Long non-coding RNA MSTRG.5970.28 regulates proliferation and apoptosis of goose follicle granulosa cells via the miR-133a-3p/ANOS1 pathway

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ABSTRACT The development of follicles in the ovaries is a critical determinant of poultry egg production. There are existing studies on the follicular development patterns in poultry, but the specific regulatory mechanisms still need further study. In a previous study, we identified long non-coding RNA (**lncRNA**) MSTRG.5970.28, anosmin 1 (**ANOS1**), and its predicted target miR-133a-3p that may be associated with goose ovary development. However, the function of MSTRG.5970.28 in goose granulosa cells and its regulatory mechanisms affecting granulosa cell proliferation and apoptosis have not been reported. In the present study, MSTRG.5970.28 and miR-133a-3p overexpression and interference vectors were constructed. Combined with reverse-transcription real-time quantitative PCR (**RT-qPCR**), a dual luciferase activity assay, Cell Counting Kit-8 (**CCK-8**), and flow cytometric analysis, we investigated the role of the MSTRG.5970.28-miR-133a-3p-ANOS1 axis in goose follicular granulosa cells and the associated regulatory mechanisms. MSTRG.5970.28 was found to be localized in the cytoplasm and its expression was influenced by reproductive hormones. The targeting relationship among MSTRG.5970.28, ANOS1, and miR-133a-3p were verified by a dual luciferase activity assay. CCK-8 and apoptosis assays showed that MSTRG.5970.28 inhibited the proliferation and promoted apoptosis of goose granulosa cells. The regulatory role of miR-133a-3p on granulosa cell proliferation and apoptosis was opposite to MSTRG.5970.28. We found that the proliferative and apoptotic effects of granulosa cells caused by MSTRG.5970.28 overexpression were attenuated by miR-133a-3p. MSTRG.5970.28 functions as a competitive endogenous RNA that regulates ANOS1 expression by sponging miR-133a-3p and thus exerts regulatory functions in granulosa cells. In sum, the present study identified lncRNA MSTRG.5970.28 as associated with goose ovary development, which affects the expression of ANOS1 by targeting miR-133a-3p, thereby influencing the proliferation and apoptosis of goose granulosa cells.

Key words: MSTRG.5970.28, miR-133a-3p, ANOS1, granulosa cell, goose

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INTRODUCTION

Follicular development is a key determinant of egg production performance in poultry (Liu et al., 2022). Follicular development is a complex biological process with numerous genes and pathways involved in proliferation and differentiation (Johnson et al., 2008). Granulosa cell proliferation promotes follicular maturation, whereas granulosa cell apoptosis triggers follicular atresia (Aerts and Bols, 2010; He et al., 2016). It follows that granulosa cells play a determinant role in follicular development. Granulosa cell proliferation and apoptosis are regulated by a variety of influences, including those that are genetic-, nutritional-, and hormonal-based (Asselin et al., 2000).

Molecular biology technologies have become important research tools to reveal the molecular mechanisms of granulosa cell proliferation or apoptosis. LncRNAs are a series of RNA larger than 200 nt (Fang and Fullwood, 2016) that play important regulatory role at the transcriptional and post-transcriptional levels by sponging microRNA (**miRNA**) or other molecules (Li et al., 2020). Studies show that lncRNAs play regulatory roles in biological processes such as gonadal development, hormone regulation, sex determination, and embryo implantation (Gayatri et al., 2012; Mulvey Brett et al., 2014). Currently, lncRNAs have been detected in human (Li et al., 2019), murine (Hu et al., 2019), porcine (Hu et al., 2021), chicken (Peng et al., 2019), and duck (Wu et al., 2021) follicles or granulosa cells. Hu et al. (2021) identified lncRNA TCONS_00814106 in porcine granulosa cells as a competing endogenous RNA (**ceRNA**) that regulates the expression of

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transforming growth factor beta receptor 1 (**TGFBR1**) by sponge-binding miR-1343, thus exerting a regulatory function in granulosa cells. Wu et al. (2021) found in duck follicles that lnc_13814 promotes apoptosis of granulosa cells by binding with and inhibiting the expression of apla-mir-145-4 and then upregulating the expression of DNA damage inducible transcript 3 (**DDIT3**). Relative to other livestock and birds, few results have been reported on lncRNA in ovarian development in geese. Gao et al. (2021) analyzed the dynamic expression and function of lncRNAs in ovarian granulosa cells at different developmental stages in geese. Ouyang et al. (2020) analyzed the differential expression and function of ovarian stromal lncRNAs between high and low egg production Sichuan white geese and Langde geese.

Our research team constructed a differential lncRNA-miRNA-mRNA regulation network associated with goose ovary development by whole transcriptome sequencing of ovarian tissues of Yili geese at the pre-laying, laying, and ceased-laying period. The expression of lncRNA MSTRG.5970.28 is significantly upregulated in Yili goose when it enters the ceased-laying from laying period. MSTRG.5970.28 has a predicted targeting relationship with differentially expressed miR-133a-3p, ANOS1 (Zhao et al., 2022). This study intends to explore the regulatory role of the MSTRG.5970.28-miR-133a-3p-ANOS1 axis in goose follicular granulosa cells by constructing lncRNA MSTRG.5970.28 overexpression vectors and siRNA to reveal the function of lncRNA MSTRG.5970.28.

MATERIALS AND METHODS

Cell Culture and Nuclear-Cytoplasmic Fractionation

Sample collection and cell treatment involved were approved by the Animal Welfare and Ethics Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China (Approval number 2019004). Healthy female geese kept under the same conditions and at peak egg production were selected. All selected geese were euthanized by carbon dioxide inhalation and cervical dislocation. Ovaries were removed immediately after slaughter and washed several times with PBS. According to the methods of Kang et al. (2017), hierarchical follicles (F1-F6) were rapidly collected from the ovaries of the geese and primary granulosa cells were isolated. The pelleted cells were resuspended in an M199 (G4620, Servicebio, Wuhan, China), complete medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution, and the cell density was measured and then seeded in 6-well dishes and incubated for 24 h at 37℃ in a 5% CO_2 incubator. The isolated granulosa cells were identified by FSHR immunofluorescence. Nucleocytoplasmic fractionation of follicular granulosa cells was performed using a Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek, Canada), and total RNA was extracted from the nucleus and cytoplasm of the cells using TRIzol reagent (Life Technologies, Carlsbad, CA).

Cell Transfection

The mimics-miR-133a-3p, inhibitor-miR-133a-3p, OE-MSTRG.5970.28, Si-MSTRG.5970.28, and empty vector required for the assay were synthesized by Shanghai Jima Pharmaceutical Technology Company (Genepharma, Shanghai, China). All constructs were verified by sequencing. Sequence information is shown in Supplementary Table S1. For plasmid transfection, granulosa cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) transfection reagents following the product manual. Cells were collected 24 h after transfection.

FSH and hCG Hormone Treatment

After 48 h of incubation, the granulosa cells were treated with 10 IU/mL follicle-stimulating hormone (**FSH**, Solaibao, Beijing, China) or 5 IU/mL human chorionic gonadotropin (**hCG**, Solaibao); the control group was treated with an equal volume of PBS for 24 h. The cells were collected after 24 h, total RNA was extracted, and the expression of lncRNA MSTRG.5970.28 was determined.

Dual-Luciferase Reporter Assay

The psiCheck2-MSTRG.5970.28-WT, psiCheck2-MSTRG.5970.28-MUT, psiCheck2-ANOS1-WT, psiCheck2-ANOS1-MUT expression vectors were constructed by He Fei Yuanen Biotechnology Company (Hefei, Anhui, China). The 293T cells were seeded in a 24-well plate (5×10^5 cells/well). These vectors were cotransfected into cells with NC or miR-133a-3p mimics using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA). At 48 h after transfection, cell lysates were prepared and a dual luciferase reporter assay kit, following the manufacturer's instructions (RG027, Beyotime Institute of Biotechnology), was used to measure luciferase activities. The relative luciferase activities were calculated by comparing the Firefly/Renilla luciferase activity ratio.

Reverse-Transcription Real-Time Quantitative PCR

TRIzol reagent (Life Technologies, Carlsbad, CA) was used to extract total RNA from granulosa cells. PrimeScript RT reagent Kit with gDNA Eraser kit (Takara, Dalian, China) was used for the reverse transcription of cDNA; qPCR was performed using 2 × SYBR Green qPCR Master Mix (Servicebio) and run on an ABI StepOnePlus system (ABI, Foster City, CA). The qPCR volume included 3 μL of cDNA template, upstream and downstream primers (0.4 μL each), 10 μL of 2×SYBR Green qPCR Master Mix (G3322-05, Servicebio), and RNase-free water to the final volume of

20 μL. Primers were designed using Primer Premier 5.0 (Premier, Canada). β-actin was used as the internal reference for lncRNA and mRNA, and U6 was used as an internal reference for miRNA expression analysis. The information on primer sequences is given in Supplementary Table 1. All primers were designed and synthesized by General Biosystems, Inc. (Anhui, China). The $2^{-\Delta\Delta CT}$ method was used to calculate relative gene expression levels.

Cell Counting Kit-8 Proliferation Assay

The granulosa cells were seeded into a 96-well plate (1×10^4 cells/well). Cells were transfected for 48 h. Cell proliferation assays were performed using the CCK-8 assay kits (Servicebio) following the manufacturer's instructions. The optical density (**OD**) value was checked under an enzyme-linked immunoassay analyzer at 450 nm, and blank wells were set up to calculate the cell proliferation viability.

Apoptosis Assays

Cells were seeded in 6-well plates, and transfection was carried out when cells approached 80% confluence. After a 24 h culture, the cells were collected and digested with pancreatic enzymes. An Annexin V-FITC apoptosis detection kit (CA1020, Servicebio) was used to detect apoptosis by flow cytometry. Apoptotic cells were detected by NovoCyte Flow Cytometer (Agilent, Santa Clara, CA) and analyzed using NovoExpress software.

Western Blotting Analysis

Cells were collected and centrifuged at 14,000 rpm for 10 min at 4℃. A sample of 100 μL of RIPA cell lysis solution (containing 1 mM PMSF; Biosharp, Hefei, China) was added to each 6-well plate and lysed on ice for 30 min. The product was centrifuged at 12,000 r/min for 15 min, and the supernatant was obtained for total protein determination using the BCA method. A sample containing 10 μg total proteins was separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. The blots were then blocked with 5% fat-free dry milk for 2 h at room temperature. The cells were incubated with the primary antibody goat anti-mouse IgG, goat anti-rabbit IgG, ANOS1 (1:500), and β-actin (1:1,000) overnight at 4℃, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:10,000) for 2 h. Protein bands were visualized using a hypersensitive ECL chemiluminescence kit (Thermo Scientific) and densitometric analysis of western blots was performed with Image J analysis software.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 (IBM Corp, Armonk, NY) and GraphPad Prism 8.0. We compared data between 2 groups with a nonparametric t test and those between more than2 groups with a one-way ANOVA. Each experiment was repeated ≥3 times, and data are expressed as mean ± standard error of the mean (**SEM**). $P < 0.05$ values were deemed significant.

RESULTS

Identification, Nucleocytoplasmic Separation, and Hormonal Treatment of Goose Ovarian Granulosa Cells

After 48 h, goose granulosa cells became adherent, and exhibited fusiform or irregular triangles (Figure 1A).

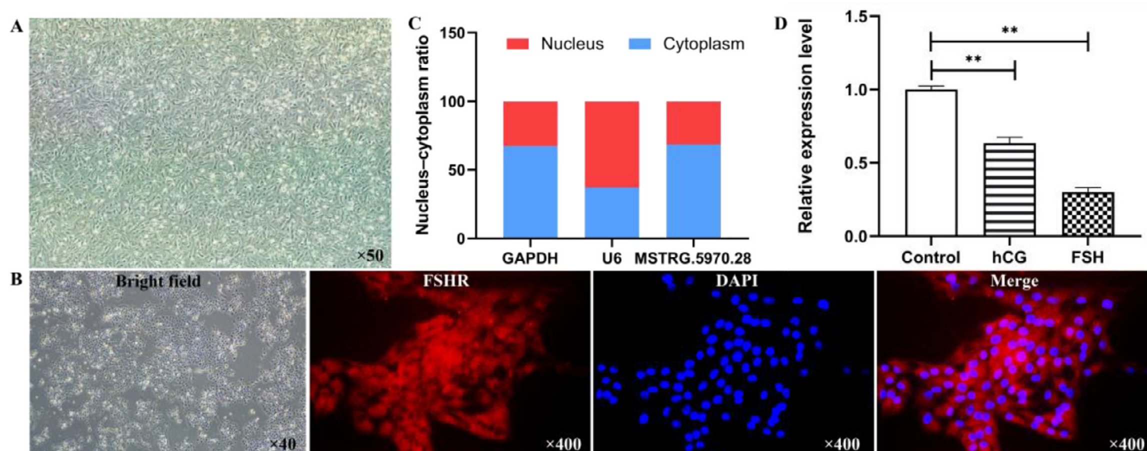

Figure 1. Identification, nucleocytoplasmic separation, and hormonal treatment of goose ovarian granulosa cells. (A) Untreated goose granulosa cells (control) cultured for 12 to 24 h. (scale bar: 200 μm, magnification 50×). (B) Identification of ovarian granulosa cells by FSHR immunofluorescence. (Bright-field microscopy images were recorded at 40 magnification with scale bar: 100 μm. FSHR immunofluorescence images were recorded at 400 magnification with scale bar: 50 μm.) (C) Nucleus/cytoplasm ratio of lncRNA MSTRG.5970.28 and GAPDH were assessed by RT-qPCR. (D) Effect of FSH or hCG on the expression of lncRNA MSTRG.5970.28. Statistically significant differences were analyzed with Student's t test (compared with the control group). All experiments were performed three times. Data are shown as the mean±SD, $^{*}P < 0.05$ and $^{**}P < 0.01$. Abbreviation: FSHR, follicle-stimulating hormone receptor.

FSHR antibodies were used to identify granulosa cells. As shown in Figure 1B, after staining, the granular cell membrane can be observed to fluoresce in red and the nucleus is stained in blue. The expression of lncRNA MSTRG.5970.28 in the nucleus and cytoplasm of granulosa cells was detected by quantitative real-time PCR assays, and MSTRG.5970.28 was found to be expressed in both the nucleus and cytoplasm, and was highly expressed in the cytoplasm (Figure 1C). After treatment of goose granulosa cells with hCG (5 IU/mL) or FSH (10 IU/mL), the expression of MSTRG.5970.28 in pellet cells significantly decreased ($P < 0.01$) under both hormonal treatments (Figure 1D).

Validation of lncRNA MSTRG.5970.28 and miR-133a-3p Targeting

RNAhybrid analysis showed that MSTRG.5970.28 has a predicted binding site to miR-133a-3p (Figure 2A). To verify the targeting relationship between MSTRG.5970.28 and miR-133a-3p, MSTRG.5970.28-wt, MSTRG.5970.28-mut plasmids containing miR-133a-3p binding sites were constructed (Figure 2B). Subsequently, wild-type or mutant plasmids were co-transfected into 293T cells with NC or miR-133a-3p mimics. Dual luciferase activity analysis showed that miR-133a-3p mimics co-transfected with MSTRG.5970.28-wt significantly reduced the fluorescence activity of the wild-type plasmid ($P < 0.05$), but had no significant effect on the mutant plasmid ($P > 0.05$; Figure 2C). To further verify whether miR-133a-3p directly regulates the expression of MSTRG.5970.28, miR-133a-3p overexpression and interference vectors were constructed and were transfected into granulocytes. To further verify whether miR-133a-3p directly regulates the expression of MSTRG.5970.28, an overexpression vector for miR-133a-3p, mimics-miR-133a-3p, and a suppressor vector, inhibitor-miR-133a-3p, were constructed and transfected into granulosa cells. Compared with the NC group, the expression of miR-133a-3p in the mimics-miR-133a-3p group was significantly increased ($P < 0.01$) and the expression of miR-133a-3p in the inhibitor-miR-133a-3p group was significantly decreased ($P < 0.01$), indicating that miR-133a-3p overexpression and inhibition plasmid construction were successful (Figure 2D). When miR-133a-3p was overexpressed, the expression of MSTRG.5970.28 was significantly decreased ($P < 0.01$), and when miR-133a-3p was inhibited, the expression of MSTRG.5970.28 was significantly increased ($P < 0.01$; Figure 2E).

lncRNA MSTRG.5970.28 Regulates the Proliferation and Apoptosis of Goose Follicle Granulosa Cells Through miR-133a-3p

As shown in Figure 3A, compared with the NC group, MSTRG.5970.28 expression was significantly higher in the OE-MSTRG.5970.28 group ($P < 0.01$); MSTRG.5970.28

Figure 2. Validation of lncRNA MSTRG.5970.28 and miR-133a-3p targeting. (A) Predicted binding information of MSTRG.5970.28 to miR-133a-3p. (B) MSTRG.5970.28 and miR-133a-3p binding site prediction and vector construction. (C) Dual luciferase activity assay of the MSTRG.5970.28-wt and MSTRG.5970.28-mut vectors in 293T cells co-transfected with miR-133a-3p mimics or negative control (NC) mimics. The relative luciferase activity was measured at 24 h after transfection, and the standard luciferase activity of the control group was set to 1. (D) miR-133a-3p overexpression and interference efficiency assay. (E) Expression changes of MSTRG.5970.28 after overexpression/repressed expression of miR-133a-3p. mimics-NC, inhibitor-NC, miR-133a-3p mimics or miR-133a-3p inhibitor were transfected into goose GCs. The level of MSTRG5970.28 was detected at 48 h. β-actin was used as an internal reference. All experiments were performed three times. Data are shown as the mean±SD, $^{*}P < 0.05$ and $^{**}P < 0.01$.

expression was significantly lower in the Si-1, Si-2, and Si-3 groups ($P < 0.01$), among which MSTRG.5970.28 expression was lower in the Si-3 group. These results indicated that the lncRNA MSTRG.5970.28 overexpression and interference plasmid were successfully constructed. Subsequently, we analyzed the effect of lncRNA MSTRG.5970.28 on the proliferation and apoptosis of granulosa cells. The results showed that the cell proliferation activity was significantly lower ($P < 0.01$) and the apoptosis rate was significantly higher ($P < 0.01$) in the OE-MSTRG.5970.28 group compared with the NC group, while the cell proliferation activity was significantly higher ($P < 0.01$) and the apoptosis rate was significantly lower ($P < 0.01$) in the Si-MSTRG.5970.28 group (Figures 3B and 3C). By analyzing the effects of miR-133a-3p on the proliferation and apoptosis of granulosa

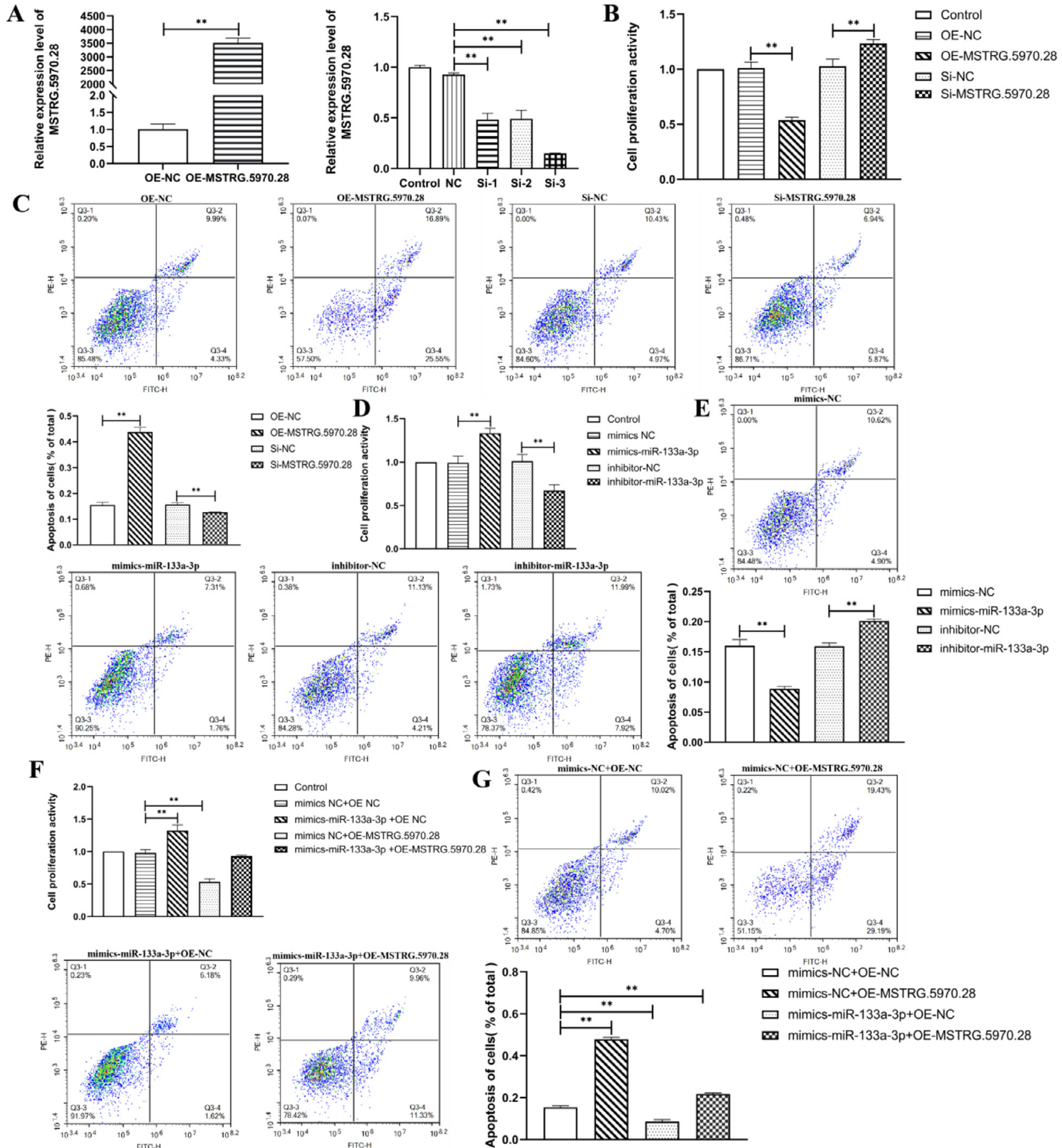

Figure 3. lncRNA MSTRG.5970.28 regulates the proliferation and apoptosis of goose follicle granulosa cells through miR-133a-3p. (A) lncRNA MSTRG.5970.28 overexpression and interference efficiency assay. (B) Effect of overexpression/repressed expression of MSTRG.5970.28 on the proliferation of granulosa cells was measured by CCK-8 assay. (C) Effect of overexpression/repressed expression of MSTRG.5970.28 on apoptosis of granulosa cells was detected by flow cytometry. (D) Effect of overexpression/repressed expression of miR-133a-3p on the proliferation of granulosa cells was measured by CCK-8 assay. (E) Effect of overexpression/repressed expression of miR-133a-3p on apoptosis of granulosa cells was detected by flow cytometry. (F) Effect of overexpression of MSTRG.5970.28 and miR-133a-3p on the proliferation of granulosa cells was measured by CCK-8 assay. (G) Effect of overexpression of MSTRG.5970.28 and miR-133a-3p on apoptosis of granulosa cells was detected by flow cytometry. All experiments were performed three times. Data are shown as the mean±SD, $^{*}P < 0.05$ and $^{**}P < 0.01$.

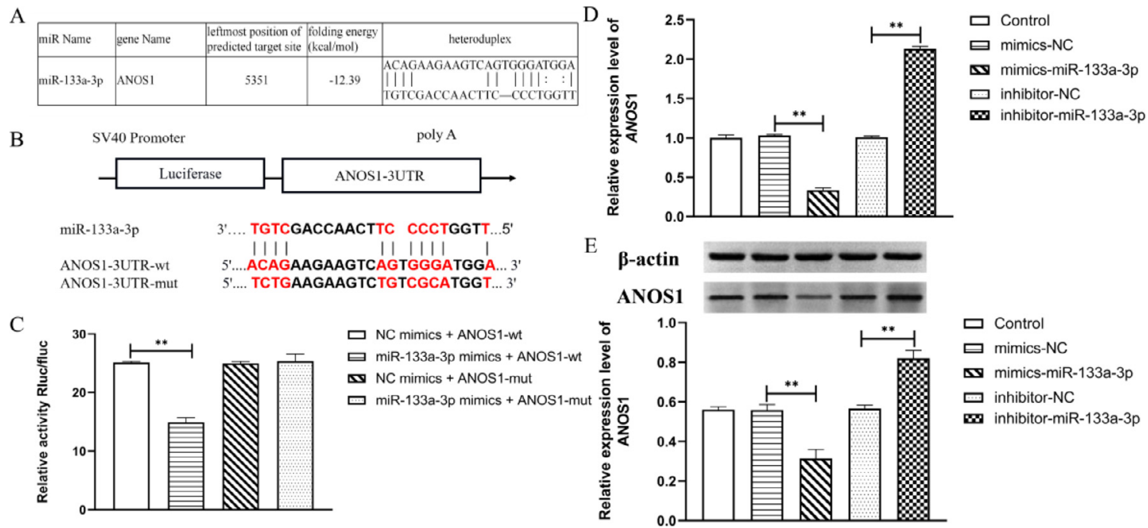

Figure 4. Validation of miR-133a-3p targeting with ANOS1. (A) Predicted binding information of miR-133a-3p to ANOS1. (B) Prediction of miR-133a-3p and ANOS1 binding site and vector construction. (C) Dual luciferase activity assay of the ANOS1-wt and ANOS1-mut vectors in 293T cells co-transfected with miR-133a-3p mimics or negative control (NC) mimics. The relative luciferase activity was measured at 24 h after transfection, and the standard luciferase activity of the control group was set to 1. (D, E) ANOS1 mRNA and protein expression levels in goose granulosa cells, transfected with miR-133a-3p mimics or miR-133a-3p inhibitor. All experiments were performed three times. Data are shown as the mean±SD, $*P < 0.05$ and $**P < 0.01$.

cells, it was found that the proliferative activity of cells in the mimics-miR-133a-3p group was significantly higher ($P < 0.01$) and apoptosis rate was significantly lower ($P < 0.01$) compared with NC. The proliferative activity of cells in the inhibitor-miR-133a-3p group was significantly lower ($P < 0.01$) and the apoptosis rate was significantly higher ($P < 0.01$) in the inhibitor-miR-133a-3p group (Figures 3D and 3E).

To further analyze the effect of lncRNA MSTRG.5970.28 on the function of goose follicle granulosa cells via miR-133a-3p, we transfected overexpressed MSTRG.5970.28 with miR-133a-3p plasmid into granulosa cells. The results showed that, compared with the NC group, the mimics-miR-133a-3p+OE-NC group showed a significant increase in cell proliferation activity ($P < 0.01$) and a significant decrease in apoptosis rate ($P < 0.01$), and the mimics-NC+OE-MSTRG.5970.28 group showed a significant decrease in cell proliferation activity ($P < 0.01$) and a significantly increase in apoptosis rate ($P < 0.01$). The co-transfection of mimics-miR-133a-3p+OE-MSTRG.5970.28 group reduced the inhibitory effect of granulocyte proliferation caused by lncRNA MSTRG.5970.28 overexpression and the apoptosis-promoting effect (Figures 3F and 3G).

Validation of miR-133a-3p Targeting With ANOS1

RNAhybrid analysis showed that miR-133a-3p had a predicted binding site to ANOS1 (Figure 4A). To verify the targeting relationship between miR-133a-3p and ANOS1, ANOS1-wt, and ANOS1-mut plasmids containing miR-133a-3p binding sites were constructed (Figure 4B). Subsequently, wild-type or mutant plasmids were co-transfected into 293T cells with NC or miR-133a-3p mimics. Dual luciferase activity analysis showed that co-transfection of miR-133a-3p mimics and the ANOS1-wt group reduced the fluorescence activity of wild-type plasmids significantly ($P < 0.01$), but had no significant effect on the mutant plasmid ($P > 0.05$; Figure 4C). To further verify whether ANOS1 is a functional target of miR-133a-3p, the overexpression vector mimics-miR-133a-3p or the inhibitor vector inhibitor-miR-133a-3p of miR-133a-3p was transfected into granulosa cell. Compared with the NC group, both mRNA and protein expression of ANOS1 were significantly decreased in the mimics-miR-133a-3p group ($P < 0.01$), and both mRNA and protein expression of ANOS1 were significantly increased in the inhibitor-miR-133a-3p group ($P < 0.01$; Figures 4D and 4E).

lncRNA MSTRG.5970.28 Regulates ANOS1 Expression by Acting as a ceRNA for miR-133a-3p

To further analyze if the lncRNA MSTRG.5970.28 regulates the expression of ANOS1 by acting as a ceRNA for miR-133a-3p, MSTRG.5970.28 was co-transfected with the overexpression plasmid of miR-133a-3p into granulosa cells. The results showed that compared with the NC group, the mRNA and protein expression of ANOS1 in the Control+mimics-miR-133a-3p+OE-NC group were both significantly decreased ($P < 0.01$), and the mRNA and protein expression of ANOS1 in the Control+mimics-NC+OE-MSTRG.5970.28 group were significantly increased ($P < 0.01$). In the co-transfection group of Control+mimics-miR-133a-3p+OE-MSTRG.5970.28, both mRNA and protein expression of ANOS1 were found to be significantly higher than in the NC group ($P < 0.01$), but significantly lower than in the

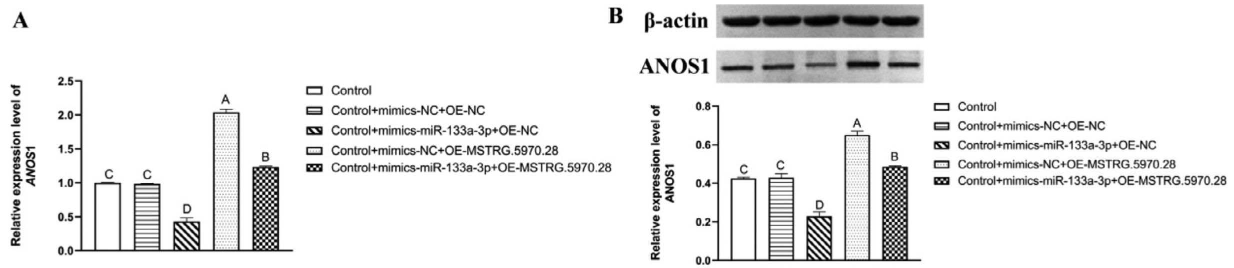

Figure 5. lncRNA MSTRG.5970.28 regulates ANOS1 expression by acting as a ceRNA for miR-133a-3. (A) Changes in mRNA expression of ANOS1 after co-transfection of MSTRG.5970.28 with the miR-133a-3p overexpression plasmid. (B) Protein expression changes of ANOS1 after co-transfection of MSTRG.5970.28 with the miR-133a-3p overexpression plasmid. All experiments were performed three times. Data are shown as the mean±SD, different capital letters denote significant differences between groups ($P < 0.01$).

Control+mimics-NC+OE-MSTRG.5970.28 group ($P < 0.01$; Figures 5A and 5B).

DISCUSSION

The development of follicles in the ovaries of poultry is an important factor in determining egg production, and it is a very complex process that is influenced by a variety of factors (Cogburn et al., 2007; Wu et al., 2021). It is important to explore the specific regulatory mechanisms that clarify follicular development in geese to understand ovarian function and improve egg production in geese. Studies show that lncRNAs can play a regulatory role as ceRNAs for miRNAs (Cogburn et al., 2007; Wu et al., 2021). Previous studies by our research group have found that the lncRNA MSTRG.5970.28 in Yili goose ovary tissue may act as a ceRNA for miR-133a-3p to regulate the expression of ANOS1, which in turn affects the developmental of Yili goose ovaries. Given that lncRNA MSTRG.5970.28 may play an important regulatory role in goose reproduction, this study verified the interaction of lncRNA MSTRG.5970.28 with miR-133a-3p and its effect on goose granulosa cell function through a series of in vitro functional assays.

Follicle-stimulating hormone receptor (**FSHR**) is a protein specifically expressed in the follicular granulosa cells of female animals, and granulosa cells are the only cells that express FSHR (Verbraak et al., 2011; Du et al., 2016). In this study, immunofluorescence identification of follicular granulosa cells by FSHR antibodies revealed positive expression of cytosolic FSHR in follicular granulosa cells. The expression of lncRNA MSTRG.5970.28 in the nucleus and cytoplasm of granulosa cells was determined and it was found that MSTRG.5970.28 may function mainly in the cytoplasm, which suggested that its mechanism functions at the post-transcriptional level. Reproductive hormones are known to play an important role in the regulation of follicle development (Peretz et al., 2011). Heng et al. (2019) showed a significant increase in the expression of OCT4 in granulosa cells after treatment of mice with FSH. Li et al. (2017) found that hCG treatment increased the expression of Enpp3 mRNA in rat ovarian granulosa cells in vitro. In the present study, we also found that the expression of MSTRG.5970.28 in goose granulosa cells was regulated by the reproductive hormones hCG and FSH, suggesting that MSTRG.5970.28 may be involved in the regulation of follicular development in geese.

It has been shown that miR-133 is involved in the regulation of oocyte meiosis (Li et al., 2017) and ovarian cancer cell proliferation (Luo et al., 2014). We previously found that lncRNA MSTRG.5970.28 may be one of the targets of miR-133a-3p (Zhao et al., 2022). To verify, this study revealed, by a dual luciferase activity assay, the presence of a targeting binding site for miR-133a-3p in MSTRG.5970.28. In addition, the expression of MSTRG.5970.28 showed significant changes when both miR-133a-3p were overexpressed or inhibited, further indicating that MSTRG.5970.28 is one of the targets of miR-133a-3p. Subsequently, we found overexpression of MSTRG.5970.28 significantly inhibited the proliferation and promoted the apoptosis of granulocytes by cell proliferation and apoptosis assays. By contrast, when MSTRG.5970.28 was interfered with, the proliferative capacity of granulosa cells was significantly increased and the apoptosis rate was significantly decreased. Thus, it may be speculated that MSTRG.5970.28 has an inhibitory effect on follicle development in geese. When we overexpressed or interfered with miR-133a-3p, its proliferative and apoptotic effects on granulosa cells were opposite to those of MSTRG.5970.28. Jiang et al. (2021) found that lncRNA CDKN2B-AS1 can upregulate the expression of GDNF, inhibit neuronal cells apoptosis by acting as a sponge to adsorb miR-133. Meanwhile, it has also been pointed out that miR-133 is involved in the proliferation regulatory process of myogenic cells (Yang et al., 2019). The results of the present study confirm that miR-133a-3p is involved in the regulation of proliferation and apoptosis of goose granulosa cells, which was consistent with the above findings (Jiang et al., 2021; Yang et al., 2019). This study found that the proliferative and apoptotic effects of granulosa cells caused by MSTRG.5970.28 overexpression are attenuated by miR-133a-3p. The above findings indicated that MSTRG.5970.28 is capable of binding to miR-133a-3p to affect the proliferation and apoptotic processes of granulosa cells.

The ANOS1 gene, located on the X chromosome (Qi et al., 2017), has been extensively studied in human

diseases and was the first gene found to be associated with the development of Kallmann syndrome (Franco et al., 1991). It was noted that ANOS1 is expressed in human skeletal muscle cells (Gianola et al., 2009), skin basal lamina(Raju and Dalakas, 2005), dermal cells (Tengara et al., 2010), and in both granulosa cells and oocytes of the gonads, suggesting that ANOS1, in addition to its regulatory role in GnRH neuronal migration, may also play a direct role locally in the gonads, regulating spermatogenesis, and folliculogenesis (Hu and Bouloux, 2011). Gach A et al. (2020) found that ANOS1 plays an important role in the FGFR1 signaling pathway and that FGFR1 dysfunction causes severe reproductive abnormalities. Based on previous research on ovarian development in Yili geese, we found that the ANOS1 gene was significantly upregulated in ovarian tissue and negatively correlated with the expression of miR-133a-3p when Yili geese entered the ceased-laying phase of the laying period. In this study, the presence of a target binding site for miR-133a-3p in ANOS1 was identified by a dual luciferase activity assay. When miR-133a-3p was overexpressed, both mRNA and protein expression of ANOS1 in granulosa cells significantly decreased; interference with miR-133a-3p increased both mRNA and protein expression of ANOS1 in granulosa cells, further indicating that miR-133a-3p binds directly to the 3′-UTR of the goose ANOS1 gene and that ANOS1 is negatively regulated by miR-133a-3p. The above findings suggest that miR-133a-3p may be involved in the regulation of granulosa cell function by negatively regulating ANOS1 and affecting signaling pathways such as FGFR1.

Studies show that lncRNAs can act as ceRNAs to bind specific miRNAs and regulate their functions (Sadeghi et al., 2022). As a potent microRNA sponge, regulation of RNA activity by competitive binding of microRNA response elements (**MREs**) is one of the most studied mechanisms of lncRNA. Studies of ceRNA-based mechanisms regulating cell proliferation and apoptosis have also been reported in lung adenocarcinoma (Zhu et al., 2022) and cervical cancer (Jiang et al., 2022). In the present study, given that miR-133a-3p can directly interact with MSTRG.5970.28 and ANOS1, we co-transfected the overexpression of MSTRG.5970.28 and miR-133a-3p plasmid into granulosa cells and found that MSTRG.5970.28 acts as a ceRNA to regulate ANOS1 expression by sponging miR-133a-3p in granulosa cells. In other words, MSTRG.5970.28 can compete with the ANOS1 transcript for the miR-133a-3p, thereby reducing the negative regulatory effect of miR-133a-3p on ANOS1. Thus, we speculated that high levels of ANOS1 may promote goose granulosa cells apoptosis.

In summary, this study identified a lncRNA MSTRG.5970.28 associated with goose ovary development, and MSTRG.5970.28 inhibited the proliferation of granulosa cells and promoted apoptosis. Our study provides direct evidence for ceRNA regulatory mechanisms in goose granulosa cells, revealing that the MSTRG.5970.28/miR-133a-3p/ANOS1 axis plays an important role in the proliferation and apoptosis of granulosa cells. As such, the results of this study provide new insights into the regulatory mechanisms of follicular development. The development of new targeted drugs for MSTRG.5970.28, miR-133a-3p, and the ANOS1 regulatory relationship will be an important step to inhibit the development of follicular atresia caused by granulosa cell apoptosis.

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Ethics statement: The animal study was reviewed and approved by the Animal Welfare and Ethics Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China (Approval number 2019004).

DISCLOSURES

All authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102451.

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