs in Sertoli cells are not fully elucidated. In the present study, we report that miR-499 promotes immature porcine Sertoli cell growth through the PI3K/AKT pathway by targeting the *PTEN* gene.

Our previous study and that of others showed that miR-499 exhibits a higher expression level during the fetal and prepubertal periods than in the pubertal period in porcine testicular tissues (Ran et al. 2015, Zhang et al. 2015). It was further reported that miR-499 targets the *QKI* gene and represses its expression in immature porcine Sertoli cells (Zhang et al. 2015). Therefore, we speculated that miR-499 might play regulatory roles in immature porcine Sertoli cell growth. Recently, miR-499 has been reported to participate in cell proliferation, apoptosis, and differentiation in multiple cell types by targeting various genes, such as human tumor cells (i.e., SGC-7901, A-549, SW-480, and HepG-2s) (Long & Pi 2018, Wan et al. 2018), mouse C2C12 and skeletal

Figure 7 PI3K/AKT signaling pathway inactivation inhibits immature porcine Sertoli cell proliferation and promotes cell apoptosis. DMSO and LY294002 were added to the cell culture medium to function as NC and PI3K/AKT signaling pathway inactivation, respectively. (A and C) The cell cycle was detected (A) and counted (C) using a FACSCanto II flow cytometer ($n=3$). (D) The mRNA relative expression of cell cycle-related genes *c-MYC*, *CNNE1*, *CCND1*, and *CDK4* were detected using a qPCR assay ($n=3$). The *pig-TBP* gene was used as the internal control. (E and F) The cell proliferation index and mitotic activity were measured using the CCK-8 (E) and EdU incorporation assays (F), respectively ($n=3$). (B and G) Cell apoptosis rate was detected (B) and counted (G) using the Annexin V-FITC/PI staining assay ($n=3$). (H) The relative ATP level was measured using an ATP assay kit. (I) The protein expression of Bcl2, BAX, Caspase-3, p-PI3K, and p-AKT were detected using a Western blot assay. The β -actin gene was used as the internal control. Data are presented as the mean $\pm$ s.d. * $P<0.05$ and ** $P<0.01$.

muscle satellite cells (Jiang *et al.* 2018, Wu *et al.* 2019), and rat H9c2 cardiomyocytes (Ding *et al.* 2018). However, the regulatory roles of miR-499 in immature porcine Sertoli cells have not been elucidated. Our results illustrated that miR-499 overexpression promoted cell cycle progression, enhanced proliferation activity, decreased the cell apoptosis rate, and increased the relative ATP level in immature porcine Sertoli cells, whereas inhibition of miR-499 had an opposite effect.

In the present study, the *PTEN* gene was identified as a target of miR-499 using bioinformatics analysis and the luciferase assay, and the protein translation process of the

PTEN gene was also repressed by miR-499 in immature Sertoli cells. It has been shown that the *PTEN* gene is negatively regulated to immature mouse Sertoli cell proliferation, testis size, and sperm production *in vivo* (Neirijnck *et al.* 2019) and participates in bisphenol-A induced rat Sertoli cell apoptosis (Wang *et al.* 2015). In addition, various miRNAs promote proliferation and inhibit apoptosis by targeting the *PTEN* gene in several cell types (Li *et al.* 2018c), such as miR-374b in human gastrointestinal stromal tumor cells (Long *et al.* 2018), miR-524 and miR-93 in human osteosarcoma cells (Kawano *et al.* 2015, Zhuang *et al.* 2018), and miR-3142

in chronic myeloid leukemia cells (Zhao *et al.* 2017). In the present study, siRNA-induced *PTEN* knockdown promoted proliferation and repressed apoptosis in immature porcine Sertoli cells, which was consistent with the effects of miR-499 overexpression. In addition, the effects of miR-499 inhibition were reversed by the *PTEN* knockdown. These results demonstrated that miR-499 promotes proliferation and inhibits apoptosis in immature porcine Sertoli cells through targeting the *PTEN* gene.

Phosphatidylinositol 3,4,5-trisphosphate (PIP3), a product of PI3K, is the key substrate of PTEN and the mediator of AKT (Matsuoka & Ueda 2018). PTEN maintains PIP3 at a low expression level through dephosphorylating the PIP3. It has been widely established that the *PTEN* gene is a key negative regulator of the PI3K/AKT signaling in multiple processes, including cell progression, proliferation (Neirijnck *et al.* 2019), apoptosis, differentiation, migration, and autophagy (Wang *et al.* 2018). Several hormones participate in male Sertoli cell processes through regulating the PI3K/AKT signaling pathway. For example, thyroid hormone inhibits the proliferation of piglet Sertoli cells via the suppression of the PI3K/AKT signaling pathway (Sun *et al.* 2015). Follicle-stimulating hormone (FSH) promotes the proliferation of immature rat Sertoli cells through the activation of the PI3K/AKT signaling pathway (Riera *et al.* 2012, Nascimento *et al.* 2016). 17 β -estradiol regulates immature boar Sertoli cell proliferation by activating the PI3K/AKT signaling pathway (Yang *et al.* 2017). Our results demonstrated that miR-499 overexpression inhibited *PTEN* mRNA and protein expression, and both miR-499 overexpression and *PTEN* knockdown increased the p-PI3K and p-AKT protein expression. These results showed that the miR-499/*PTEN*/PI3K-AKT axis exists in immature porcine Sertoli cells, although its regulatory roles in immature porcine Sertoli cells remain unknown. Therefore, LY294002 was added into the cell medium to inhibit the activity of the PI3K/AKT signaling pathway. The results indicated that the inhibition of the PI3K/AKT signaling pathway repressed proliferation and promoted apoptosis in immature porcine Sertoli cells. These effects were similar to that of the miR-499 knockdown, whereas they were opposite to those of miR-499 overexpression and the *PTEN* knockdown.

In conclusion, this study proved that miR-499 promotes immature porcine Sertoli cell growth through the PI3K/AKT pathway by targeting the *PTEN* gene. We suspected that miR-499, *PTEN*, and the PI3K/AKT signaling pathway might play crucial regulatory roles in determining the fate of immature Sertoli cells.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0303>.

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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.

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Author contribution statement

Ran M L and Chen Bin contributed to experimental conception and design. Gao H, Ran M L, Luo H, Weng B, Tang X F, Chen Y, and Yang A Q performed the experiments. Ran M L and Gao H analyzed the data. Ran M L and Gao H wrote the first draft of the manuscript. All authors reviewed and approved the final manuscript.

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