

At the crossroads of fertility and metabolism: the importance of AMPK-dependent signaling in female infertility associated with hyperandrogenism

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STUDY QUESTION: What biological processes are linked to the signaling of the energy sensor 5'-AMP-activated protein kinase (AMPK) in mouse and human granulosa cells (GCs)?

SUMMARY ANSWER: The lack of α IAMPK in GCs impacted cell cycle, adhesion, lipid metabolism and induced a hyperandrogenic response.

WHAT IS KNOWN ALREADY: AMPK is expressed in the ovarian follicle, and its activation by pharmacological medications, such as metformin, inhibits the production of steroids. Polycystic ovary syndrome (PCOS) is responsible for infertility in approximately 5–20% of women of childbearing age and possible treatments include reducing body weight, improving lifestyle and the administration of a combination of drugs to improve insulin resistance, such as metformin.

STUDY DESIGN, SIZE, DURATION: AMPK signaling was evaluated by analyzing differential gene expression in immortalized human granulosa cells (KGNs) with and without silencing α IAMPK using CRISPR/Cas9. *In vivo* studies included the use of a α IAMPK knock-out mouse model to evaluate the role of α IAMPK in folliculogenesis and fertility. Expression of α IAMPK was evaluated in primary human granulosa-luteal cells retrieved from women undergoing IVF with and without a lean PCOS phenotype (i.e. BMI: 18–25 kg/m²).

PARTICIPANTS/MATERIALS, SETTING, METHODS: α IAMPK was disrupted in KGN cells and a transgenic mouse model. Cell viability, proliferation and metabolism were evaluated. Androgen production was evaluated by analyzing protein levels of relevant enzymes in the steroid pathway by western blots, and steroid levels obtained from *in vitro* and *in vivo* models by mass spectrometry. Differential

gene expression in human GC was obtained by RNA sequencing. Analysis of *in vivo* murine folliculogenesis was performed by histology and immunochemistry, including evaluation of the anti-Müllerian hormone (AMH) marker. The α IAMPK gene expression was evaluated by quantitative RT-PCR in primary GCs obtained from women with the lean PCOS phenotype ($n = 8$) and without PCOS ($n = 9$).

MAIN RESULTS AND THE ROLE OF CHANCE: Silencing of α IAMPK in KGN increased cell proliferation ($P < 0.05$ versus control, $n = 4$), promoted the use of fatty acids over glucose, and induced a hyperandrogenic response resulting from upregulation of two of the enzymes involved in steroid production, namely 3β -hydroxysteroid dehydrogenase (3β HSD) and P450 side-chain cleavage enzyme (P450scc) ($P < 0.05$, $n = 3$). Female mice deficient in α IAMPK had a 30% decrease in their ovulation rate ($P < 0.05$, $n = 7$) and litter size, a hyperandrogenic response ($P < 0.05$, $n = 7$) with higher levels of 3β HSD and p450scc levels in the ovaries, and an increase in the population of antral follicles ($P < 0.01$, $n = 10$) compared to controls. Primary GCs from lean women with PCOS had lower α IAMPK mRNA expression levels than the control group ($P < 0.05$, $n = 8-9$).

LARGE SCALE DATA: The FastQ files and metadata were submitted to the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB46048.

LIMITATIONS, REASONS FOR CAUTION: The human KGN is a not fully differentiated, transformed cell line. As such, to confirm the role of AMPK in GC and the PCOS phenotype, this model was compared to two others: an α IAMPK transgenic mouse model and primary differentiated granulosa-lutein cells from non-obese women undergoing IVF (with and without PCOS). A clear limitation is the small number of patients with PCOS utilized in this study and that the collection of human GCs was performed after hormonal stimulation.

WIDER IMPLICATIONS OF THE FINDINGS: Our results reveal that AMPK is directly involved in steroid production in human GCs. In addition, AMPK signaling was associated with other processes frequently reported as dysfunctional in PCOS models, such as cell adhesion, lipid metabolism and inflammation. Silencing of α IAMPK in KGN promoted folliculogenesis, with increases in AMH. Evaluating the expression of the α IAMPK subunit could be considered as a marker of interest in infertility cases related to hormonal imbalances and metabolic disorders, including PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is one of the leading causes of female infertility, occurring in 5–20% of women of childbearing age (Joham et al., 2015; El Hayek et al., 2016). It is characterized by hyperandrogenism, infertility, anovulation and/or ovarian cysts, with a broad phenotypic expression (including hirsutism and acne) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Nearly 40–60% of patients with PCOS are either overweight or obese (Ehmann et al., 2006). As such, intervention therapies for PCOS include reducing body weight, improving lifestyle and administering a combination of drugs (e.g. clomiphene citrate with metformin) to overcome insulin resistance and attenuate PCOS-related endocrine and infertility issues (Fulghesu et al., 2012).

Metformin therapy has been used to realign metabolic and hormonal dysfunctions, with some positive outcomes after IVF and IVF/ICSI interventions in women with PCOS (Witchel et al., 2019; Abdalla et al., 2020; Wu et al., 2020). Deciphering the mechanisms of metformin's actions in the ovaries has pointed to a critical kinase activated by metformin called the 5'-AMP-activated protein kinase (AMPK) (Tosca et al., 2005, 2007b; Hardie et al., 2016; Faure et al., 2018). Recently, decreases in AMPK protein levels in the ovaries were reported in a dehydroepiandrosterone (DHEA)-induced PCOS mouse model (Tao et al., 2017), whereas AMPK activators (5-aminoimidazole-4-carboxamide riboside

(AICAR), resveratrol, metformin) improved fertility. Moreover, a close functional link was reported for androgen production and AMPK activity in the ovaries from a transgenic mouse model deficient in LKB1, an AMPK activating kinase gene (Tao et al., 2017). Collectively, these studies support the notion of a critical role of AMPK in granulosa cells (GCs) linked to both metabolism and fertility.

AMPK is a serine/threonine heterotrimeric kinase composed of a catalytic α -subunit and two regulatory subunits, β and γ . All three subunits of AMPK are highly conserved in eukaryotic species (Hardie et al., 2003; Ghillebert et al., 2011). This kinase is activated when energy reserves are depleted to produce more energy to maintain ATP homeostasis. Activation of AMPK entails the phosphorylation of the α -subunit at Thr172. All three subunits of AMPK are expressed in the ovaries (Tosca et al., 2005; Dupont et al., 2012; Tartarin et al., 2012) as well as in different follicle cell types (GCs, theca cells and oocytes) and corpora lutea. The expression and localization of AMPK in the ovary are conserved across species, ranging from invertebrates to vertebrates (Guévelou et al., 2013), and within vertebrates, from mammals to birds (Tosca et al., 2006), cows (Tosca et al., 2007a), pigs (Mayes et al., 2007), rodents (Tosca et al., 2005; Downs et al., 2010) and humans (Pellatt et al., 2011).

In a related but separate role, AMPK may control the crosstalk between energy balance and reproduction, both processes affected in

PCOS (Bertoldo *et al.*, 2014). For instance, glucose metabolism dysregulation can trigger defects in ovulation and lower oocyte quality (Downs and Mastropolo, 1994; Pesant and Baillargeon, 2011). Few studies have explored the role of AMPK in fertility despite that silencing of oocyte-specific α 1AMPK leads to a 30% litter size reduction (Bertoldo *et al.*, 2015). The specific deletion of α 1AMPK in murine oocytes altered junctional protein expression and some aspects of mitochondrial physiology (Bertoldo *et al.*, 2015). Pharmacological activation of AMPK inhibited the production of progesterone and oestradiol (Tosca *et al.*, 2005, 2007b), and lower steroidogenesis associated with decreased 3β -hydroxysteroid dehydrogenase (3β -HSD) and P450 side-chain cleavage enzyme (p450_{scc}) gene and protein expression (Tosca *et al.*, 2005, 2007b, 2010). However, interesting these studies are, they included the use of cultured GCs obtained from animal models (rodent and bovine species) and the use of AMPK activators that may lack target specificity. As such, no studies have shown a direct role of AMPK signaling in ovarian granulosa steroidogenesis *in vivo*.

To bridge this gap in knowledge, this study assessed the role of AMPK in steroids production, cell proliferation and survival in *in vitro* and *in vivo* models by silencing the main catalytic subunit of AMPK, α 1AMPK, in human GCs, and by utilizing a transgenic mouse model deficient in α 1AMPK.

Material and methods

Animals

Adult animals (10- to 16-week-old mice) that were wild-type (α 1AMPK^{+/+}) or global α 1AMPK knock-out mice (α 1AMPK^{-/-}) were described in Jørgensen *et al.* (2004). Briefly, an IRES- β geo cassette (internal ribosomal entry site- β -galactosidase and neomycin phosphotransferase fusion gene) was inserted to delete a part of the catalytic domain of α 1AMPK subunit from amino acids 97–157. Mice were maintained under standard conditions of light (12 h light and 12 h darkness) and temperature with *ad libitum* access to food and water. All animal procedures followed the European legislation for animal experimentation (Directive 86/609/EEC) for animal experiments and the French legislation on animal research. The procedure using α 1AMPK-deficient mice was approved by the Directeur Départemental des Services Vétérinaires of the Préfecture de Police de Paris (agreement n° 75-886) and the Paris Descartes University ethics committee (agreement n° CEEA34.BV.157.12). The fertility assessment was performed by crossing a 3-month-old wild-type male mouse with two α 1AMPK-deficient female mice at the same age for 2 weeks in the same cage. When a vaginal plug was detected, the mated female was moved to another cage, and then the litter size was counted at birth.

To recover blood and ovarian tissue, 10- to 16-month-old wild-type female animals were killed by cervical dislocation. Ovaries were fixed in Bouin solution for histological studies or directly stored at -80°C for molecular biology analysis. Five days after a vaginal plug, a few females were also euthanized to recover embryos (5-day post-coitum (dpc)).

To assess ovaries' response to gonadotrophins, oocytes were retrieved following superovulation induction. Mice were injected i.p. with 5 I.U. of equine chorionic gonadotropin; 46 h later, they were injected

with 5 I.U. hCG (Intervet, Boxmeer, Holland). At 12 h later, the oocytes were retrieved and counted.

Human primary granulosa cells

Primary human granulosa-lutein cells (GCs) were obtained from lean women (i.e. BMI: 18–25 kg/m²) with PCOS and without PCOS undergoing IVF protocols. The cells were collected from pre-ovulatory follicles during oocyte retrieval. GC were centrifuged (400×g, 10 min) and most of the red blood cells were discarded from the pellet using two layers of discontinuous Percoll gradient (40%, 60% in Ham's medium; GIBCO-BRL, Life Technologies, Cergy Pontoise, France) and centrifugation (400×g, 20 min). After centrifugation, the pellet was washed with fresh medium, counted in a hemocytometer and cell viability was determined using trypan blue dye exclusion and used for culture, or the pellets were frozen at -80°C to analyze α AMPK expression (Table I). The ovarian stimulation procedure was described in detail previously (Roche *et al.*, 2016). Ethical approval for this study was obtained from the Research Ethics Board of the CHRU Bretonneau and written informed consent was obtained from all patients. No patients received any monetary compensation for participating in the study, and all patients gave written informed consent before cells were used for research purposes. The diagnosis of PCOS was established according to the Rotterdam criteria (oligomenorrhea and/or anovulation, clinical and/or biochemical signs of hyperandrogenism and polycystic ovaries on ultrasonography scan, as well as the exclusion of other etiologies that mimic the PCOS phenotype) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004; Teede *et al.*, 2018) by qualified personnel. The non-obese women without PCOS underwent fertility treatments owing to male hormonal imbalances or tubal obstruction.

Immortalized granulosa cells

Immortalized human granulosa cells (KGNs) were kindly provided by Drs. Masatoshi Nomura and Hajime Nawata, Kyushu University, Japan (Nishi *et al.*, 2001). KGN cells maintain most of the physiological characteristics of primary GCs, including functional FSH receptors and aromatase expression. KGN cells were cultured in Dulbecco's minimal essential medium (DMEM)/F12 medium (Sigma, St. Louis MO, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma, l'Isle d'Abeau Chesnes, France)) in a 5% CO₂ atmosphere at 37°C. Only early passages (under 30) were used. The cells were cultured at 1 million cells/culture flasks surface area 75 cm² for quantitative PCR or western blot analysis or in 96-well dishes (5000 cells/well for immunocytochemistry and 10 000 cells/well for other assays). The culture medium was changed every 48 h. Silencing of human α 1AMPK in KGN cells was performed after infection with lentiviral particles expressing small guide RNA (sgRNA) guides targeting the *PRKAA1* gene (encoding α 1AMPK) (5'GAATGGTACTCTTTTCAGGAT-3'), establishing KO-KGN cells, and a non-targeted guide (5'GTAGCGCGCCGCTCTCTAC-3') creating the control cells, called Scr-KGN cells. The sgRNA guides and lentivirus generation were as previously described (Sujobert *et al.*, 2015) (Grenier *et al.*, 2018). Successfully transfected KGN cells were selected by puromycin. Clones were tested for α 1AMPK expression by western blotting and sequencing amplicons surrounding the target sequence on *PRKAA1* exon 7 (sequencing primers: forward:

Table 1 Biological parameters of patients from control and PCOS groups.

	Control (n = 8)	PCOS (n = 9)	P
Age (years)	30.63 ± 1.48 (26–37)	29.56 ± 1.26 (22–34)	NS
BMI (kg/m ²)	21.90 ± 0.83 (19–25)	20.43 ± 0.70 (18–25)	NS
Testosterone (mmol/l)	1.39 ± 0.57 (0–5)	4.11 ± 1.48 (1–15)	<0.05
Anti-Müllerian hormone (ng/ml)	3.03 ± 0.74 (1–7)	22.45 ± 6.02 (7–60)	<0.0001
FSH (UI/l)	7.38 ± 1.33 (3–15)	5.76 ± 0.35 (3–7)	NS
LH (UI/l)	4.41 ± 0.32 (3–6)	8.73 ± 1.44 (4–15)	<0.05
Estradiol (ng/l)	40.74 ± 5.56 (25–73)	46.01 ± 4.33 (32–72)	NS
Cycle duration (days)	28.25 ± 0.87 (26–34)	105.06 ± 23.86 (31–180)	<0.001
Number of total follicles (determined by ultrasound using Rotterdam criteria)	16.25 ± 1.94 (7–22)	51.00 ± 5.86 (40–80)	<0.001
Number of mature oocytes retrieved	4.50 ± 0.71 (2–7)	7.67 ± 1.71 (3–16)	NS

Data are expressed as mean ± SEM (range).
Unpaired Student's *t*-test was used to compare the two groups.
NS, not significant.

5'-ATCACCAGGATCCTTTGGCA-3'; reverse: 5'-TGCTTTCCTTACACCTTGGTG-3').

A second mutant KGN cell line was established after infection with lentiviral particles expressing firefly luciferase reporter gene under the control of a cAMP-responsive element (Cignal Lenti CRE-Luciferase Reporter, S.A. Biosciences, Qiagen, France) named hereafter KGN-CRE-Luc cells. The cAMP-dependent activity was followed by evaluating the production of cAMP (Promega Luciferase Assay Kit, Promega, Charbonnières, France) in KGN-CRE-Luc cells after 48 h of treatment with an activator (1 mM dibutyl AMP) or inhibitor of AMPK (Compound C, Sigma, l'Isle d'Abeau Chesnes, France). Values were presented as relative luminescence unit/μg protein and expressed as mean ± SEM.

Immunohistochemistry on fixed cells or tissue

Cell proliferation was evaluated by bromodeoxyuridine (BrdU) staining. Cells were labeled with 10 μM BrdU for 24 h (Sigma, l'Isle d'Abeau Chesnes, France), then fixed in 4% paraformaldehyde/PBS, and BrdU-positive cells were visualized under indirect immunofluorescence. For labeling polarized mitochondria, living cells were incubated for 30 min at 37°C with 200 nM MitoTracker Orange CM-H2TMRos (Invitrogen, Fisher Scientific, Illkirch, France). Afterward, they were fixed, and nuclei were stained with 4',6-diamidino-2-phenylindole.

For all other protocols, fixed cells were incubated with PBS/0.1 M glycine for 15 min at room temperature, permeabilized with 0.1% Triton X-100 (w/v) in PBS for 15 min, and non-specific binding sites were blocked in 2% bovine serum albumin (BSA)/PBS for 15 min. Then, cells were incubated overnight at 4°C with the following primary antibodies against phospho-ERK (extracellular signal-regulated kinase), Glut4, β-cadherin (Cell Signaling Technologies, Beverly, MA, USA), BrdU and phalloidin (Sigma, l'Isle d'Abeau Chesnes, France), p53 and anti-Müllerian hormone (AMH) (Santa Cruz Biotechnology, CA, USA). Mouse or rabbit IgG (Sigma-Aldrich, l'Isle d'Abeau Chesnes, France) was used as a negative control. All primary antibodies were diluted at 1:100 in 1% BSA/PBS. After incubating with the primary antibody, cells

were incubated for 45 min with goat anti-rabbit IgG Alexa Fluor® 555 or anti-mouse IgG Alexa Fluor® 488 antibodies (Invitrogen, Fisher Scientific, Illkirch, France diluted at 1:500 in 1% BSA/PBS). Cells were counterstained with Hoechst 33342 (Sigma-Aldrich, l'Isle d'Abeau Chesnes, France) and examined using standard immunofluorescence microscopy or a high content screening (HCS) imaging system on the ArrayScan VTI HCS Reader (Thermo Fisher Scientific—Cellomics, Pittsburgh, PA, USA). Analysis was performed on three independent biological replicates, with a minimum of 3000 cells per well. Analysis of the surface of cells was quantified using a phase-contrast microscope and image analysis software (ImageJ Fiji software, version 1.52, 2018, National Institutes of Health, MD, USA). A minimum of 100 cells in three different experiments (n = 3) were analyzed per genotype.

Immunohistochemistry was performed on ovaries embedded in paraffin and sectioned at 7-μm thickness. Deparaffinized sections were hydrated, microwaved for 5 min in an antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France), and cooled at room temperature. After two washes with PBS for 5 min each, non-specific background was removed by incubation in 2% BSA/PBS for 30 min. Sections were incubated overnight at 4°C with PBS containing primary antibody (1:100) against β-catenin, N-cadherin (Cell Signaling Technologies, Beverly, MA, USA), and AMH (Santa Cruz Biotechnology, CA, USA). The following day, sections were incubated with a second fluorescent antibody (goat anti-rabbit IgG Alexa Fluor® 555 or anti-mouse IgG Alexa Fluor® 488 antibodies (Invitrogen, Fisher Scientific, Illkirch, France diluted at 1:500 in 1% BSA/PBS)). Measurements from different sections per animal (three animals per genotype) were analyzed using ImageJ Fiji software.

Colony formation assay and migration assay

A total of 10 000 KGN cells were plated in 6-well plates, and cultured in 10% FCS, DMEM/F12. Cells were allowed to form colonies for up to 1–2 weeks, with media being replaced every 48 h. Colonies were stained with 0.2% crystal violet in methanol, dried and the colony surface was analyzed with ImageJ Fiji software.

Migration of KGN cells was assessed using a scratch assay. Cells were seeded into 12-well tissue culture dishes at 5×10^5 cells per well. At confluence, a linear scratch was generated by using a sterile tip. Then, cells were incubated with fresh culture medium containing 10% FCS for 4, 8 and 24 h at 37°C with 5% CO₂. The data were analyzed using ImageJ Fiji software to estimate the migration distance (μ m).

Transepithelial electrical resistance

KGN cells were grown to confluence in 24-well tissue culture inserts (0.4 μ m pore size, WWR, Fontenay sous Bois, France). Transepithelial electrical resistance (TEER) was measured with the Millicell-ERS apparatus and MERSTX01 electrodes (Millipore, Fisher Scientific, Strasbourg, France). The culture was equilibrated at room temperature before the evaluation. Blanks were considered to be wells with Hank's buffered salt solution (Invitrogen, Cergy Pontoise, France), and the background comprised empty wells. TEER was calculated according to the following equation: $TEER (Ohm/cm^2) = (R_{total} - R_{control}) \times A^{-1}$. 'R_{total}' is the resistance recorded, 'R_{control}' is the resistance of the background, and 'A' is the surface of the insert (i.e. 0.33 cm²). Four independent biological replicates and technical triplicates were performed in total.

Cell apoptosis and reactive oxygen species assay

Apoptosis of KGN cells was determined by evaluating the activity of the cleaved form of caspase-3, a mediator of programmed cell death, by using the commercial Caspase-Glo™ 3/7 Assay (Promega, Charbonnières, France). Analysis was performed on six different cultures in duplicate for each condition.

The ROS-Glo™ H₂O₂ Assay (Promega, Charbonnières, France) was used to evaluate oxidative stress by measuring reactive oxygen species (ROS) in cells after 48 h of culture by using a luminescent microplate reader (Luminoskan Ascent, V.W.R. International, France).

Metabolites and steroid analysis

Glucose uptake was evaluated by using the Glucose Uptake-Glo™ Assay (Promega, Charbonnières, France). Lactate concentrations in the culture medium and pyruvate and cholesterol contents in GCs were evaluated by using commercial spectrophotometric assays spectrophotometric assays, respectively (Lactate assay, Sigma, l'Isle d'Abeau Chesnes, France; Pyruvate assay, Clinisciences, Nanterre, France; Cholesterol assay, Biolab, Maizy, France). Analysis was performed on at least three different cultures in duplicate for each condition. The results of each assay were normalized per 10⁶ cells.

Steroid (pregnenolone, 17 α -hydroxypregnenolone, DHEA, 17 α -hydroxyprogesterone, Δ 4 androstenedione, testosterone and dihydrotestosterone (DHT)) levels were determined by LC-MS/MS (mmol/l) as described by Meunier et al. (2015).

The serum AMH level was evaluated on five control and three transgenic mice by using an ELISA (AMH Gen II assay®) and read with a Beckman Coulter® (France) as already described in mice (Valeri et al., 2020). The values are expressed in pmol/l.

Mitochondrial oxygen consumption (JO₂)

Live, cultured KGN cells were resuspended at a density of 1×10^6 cells $\times$ ml⁻¹. The mitochondrial oxygen consumption (JO₂ (pmol O₂ $\times$ (s $\times$ 10⁶ cells)⁻¹) was recorded at 37°C with a high-resolution Oxygraph-2K respirometer (Oroboros, Innsbruck, Austria), and data were acquired with DatLab4 software. The cell's basal respiration rate was determined by measuring the linear velocity of oxygen consumption, which reflects the cell's aerobic metabolic activity with physiological substrates in the culture medium. Oligomycin (0.5 μ g/ml) was then added to determine the respiration uncoupled to ATP synthesis JO₂ (basal JO₂ minus oligomycin-insensitive JO₂). The uncoupled (maximum) respiratory rate was assessed by stepwise additions of carbonyl cyanide *m*-chlorophenylhydrazone (FCCP; 1–2 μ M) up to an optimal concentration that represented the capacity of the respiratory chain without the regulation of ATP synthase.

Western immunoblotting

Whole ovaries, embryos at 5-dpc, oocytes and GCs were lysed and exposed to three freeze/thaw cycles in a lysis buffer. After centrifugation, the supernatant protein concentration was determined using a colorimetric assay (DC assay kit; Uptima Interchim, Montmuçon, France). Equal amounts of proteins were analyzed using 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis under reducing conditions then transferred onto nitrocellulose membranes (Schleicher & Schuell, Ecqueville, France) by following the protocol described previously (Tartarin et al., 2012). Membranes were then incubated overnight at 4°C with the following primary antibodies: phospho- α AMPK (Thr172), proliferating cell nuclear antigen (PCNA), phospho-CREB (Ser 133), β -catenin (Cell Signaling Technologies, Beverly, MA, USA), α 1AMPK (Upstate Biotechnology Inc, Lake, Placid, NY, USA), vinculin (Sigma-Aldrich, l'Isle d'Abeau Chesnes, France), AMH and β HSD (Santa Cruz, CA, USA). Steroidogenic acute regulatory protein (StAR) and anti-p450 side-chain cleavage (p450_{sc}) antibodies were generously provided by Dr. Dale Buchanan Hales (Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL, USA). All commercial antibodies were used at 1:1000 dilution for western blotting, except β HSD antibodies at 1:500 dilution. Signal was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Orsay France), and the band densities were quantified using ImageJ Fiji software (NIH, MD, USA). The results are expressed as the intensity signal in arbitrary units after normalization using an internal standard (α 1AMPK protein or vinculin) and correspond to the average of three different experiments.

High-throughput RNA sequencing

The 20 RNA sequencing (RNAseq) libraries were constructed from 1 μ g of total RNA extracted from Scr-KGN cells or KO-KGN cells using Illumina TruSeq RNA Sample Prep (Illumina, San Diego, CA, USA). Sequencing was performed on an Illumina HiSeq 3000 apparatus using the Illumina TruSeq Standard mRNA kit v2 to obtain stranded paired-end reads (2 $\times$ 150 bp). The analysis pipeline of sequence data was performed using the local instance of Galaxy (<https://galaxyproject.org>) at the Toulouse Midi-Pyrénées bioinformatics platform (<http://signae-workbench.toulouse.inra.fr/>), as already described (Talebi et al., 2018). The FastQ files and metadata were submitted to the

European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB46048 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB46048>).

The cleaned paired-end reads were combined and mapped using STAR2 against the human genome assembly (GRCh38.p13) annotated with the Ensembl *Homo sapiens* GRCh38.104 annotation version. For each time condition (Scr-KGN cells and KO-KGN cells), the 20 files ($n = 10$ for Scr-KGN samples and $n = 10$ for KO-KGN samples) with raw counts were merged to obtain a final count file for statistical analysis of differential gene expression under AMPK depletion.

Differentially expressed gene analysis

SARTools R package 1.3.2 (Varet et al., 2016) was used for the descriptive and diagnostic analyses, keeping all samples. Raw counts were pre-filtered for low counts by keeping genes with at least 1 count per million across 10 out of 20 samples, and counts were normalized for library size. A generalized linear model was fitted on the normalized raw count data using the DESeq2 R package version 1.12.3 (Love et al., 2014) to identify the differentially expressed genes (DEGs) between control Scr-KGN ($n = 10$) and KO-KGN ($n = 10$) cells. The raw P -values were adjusted for multiple testing using the Benjamini–Hochberg procedure to control for the false discovery rate (FDR) (Benjamini and Hochberg, 1995). Genes with an adjusted P -value lower than 0.05 (FDR < 5%) were considered as DEGs and selected for subsequent analyses using Metascape (<http://metascape.org/>). Metascape is a gene annotation and analysis resource (Zhou et al., 2019) to perform functional enrichment analysis, including a canonical, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathway analysis of the overlapping (DEGs) in all three databases.

RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA from GCs was extracted with Trizol reagent (Invitrogen, Cergy Pontoise, France). Total RNA (1 μ g) was denatured and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase, 15 U (Promega, Charbonnières, France) in a 20 μ l reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 200 μ M dNTP (Amersham, Piscataway, NJ, USA), 50 pmol of oligo(dT)15 and 5 U of ribonuclease inhibitor at 37°C for one h. Targeted cDNAs were quantified by real-time PCR using SYBR Green Supermix (Bio-Rad, Marnes la Coquette, France) and 250 nM of specific primers (Table II). Each reaction was performed in duplicate and included a negative control (without template DNA). The cycle threshold (Ct) values were calculated automatically and analyzed using CFX Manager™ Software (version 3.1, Biorad, USA). The relative cDNA number was calculated using the geometric mean of the Ct values from the following normalizing genes ACTB (actin beta), EEF1A1 (eukaryotic translation elongation factor 1 alpha 1) and GAPDH (Vandesompele et al., 2002).

Statistical analysis

All data are presented as means $\pm$ SEM. Unpaired Student's t -test was used to compare the two genotypes (KO-KGN cells versus Scr-KGN cells or wild-type mice versus KO mice). In the case of variance heterogeneity, the Mann and Whitney U test was used to compare

means between two groups. Statistical analysis was performed by using GraphPad Prism 6 (La Jolla, CA, USA). Values were determined to be significant when $P \leq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

Confirmation of the silencing of α IAMPK in human granulosa cells

To assess the role of AMPK on GCs, we silenced the main catalytic subunit of AMPK α IAMPK in human KGN cells by using the sgRNA system developed by Tamburini (Grenier et al., 2018). Sequencing the *PRKAA1* gene confirmed the presence of a sgRNA-induced mutation in exon 7 (Fig. 1A). The KO-KGN clone showed negligible α IAMPK Thr172 phosphorylation and α IAMPK protein levels (Fig. 1B).

In vitro silencing α IAMPK stimulates proliferation, adhesion and faster migration in KGN cells

We first investigated the effects of silencing α IAMPK on cell viability and proliferation. While KO-KGN cells did not undergo apoptosis or stabilize tumor protein 53 (*TP53*; Supplementary Fig. S1), they showed higher proliferation, as shown by the greater cell colony area (1.4-fold higher; $P < 0.01$, Fig. 2A); higher number of BrdU⁺ positive cells (2-fold; $P < 0.05$) (Fig. 2B); higher percentage of cells with phosphorylated-ERK (Fig. 2C); and higher content of PCNA ($P < 0.05$) (Fig. 2D). The hyperproliferation was associated with a reduction of the size of GCs (Fig. 2E) and a migration estimated (by the scrap assay) to be 16% faster at 24 h than that in Scr-KGN cells ($P < 0.05$) (Fig. 2F). Additionally, the cell–cell adhesion of KO-KGN cells was more robust than that of Scr-KGN cells, supported by the increased electrical resistance of the cell layer evaluated by TEER (Fig. 2G) and the higher β -catenin content observed by immunochemistry and western blot (Supplementary Fig. S1).

Silencing α IAMPK alters glucose metabolism in KGN

It has been reported that hyperproliferation is associated with lower mitochondrial energy metabolism, such as described for cancer cells (Faubert et al., 2015). In support of this concept, hyperproliferative KO-KGN cells had a modestly lower polarized mitochondria density per cell (13% decrease, $P < 0.05$) than Scr-KGN cells (Fig. 3A), suggesting that silencing AMPK did influence mitochondrial mass significantly. However, the ATP-linked oxygen consumption rate under basal conditions in KO-KGN cells was half than that of Scr-KGN cells, and the uncoupled or maximal rate in KO-KGN cells was 0.7-fold that of Scr-KGN cells ($P < 0.05$; Fig. 3B). The lower electron transport capacity (phosphorylating and non-phosphorylating) was associated with a 40% reduction in the levels of hydrogen peroxide (H_2O_2) and ROS ($P < 0.01$), as well as a 20% reduction in CellroX immunostaining, a fluorescence probe activated by ROS ($P < 0.05$) (Supplementary Fig. S2). These results indicated that silencing AMPK signaling had a more profound impact on mitochondrial function (30–50%) than on mitochondrial mass (13%).

Table II Primers for quantitative real-time PCR.

Name	Accession no	Forward primer 5' to 3'	Reverse primer 5' to 3'
α1AMPK	NM_006251.6	TGCGTGACGAAGGAAGAATCC	TGTGACTTCCAGGTCTTGGAGT
α2AMPK	NM_006252.4	GGGTGAAGATCGGACACTACGT	TTGATGTTCAATCTTCACTTTG
Interleukin 6 (IL6)	NM_001318095.2	CCAGCTATGAACTCCTTCTC	GCTTGTTCCTCACATCTCTC
Superoxide dismutase 2 (SOD2)	NM_001322819.2	GACAAACCTCAGCCCTAACG	GAAACCAAGCCAACCCCAAC
Pentraxin 3	NM_002852.4	CGG TGC TAG AGG AGC TG	TAGCTGTTTCACAACT
Collagen type IV alpha 6 chain (COL4a6)	NM_001847	CACTATGCCAGGCGCAATG	CACACACAGAGCAGCGGCT
Matrix metalloproteinase 11 (MMP11)	NM_005940.5	ATTTGGTTCTTCCAAGGTGCTCAGT	CCTCGGAAGAAGTAGATCTTGTCT
Creatine kinase (CKB)	NM_001823	GCC TCA CTC AGA TCG AAA CTC	GGC ATG TGA GGA TGT AGC CC
Actin beta (ACTB)	NM_001101.5	ACGGAACCACAGTTTATCATC	GTCCCAGTCTTCAACTATACC
Eukaryotic translation elongation factor I alpha I (EEF1A1)	NM_001402.6	AGCAGACTTTGTGACCTTGCC	TCACATGAGACAGACGGTTGC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046.7	ACGGATTTGGTCGTATTGGG	TGATTTTGGAGGATCTCGC

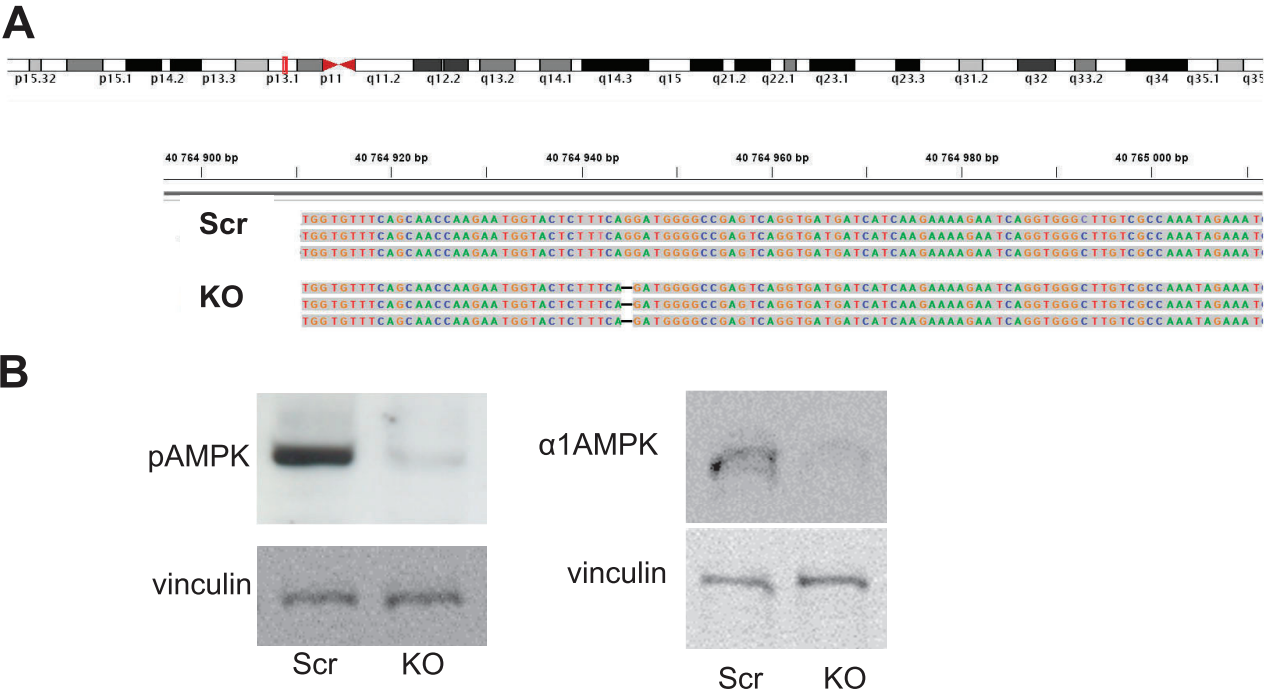


Figure 1. Mutation of α1AMPK. (A) Sequence of part of exon 7 of the PRKAA1 (Protein Kinase AMP-Activated Catalytic Subunit Alpha 1) gene from KGN genomic DNA (location: 40765238 to 40764752). (B) Western blot analysis of phospho-AMPK-alpha (Thr172) and α1AMPK in KO-KGN (KO, mutant cells) and Scr-KGN granulosa cells (Scr, control cells). Vinculin was used as control. Results are representative of three independent experiments.

In addition, lower glucose uptake was observed in KO-KGN cells (by 70%; $P < 0.05$) associated with a reduction in the expression of the glucose transporter insulin-dependent GLUT4 (by 30%; $P < 0.01$) (Fig. 3C and D). Despite the lower glucose uptake, activation of glycolysis is inferred by the substantial increase in pyruvate and lactate in KO-KGN cells compared to controls (1.8-fold). The higher pyruvate levels in KO-KGN cells compared to Scr-KGN cells is suggestive of lower activity of the pyruvate dehydrogenase complex (Fig. 3E and F),

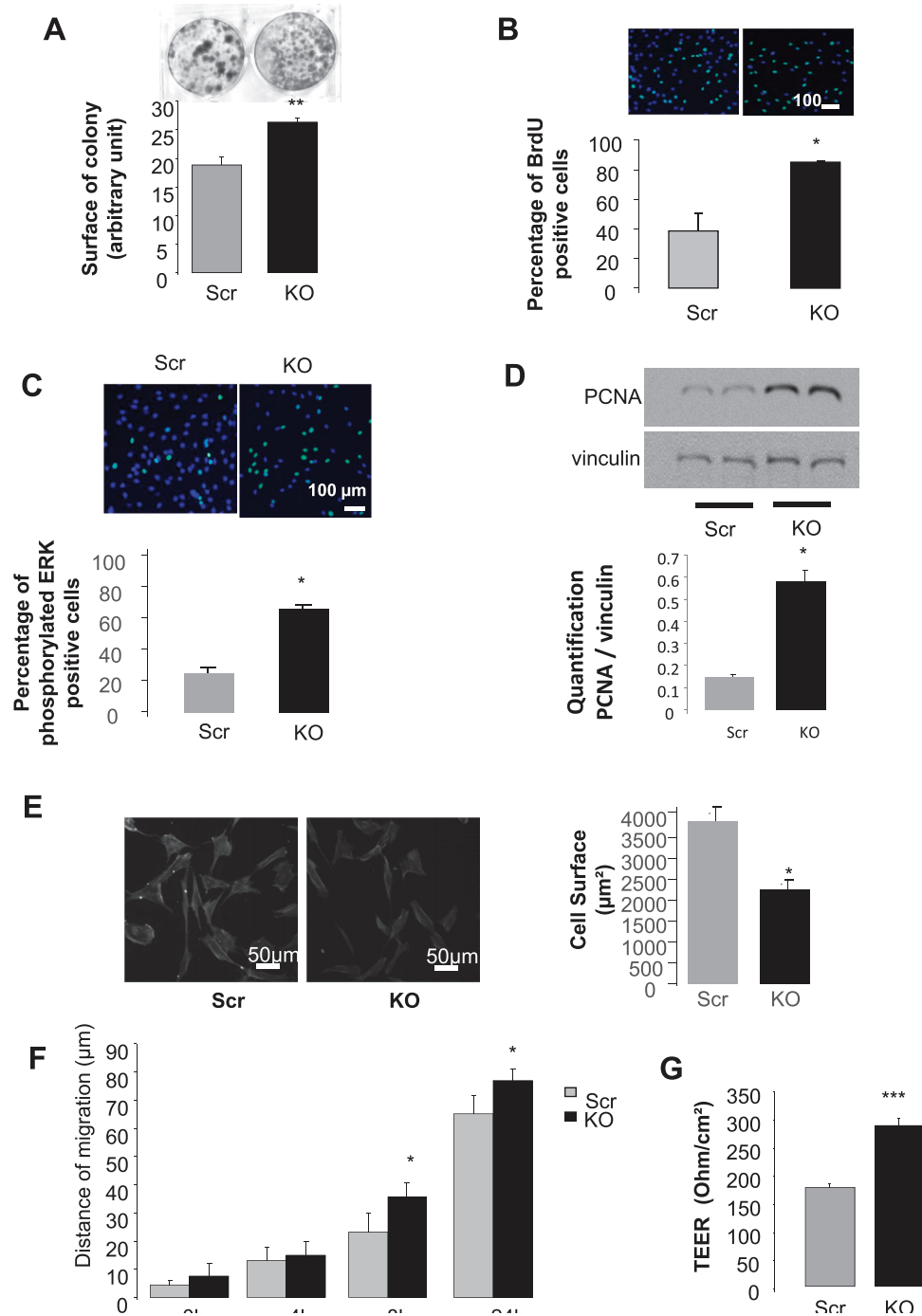


Figure 2. *In vitro* proliferation ability of KO-KGN cells. **(A)** Comparison of the KO-KGN (KO, mutant cells) and Scr-KGN cells (Scr, control cells) granulosa cells colony formation, cells were plated at a very low density (1.5×10^3 cells/well) and 2 weeks later, the surface occupied by foci were quantified ($n = 4$ independent experiments). Representative fields are presented below. **(B)** KGN were cultured in presence of $10 \mu\text{M}$ Bromodeoxyuridine (BrdU) for 24 h. The percentage of BrdU positive cells was analyzed by high content screening. Results are representative of six independent experiments in triplicate with at least 3000 cells counted per well. **(C)** Percentage of phosphorylated ERK positive cells analyzed by high content screening ($n = 3$ independent experiments). **(D)** Western blot analysis of PCNA in KO-KGN and Scr-KGN granulosa cells. Vinculin was used as control. Results are representative of three independent experiments. **(E)** Representative microscopic fields of KO-KGN and Scr-KGN cell surface. Scale bar = $50 \mu\text{m}$. (right panel), Quantification of cell surface (μm^2) by ImageJ software (measured on at least 300 cells from 3 independent cultures). **(F)** Migration assays. KGN cells were scraped from the culture dish and the mean migration distance of cells was assessed for 24 h. Quantification of the migration distance for KGN cells is shown as (μm) ($n = 4$). **(G)** KGN cells were cultured at confluence in 24-well tissue culture inserts ($0.4 \mu\text{m}$ pore size) The Trans-epithelial electrical resistance (TEER) was analyzed and reported as (Ohm/cm^2) Four different cultures were performed. Values are the mean $\pm$ SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

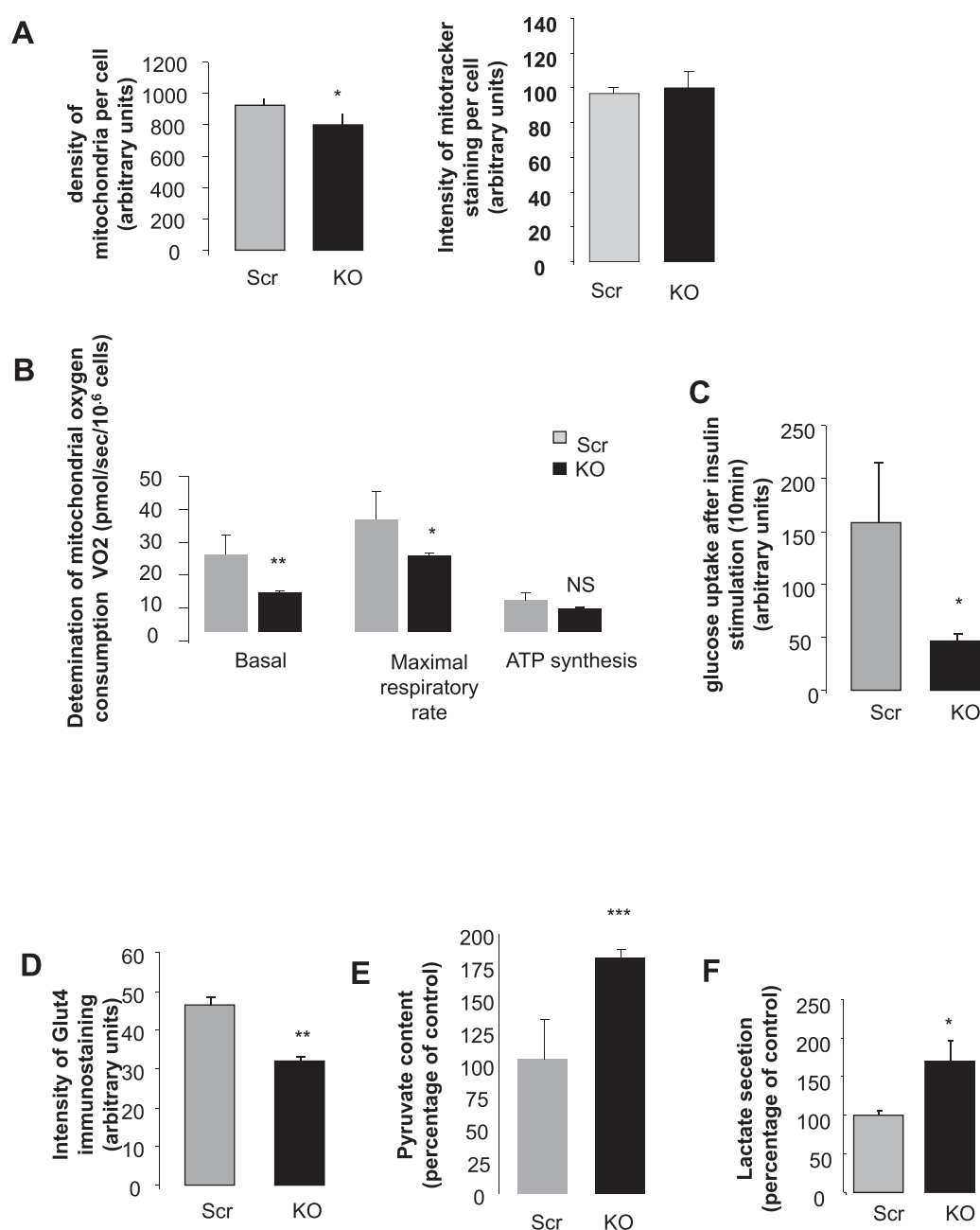


Figure 3. Mitochondria activity in KO-KGN cells. (A) The mitochondrial membrane potential in KO-KGN (KO, mutant cells) and Scr-KGN cells (Scr, control cells) granulosa cells was analyzed by mitotracker dye. The density and intensity of mitotracker in each cell was quantified by high content screening. Results are representative of three independent experiments. (B) KGN suspension (1×10^6 cells/ml) was transferred into a stirrer 2-ml oxygraph vessel where mitochondrial oxygen consumption (VO_2) was recorded before and after the successive addition of $0.5 \mu\text{g/ml}$ oligomycin and $1\text{--}2 \mu\text{M}$ carbonyl cyanide *m*-chlorophenylhydrazone. Data are represented as means $\pm$ sem of $n = 4$ different KGN isolations. (C) Glucose uptake in KGN granulosa cells was quantified using the Glucose Uptake-Glo Assay after medium change, insulin (100 ng/ml) and 30 min incubation with 2DG (2-DeoxyGlucose) ($n = 4$ independent experiments). (D) Intensity of Glut4 immunostaining in each cell was quantified by high content screening. Results are representative of three independent experiments. (E) Pyruvate intracellular concentration (ng) per μg protein content ($n = 3$). (F) Lactate secretion measured in culture medium conditioned by KO-KGN and Scr-KGN granulosa cells for 48 h (percentage of control normalized per 200 000 cells/well) ($n = 7$). Values are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

probably as a result of higher PDH kinase activity and/or lower mitochondrial function. Indeed, *in vivo* studies suggested that AMPK regulates pyruvate entry into the mitochondrion (Klein et al., 2007; Buresova et al., 2020).

Silencing AMPK stimulates steroidogenesis in KGN cells

The primary function of GCs is the production of steroids, which evolve during follicular maturation. The cholesterol content, the precursor molecule of steroid synthesis, was 30% higher in KO-KGN cells (Fig. 4A). This higher cholesterol content seemed to facilitate androgen production (6-fold), supported by the higher levels of Delta4 androstenedione, testosterone, and DHT ($P < 0.01$) (Fig. 4B). This hyperandrogenism was associated with higher expression of two enzymes involved in steroid production (p450scc and 3β HSD), 1.2- and 1.7-fold, respectively, and also in the expression of the cholesterol carrier StAR in KO-KGN cells compared to Scr-KGN cells ($P < 0.05$) (Fig. 4C and D). Taken together these results show that silencing AMPK increases the content of steroid enzymes. This is also supported by the 3-fold increase in a cAMP-dependent factor, the pSer-133 CREB transcription factor ($P < 0.01$) (Fig. 4C and D), and the induction of the reporter gene (luciferase) driven by the cAMP response element (the response element for CREB) under both basal and dbAMP-stimulated conditions ($P < 0.05$) (Fig. 4E) when AMPK was inhibited by Compound C.

Silencing AMPK alters the transcriptome of KGN cells

In order to get a global view of the consequences of the absence of AMPK, we performed RNA sequencing. RNAseq analysis has revealed 1237 DEGs (541 DEG downregulated in KO group, 696 DEG upregulated in KO group) with a $P < 0.05$. The DEGs are involved in the cytoskeleton, extracellular matrix, cell cycle, cell proliferation, cell locomotion, monocarboxylic acid metabolism, lipid metabolism, steroidogenesis and inflammatory response (Fig. 5A; volcano-plot, see Supplementary Fig. S3). The results were confirmed and validated by following the gene expression of six candidate genes (IL-6, superoxide dismutase 2, Pentraxin 3, collagen type IV alpha 6 chain, matrix metalloproteinase 11 and creatine kinase; Supplementary Fig. S4). Biological processes enriched in the KO-KGN cells showed the interaction between cytoskeleton reorganization, metabolism, lipid processes and immunity processes (Fig. 5B). Relevant genes associated with lipid and steroid production were ELOVL7, HSD11B1, CPT1A and perilipin 2, including androgen-induced genes, such as vanin 1, genes related to the extracellular matrix (COL4A5 and COL4A6) and pro-inflammatory genes (IL6, IL1B, CCL20, C-C motif chemokine ligand 20 and CXCL1). Interestingly, a total of 42 downregulated genes and 14 upregulated genes in KO-KGN cells have already been reported in a PCOS database developed from the literature (PCOSbase) (Supplementary Tables SI and SII). These changes in gene expression have been confirmed in human primary granulosa-lutein cells exposed to AMPK inhibitor and also rescued after AMPK re-expression (Supplementary Figs S5, S6 and S7).

Fertility of α 1AMPK^{-/-} female mice is reduced

To place the effects of AMPK in a physiological context, we investigated the fertility of female α 1AMPK-deficient mice (Fig. 6A). The number of live pups per litter was decreased by 30% in α 1AMPK^{-/-} female mice in comparison to controls ($P < 0.05$) (Fig. 6B), and there was a similar decrease in the number of corpora lutea (Fig. 6C). To evaluate the ovary's response independently of the hormones of the hypothalamic-pituitary system, oocytes were collected and counted after superovulation. The oocyte number was lower in α 1AMPK^{-/-} mice (21.4 ± 2.4 oocytes) than in control mice (34.2 ± 1.7 oocytes; $P < 0.05$) (Fig. 6D). Histology of ovaries in α 1AMPK^{-/-} mice has shown the presence of all types of follicles but more secondary follicles formed a ring around the ovary (Fig. 6E). Analysis of the estrous cycle in transgenic mice revealed a delay between two cycles with no change in estrous length (Fig. 6F and G).

The drop in the ovulation rate allowed us to further evaluate folliculogenesis. Primary and secondary antral follicles in α 1AMPK^{-/-} female mice were increased by 1.3- and 1.5-fold, respectively ($P < 0.01$) (Fig. 7A). Analysis of atretic follicles by visualization of pycnotic bodies in follicles revealed only an increase in antral follicles (200–300 μ m), but not in primary follicles (50–199 μ m) or pre-ovulatory follicles (Fig. 7A and Supplementary Fig. S8). This result was confirmed by a high proportion of AMH-positive follicles, a marker of large preantral and small antral follicles, in α 1AMPK^{-/-} ovarian tissue ($P = 0.05$) (Fig. 7B and C), and elevated AMH levels in the blood (Fig. 7D); however, the number of pre-ovulatory follicles was limited in α 1AMPK^{-/-} mice (Fig. 7A). Expression of β -catenin, which is involved in tissue architecture, was decreased in secondary antral follicles from α 1AMPK^{-/-} female mice. This was evidenced by changes in the shape and structure of GCs layers, which were not cuboidal as in control mice and had a non-linear distribution (Fig. 7E).

Consistent with our results with KO-KGN cells, higher levels of steroids and androgens were observed in α 1AMPK^{-/-} ovarian tissue and blood compared to those in controls, including testosterone, pregnenolone, 17α -hydroxyprogesterone and Δ 4-androstenedione (Fig. 8A–F). The expression of enzymes involved in steroid production (p450scc and 3β HSD) was also higher in α 1AMPK^{-/-} ovarian tissue (Fig. 9A–C). These results confirmed the validity of using the KGN based on the overlap of outcomes observed with the α 1AMPK^{-/-} mice.

α 1AMPK content is lower in primary human granulosa cells from lean patients with PCOS

To compare the results obtained *in vitro* with those *in vivo*, we evaluated the gene expression of α 1AMPK and α 2AMPK in primary human GCs retrieved from non-obese women with and without PCOS, both obtained through IVF protocols. As shown in Fig. 10A, testosterone level in the follicular fluid is higher in lean patients with PCOS in comparison to control patients and is associated with a significant 63% reduction in α 1AMPK mRNA expression in GCs from the same group of lean patients with PCOS in comparison to control group ($P < 0.05$), with no statistical differences in the gene expression of α 2AMPK. The

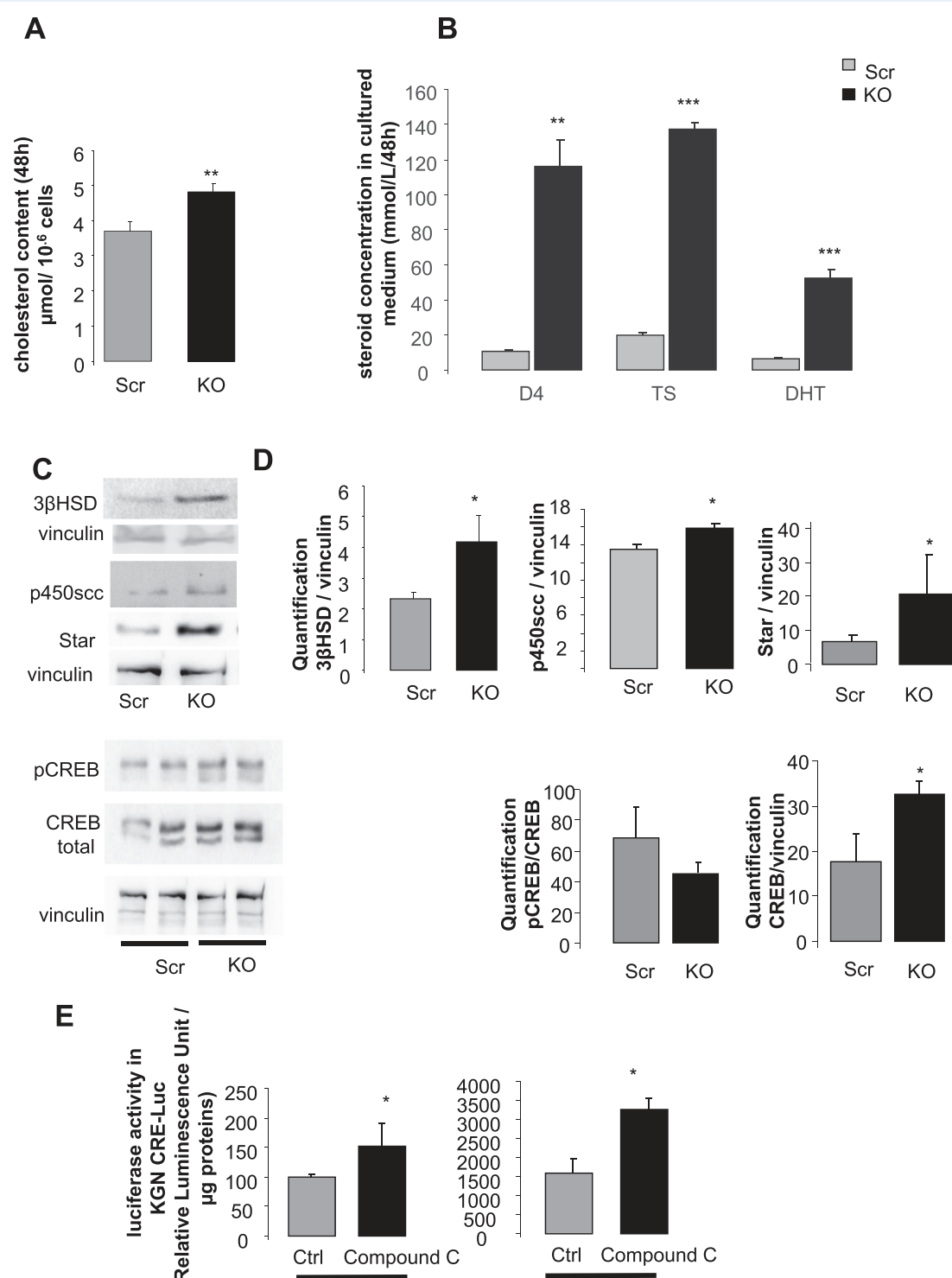


Figure 4. Molecular alteration in steroidogenesis in KO-KGN cells. (A) Intracellular concentrations of cholesterol in KO-KGN (KO) and Scr-KGN (Scr) granulosa cells ($n = 3$). (B) KGN granulosa cells were incubated for 48 h with dehydroepiandrosterone (DHEA) and the following steroids were quantified by mass spectrometry: $\Delta 4$ androstenedione (D4), testosterone (TS), dihydrotestosterone (DHT) ($n = 3$). (C) Western blot analysis and (D) quantification of phospho-cAMP responsive element binding protein (CREB), CREB, 3 β -hydroxysteroid dehydrogenase (3 β HSD); steroidogenic acute regulatory protein (Star); p450 side-chain cleavage (p450scc) expression in KO-KGN and Scr-KGN granulosa cells, normalized to vinculin ($n = 3$ –5). (E) Wild-type KGN cells expressing luciferase under the control of cAMP response element (Signal Lenti Reporters, Qiagen) (KGN-CRE-Luc) were stimulated for 48 h with dibutyl AMP (dbAMP) 1 mM and/or with the AMPK inhibitor (10 μM Compound C). Luciferase assay was performed, and promoter activity values are expressed as relative luminescence units per μg of proteins content ($n = 3$). Values are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

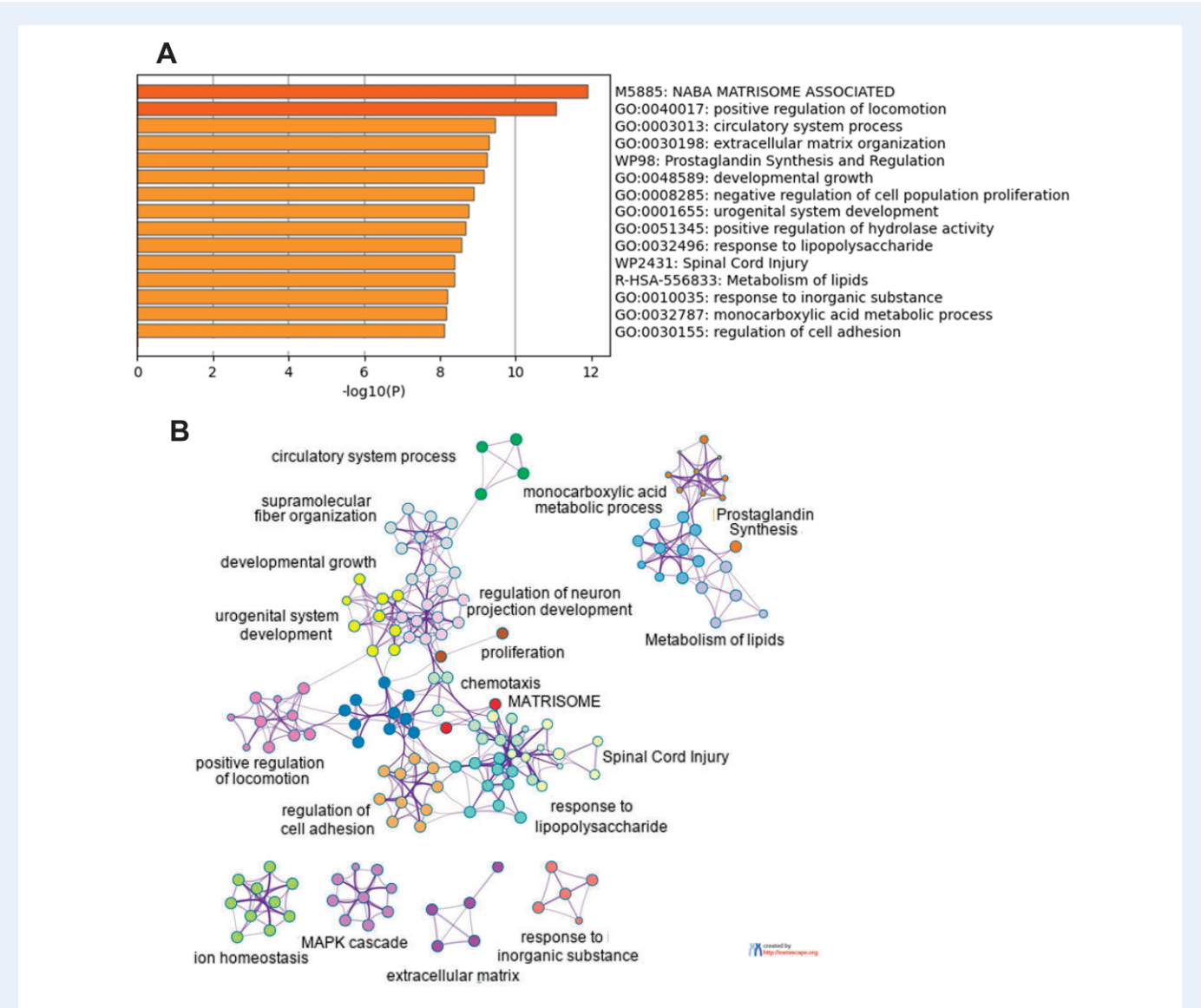


Figure 5. Gene enrichment analysis in KO-KGN cells. Biological function processes or networks of related pathways enriched were constructed using the Metascape software. **(A)** Heatmap of the top15 of GO enriched terms colored by P -values. **(B)** Network of biological processes or related pathways enriched in the KO-KGN cells samples ($n = 5$) which were closely correlated.

α IAMPK protein content was slightly but significantly lower in PCOS GCs (by ~25%) (Fig. 10B).

Discussion

Our work illustrates the importance of α IAMPK for granulosa cell functions and a potential link with the development of the PCOS phenotype. *In vitro* inactivation of α IAMPK in human GCs leads to changes in the levels of proteins involved with the cytoskeleton, extracellular matrix, cell cycle, immune system, monocarboxylic acids and lipid metabolism. This difference in protein levels may explain the issues observed with cell maturation, glucose metabolism, inflammation and steroid production (Fig. 11). This

phenotype obtained with a disrupted AMPK axis overlaps with those described in women with PCOS (e.g. formation of cysts in the ovary, hyperandrogenism and insulin resistance (Sirmans and Pate, 2013)).

To decipher more precisely the role of AMPK in GCs function, we chose to limit the use of pharmacological interventions which, despite their inhibitory effect on AMPK, may have unintended effects on other kinases. Instead, we developed a strategy to mutate the α IAMPK gene in human GCs, thereby conserving some properties of the primary cells such as FSH-sensitivity, steroid production, and expression of AMH. These cells have the disadvantage of being a transformed tumor cell line suggesting that some genes associated with the cell cycle are mutated. Primary GCs from transgenic mice or human granulosa-lutein cells might also have been available for our study but they

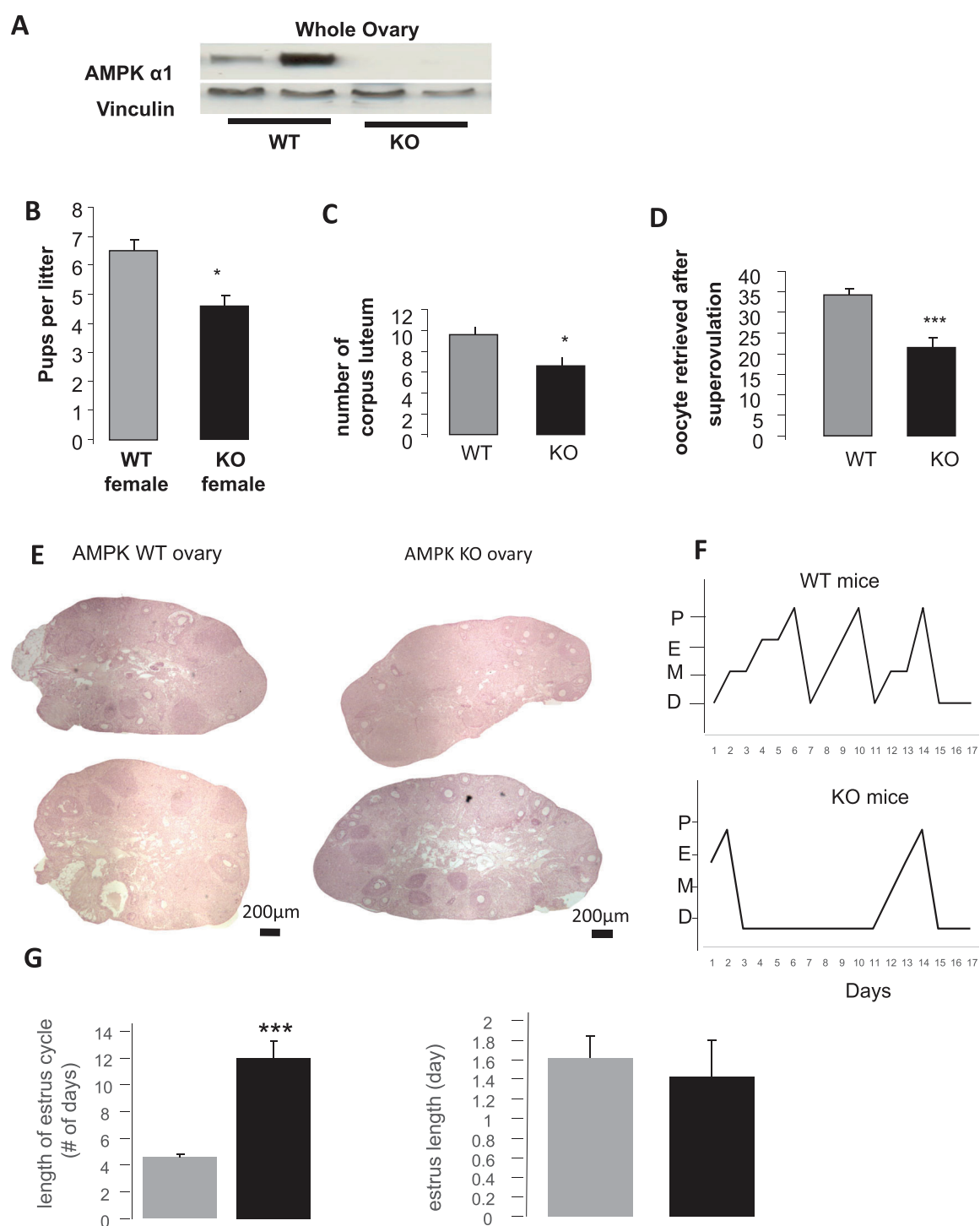


Figure 6. Fertility of $\alpha 1$ AMPK $^{-/-}$ female mice. (A) Western blot analysis of the $\alpha 1$ AMPK subunit in whole ovary from total knock-out animals (KO, $\alpha 1$ AMPK $^{-/-}$) versus wild-type mice (WT, $\alpha 1$ AMPK $^{+/+}$) ($n = 4$). (B) Fertility analysis of $\alpha 1$ AMPK $^{+/+}$ and $\alpha 1$ AMPK $^{-/-}$ females mated with wild-type males ($n = 29$ (KO), $n = 43$ (WT)). (C) Number of corpora lutea per female from both genotypes ($n = 7$). (D) Number of oocytes recovered following superovulation treatment in $\alpha 1$ AMPK $^{+/+}$ and $\alpha 1$ AMPK $^{-/-}$ females ($n = 7$ per genotype). (E) Histology of ovaries of nontransgenic (left) and homozygotes $\alpha 1$ AMPK transgenic mice (right). Bar. 200 μ m. (F) Estrous cycle pattern in a representative females. D, diestrus; E, estrus; M, metestrus; P, proestrus. (G) Average length of estrus cycle and estrus length. Analysis of the AMH serum level. Values are the mean $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$.

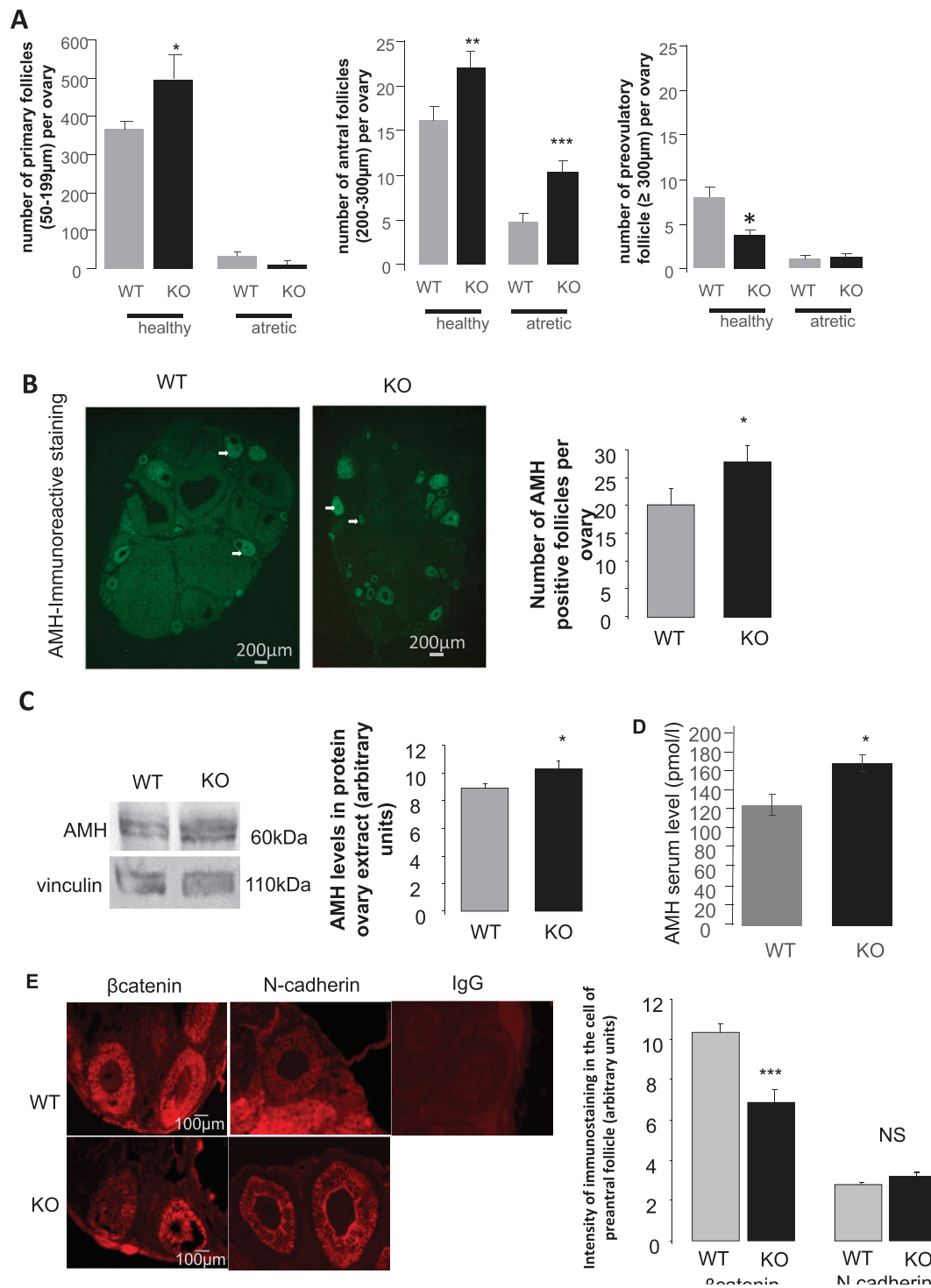


Figure 7. Decrease in pre-ovulatory follicles population in α IAMPK $^{-/-}$. (A) Representative picture of atretic and healthy follicle. Bar 100 μ m. Quantification of healthy and atretic follicles in the following range of follicles: primary follicles (50–199 μ m), antral follicles (200–300 μ m) and pre-ovulatory follicles ($\geq$ 300 μ m) per ovary were counted in α IAMPK $^{+/+}$ (WT, wild type mice) and α IAMPK $^{-/-}$ (KO) ovary (n = 10 mice per genotype). (B) Immunostaining and quantification of anti-Müllerian hormone (AMH) in α IAMPK $^{+/+}$ and α IAMPK $^{-/-}$ ovary (n = 5 mice per genotype). (C) AMH protein content in whole ovary lysates from α IAMPK $^{+/+}$ and α IAMPK $^{-/-}$, normalized to vinculin. Quantification was shown in the side of the western blot. (n = 4 mice per genotype). (D) AMH serum level in α IAMPK transgenic mice. (E) Immunostaining and quantification of β catenin and N-cadherin in preantral follicles. IgG was used as negative control. Quantification of intensity was performed by ImageJ software (n = 5 mice per genotype). Values are the mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

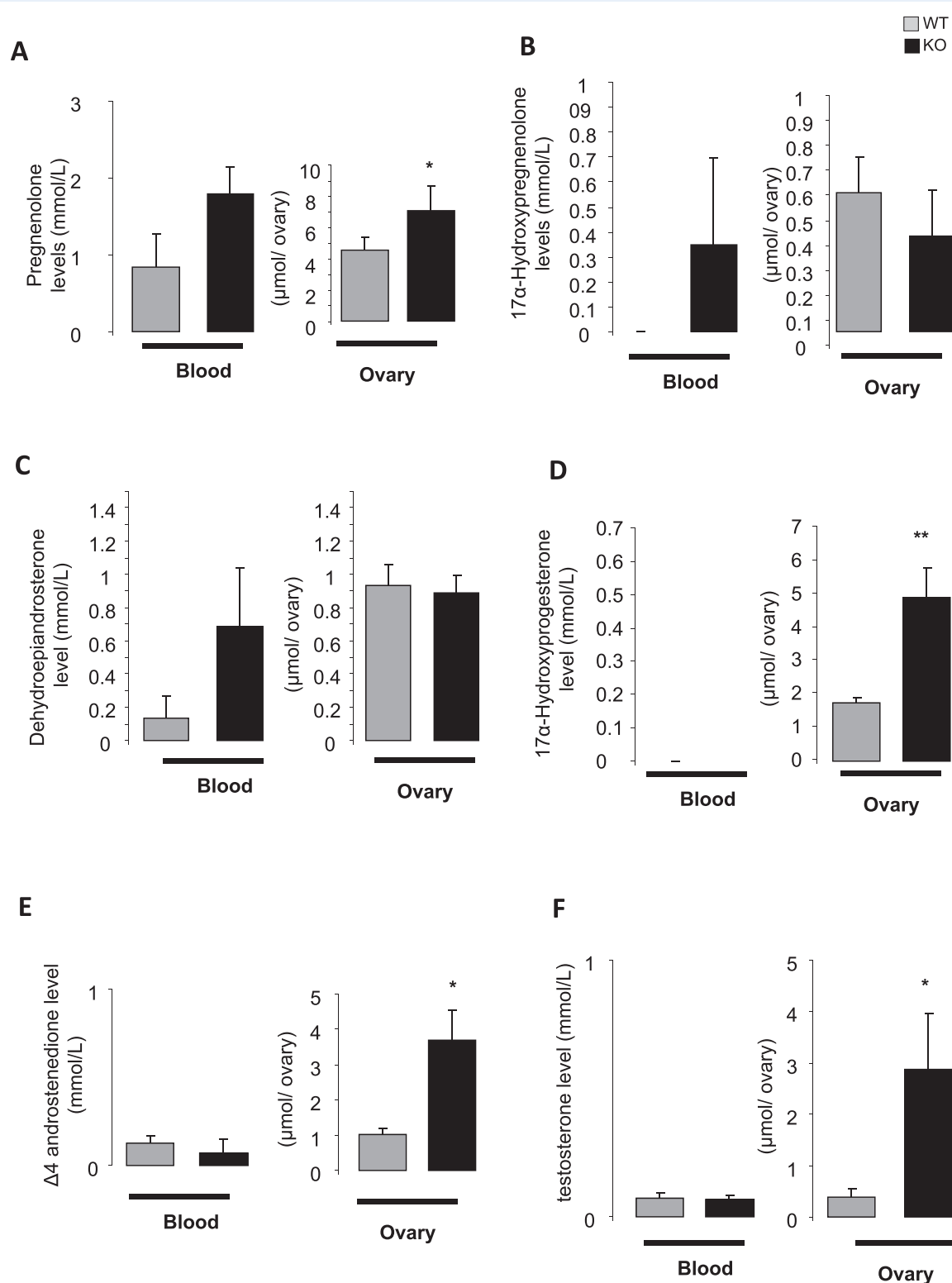


Figure 8. Steroid analysis in α 1AMPK^{-/-} female mice. The following steroids were analyzed in blood and whole ovary extract from α 1AMPK^{+/+} (WT) and α 1AMPK^{-/-} (KO) adult females. (A) Pregnenolone, (B) 17 α -Hydroxypregnenolone, (C) Dehydroepiandrosterone, (D) 17 α -Hydroxyprogesterone, (E) Δ 4 androstenedione, (F) testosterone (n = 7 mice per genotype). Values are in mmol/L or μ mol/ovary extract as a mean $\pm$ SEM. * p < 0.05, ** p < 0.01.

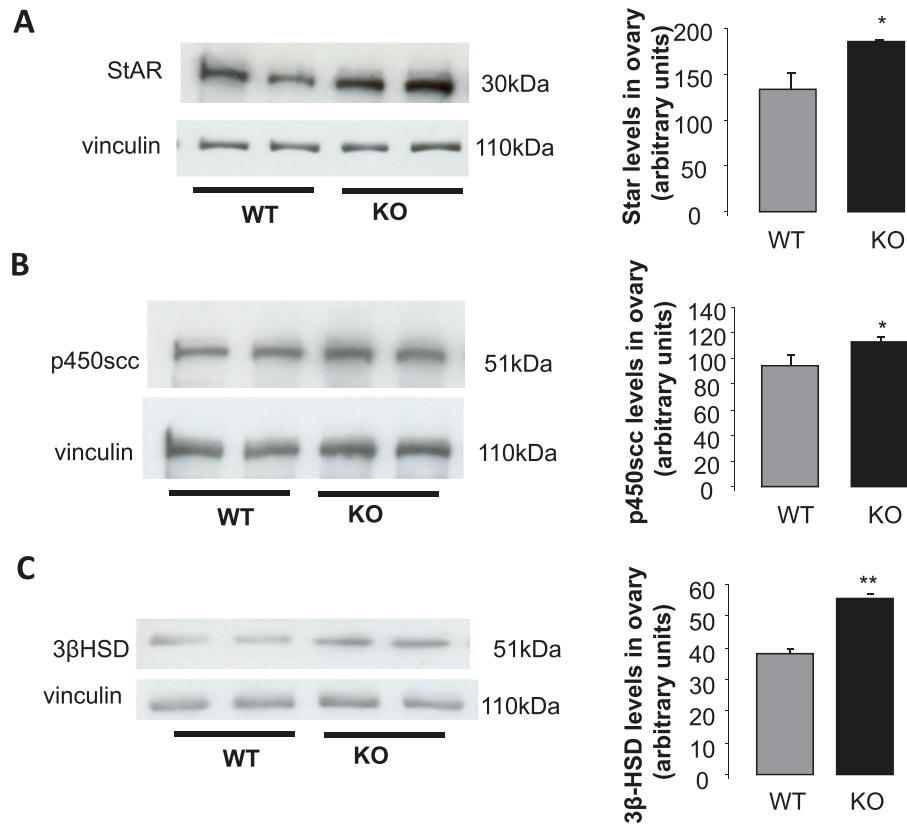


Figure 9. Expression of steroidogenic enzymes in α IAMPK^{-/-} female mice. Steroidogenic acute regulatory protein (StAR) p450 side-chain cleavage (p450scc), 3 β -hydroxysteroid dehydrogenase (3 β HSD) expression in whole ovary lysates from α IAMPK^{+/+} (WT) and α IAMPK^{-/-} (KO), normalized to vinculin. Quantification of the expression of StAR, p450scc and 3 β HSD in both genotypes is shown to the side of the western blot (n = 3 mice per genotype). Values are the mean $\pm$ SEM. *P < 0.05, **P < 0.01.

present some disadvantages as well. Human granulosa-lutein cells are retrieved after hormonal treatment and are differentiated cells with high steroid production and low proliferation rates. The mouse primary GCs proliferate slowly, and cell preparation consistency across mice is an issue, as well as obtaining follicles at the same stage of differentiation at the moment of retrieval. The use of primary cells here overcomes the disadvantage of using heterogeneous cell populations that are associated with lower efficiency in gene manipulation resulting in a mosaic population, which may mask the actual effect of the silencing. We confirmed the results obtained with KGN by using a pharmacological inhibitor drug (compound C or SBI-0206965) on primary GC in culture (Supplementary Figs S5, S6 and S7) and *in vivo* with ovaries from transgenic α IAMPK^{-/-} mice, confirming the disruption of steroid production and adhesion or migration of GCs.

Transcriptomics and biological approaches in KGN cells showed the impact of the deficiency in α IAMPK on three main functions: the extracellular matrix and cell proliferation, energy metabolism which has repercussions on steroid production, and immune function. Immune function, not explored here, is worthy of more studies in the future. RNAseq analysis showed dysregulation of genes related to cytoskeleton reorganization (collagen type IV, matrix metalloproteinase 11, CD9 molecule, laminin A/C, destrin, an actin-depolymerizing factor).

Laminins and collagen proteins are the most abundant extracellular matrix components of the basal lamina and granulosa cell layers of ovarian follicles (Rodgers et al., 2001). Modifying the cytoskeleton in ovine GCs using pharmacological drugs induced cell rounding, inhibition of proliferation, and progesterone secretion. These observations may support the hypothesis that the cytoskeleton and extracellular matrix play an essential role in granulosa cell luteinization (Le Bellego et al., 2005). Supporting this notion, several studies have shown that rigidity of the extracellular matrix has negative consequences on follicular activation and growth (West et al., 2007) and the capacity for migration and adhesion of the cells is critical for the formation of the corpus luteum (Rolaki et al., 2007; Franz et al., 2013). In a mouse model of PCOS, GCs have been noted to be arranged loosely in fewer (only 1–3) layers (Tao et al., 2017), as we have observed in our transgenic mouse model. In cultured bovine GCs, among 30 differentially regulated genes related to cell–cell communication were identified by the ingenuity pathway analysis tool, with genes affected by AMPK signaling when density changes occurred (Baufeld et al., 2017). Also, in our study conditions, it is possible that the absence of α IAMPK changed the extracellular matrix and induced signals to stimulate granulosa cell proliferation by stimulating follicular activation, as attested by the higher population of pre-antral follicles in α IAMPK^{-/-} mice.

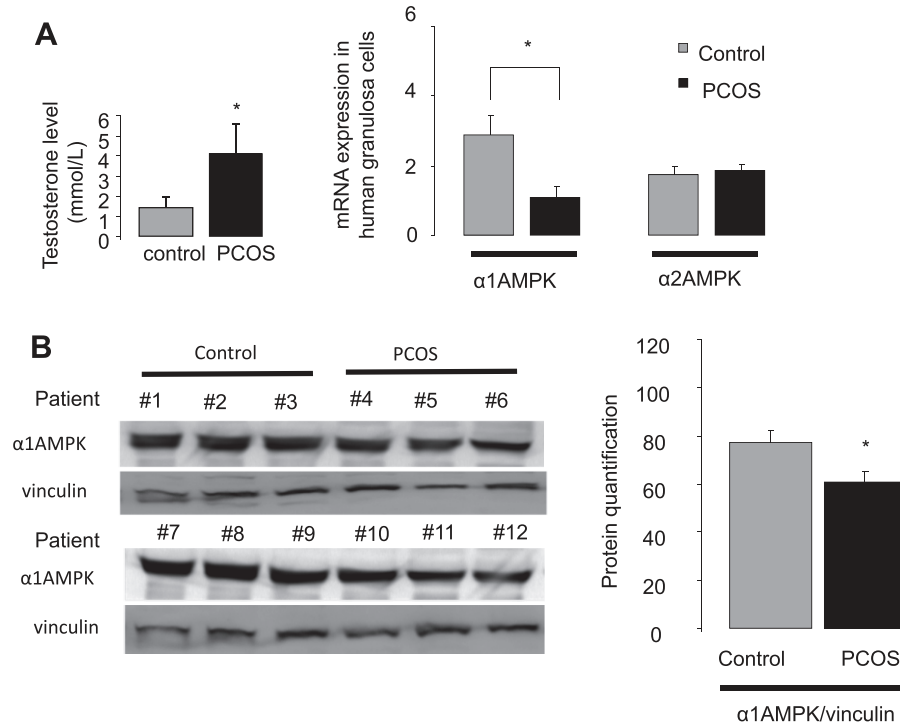


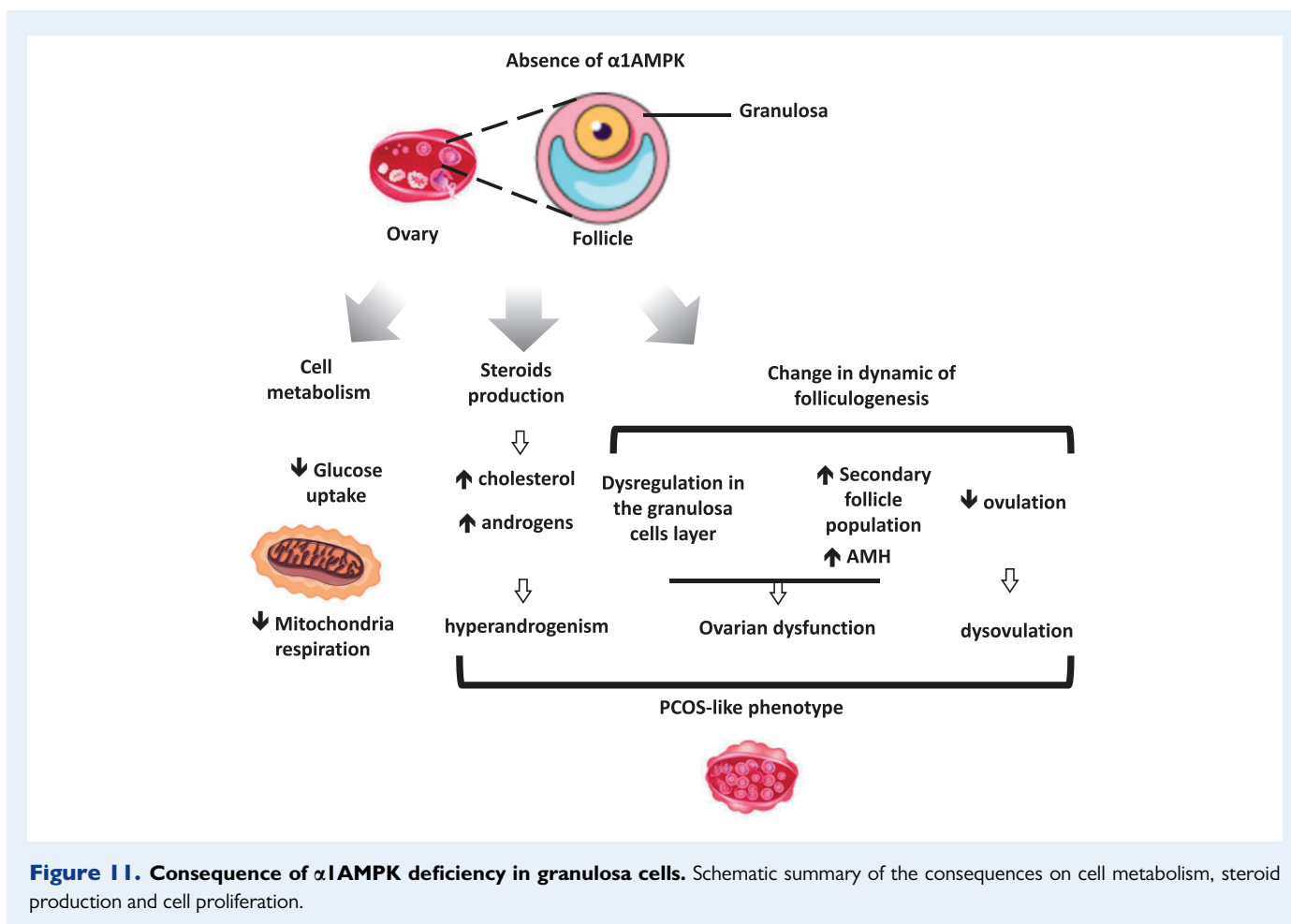
Figure 10. Expression of AMPK in primary human granulosa cells. (A) Left panel: Testosterone serum level in the control and lean patients with PCOS patients (BMI ≤ 25 kg/m²). Right panel: mRNA expression of α 1AMPK and α 2AMPK by real-time quantitative PCR (RT-qPCR) in human granulosa cells retrieved from a group of control lean patients (n = 8 patients) and a group of lean PCOS patients (BMI ≤ 25) (n = 9 patients). (B) Western blot analysis of α 1AMPK in human granulosa cells retrieved from lean control or lean PCOS patients. Vinculin was used as control. Quantification of total α 1AMPK was shown below the western blot (n = 6 different patients per group). The results are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

Furthermore, a study using an AMPK inhibitor described that incubation of primordial follicles *in vitro* with Compound C increased the follicle growth (Jiang *et al.*, 2016). We can compare these data to a recent study in an ovine model that reported a disruption in the extracellular matrix structure in the ovaries induced by a maternal environment rich in androgens, and culminated in the acceleration of follicular growth (Monniaux *et al.*, 2020). Similarly, in our α 1AMPK^{-/-} mice, a high number of large preantral and small antral follicles were observed with a higher population of AMH-positive follicles in the ovaries. AMH is a well-established specific marker of this subpopulation of follicles, which is also elevated in women with PCOS (Visser *et al.*, 2006; Torres-Rovira *et al.*, 2014) and in PCOS-induced mouse models. Consistent with this view, polycystic ovaries present some abnormalities in the dynamics of follicles and contain higher numbers of primary and secondary follicles (Franks *et al.*, 2008). AMH levels and ovary follicular development could be improved by administering an AMPK agonist, metformin, associated with resveratrol or pioglitazone treatments (Stracquandano *et al.*, 2018).

As expected, energy metabolism was affected in α 1AMPK^{-/-} models (mice and of KO-KGN cells). In KO-KGN cells, we reported a decrease in glucose uptake, and a lower content of the insulin-regulated glucose transporter, GLUT4, as well as lower mitochondrial metabolism associated with elevated lactate and pyruvate levels. It is now

widely recognized that insulin resistance is present in obese or overweight women and often in lean women with PCOS. The data obtained from KO-KGN cells are consistent with other studies showing that the levels of GLUT1 and GLUT3 are lower in induced pluripotent stem cells from patients with PCOS (Min *et al.*, 2018), and a lower mitochondria content in blood cells of patients with PCOS, which is inversely correlated to the level of insulin resistance (Lee *et al.*, 2011). It is known that metabolic disorders are clinically associated with declining fertility (Estienne *et al.*, 2019). In the present study, we showed a change in the expression of glucose and lipid metabolism enzymes, such as pyruvate dehydrogenase kinase 4 (PDK4: entry of pyruvate to Krebs' cycle) and transaldolase I (pentose phosphate shunt) by the RNAseq analysis of α 1AMPK-deficient human GCs. PDK4 is known to be induced by AMPK (Houten *et al.*, 2009) which, by phosphorylating PDK, inhibits its activity thereby explaining the higher lactate and pyruvate concentrations in KO KGN cells. Transaldolase I is a key enzyme of the pentose phosphate pathway that generates NADPH for DNA and RNA syntheses related to proliferation and growth. Other genes of metabolism such as vanin 1 (VNN1), which are regulated by androgens, should be indirectly modified by hyperandrogenism.

Interestingly, the most upregulated gene was ELOVL fatty acid elongase 7 (ELOVL7), which catalyzes the formation of neutral lipids and



cholesterol esters as a source of *de novo* steroid synthesis. ELOVL7 is also regulated by the androgen pathway through SREBP1 (Tamura et al., 2009). We have confirmed the high cholesterol content in GCs and the overall consequence on steroid production both *in vitro* and *in vivo* in $\alpha 1$ AMPK^{-/-} transgenic mice. We also noticed a direct consequence on the hyperactivation of the transcription factor CREB and enzymes involved in steroid production. Several studies on granulosa cells using pharmacological approaches described an inhibition of the production of progesterone and oestradiol in rodent and bovine cells by the two AMPK activators: AICAR or metformin (Bowdridge et al., 2015). Metformin is also used as a treatment for patients with PCOS, restoring ovulation by inhibiting androgen synthesis (Palomba et al., 2005). This inhibition was partly explained by a decrease in the expression of key enzymes, such as $\beta 3$ HSD, p450scc and StAR (Tosca et al., 2005), leading to a reduction of androgen synthesis by thecal cells (Attia et al., 2001; Mansfield et al., 2003). More recently, the secretion of progesterone by human mural GCs was shown to be resistant to AMPK inhibition (Bowdridge et al., 2017).

To investigate the '*in vivo*' possibility of a potential genotype-phenotype correlation, we examined the fertility of transgenic $\alpha 1$ AMPK-deficient mice because the phenotype presents several PCOS traits, and we have determined the expression of the $\alpha 1$ AMPK gene in purified GCs from patients carrying the PCOS phenotype. Indeed, the $\alpha 1$ AMPK^{-/-} transgenic mouse model showed irregular cycles,

ovulatory dysfunction, dysregulation in folliculogenesis associated with elevated AMH level and excess in androgen production by the ovaries. Interestingly, this phenotype is quite similar to those described in rodent model of PCOS induced by DHEA, where the authors still observed phospho- α AMPK, but lower total α AMPK concentrations in the ovaries in comparison to control mice (Tao et al., 2017). Another recent study found a reduction in AMPK immunoreactivity in follicles from a group of DHEA-induced PCOS rats compared to a control group (Furat Rencber et al., 2018). The expression of α AMPK could be upregulated by treatment with metformin and resveratrol and was associated with an improvement of the PCOS phenotype in rat (Furat Rencber et al., 2018). These results suggest that a lower expression of $\alpha 1$ AMPK could be associated with the etiology of PCOS.

However, although the human PCOS syndrome is associated with multiple factors, several PCOS rodent models have been developed, through different strategies that tend to mimic PCOS traits. First, hyperandrogenism, induced by androgen (DHEA, DHT) injection or using an aromatase inhibitor, is one of the most important features to induce a PCOS phenotype (Caldwell et al., 2014; Abbott et al., 2019; Rodriguez Paris et al., 2021). More recently, high AMH levels during the embryonic stage have been shown to induce a PCOS phenotype during adulthood. Indeed, elevated AMH resulted in maternal neuroendocrine-driven testosterone excess and masculinized the female fetus (Tata et al., 2018). Second, the PCOS phenotype can be

mimicked by changing diet or in stressful environmental conditions and, third, by gene mutation, as in transgenic animal models over-expressing LH or nerve growth factor, or presenting a deletion of PTEN or plasminogen activator inhibitor-1 in follicle (Ryu et al., 2019).

In our model, we can hypothesize that a deficiency in α 1AMPK in female mice increases androgen levels through higher expression of enzymes involved in steroid production, as observed in DHEA- or DHT-induced PCOS mouse models and higher AMH levels during pregnancy. These data provide further evidence for the potential to use AMPK activators, e.g. adiponectin agonist/inducer, metformin in the treatment of PCOS or to personalize treatment according to the activity of AMPK in GCs, in order to determine the sensitivity of the patient to the treatment and to adapt it. Furthermore, this model is also of interest for studying the AMPK pathway and using AMPK activators to investigate the etiology of granulosa cell cancer or the development of a therapy. It will be interesting to use the strategy of overexpression of AMPK in ovarian follicles of a mouse PCOS model, in order to evaluate the importance of AMPK signaling in the treatment of PCOS traits. Moreover, creating an inducible deletion of α 1AMPK specifically in GCs could help us to decipher the consequences on folliculogenesis if AMPK is absent at an early stage (embryo stage) or during adulthood.

In summary, GCs from lean patients with PCOS exhibit lower gene expression of α 1AMPK. Similarly, a disruption of the α 1AMPK gene in both *in vitro* human GCs and *in vivo* in transgenic mice results in a PCOS-like phenotype with irregular cycles, changes in follicular dynamics and hyperandrogenism. These changes in folliculogenesis trigger a reduction in ovulation rate as observed in patients with PCOS or in PCOS-induced mice (Tao et al., 2017). Our study suggests the involvement of AMPK in granulosa cell functions, and a dysregulation of AMPK could be implied in the etiology of the PCOS phenotype. Therefore, novel insights into the mechanisms involved in the association of AMPK with PCOS have to be identified. The development of specific therapeutic targets of AMPK could be of interest to improve the subfertility associated with a metabolic syndrome, such as PCOS.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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Authors' roles

Conceptualization, funding acquisition: P.F., I.P. and J.D.; intellectual input, writing—review and editing, C.G.; investigation, P.F., S.F., N.I.M., S.H., C.Ra., C.Ro., J.G., C.C., R.G. and P.M.; formal analysis: Y.B. and R.K.; plasmids construction, mice and biological samples elaboration: R.A., F.G., J.T., B.V. and M.F.; All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors report no intellectual or financial conflicts of interest related to this work. R.K. is identified as personnel of the International Agency for Research on Cancer/World Health Organization. R.K. alone is responsible for the views expressed in this article and she does not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/World Health Organization.

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