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Original Article

MiR-145-5p regulates granulosa cell proliferation by targeting the *SET* gene in KGN cells

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Abstract. MiR-145-5p has been implicated in the development and progression of various disorders, and it is primarily recognized as a tumor suppressor in numerous cancers types. Its expression has been reported to decrease in the granulosa cells of patients with polycystic ovarian syndrome (PCOS). This study aimed to investigate whether miR-145-5p plays a role in granulosa cell proliferation and to shed light on the underlying pathological mechanisms of follicular development in patients with PCOS. Follicular fluid samples were collected from patients with PCOS and healthy individuals. The Cell Counting Kit-8 and bromodeoxyuridine assays were performed to assess KGN cell proliferation. The expression of miR-145-5p was significantly decreased in PCOS granulosa cells than in control cells, whereas the expression of *SET* was increased. Furthermore, miR-145-5p suppressed the proliferation of KGN cells. *SET* was identified as a direct target of miR-145-5p. Additionally, *SET* promoted the proliferation of KGN cells, and knockdown of *SET* counteracted the effect of the miR-145-5p inhibitor. Therefore, miR-145-5p regulates granulosa cell proliferation by targeting the *SET* in KGN cells; this process may be a potential pathological pathway that contributes to follicular developmental disorders in PCOS.

Key words: Granulosa cell proliferation, MiR-145-5p, *SET*

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Polycystic ovarian syndrome (PCOS) is one of the most common endocrine and metabolic disorders, affecting approximately 5–10% of women in their reproductive years [1]. It is primarily characterized by oligo-ovulation or anovulation, biochemical or clinical hyperandrogenism, and polycystic ovarian morphology. PCOS is the most common cause of anovulatory infertility. However, the precise mechanisms underlying these abnormalities in anovulatory PCOS remain unknown.

Abnormal follicular development is a primary pathological characteristic of PCOS. In a previous study, the number of primordial follicles in the ovaries of patients with PCOS was normal, whereas the number of preantral and antral follicles was notably elevated, ranging from two to three times that observed in normal ovaries [2]. Despite the increased presence of preantral and antral follicles, PCOS ovaries fail to progress to dominant follicles, leading to anovulation. Follicular dysplasia observed in PCOS has been confirmed to be related to the abnormal proliferation and apoptosis of granulosa cells. Compared with normal individuals, anovulatory patients with PCOS have exhibited a significant increase in the proliferation of granulosa cells and a decrease in the apoptosis of these cells [3]. However, the precise etiology and pathophysiological mechanisms underlying PCOS remain unclear. Genetic, environmental, and epigenetic factors play pivotal roles in the onset and progression of PCOS.

MicroRNAs (miRNAs) are highly conserved small non-coding RNAs of approximately 22 nucleotides. They exert their regulatory effects by binding to the 3′ untranslated region (3′ UTR) of target mRNA, thereby modulating gene expression of target genes. These miRNAs are involved in various biological processes, including cell proliferation, migration, differentiation, and apoptosis. In recent years, an increasing number of miRNAs have been confirmed to be implicated in the pathogenesis of PCOS [4]. For instance, miR-483 was found to be reduced in the ovarian cortex of patients with PCOS. In the human ovarian granulosa tumor cell line KGN, miR-483 has been shown to inhibit cell growth and induce cell-cycle arrest by interacting with IGF1 [5]. Conversely, miR-93 expression has been found to be elevated in the ovarian cortex of patients with PCOS. Overexpression of miR-93 promotes granulosa cell proliferation and cell-cycle progression in patients with PCOS by binding to CDKN1A [6]. Additionally, overexpression of miR-182 in granulosa cells from patients with PCOS and from KGN cell lines was observed to suppress cell proliferation and cell-cycle progression by targeting SGK3, while promoting apoptosis [7]. Furthermore, miR-145-5p expression has been reported to be decreased in PCOS granulosa cells [8].

Previous gene chip results have shown that the expression of *SET* is upregulated in the ovaries of patients with PCOS, with its expression level being 2.01 times that in normal ovaries [9]. SET is a multitasking protein involved in various biological processes such as cell-cycle regulation, cell proliferation, apoptosis, DNA repair, gene transcription, and epigenetics. The SET protein regulates cell proliferation and apoptosis via multiple pathways in various cell types. In non-small cell lung cancer, knockout of the *SET* gene inhibits cell proliferation by upregulating the activity of protein phosphatase 2A (PP2A), which in turn inhibits the AKT and ERK signaling pathways [10]. *SET* has been found to regulate cell proliferation via the PI3K/AKT signaling pathway in prostate cancer [11], cell proliferation and viability via the ERK signaling pathway in squamous cell carcinoma cell lines of the head and neck [12], and apoptosis through both caspase-independent and caspase-dependent pathways

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[13]. In HEK293T cells, overexpression of the SET protein inhibited the activity of caspase 9 and caspase 3, suppressing the intrinsic apoptosis pathway [14]. Overexpression of *SET* gene in mouse oocytes promoted oocyte maturation and inhibited oocyte apoptosis [15]. Bioinformatics predictions suggest that *SET* may be a target gene of miR-145-5p. Therefore, this study aimed to investigate whether miR-145-5p regulates granulosa cell proliferation by targeting the *SET*, thereby participating in the pathological and physiological processes of follicular developmental disorders in PCOS.

Materials and Methods

Patient samples

The follicular fluid samples from patients with PCOS and control individuals were collected at the Northern Jiangsu People's Hospital Affiliated to Yangzhou University. All patients underwent *in vitro* fertilization, and oocyte retrieval was performed under transvaginal ultrasound guidance to collect follicular fluid. Patients with PCOS were diagnosed on the basis of the Rotterdam criteria. The control group consisted of patients undergoing *in vitro* fertilization owing to male factor infertility or isolated tubal factor infertility who exhibited normal ovarian function, regular menstrual cycles, normal basal hormone levels, and normal follicular numbers in both ovaries. The following patients were excluded: those over 35 years of age and those with ovarian cysts; ovarian tumors; or a history of ovarian surgery, ovarian radiotherapy or chemotherapy, endometriosis, thyroid dysfunction, hyperprolactinemia, or other endocrine diseases and chromosomal abnormalities. All patients signed informed consent forms that were approved by the hospital's ethics committee.

Human granulosa cell isolation and culture

Human granulosa cells were obtained from the follicular fluid of patients undergoing *in vitro* fertilization. Granulosa cells were isolated according to a previously established protocol [16]. Briefly, granulosa cells were separated from red blood cells in follicular aspirates using centrifugation in 50% Percoll solution (Sigma, St. Louis, MO, USA), followed by washing and suspension in DMEM/F12 medium (HyClone, Logan, UT, USA). Cell viability was assessed using the trypan blue (Beyotime, Shanghai, China) exclusion method to ensure that the percentage of live cells exceeded 90% in each experiment. The human granulosa cells (with a purity exceeding 95%) were subsequently seeded into culture plates (Corning, Corning, NY, USA) containing DMEM/F12 medium (HyClone) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. KGN cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and cultured in DMEM/F12 medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 37°C humidified environment with 5% CO_2.

Recombinant adenovirus generation and infection

AdCMV-SET and AdH1-SiRNA/SET adenoviruses were constructed as described in our previous work to overexpress or knockdown SET expression, respectively. The efficacies of SET knockdown and overexpression have been validated at both mRNA and protein levels [17]. AdCMV-GFP was used as the control for AdCMV-SET and AdH1-SiRNA/NS as the control for AdH1-SiRNA/SET.

Cell Counting Kit-8 assay

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Beyotime) according to the manufacturer's instructions. To determine the effect of miR-145-5p or *SET* on cell proliferation, cells were seeded in 96-well plates at approximately 1×10^4/150 µl/well and transfected or infected. After culturing for 48 h, the CCK-8 solution was added to each well. After incubation for another 3 h, the absorbance was measured using a microplate reader at 450 nm. This assay was performed in triplicate and repeated three times.

Bromodeoxyuridine assay

To assess the effect of miR-145-5p and *SET* on the DNA synthesis capability of granulosa cells, BrdU incorporation was performed using a BrdU cell proliferation enzyme-linked immunosorbent assay kit (BeyoClick, Shanghai, China). Briefly, cells treated with miRNAs or adenoviruses were seeded into 96-well plates and cultured for 24 h. Subsequently, BrdU solution (10 ml/well), fixing/denaturing solution (100 ml/well), peroxidase-labeled sheep anti-mouse IgG solution (100 ml/well), and tetramethylbenzidine substrate (100 ml) were added sequentially. The mixtures were incubated according to the manufacturer's instructions. After the indicated incubation period, BrdU incorporation into the cells was quantified at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Each experiment was repeated three times in triplicate.

Dual-luciferase reporter assay

The SET-3′ UTR and the SET-mutated-3′ UTR fragments were amplified by PCR. Subsequently, the fragments were cloned into the pGL3 luciferase promoter vector (Promega, Madison, Wisconsin, USA) to construct the Luc-pGL3-SET-3′ UTR and Luc pGL3-SET-mut-3′ UTR vectors. The putative binding site for human miR-145-5p in the 3′ UTR of SET mRNA is 3′ UGACCU. The seed match region of SET-WT was 5′ ACUGGAA, and seed mutant region of SET-Mut was designed as 5′ CGAUUG. 293T cells were used for the luciferase assay. Cells seeded in 24-well plates were co-transfected with Luc-pGL3-SET-3′ UTR or Luc-pGL3-SET-mut-3′ UTR vector, along with miR-145-5p mimics or scrambled miRNA, using Lipofectamine 2000 reagent (Invitrogen), following the manufacturer's instructions. After 48 h post-transfection, firefly and Renilla luciferase activities were measured using a dual-luciferase reporter assay kit (Promega). The results are expressed as relative luciferase activity (firefly luciferase/Renilla luciferase). Each experiment was conducted three times with triplicate samples.

Real-time RT-PCR

Total mRNA was extracted using the TRIZOL reagent (Invitrogen). Total RNA was reverse-transcribed using the PrimeScript Reverse Transcription Reagent Kit (Perfect Real Time, Takara, Dalian, China) according to the manufacturer's instructions. The primers for the human miR-145-5p, U6, *SET*, and GAPDH gene were as follows: 5′miR-145-5p: ACACTCCAGCTGGGGTCCAGTTTTCCCAGGAA, 3′miR-145-5p: GTTCGCTGAGATGAAGCACTG, 5′U6: CTCGCTTCGGCAGCACA, 3′U6: TGGTGTCGTGGAGTCG, 5′SET: GTCCACCGAAATCAAATGGAAATC, 3′SET: GCACCTGCATCAGAATGGTCA; 5′GAPDH: AGGTTGTCTCCTGCGACTTCA, 3′GAPDH: GGGTGGTCCAGGGTTTCTTACT. Real-time PCR was performed using an ABI Prism 7300 Sequence Detection System (PerkinElmer Applied Biosystems, Foster City, CA, USA). Melting curve analysis was performed to confirm product specificity. Data were calculated according to the $2^{-\Delta\Delta CT}$ method and presented as the fold change

relative to the corresponding data for the GAPDH internal reference gene.

Western blot analysis

Cells from different treatment groups were collected and immediately sonicated in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, and 1% protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). After 30 min of incubation on ice with intermittent agitation, the lysates were centrifuged at 12000 *g* at 4°C for 10 min. The suspended proteins were collected, separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (GE Healthcare, San Francisco, CA, USA). Following the transfer, the membranes were blocked in Tris-buffered saline (TBS) containing 5% skim milk for 1 h and incubated overnight at 4°C with 1:1000 rabbit anti-SET polyclonal antibody (sc-25564, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Loading levels were normalized using a 1:2000 rabbit anti-tubulin antibody (ab6046; Abcam, Cambridge, MA, USA). The results were analyzed using Quantity One version 4.62 (Bio-Rad).

Statistical analysis

All data were expressed as the means ± SD, which were calculated from at least three independent experiments. Normal distribution was assessed using the Shapiro–Wilk test. Statistical analyses were performed using the Stata 10 software (StataCorp LP, College Station, TX, USA). Differential analysis was performed using Student's *t*-test or one-way analysis of variance. Quantitative differences were considered statistically significant if the p-value reached $P < 0.05$.

Results

The expression of miR-145-5p and SET in granulosa cells from patients with PCOS and control individuals

To validate whether miR-145-5p and *SET* were associated with PCOS, luteinized human granulosa cells were isolated from the follicular fluid of women with PCOS and control women who underwent *in vitro* fertilization because of male or tubal factor infertility. The expression levels of miR-145-5p and *SET* in granulosa cells from patients with PCOS and healthy women were measured using real-time PCR. As depicted in Fig. 1A, the expression of miR-145-5p was significantly lower in PCOS granulosa cells than in the controls ($P < 0.05$). The expression of *SET* in PCOS granulosa cells was higher than that in the controls (Fig. 1B).

Effect of miR-145-5p on granulosa cell proliferation

To explore the function of miR-145-5p in granulosa cells, miR-145-5p mimics and inhibitor were used to overexpress and knockdown its expression in KGN cells, respectively. The CCK-8 assay showed that the viability was significantly decreased in KGN cells transfected with miR-145-5p mimics 72 h post-transfection (Fig. 2A), whereas the miR-145-5p inhibitor significantly increased viability in KGN cells 48 h and 72 h post-transfection (Fig. 2B). The EdU assay was performed to validate the results of the CCK-8 assay. Consistent with the CCK-8 assay results, cell proliferation was decreased in KGN cells treated with miR-145-5p mimics and was increased in cells treated with the miR-145-5p inhibitor 48 h post-transfection (Fig. 2C). Collectively, these results suggest that miR-145-5p suppressed KGN cell proliferation.

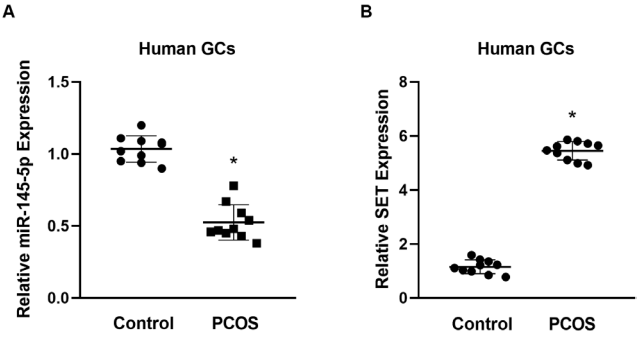

Fig. 1. The expression of miR-145-5p and *SET* in granulosa cells from patients with PCOS and control individuals. (A) Real-time PCR analysis of miR-145-5p in granulosa cells from patients with PCOS and control individuals. The expression of miR-145 is significantly declined in PCOS granulosa cells compared to that in the controls ($P < 0.05$). (B) Real-time PCR analysis of *SET* in granulosa cells from patients with PCOS and control individuals. The expression of *SET* in PCOS granulosa cells is increased compared to that in the controls. Results are presented as the mean ± SD from at least 3 independent experiments. The Student's *t*-test was used to compare the mRNA levels. * $P < 0.05$ and ** $P < 0.01$.

MiR-145-5p directly inhibited SET expression by binding to its 3′ UTR

Bioinformatics analysis predicted that *SET* could be a target gene of miR-145-5p (Fig. 3A, TargetScan and miRDB databases). To explore whether miR-145-5p directly targets and regulates *SET* expression in granulosa cells, dual-luciferase reporter assay, real-time PCR, and western blotting were performed. The relative luciferase activity of the pGL3-SET-3′ UTR-WT in 293T cells transfected with miR-145-5p mimics was markedly declined compared with that of the scramble control (Fig. 3B). In contrast, no significant change was observed in the relative luciferase activity between the 293T cells transfected with pGL3-SET-3′ UTR-Mut and those transfected with miR-145-5p mimics. Moreover, miR-145-5p mimics significantly reduced *SET* mRNA expression, whereas the miR-145-5p inhibitor increased *SET* mRNA expression (Fig. 3C). Similarly, miR-145-5p mimics markedly reduced SET protein expression, whereas the miR-145-5p inhibitor elevated *SET* protein expression (Fig. 3D) in KGN cells. These findings suggested that the *SET* is a direct target of miR-145-5p.

Effect of SET on granulosa cell proliferation

To assess the impact of *SET* on human granulosa cells, AdCMV-SET and AdH1-SiRNA/SET adenoviruses were used to overexpress and knockdown *SET* expression in KGN cells, respectively. CCK-8 and EdU assays were performed to evaluate the effect of *SET* on granulosa cell proliferation. AdCMV-SET and AdH1-SiRNA/SET adenoviruses effectively elevated and decreased SET protein levels in KGN cells, respectively, after 48 h of infection (Fig. 4A). As expected, viability was significantly increased in KGN cells infected with AdCMV-SET adenoviruses but was significantly decreased in those infected with AdH1-SiRNA/SET adenoviruses (Fig. 4B). Furthermore, KGN cells infected with AdCMV-SET adenoviruses exhibited an increase in EdU-positive cells compared with those infected with AdCMV-GFP. Conversely, KGN cells infected with AdH1-siRNA/SET adenoviruses displayed a decrease in the number of EdU-positive cells compared to those infected with AdH1-siRNA/NS (Fig. 4C). These results suggested that *SET* promoted the proliferation of KGN cells.

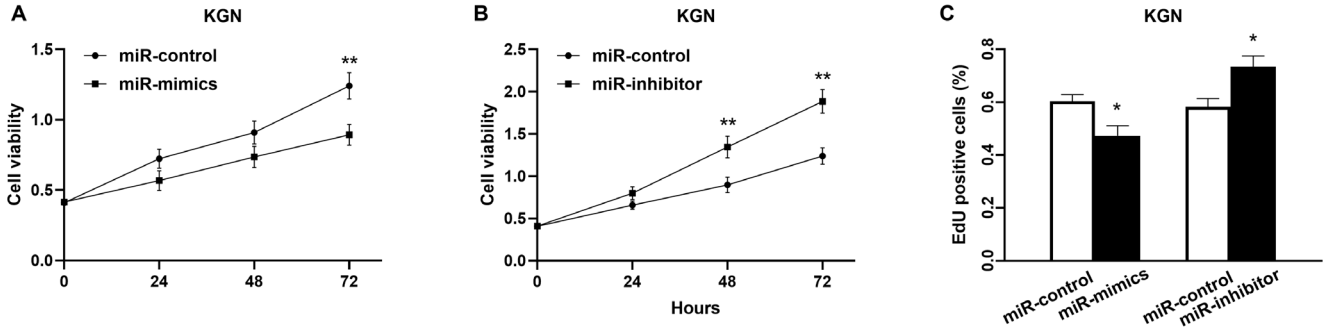

Fig. 2. Effect of miR-145-5p on granulosa cell proliferation. (A) KGN cells were transfected with miR-145-5p mimics or miR-control. The cell viability is significantly decreased in KGN cells transfected with miR-145-5p mimics compared with that in KGN cells transfected with miR-control, at 72 h post-transfection, according to CCK-8 assay. (B) KGN cells were transfected with miR-145-5p inhibitor or miR-control. The cell viability is significantly decreased in KGN cells transfected with miR-145-5p inhibitor compared with that in KGN cells transfected with miR-control, at 48 h and 72 h post-transfection, according to CCK-8 assay. (C) KGN cells were transfected with miR-145-5p mimics or miR-control for 48 h. The cell proliferation is significantly decreased in KGN cells transfected with miR-145-5p mimics compared with that in KGN cells transfected with miR-control, according to EdU assay. The cell proliferation is significantly increased in KGN cells transfected with miR-145-5p inhibitor compared with that in KGN cells transfected with miR-control, according to EdU assay. Results are presented as the mean ± SD from at least 3 independent experiments. The Student' *t*-test was used to compare the mRNA levels. * $P < 0.05$ and ** $P < 0.01$.

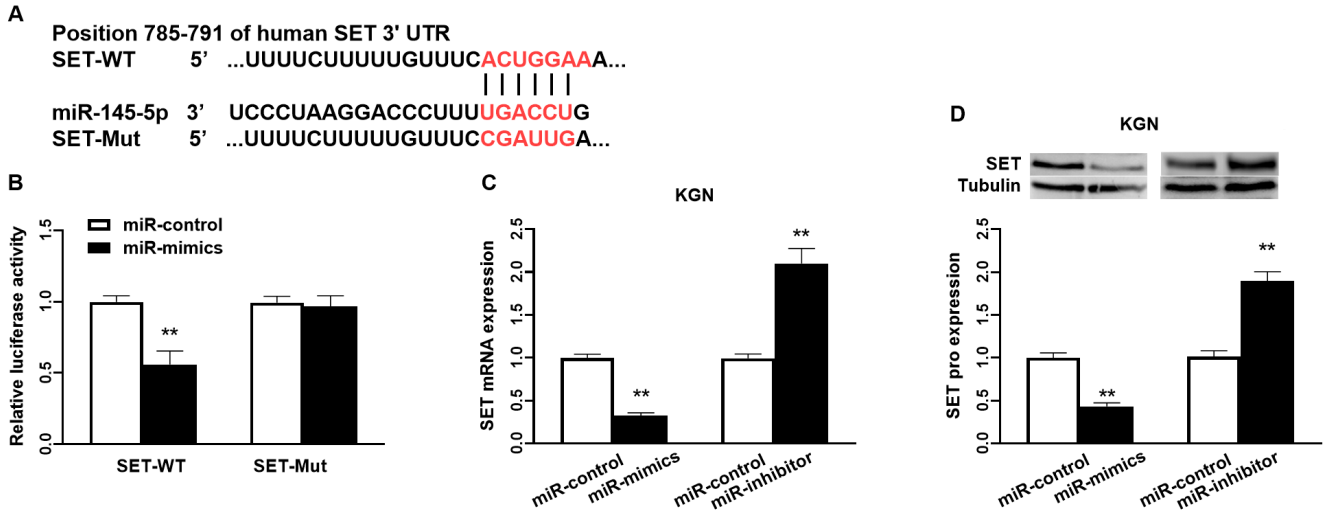

Fig. 3. MiR-145-5p directly inhibits *SET* expression by binding to its 3′ UTR. (A) Putative binding sites for human miR-145-5p in the 3′ UTR of *SET* mRNA, seed match region, and seed mutant region are shown in red. (B) KGN cells were transfected with miR-145-5p mimics or miR-145-5p control. The luciferase activity of the human *SET* gene 3′ UTR is inhibited by miR-145-5p mimics, whereas they have no effect on the activity of luciferase fused with the *SET* 3′ UTR mutant. (C) *SET* mRNA expression is down-regulated in KGN cells transfected with miR-145-5p mimics for 24 h compared to that in the miR-control group. Similarly, *SET* mRNA expression is increased in KGN cells transfected with miR-145-5p inhibitor compared to that in the miR-control group. (D) *SET* protein expression is reduced in KGN cells transfected with miR-145-5p mimics for 48 h compared to that in the miR-control group. Similarly, *SET* protein expression is elevated in KGN cells transfected with miR-145-5p inhibitor compared to that in the miR-control group. Results are presented as the mean ± SD from at least 3 independent experiments. The Student's *t*-test was used to compare the mRNA levels. * $P < 0.05$ and ** $P < 0.01$.

Knockdown of SET restored the effects of miR-145-5p inhibitor on cell proliferation

To further determine the functional relationship between the miR-145-5p/*SET* axis and granulosa cell proliferation, miR-inhibitor-treated KGN cells were infected with AdH1-SiRNA/SET or AdH1-SiRNA/NS adenoviruses. The knockdown of miR-145-5p markedly elevated the protein level of SET in KGN cells, whereas co-infection with AdH1-SiRNA/SET adenoviruses reversed this effect (Fig. 5A). Furthermore, the effect of miR-145-5p inhibitor on cell viability was significantly reversed by *SET* knockdown (Fig. 5B). Similarly, the effect of the miR-145-5p inhibitor on DNA synthesis was significantly abrogated by *SET* knockdown (Fig. 5C). These results indicate that the ability of miR-145-5p to regulate cell proliferation in human granulosa cells is specifically attributed to its ability to inhibit *SET*.

Discussion

MiR-145-5p is encoded by the *MIR145* gene, which is located on Chromosome 5: 149,430,646–149,430,733 forward strand and reported to play a role in the onset and progression of various disorders. This miRNA is mainly recognized as a tumor suppressor in numerous types of cancers. In a previous study, miR-145-5p expression was found to be decreased in bladder cancer; when its expression was restored, it inhibited cell growth, migration, and invasion while promoting

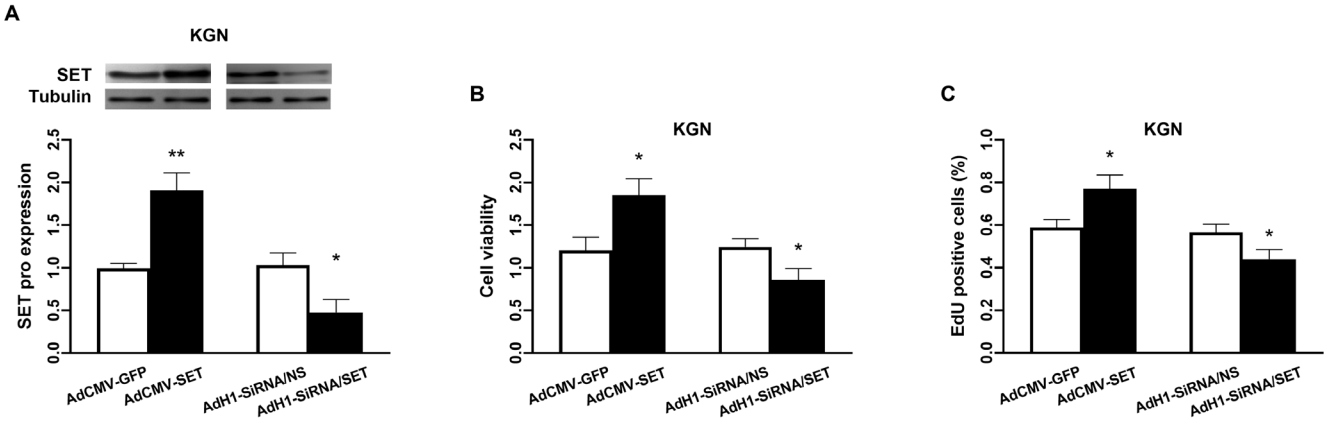

Fig. 4. Effect of *SET* on granulosa cell proliferation. (A) KGN cells were infected with AdCMV-*SET* or AdCMV-GFP adenoviruses for 48 h. AdCMV-SET adenoviruses effectively elevate SET protein levels in KGN cells, according to western blot analysis. KGN cells were infected with AdH1-SiRNA/*SET* or AdH1-SiRNA/NS adenoviruses for 48 h. AdH1-SiRNA/SET adenoviruses effectively decrease SET protein levels, according to western blot analysis. (B) The cell viability is elevated in KGN cells infected with AdCMV-*SET* adenoviruses compared with that in KGN cells infected with AdCMV-GFP adenoviruses, according to CCK-8 assay. The cell viability is decreased in KGN cells infected with AdH1-SiRNA/*SET* adenoviruses compared with that in KGN cells infected with AdH1-SiRNA/NS adenoviruses after 48 h, according to CCK-8 assay. (C) EdU-positive cells are increased in KGN cells infected with AdCMV-*SET* adenoviruses compared to those infected with AdCMV-GFP, according to EdU assay. EdU-positive cells are reduced in KGN cells infected with AdH1-SiRNA/*SET* adenoviruses compared to those infected with AdH1-SiRNA/NS, according to EdU assay. Results are presented as the mean ± SD from at least 3 independent experiments. The Student's *t*-test was used to compare the mRNA levels. * $P < 0.05$ and ** $P < 0.01$.

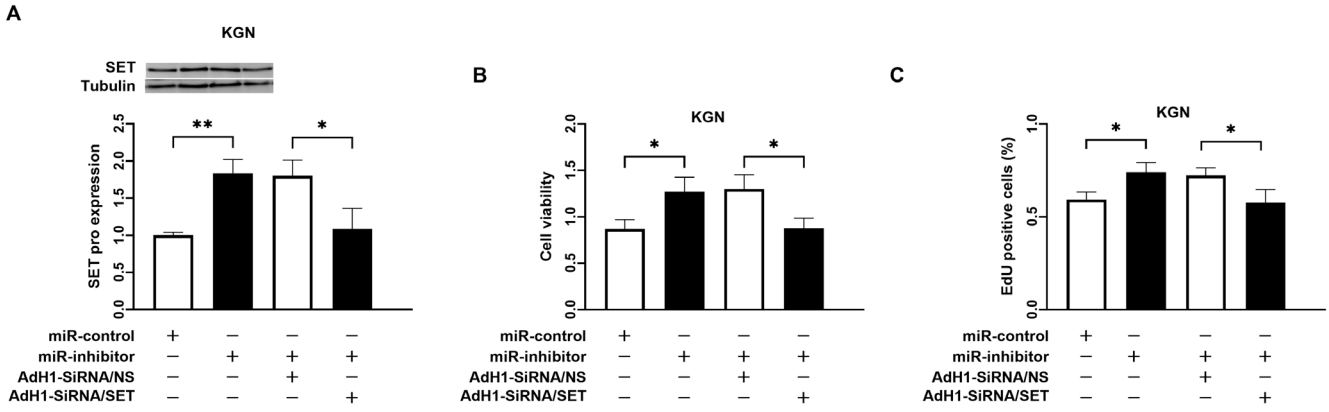

Fig. 5. Knockdown of SET restores the effects of miR-145-5p inhibitor on cell proliferation. (A) MiR-inhibitor-treated KGN cells were infected with AdH1-SiRNA/SET or AdH1-SiRNA/NS adenoviruses for 48 h. Knockdown of miR-145-5p markedly elevates the protein level of SET in KGN cells, while co-infection with AdH1-SiRNA/SET adenoviruses reverses this effect. (B) CCK-8 assay shows that the effect of miR-145-5p inhibitor on cell viability is reversed by *SET* knockdown. (C) EdU assay shows that the effect of miR-145-5p inhibitor on DNA synthesis levels is significantly abrogated by *SET* knockdown. Results are presented as the mean ± SD from at least 3 independent experiments. The Student's *t*-test was used to compare the mRNA levels. * $P < 0.05$ and ** $P < 0.01$.

apoptosis by targeting UHRF1 [18]. Similarly, in breast cancer, miR-145-5p expression has been shown to be downregulated, with restoration of its expression leading to Ago2 induction and inhibition of cell migration [19]. According to another study, miR-145-5p showed decreased levels in cervical cancer, and the restoration of its expression suppressed cell proliferation, migration, and invasion by downregulating KLF5 [20]. Furthermore, it has been reported to be decreased in esophageal carcinoma, and upregulation of miR-145-5p via ABRACL targeting has been shown to suppress cell proliferation, migration, and invasion [21]. Moreover, miR-145-5p has been shown to affect the pathogenesis of several non-malignant conditions such as asthma, aplastic anemia, diabetic nephropathy, and rheumatoid arthritis. In a study on aplastic anemia, miR-145–5p expression was downregulated, which led to increased T cell activation through upregulation of MYC expression and promotion of T cell proliferation [22]. In asthmatic mice, miR-145–5p levels were elevated, thereby promoting chemokine and inflammatory factor release while inhibiting epithelial repair by targeting KIF3A [23]. In mouse granulosa cells, microRNA-145 has been confirmed to inhibit cell proliferation by targeting activin receptor IB [24]. Moreover, microRNA-145 negatively regulated cell proliferation by targeting IRS1 in ovarian granulosa cells isolated from patients with PCOS [25]. In our study, we validated that microRNA-145-5p suppressed the proliferation of human granulosa cells by directly targeting *SET*, which suggests that microRNA-145-5p can regulate cell proliferation by targeting different downstream genes.

The *SET* gene, which is involved in various cellular processes including cell proliferation and apoptosis, has been implicated in

several cancers and other diseases. In a previous study, when the expression of *SET* gene was suppressed in glioblastoma cells, apoptosis was significantly increased, accompanied by a notable inhibition of cell proliferation and a marked decrease in cell migration [26]. Accumulation of the SET protein was found to increase cellular proliferation in HEK293 and HNSCC cell lines [27]. Introducing exogenous *SET* into HTO cells led to the suppression of cell proliferation. Similarly, depletion of *SET* through siRNA resulted in enhanced cell proliferation [28]. The present study demonstrated that *SET* promoted the proliferation of KGN cells. Furthermore, this study expands the understanding of the function of *SET* by demonstrating its regulation by miRNA-145-5p in granulosa cells.

MiR-145-5p has been shown to be sponged by several lncRNAs and circRNAs. CircRNAs are a family of non-coding RNAs with covalently closed loop structures that participate in gene expression regulation at both the transcriptional and post-transcriptional levels by serving as miRNA sponges, interacting with RNA-binding proteins, and regulating host gene expression. Among them, most circRNAs act as miRNA sponges along the circRNA–miRNA axis. CircSTAG2 has been confirmed to modulate the progression of bladder cancer cell lines by sponging the tumor suppressor miR-145-5p, resulting in the activation of TAGLN2 [29]. CircCPA4 has been found to bind to miR-145-5p, thereby regulating cell proliferation, migration, invasion, and apoptosis in non-small cell lung cancer *in vitro* [30]. CircRNA-02191 has been reported to regulate unsaturated fatty acid synthesis by adsorbing miR-145 to enhance CD36 expression in the bovine mammary gland [31]. Hsa_circ_0001326 has been shown to inhibit the proliferation, migration, and invasion of trophoblasts via the miR-145-5p/TGFB2 axis [32]. Circ_0015756 has been found to promote ovarian cancer progression by sponging miR-145-5p [33]. Currently, only a few circRNAs have been identified to be aberrantly expressed in PCOS and involved in its pathological processes. Previous studies have shown that circ_0118530 was upregulated in the granulosa cells of patients with PCOS and that downregulation of circ_0118530 in the KGN cell line inhibits cell viability and migration by selectively adsorbing miR-136, leading to apoptosis [34]. Additionally, circ_0043532 expression has been reported to be elevated in patients with PCOS, and interference with circ_0043532 has been shown to inhibit cell proliferation and induce cell-cycle arrest and apoptosis by regulating the miR-182/SGK3 axis [7]. CircPUM1 has been shown to be strongly expressed in the granulosa cells of patients with PCOS and to regulate granulosa cell proliferation and apoptosis by sponging miR-760 [35]. Microarray analysis has shown that circ_0043314 is significantly upregulated in the granulosa cells of patients with PCOS [36]. Bioinformatics predictions have suggested that miR-145-5p could serve as a target gene of circ_0043314. Therefore, our future studies will aim to validate whether circ_0043314 participates in the regulation of granulosa cell function by acting as a sponge for miR-145-5p.

MiR-145-5p has been observed to modulate apoptosis in various cell types. Overexpression of miR-145-5p repressed apoptosis in high-glucose-induced HK-2 cells by targeting VASN [37]. Additionally, miRNA-145-5p has been shown to suppress apoptotic responses in LPS-activated Caco-2 cells [38]. Decreased levels of miRNA-145-5p have been associated with prostate cancer bone metastasis, where miRNA-145-5p induction promoted apoptosis [39]. Similarly, high expression of miRNA-145 in thyroid cancer cells has been reported to inhibit cell proliferation and migration, while promoting apoptosis [40]. However, reports are unclear on whether miR-145-5p is involved in granulosa apoptosis. Further experiments will be conducted to address this question.

Dysregulation of granulosa cell proliferation is a hallmark of PCOS, and it contributes to the characteristic follicular abnormalities observed in affected individuals. By exploring the potential role of miRNA-145-5p and its target gene *SET* in this process, our findings provide insights into the molecular mechanisms that may contribute to the pathogenesis of PCOS. Furthermore, our study contributes to the broader understanding of miRNA-mediated gene regulation in reproductive biology. The identification of miRNA-145-5p as a regulator of granulosa cell proliferation highlights the intricate network of molecular interactions involved in ovarian function. Future research directions include investigating the therapeutic potential of targeting miRNA-145-5p or the *SET* gene in the management of PCOS. Further studies are warranted to explore the downstream and upstream signaling pathways and molecular targets influenced by miRNA-145-5p-mediated regulation of granulosa cell proliferation.

In conclusion, our findings provide novel insights into the molecular mechanisms underlying granulosa cell proliferation in PCOS, implicating miRNA-145-5p and its target gene *SET* as potential key regulators of this process. This study contributes to the growing body of knowledge regarding the role of miRNAs in the pathogenesis of PCOS and may pave the way for the development of targeted therapeutic interventions for this common endocrine disorder.

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