The HMGA2-IMP2 Pathway Promotes Granulosa Cell Proliferation in Polycystic Ovary Syndrome

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Context: The high mobility group AT hook 2 (*HMGA2*) gene was previously identified in a genome-wide association study as a candidate risk gene that might be related to polycystic ovary syndrome (PCOS). Whether HMGA2 contributes to promoting granulosa cell (GC) proliferation in PCOS remains unknown.

Objective: We sought to determine whether HMGA2 is involved in the ovarian dysfunction of PCOS and in the mechanism of increased GC proliferation.

Patients and Cells: mRNA expression was analyzed in ovarian GCs from 96 women with PCOS and 58 healthy controls. Immortalized human GCs (KGN and SVOG cells) were used for the mechanism study.

Main Outcome Measures: mRNA expression in ovarian GCs was measured using quantitative RT-PCR, and KGN cells were cultured for proliferation assays after overexpression or knockdown of target genes. Protein expression analysis, luciferase assays, and RNA binding protein immunoprecipitation assays were used to confirm the mechanism study.

Results: HMGA2 and IGF2 mRNA binding protein 2 (IMP2) were highly expressed in the GCs of women with PCOS, and the HMGA2/IMP2 pathway promoted GC proliferation. Cyclin D2 and SERPINE1 mRNA binding protein 1 were regulated by IMP2 and were highly expressed in women with PCOS.

Conclusions: The HMGA2/IMP2 pathway was activated in women with PCOS and promoted the proliferation of GCs. This might provide new insights into the dysfunction of GCs in PCOS. (***J Clin Endocrinol Metab*** **104: 1049–1059, 2019)**

Polycystic ovary syndrome (PCOS) is a complex genetic disease, affecting up to 15% of reproductive-age women worldwide depending on the diagnostic criteria applied (1). PCOS is characterized by hyperandrogenism, menstrual irregularity, and polycystic ovarian morphology. Its pathogenesis is still not fully understood, but recent

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Abbreviations: CCND2, cyclin D2; GC, granulosa cell; GWAS, genome-wide association study; hCG, human chorionic gonadotropin; HMGA2, high mobility group AT hook 2; IMP2, IGF2 mRNA binding protein 2; P4, progesterone; PCOS, polycystic ovary syndrome; RIP, RNA immunoprecipitation; SERBP1, SERPINE1 mRNA binding protein 1; siRNA, small interfering RNA; T2DM, type 2 diabetes mellitus.

genome-wide association studies (GWASs) have provided insights into the biologic pathways that might be disrupted. For example, *DENND1A*, a GWAS susceptibility locus in Han Chinese patients with PCOS (2) that was subsequently replicated in European ancestry cohorts, plays a central role in theca cell androgen biosynthesis (3). Another Han Chinese GWAS susceptibility locus contained high mobility group AT-hook 2 (*HMGA2*) (4).

HMGA2 is a member of the *HMGA* gene family that contains three DNA binding domains (AT hooks that interact with AT-rich stretches in the narrow minor groove of DNA) and an acidic C-terminal tail (5). HMGA2 has been shown to be associated with adult stature (6) and type 2 diabetes mellitus (T2DM) (7). HMGA2 is highly expressed during embryonic development and in various cancers, implying that it might play a role in controlling cell proliferation (8). HMGA2 also enhances E2F activity and promotes cell proliferation in pituitary adenomas (9). Disruption of both *Hmga2* alleles results in the pygmy mouse that has substantially lower body weight as well as infertility in both sexes (10). *Hmga2*-null and heterozygous mice are resistant to both diet-induced and genetic obesity due to the retarded proliferative capacity of mesenchymally derived preadipocytes (11). Taken together, these studies suggest that HMGA2 is associated with proliferation and reproduction, making it a plausible PCOS candidate gene.

IGF2 mRNA binding protein 2 (*IMP2*) is an HMGA2 target gene during early embryonic development (12). The IMP2 protein contains six characteristic RNA binding modules, including two *N*-terminal RNA recognition motifs (RRM1 and RRM2) and four C-terminal hnRNP K-homology (KH1 to KH4) domains (13). A GWAS has identified the association between *IMP2* genetic variants and the risk of T2DM (14). $Imp2^{-/-}$ mice have improved glucose tolerance, insulin sensitivity, and resistance to diet-induced obesity (15). However, the role of IMP2 in granulosa cell (GC) proliferation has not been previously reported.

Throughout oocyte development, there is an interdependence between the oocyte and its surrounding GCs. It has been demonstrated that GCs provide the oocyte with nutrients and growth regulators, whereas the oocyte promotes the growth and differentiation of GCs, and abnormal GC proliferation might lead to abnormal ovulation (16). The ovaries of women with PCOS contain extra numbers of growing follicles at all stages of development (17), and increased numbers of growing follicles are associated with increased GC proliferation in the primate model of PCOS (18). Clinical studies have also shown that GC proliferation is increased in the ovaries of anovulatory women with PCOS compared with normal ovulatory women with PCOS (19, 20). In this study, we investigated the mechanism of HMGA2-IMP2 signaling in GC proliferation in PCOS, and our results suggest that the HMGA2-IMP2 pathway promotes the proliferation of GCs in women with PCOS.

Materials and Methods

Clinical samples

A total of 154 Han Chinese women were enrolled from the Center for Reproductive Medicine, Shandong University, Jinan, China, between October 2015 and June 2016. Of the 154 women, 96 had PCOS and 58 were controls. Women with PCOS were selected in accordance with the Rotterdam criteria (21), including oligoovulation and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovarian morphology by ultrasound with the exclusion of other causes of hyperandrogenism such as hyperprolactinemia, androgen-secreting tumors, Cushing syndrome, and nonclassical congenital adrenal hyperplasia. All women were 20 to 35 years old and with at least two serum concentrations of FSH <10 IU/L and anti-Müllerian hormone >1 ng/mL obtained at least 1 month apart. Control subjects were women of comparable age with regular menses and normal basic endocrine parameters and bilateral antral follicle counts (single antral follicle count = 6 to 10) who had received IVF-ET/ICSI (in vitro fertilization-embryo transfer/intracytoplasmic sperm injection) for male and/or tubal factor infertility and who had no family history of T2DM. Exclusion criteria included current or recent treatment with glucocorticoids, congenital adrenal hyperplasia, hyperprolactinemia, thyroid dysfunction, pregnancy, or oral contraceptive use within 3 months prior to recruitment. Those individuals who had a homeostasis model assessment of insulin resistance (= fasting insulin $\times$ fasting plasma glucose / 22.5) score $\geq$2.57 were classified in the insulin-resistant group.

Ovarian stimulation and GC collection

Ovarian stimulation and oocyte retrieval were performed using the long gonadotropin-releasing hormone agonist protocol. Ultrasound for follicle development and blood sampling for estradiol and progesterone (P4) levels were performed every 1 to 3 days. After adequate follicle development (the presence of two or more follicles measuring 18 mm or more), human chorionic gonadotropin (hCG) was administered. Oocyte retrieval was performed 36 hours after hCG administration by transvaginal ultrasound-guided needle puncture for follicles $>$15 mm in diameter. No more than three embryos were implanted into the uterine cavity 3 days after the oocyte retrieval. Patients received hCG or P4 treatment from the day of oocyte extraction. Urine and serum tests for hCG were performed 14 days after embryo transfer, and in case of a positive hCG test, ultrasonic examination was conducted 2 weeks later to determine clinical pregnancy. At the time of oocyte retrieval, follicular fluid aspirates were collected in sterile tubes and centrifuged. GCs were isolated with Ficoll-Percoll (Solarbio-Life-Sciences, Beijing, China) as previously described (22).

Cell culture

Animals were maintained in the Laboratory Animal Center of Shandong University. Mouse primary ovarian GCs were isolated from the ovaries at postnatal day 21, and these were

subjected to pregnant mare serum gonadotropin treatment after culturing for 24 hours.

The HEK293 cell line was grown in DMEM High Glucose (HyClone, Logan City, UT) supplemented with 10% fetal bovine serum (BI, Beit-Haemek, Israel) and 100 U/mL penicillin G and 0.1 mg/mL streptomycin sulfate (SV30010; HyClone, Logan City, UT). The SVOG cell line (a gift from Prof. Peter C. K. Leung of the University of British Columbia) and the steroidogenic human granulosa–like KGN tumor cell line (a gift from Professor Toshihiko Yanase of Fukuoka University, Japan) were cultured in DMEM/F12 medium (HyClone) supplemented with 10% fetal bovine serum (HyClone) and 100 U/mL penicillin G.

Immunohistochemistry and immunofluorescence

Immunohistochemistry analysis was performed on ovarian sections from postnatal day 21 mice to determine the localization of HMGA2. Briefly, the ovaries were fixed with 4% paraformaldehyde for 24 hours and then washed with PBS and stored in 70% ethanol. The samples were embedded in paraffin, and 5-μm sections were prepared. After deparaffinization and rehydration through a graded ethanol series, the slides were incubated with 5% BSA for 30 minutes at room temperature and incubated with anti-HMGA2 (20795-1-AP; Proteintech, Wuhan, China) antibody at 1:100 dilution overnight at 4°C.

Immunofluorescence assays were performed using the cultured GCs. The cells were fixed with 4% paraformaldehyde and blocked with 5% BSA and then incubated with anti-HMGA2 antibody (ab184616; Abcam, Cambridge, United Kingdom) and anti-IMP2 antibody (14672; Cell Signaling, Danvers, MA) at 1:100 dilutions overnight at 4°C. The cells were then incubated with fluorescence-labeled secondary antibody for 1 hour.

Adenoviral and lentiviral vector construction

Adenoviruses expressing HMGA2 (VH893703; Vigene Bioscience, Jinan, China), lentiviruses expressing IMP2 (CH884509; Vigene Bioscience, Jinan, China), and green fluorescent protein (CV0001; Vigene Bioscience, Jinan, China) were purchased from Vigene Bioscience Company (Jinan, China).

Small interfering RNA and transfection

ON-TARGET plus *SMARTpool* small interfering RNA (siRNA) targeting HMGA2 and IMP2 and ON-TARGET plus Non-Targeting pool siRNA controls (Dharmacon, GE Healthcare Life Technologies, Marlborough, MA) were transfected at 50 nM. Cells were transfected with siRNA using X-treme GENE siRNA Transfection Reagent (Roche, Germany) for 48 hours.

Real-time RT-PCR

Total RNA was extracted from cultured GCs using TRIzol reagent (Takara Bio, Inc., Dalian, China) and was reverse transcribed into cDNA using the Prime Script RT reagent Kit with gDNA Eraser (Takara Bio, Inc., Dalian, China). The PCRs were performed using SYBR Premix Ex Taq (Takara Bio, Inc., Dalian, China) according to the manufacturer's instructions, and the primer sequences are shown in Supplemental Table 2. Real-time PCRs were carried out using a Roche LightCycle 480 (Roche, Penzberg, Germany). The expression of the housekeeping gene *ACTB* was used to normalize gene expression. The relative gene expression in the PCOS group was calculated using the $2^{-\Delta CT}$ method and was expressed as the fold change relative to the expression in the control group (23).

Western blot

After treatment, GCs were harvested and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride. Equal amounts of protein were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels, and the bands were transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA). Immunoreactive bands were detected and analyzed with a BIO-RAD ChemiDoc MP Imaging System and Image Laboratory Software (BIO-RAD). Relative protein levels in each sample were normalized to glyceraldehyde 3-phosphate dehydrogenase to standardize the loading variations. The primary antibodies for immunoblotting included anti-HMGA2 antibody (ab184616; Abcam, Cambridge, United Kingdom), anti-IMP2 antibody (14672; Cell Signaling, Danvers, MA), anti–cyclin D2 (CCND2) antibody (3741; Cell Signaling, Danvers, MA), anti–glyceraldehyde 3-phosphate dehydrogenase antibody (60004-1-Ig; Proteintech, Wuhan, China), and anti- SERPINE1 mRNA binding protein 1 (SERBP1) antibody (10729-1-AP; Proteintech, Wuhan, China).

Enhanced CCK8 assay

Cells transfected with virus or siRNAs for 24 hours were reseeded in 96-well plates at 1500 cells/well in a final volume of 100 μL and incubated overnight. The effects of HMGA2/IMP2 on cell growth and proliferation were determined using the CCK8 assay (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Flow cytometry

We used the BD Cycletest Plus DNA Kit (340242; BD Biosciences, Sparks, MA) for the analysis of nuclear DNA from cell suspensions. Cells transfected with siRNAs were plated in 6-well plates and incubated at 37°C for 72 hours. The cells were then collected and washed with buffer solution three times, and the cell cycle distribution was analyzed using propidium iodide staining and flow cytometry.

RNA binding protein immunoprecipitation

RNA immunoprecipitation (RIP) was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Burlington, MA) according to the manufacturer's instructions. The IMP2 antibody used for RIP was obtained from Proteintech (11601-1-AP; Proteintech, Wuhan, China). Immunoprecipitations were carried out using antibodies against IMP2 or normal human IgG with protein A magnetic beads. The beads with bound complexes were immobilized with a magnet, and unbound materials were washed away with washing buffer. RNAs that coimmunoprecipitated with the IMP2 antibody or IgG were eluted, reverse transcribed, and analyzed by quantitative real-time PCR.

Luciferase reporter assay

The 3′-UTR sequences of *CCND2* and *SERBP1* containing the IMP2 binding sites (CCND2: ACA TTC CCA TCA CAA CAT TCC TCA G; SERBP1: CTA TAG AAA ACA CCT GCT ACT CAA AAC ACA ACT TCT CAG T) were amplified by PCR and cloned into the pEZX-GA02 vector (Genecopoeia, Guangzhou, China) in front of the Gaussia luciferase gene. These plasmids were then used to cotransfect HEK293 cells along with the IMP2 expression vector (IMP2-pCDH) or control vector (pCDH) using the X-treme GENE HP DNA transfection reagent (Roche, Penzberg, Germany). Luciferase activities of cultured supernatants were measured 48 hours and 72 hours after transfection using a Secrete-Pair Dual Luminescence

Assay Kit (SPGA-G010; Genecopoeia, Guangzhou, China). The Gaussia luciferase activity was normalized to secreted alkaline phosphatase luciferase activity to measure transfection efficiency. The activity ratio of the empty vectors was arbitrarily set at 1.0, and we compared the relative luciferase activity of the different groups.

Statistical analysis

Data are presented as the median ± interquartile range from at least three independent experiments. Two-way ANOVA followed by Tukey multiple comparison tests were used for statistical comparison. Statistical significance was set at $P < 0.05$.

Ethics statement

This study received ethical approval from the Institutional Review Board of Reproductive Medicine of Shandong University (Jinan, China). All of the methods described here were carried out in accordance with the guidelines and regulations approved by the Institutional Review Board of Reproductive Medicine of Shandong University. Our study conformed to the Declaration of Helsinki.

Results

Comparison of basic clinical features between controls and women with PCOS

We first determined the clinical features related to PCOS in control and patient samples (Supplemental Table 1). The average body mass index (kg/m^2) of the women with PCOS was 24.89 ± 4.24, which was significantly higher than the control group (21.76 ± 3.17; $P < 0.05$). In women with PCOS, levels of luteinizing hormone, total testosterone, anti-Müllerian hormone, fasting plasma glucose, fasting insulin, and homeostasis model assessment of insulin resistance were higher than in the control group, whereas levels of FSH were lower. No significant differences were found for P4 between the PCOS and control groups.

HMGA2 was highly expressed in PCOS GCs from Han Chinese women

Although HMGA2 was previously reported to be expressed in mouse oocytes (24) and overexpressed in ovarian tumors (25), it remains unclear where HMGA2 localizes within the whole ovary. We examined HMGA2 expression in the ovary by immunohistochemistry in mice (Supplemental Fig. 1A, arrows), which showed that HMGA2 was exclusively expressed in GCs of follicles at various stages and oocytes rather than theca cells. HMGA proteins might modulate gene expression by altering chromatin architecture and/or by recruiting other proteins to the transcription regulatory complex (26). To determine if the HMGA2 protein functions as an architectural transcription factor, we examined HMGA2 expression in mouse GCs. We found that HMGA2 was exclusively expressed in the nuclei of GCs (Fig. 1A) and was expressed to a greater extent in the GCs of women with PCOS compared with controls (Fig. 1B and 1C).

HMGA2 overexpression promoted proliferation in the KGN and SVOG cell lines

To determine if the high expression of HMGA2 promotes GC proliferation and/or survival, we transfected KGN cells with siRNA and conducted CCK8 experiments. The siRNA in KGN cells achieved a 60% knockdown of *HMGA2* mRNA and protein in comparison with the nontargeting siRNA (Supplemental Fig. 1B and 1C). Knockdown of HMGA2 resulted in inhibition of cell proliferation as measured by the CCK8 assay (Fig. 2A). In addition, transfection of KGN cells with HMGA2-expressing adenovirus increased the levels of HMGA2 protein expression (Supplemental Fig. 1D and 1E) and increased the proliferation of GCs (Fig. 2B). We determined the effect of HMGA2 on the cell cycle distribution using flow cytometry. Compared with the control group, at 72 hours post-transfection, we observed an increased proportion of G1-phase cells and a decreased proportion of S-phase cells (Fig. 2C–2E; $P < 0.5$). Similar results were found when we conducted the experiments in SVOG cells (Supplemental Fig. 2B and 2C).

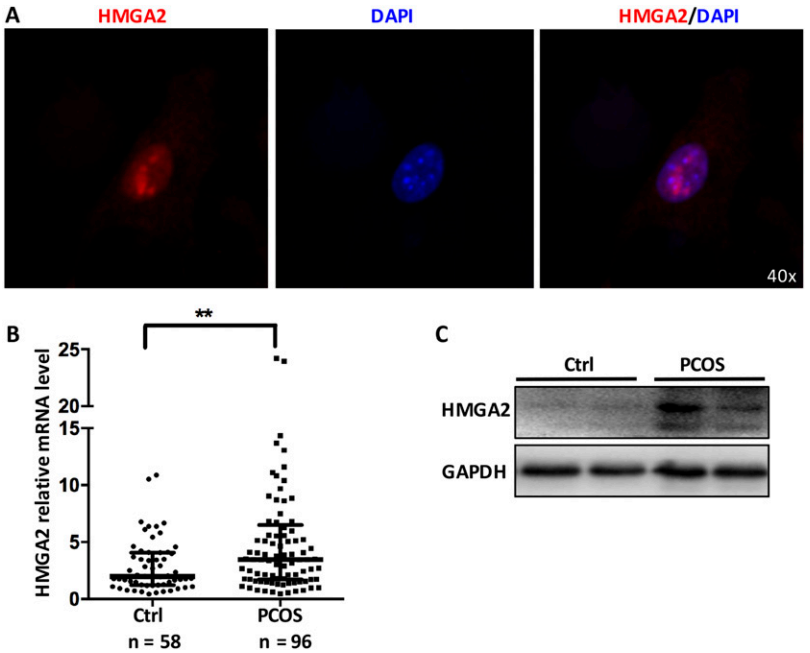

Figure 1. HMGA2 expression in PCOS GCs. (A) HMGA2 protein was localized to the nuclei of mouse GCs. Left panel, staining with anti-HMGA2 antibody; middle panel, staining with 4′,6-diamidino-2-phenylindole (DAPI); right panel, merged staining. Scale bar, 25 μm. (B, C) Comparison of *HMGA2* (B) mRNA and (C) protein expression in control and PCOS GCs. HMGA2 levels were higher in women with PCOS than in controls. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Ctrl, control. *$P < 0.05$. **$P < 0.01$.

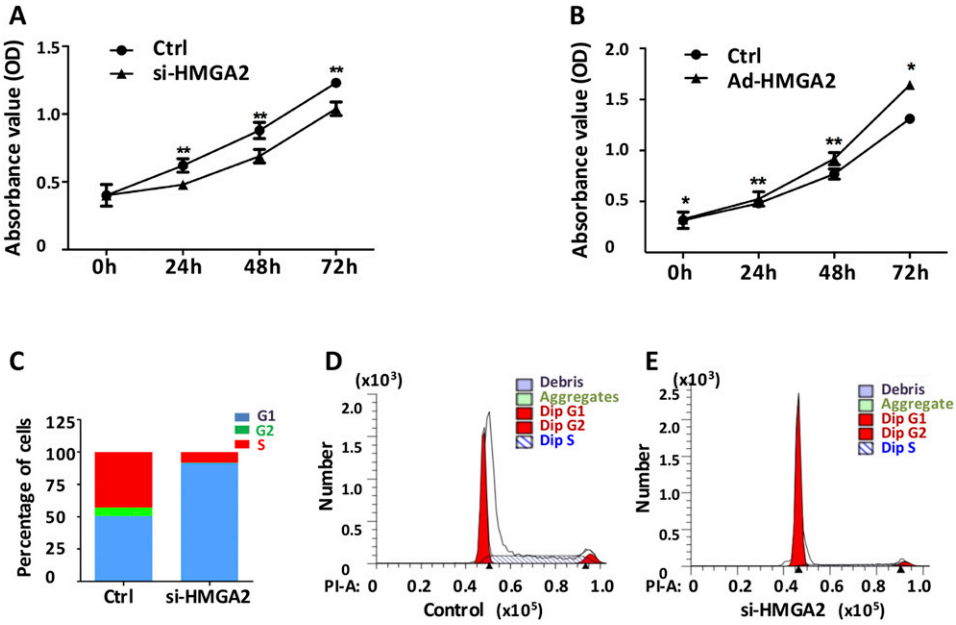

Figure 2. Effects of HMGA2 on GC proliferation. (A, B) CCK8 assay of control and experimental cells in which HMGA2 was downregulated or upregulated. (A) Knockdown of HMGA2 reduced cell proliferation, whereas (B) overexpression of HMGA2 increased cell proliferation. Each value represents the mean of three independent experiments. $*P < 0.05$. $**P < 0.01$. (C–E) Flow cytometry assay of GC cell cycle progression under (D) control and (E) knockdown conditions. (C) Knockdown of HMGA2 expression resulted in an increased proportion of G1-phase cells and a decreased proportion of S-phase cells. Ctrl, control; PI-A, propidium iodide-annexin V.

HMGA2 upregulated IMP2 expression in GCs from Han Chinese women with PCOS

To better understand the mechanism by which HMGA2 mediates GC proliferation, we studied the downstream gene pathway related to HMGA2. *Imp2* is an important target gene of HMGA2 through the AT-rich regulatory region in the first intron of the *IMP2* gene (23), and the HMGA2/IMP2 pathway is conserved in several cell lines and plays a role in embryonic development and tumorigenesis (27, 28). To determine whether *IMP2* is a target

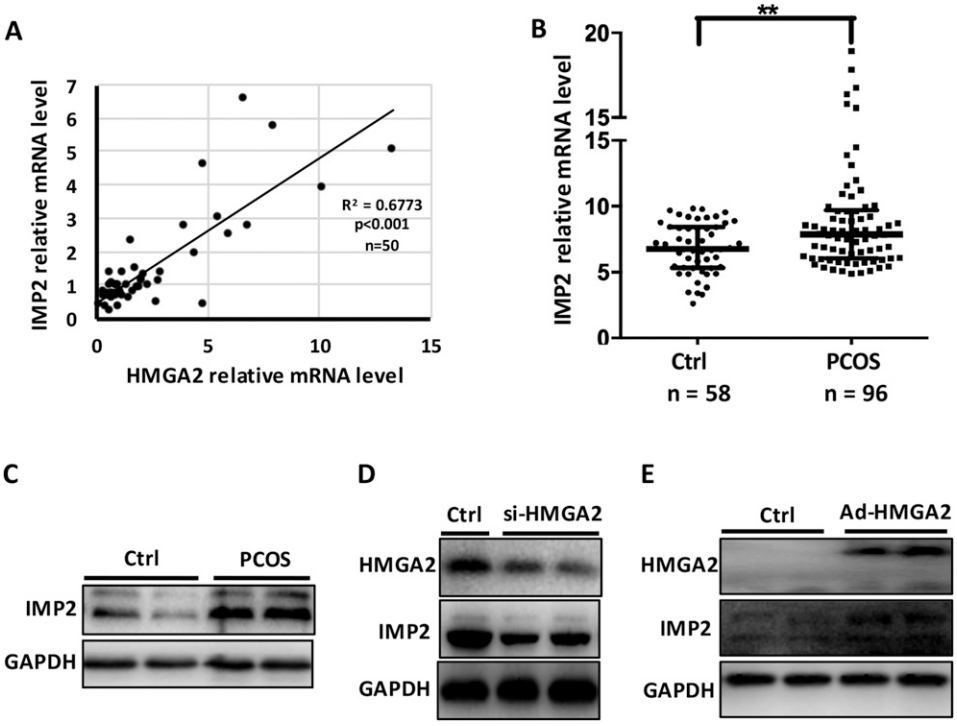

Figure 3. Correlation between the expression of HMGA2 and IMP2 in PCOS GCs. (A) *HMGA2* and *IMP2* mRNA levels were correlated in GCs from women with PCOS. $R^2 = 0.6773$. (B, C) *IMP2* mRNA and protein expression in control and PCOS GCs. IMP2 levels were higher in PCOS GCs than in control cells. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). $*P < 0.05$. $**P < 0.01$. (D, E) Western blots of IMP2 expression in KGN cells. (D) Knockdown of HMGA2 reduced IMP2 levels, whereas (E) overexpression of HMGA2 increased IMP2 levels. Ctrl, control.

gene of HMGA2 in PCOS GCs, we analyzed the *IMP2* mRNA level in clinical samples. We found that *HMGA2* mRNA and *IMP2* mRNA levels were correlated in the GCs of women with PCOS (Fig. 3A), and the *IMP2* mRNA level was higher in the GCs of women with PCOS compared with controls (Fig. 3B). The IMP2 protein level was also higher in the GCs of women with PCOS compared with controls (Fig. 3C). We found that both *IMP2* mRNA and protein were reduced upon HMGA2 knockdown in KGN cells (Fig. 3D), whereas overexpression of HMGA2 increased the expression of IMP2 in KGN cells (Fig. 3E). These results suggest that IMP2 is regulated by HMGA2.

Overexpression of IMP2 promoted proliferation in the KGN and SVOG cell lines

To determine whether IMP2 is involved in PCOS, we conducted the following experiments. First, we determined the localization of the IMP2 protein in mouse GCs by immunofluorescence and showed that IMP2 was concentrated to the cytoplasm (Fig. 4A). Second, we used the CCK8 assay to determine whether the expression of IMP2 is involved in cell proliferation. Our results demonstrated that knockdown of IMP2 reduced the proliferation of KGN cells (Fig. 4B), whereas overexpression of IMP2 (from lentivirus-expressing IMP2) increased cell proliferation (Fig. 4C). In addition, we performed cell cycle analysis by flow cytometry. We found that when IMP2 was knocked down, the percentage of S-phase cells was decreased and the percentage of G1-phase cells was increased (Fig. 4D, Supplemental Fig. 4A and 4B). Similar results were found when we repeated these experiments in SVOG cells (Supplemental Fig. 3A and 3B).

CCND2 and SERBP1 were regulated by IMP2 in KGN cells

It was next of interest to understand the mechanism by which IMP2 regulates proliferation in GCs. Therefore, we first tried to identify mRNAs bound by IMP2 in GCs. A previous study identified top RNAs, including mRNAs, tRNAs, rRNAs, and microRNAs, bound by overexpressed IMP2 in HEK293 cells, and we reanalyzed data from Hafner *et al.* (29). We found that IMP2 bound to the 3′-UTR of *CCND2* and *SERBP1* mRNA (Fig. 5A and 5B), and the IMP2 proteins stabilized their target mRNAs. To determine whether endogenous IMP2 binds to *CCND2* and *SERBP1*, we conducted RNA binding protein immunoprecipitation (RBP-IP) experiments in HEK293 cells. RIP sequence analysis in HEK293 cells showed that *CCND2* and *SERBP1* mRNAs were bound by the IMP2 protein (Fig. 5C). Overexpression of HMGA2 increased the CCND2 and SERBP1 protein levels (Supplemental Fig. 4C), whereas knockdown of HMGA2 in KGN cells reduced the CCND2 and SERBP1 levels (Fig. 5D). Furthermore, knockdown of IMP2 reduced CCND2 and SERBP1 levels, whereas the HMGA2 expression level remained the same (Fig. 5E).

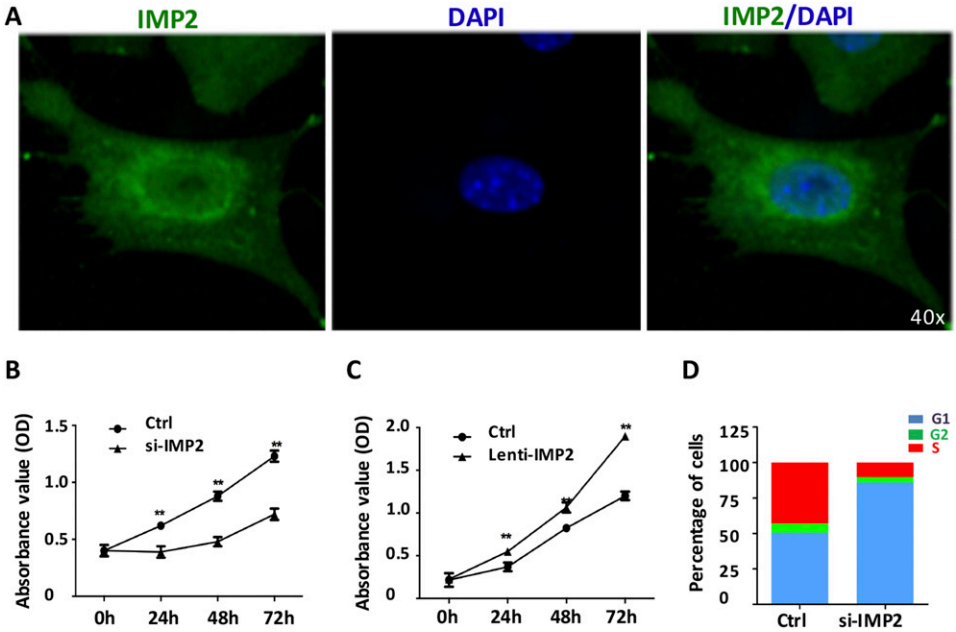

Figure 4. Effects of IMP2 on GC proliferation. (A) Localization of IMP2 in GCs. IMP2 protein was localized to the cytoplasm of mouse GCs. Left panel, staining with anti-IMP2 antibody; middle panel, staining with 4′,6-diamidino-2-phenylindole (DAPI); right panel, merged staining. Scale bar, 25 μm. (B, C) CCK8 assay of control and experimental cells in which IMP2 was downregulated or upregulated. (B) Knockdown of IMP2 reduced cell proliferation, (C) whereas overexpression of IMP2 increased cell proliferation. $^{**}P < 0.01$. (D) Flow cytometry assay of the cell cycle phase of GCs under control and knockdown conditions. Knockdown of IMP2 expression resulted in an increased proportion of G1-phase cells and a decreased proportion of S-phase cells. Each value represents the mean of at least three independent experiments. Ctrl, control.

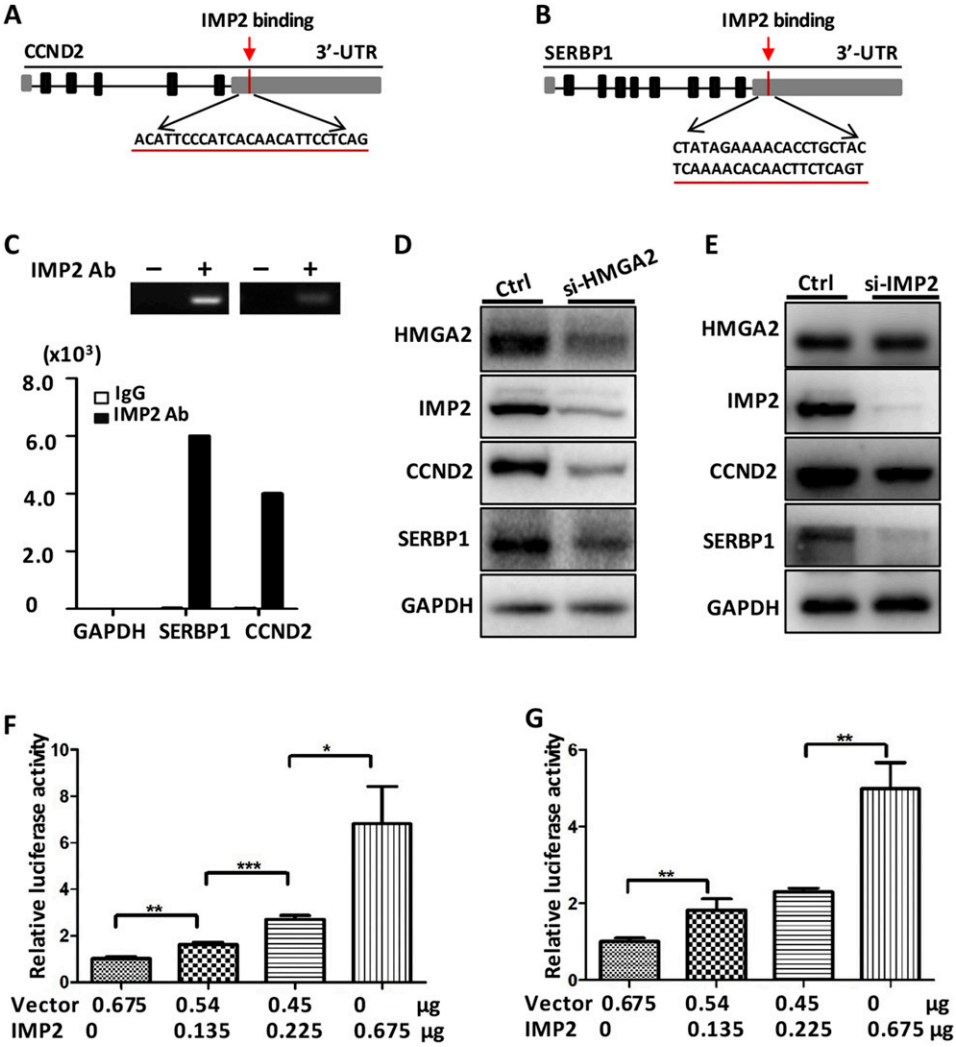

Figure 5. CCND2 and SERBP1 expression in the HMGA2-IMP2 pathway. (A, B) IMP2 binding sites in the *CCND2* and *SERBP1* genes. IMP2 bound to *CCND2* and *SERBP1* mRNAs. (C) RIP experiments in HEK293 cells. CCND2 and SERBP1 proteins were pulled down using anti-IMP2 antibodies. IgG and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as negative controls. (D) Knockdown of HMGA2 in KGN cells reduced the CCND2 and SERBP1 protein levels. (E) Knockdown of IMP2 reduced the CCND2 and SERBP1 protein levels in KGN cells. (F, G) Luciferase assays of IMP2 and downstream genes (F, *CCND2*; G, *SERBP1*) in KGN cells. In both cases, a dose-dependent increase in luciferase activity of CCND2 and SERBP1 was observed as IMP2 expression increased. $*P < 0.05$. $**P < 0.01$. Each value represents the mean of at least three independent experiments. Ctrl, control.

To determine whether IMP2 regulated mRNA stability through binding to the 3′-UTR, we cotransfected HEK293 cells with a luciferase-CCND2 3′-UTR fusion construct, a luciferase-SERBP1 3′-UTR fusion construct, and an IMP2-expressing construct. IMP2 overexpression caused a dose-dependent increase in luciferase activity (Fig. 5F and 5G), indicating that IMP2 binds to the 3′-UTR of *CCND2* and *SERBP1* mRNAs and increases the expression of these proteins.

CCND2 and SERBP1 were highly expressed in the GCs of women with PCOS

We measured the CCND2 and SERBP1 expression levels in the GCs of women with PCOS using quantitative RT-PCR and western blotting methods. *CCND2* and *SERBP1* mRNA (Fig. 6A and 6C) and protein (Fig. 6B and 6D) were increased in the GCs of women with PCOS, and this is consistent with previous findings that increased expression of CCND2 and SERBP1 increases GC proliferation (30) and decreases apoptosis (31).

Discussion

PCOS is a highly heritable, complex genetic disease (32). GWASs have enabled an unbiased interrogation of the entire genome for susceptibility loci for PCOS and its quantitative traits (33), and these studies have implicated several biological pathways in PCOS pathogenesis, including gonadotropin secretion (32) and action (2), androgen biosynthesis (34), and cell survival (35). However, most GWAS loci are in noncoding portions of the genome, and elucidating the mechanisms by which GWAS variants contribute to disease susceptibility remains quite challenging (36, 37). Two studies reported

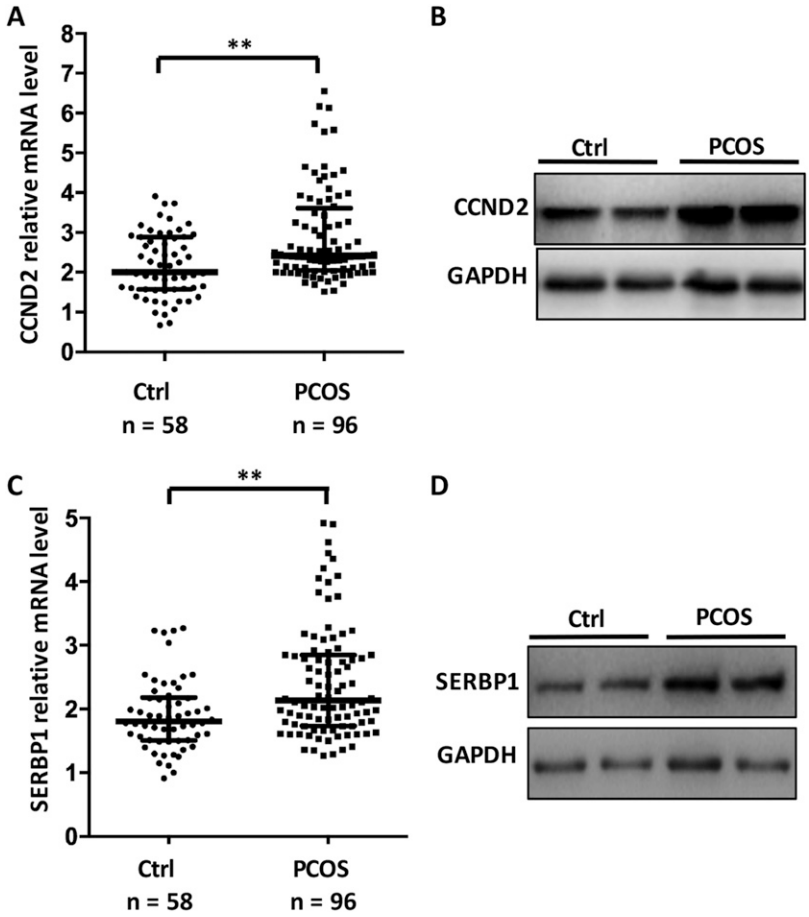

Figure 6. *CCND2* and *SERBP1* mRNA and protein expression in control and PCOS GCs. (A, B) *CCND2* mRNA and protein expression was higher in PCOS GCs than in control cells. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). $^{**}P < 0.01$. (C, D) *SERBP1* mRNA and protein expression was higher in PCOS GCs than in control cells. Data were normalized to GAPDH. $^{**}P < 0.01$. Ctrl, control.

the discovery of the fat mass and obesity-associated (*FTO*) gene as the first obesity susceptibility gene identified by GWAS, and *FTO* regulates adipogenesis through RNA modification. In this study, we have investigated potential mechanisms by which the PCOS GWAS susceptibility gene *HMGA2* might contribute to PCOS pathogenesis.

The proper proliferation and differentiation of GCs are important for follicular development, and we have shown here that HMGA2 is highly expressed in the GCs of women with PCOS and plays a role in GC proliferation. The HMGA2/IMP2 pathway is involved in several signaling-transduction pathways that mediate cell proliferation and differentiation (12, 38). However, the relevance of this pathway in GC proliferation in PCOS remains unknown. We found that activation of the HMGA2/IMP2 pathway promotes GC proliferation in both the KGN and SVOG cell lines and that IMP2 is overexpressed in the GCs of women with PCOS. We used the 293 cell line and immortalized GC cell lines to verify the mechanism study. HMGA2/IMP2 regulates the expression of CCND2 and SERBP1, and CCND2 and SERBP1 are also overexpressed in women with PCOS.

In the ovary, CCND2 is expressed mainly in the GCs and is responsive to FSH, and *Ccnd2*-deficient female mice are sterile due to the failure of the G1/S transition in GCs and subsequent preantral follicle growth arrest (30). SERBP1 is expressed in GCs and in luteal, thecal, interstitial, and surface epithelial cells and is involved in mediating P4's antiapoptotic action by binding to PGRMC1 (progesterone receptor membrane complex 1) (31). We found that overexpression of the HMGA2-IMP2 pathway increased the CCND2/SERBP1 level in the KGN and SVOG cell lines, and the target genes *CCND2* and *SERBP1* were highly expressed in GCs from women with PCOS. We concluded from these results that activation of the HMGA2-IMP2 pathway in GCs from women with PCOS leads to high expression of CCND2 and SERBP1, which in turn increases GC proliferation.

The accumulation of antral follicles in the range of 2 to 8 mm in diameter is a characteristic feature of women with PCOS (39). There is an apparent failure to select a dominant follicle, and this is assumed to be due to the developmental arrest of larger antral follicles (5 to 8 mm). Although the underlying mechanisms behind this developmental arrest remain to be examined, it is possible that lower serum concentrations of FSH result in inadequate follicle maturation (40). Our data provide new evidence, to our knowledge, for the increased proliferation of GCs in women with PCOS.

There is some controversy over GC proliferation in PCOS. It has been previously shown that miR-93 is upregulated in the GCs from women with PCOS and that it promotes the proliferation and cell cycle progression of these cells (41). In contrast, miR-145 expression levels have been shown to be markedly decreased in isolated human GCs from women with PCOS, and this has been shown to reduce the survival rate and suppress DNA synthesis in these cells (42). All of these studies have demonstrated that a high rate of GC proliferation contributes to the abnormal folliculogenesis seen in women with PCOS. It is notable, however, that some previous reports have shown decreased GC proliferation in PCOS. For example, it has been shown that miR-27a-3p is overexpressed in women with PCOS and that overexpression of miR-27a-3p in KGN cells inhibits

SMAD5, which in turn decreases cell proliferation and promotes cell apoptosis (43). The inconsistent results obtained from these human and rat experiments might be due to differences in the stages of the follicles that were examined.

A potential limitation of our study is the use of KGN and SVOG cell lines as the GC model. The KGN cell line is frequently used to assess signaling pathways, growth, and function in PCOS (44). Despite the widespread use of SVOG cells as proxies for normal granulosa cells, these cells have features of luteal as well as granulosa cells, including a tendency to differentiate into luteal cells. Therefore, further studies are needed to elucidate more mechanisms using *in vivo* models. In addition, HMGA2 is a GWAS susceptibility locus in Han Chinese women with PCOS (4). However, the two cell lines are from other ancestry groups. The KGN cell line was derived in Japan (45), and the SVOG cell line was derived in Canada (46). Accordingly, there may be racial/ethnic genetic differences that affected study outcomes.

Insulin resistance is a common feature of PCOS and confers an increased risk for T2DM (47), and *HMGA2* and *IMP2* are both T2DM GWAS susceptibility loci (7, 14). HMGA2 is overexpressed in the adipose tissues of obese humans and has a gene-dosage effect on obesity (11). HMGA2 is also highly expressed in exponentially growing 3T3-L1 preadipocytes and during the initial stages of differentiation (48). Thus, HMGA2 might play a role in initiating preadipocyte proliferation and differentiation. In addition, HMGA2 levels are higher in white adipose tissue in patients with T2DM compared with patients without T2DM (49). Therefore, the HMGA2-IMP2 pathway might play a role in the development of obesity in PCOS.

In conclusion, we found that the HMGA2-IMP2 pathway is involved in the proliferation of ovarian GCs from women with PCOS. The induction of IMP2 expression upregulated the expression of target genes, such as *CCND2* and *SERBP1*, thereby leading to increased proliferation of GCs. Our data suggest that overexpression of HMGA2 in ovarian GCs leads to increased cell proliferation and thus contributes to the polycystic ovary phenotype. To our knowledge, these results provide new insights into the mechanisms underlying the GC dysfunction seen in PCOS.

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