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Mitochondrial Protein Turnover Is Critical for Granulosa Cell Proliferation and Differentiation in Antral Follicles

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Granulosa cell (GC) proliferation is essential for follicular development. FSH is a key factor in GC proliferation, and a continuous supply of high levels of ATP is necessary for cell proliferation. However, genes encoding proteins of the glycolytic pathways are poorly expressed in GCs. Therefore, we hypothesized that mitochondrial gene expression and protein synthesis play a primary role in ATP production during GC proliferation. To test this hypothesis, we performed an *in vivo* study of GCs collected from 23-day-old mice ovaries with or without equine chorionic gonadotropin (eCG) priming. It was observed that mitochondrial activity with membrane potential, expression of protein-coding genes (*Nd1-6*, *Cytb*, *Atpase6,8*) and transcription-related genes (*Polrmt*, *Tfam*, *Tfb2m*), copy number of mitochondrial (mt-)DNA, and protein synthesis were increased in GCs after 24 hours of eCG injection and mostly maintained elevated up to 48 hours. Therefore, we performed *in vitro* culture of GCs in DMEM medium supplemented with FSH, testosterone, and serum and containing different glucose concentrations with or without D-chloramphenicol (CRP) for 24 hours. GC proliferation and ATP production were observed to be independent of glucose concentration. Furthermore, FSH-induced mitochondrial activity with membrane potential, ATP content, BrdU-incorporated cell proliferation, intensity of mt-ND1 and mt-ND6 proteins, and expressions of marker genes for proliferation and differentiation were significantly decreased by CRP treatment. These results revealed the crucial role of mitochondria in the supply of ATP and the necessity of mitochondrial gene expression and protein synthesis in not only the proliferation but also the differentiation of GCs during follicular development.

Freeform/Key Words: granulosa cells, proliferation, mitochondria, gene expression, protein synthesis

FSH is a key factor in the promotion of follicle development from the secondary follicle to the preovulatory stage [1–4]. In this process, FSH is a potent and essential regulator of the proliferation and differentiation of granulosa cells (GCs). During follicular development, numerous genes are expressed in GCs, including *Cyp19a1*, *Lhcgr*, and *Ccnd2*. *Cyp19a1* encodes aromatase that converts androgen to estrogen. Estrogen receptor β–null mice show a

Abbreviations: CDK, cyclin-dependent kinase; CRP, D-chloramphenicol; eCG, equine chorionic gonadotropin; ETC, electron transport chain; FCS, fetal calf serum; FT, FSH and testosterone; GC, granulosa cell; mt-, mitochondrial; OXPHOS, oxidative phosphorylation; TBST, Tris-buffered saline containing Tween 20.

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low number of GCs and low expression of *Lhcgr* in GCs and exhibit subfertility phenotypes [5, 6]. Additionally, mice that lack *Ccnd2* also show an infertile phenotype because of the low number of GCs in the antral follicles [7, 8], indicating that GC proliferation is an essential event in GC differentiation during follicular development.

The proliferation of GCs is dependent on cyclin D2 to activate cyclin-dependent kinase (CDK) family members CDK2, CDK4 and CDK6 [8]. These CDKs induce DNA replication (S phase), and other CDKs, including CDK1 and CDK7, are involved in cell division (M phase) via spindle formation and chromatid separation [9, 10]. In general, inhibition of mitochondrial ATP synthesis results in ATP deprivation during G_1 phase, which reduces levels of D cyclins and contributes to cell cycle arrest [11]. Moreover, Xiong *et al.* [12] observed that a reduced supply of ATP suppressed proliferation of HeLa cells. Therefore, ATP is also suggested to be critical for GC proliferation, which, in turn, is essential for follicular development; however, the relationship between ATP production and proliferation of GCs is undetermined. Glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) are two main pathways for ATP generation in mammalian cells. Genes associated with the glycolytic pathway are expressed in cumulus cells [13–16] but are poorly expressed in GCs [17–19], suggesting that mitochondrial ATP production in GCs is important to induce proliferation during follicular development.

In mitochondria, ATP is mostly produced through a sequential redox reaction in electron transport chain (ETC) complexes by electron transfer from reduced NAD or succinate or other intermediary metabolites to oxygen and ejection of protons [9]. The sequential reaction depends on numerous proteins; 13 of these are encoded by mitochondrial (mt-)DNA, and rests are encoded by the nuclear genome. Mitochondria have specific transcription and translational systems that are independent of those in the cytoplasm. Therefore, if mitochondrial ATP production is dominant in GCs, this would be essential for FSH-induced GC proliferation and differentiation during follicular development. Recently, Liu *et al.* [20] showed ATP generation in GCs during follicular development using a knockout mouse model with defective mitochondrial fusion, but mitochondrial gene expression and protein synthesis during GC proliferation and differentiation are undetermined.

Therefore, to elucidate the importance of mitochondrial gene expression and protein synthesis during the development of ovarian follicles from antral to preovulatory stage, we investigated mitochondrial functions *in vivo*, including mitochondrial activity, membrane potential, gene expression, protein synthesis, and ATP production. Follicular fluid contains a comparatively lower level of glucose than does blood serum [21–23]; therefore, we also performed an *in vitro* culture of GCs in hormone and serum-supplemented DMEM medium containing different concentrations of glucose. To explain the role of mitochondria in the proliferation of GCs, we added D-chloramphenicol (CRP), an inhibitor of mitochondrial but not cytoplasmic translation, to DMEM/F12 culture medium supplemented with hormone and serum and studied mitochondrial gene expression, protein synthesis, total activity and individual membrane potential along with cell proliferation, and ATP production.

1. Materials and Methods

A. Materials

Equine chorionic gonadotropin (eCG) was purchased from Asuka Seiyaku (Tokyo, Japan). DMEM (DMEM/F12 and DMEM/low glucose), penicillin, streptomycin, and oligonucleotide poly(dT) were from Invitrogen (Carlsbad, CA). BSA and fetal calf serum (FCS) were from Life Technologies (Grand Island, NY). Avian myeloma virus reverse transcription was from Promega (Madison, WI). CRP was from Sigma-Aldrich (St. Louis, MO). Standard chemicals and reagents were obtained from Nacalai Tesque (Osaka, Japan) or Sigma-Aldrich.

B. Animals

Immature (3-week-old) female C57BL/6 mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). Animals were housed at the Experimental Animal Center, Hiroshima University, under a 12-hour light/12-hour dark schedule and provided with food and water *ad libitum*. Animals were treated in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, as approved by the Animal Care and Use Committee at Hiroshima University.

C. Treatment of Mice and Collection of GCs

Mice were treated and GCs were harvested according to our previous study [24, 25]. Briefly, 4 IU of eCG was IP injected to each mouse on day 23 to stimulate follicular growth. Immature (23-day-old) mice ovaries contain a considerable number of antral follicles in addition to preantral follicles, and we collected GCs from antral follicles only. For *in vivo* study, GCs were collected at 0, 24, and 48 hours after eCG injection to study the follicular development process. However, for *in vivo* study, the mice were euthanized after 6 hours of eCG priming, and harvested GCs were cultured for 24 hours at 37°C in an atmosphere of 5% CO_2 and maximum humidity for further use. The eCG priming for 6 hours was done to ensure maximum number of cells as well as to inhibit spontaneous luteinization after culture. In both *in vivo* and *in vitro* studies, entire follicles were punctured and COCs were picked up and removed, keeping the membrana granulosa under a microscope. Only membrana granulosa were then collected, washed, and used for further experiment or culture.

D. In Vitro *Culture of GCs*

GCs collected from each antral follicle were cultured in high-glucose DMEM/F12 (17.51 mM D-glucose; catalog no. 11330-032), DMEM/low glucose (5.56 mM D-glucose; catalog no. 11885-084), or DMEM/no glucose (no D-glucose; catalog no. 11966-025) medium (Thermo Fisher Scientific, Waltham, MA) containing 1% (v/v) FCS in 5% (v/v) FCS-coated 96-well plates for 24 hours at 37°C in an atmosphere of 5% CO_2 and maximum humidity. The medium was supplemented with (i) no supplement (control), (ii) FSH (100 ng/mL) and testosterone (10 ng/mL) (FT), and (iii) FSH (100 ng/mL) and testosterone (10 ng/mL) plus 0.1 mM CRP (FT-CRP). For western blotting, we used an additional treatment of 0.1 mM CRP.

E. RNA Extraction and RT-PCR

DNA and RNA extraction and RT-PCR analysis were performed as previously described [26]. Briefly, total RNA was extracted from collected or cultured GCs using an RNAeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Total RNA was then reverse transcribed using 500 ng of poly(deoxythymidine) and 0 and 0.25 U of avian myeloblastosis virus reverse transcription (Promega) at 42°C for 75 minutes followed by 95°C for 5 minutes.

F. Real-Time PCR

Quantitative real-time PCR analysis was performed as previously described [27]. Briefly, cDNA and primers were added to Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) to give a 15-μL total reaction volume. PCR was then performed using the StepOne real-time PCR system (Applied Biosystems) with the following parameters: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C to 64°C. The primer sets are shown in Table 1. *L19* was used as a control for reaction efficiency and variations in concentrations of mRNA in the first real-time PCR.

G. DNA Isolation and mt-DNA Copy Number

DNA was isolated from collected GCs using QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions and our previous study [25]. Mitochondrial copy number was

Table 1. List of Primers Used for RT-PCR

Genes	Forward Primer	Reverse Primer	Size (bp)
L19	5′-GGCATAGGGAAGAGGAAGG-3′	5′-GGATGTGCTCCATGAGGATGC-3′	199
ND1	5′-CAG GATGAGCCTCAAACTCC-3′	5′-CCGGTTTGTTTCTGCTAGGG-3′	230
ND2	5′-AGGGATCCCACTGCACATAG-3′	5′-GGGATGGGTTGTAAGGAAGA-3′	239
ND3	5′-ACCTGTACACTGTTATCTTCATT-3′	5′-TCATATGGATTTGCTTTTTC-3′	109
ND4	5′-CACTGCTAATTGCCCTCATC-3′	5′-GACCCAGCAATTGGAGCTT-3′	208
ND4L	5′-TGCCATCTACCTTCTTCAACC-3′	5′-GTGATGGGGATTGGTATGGA-3′	184
ND5	5′-CTTCCCACTGTACACCACCA-3′	5′-CATGTGACAAAAAGGGCTACAG-3′	201
ND6	5′-GGTTGGTTGTCTTGGGTTAGC-3′	5′-TAGATCCCCAAGTCTCTGGA-3′	215
Cytb	5′-ACGTCCTTCCATGAGGACAA-3′	5′-GGAGGTGAACGATTGCTAGG-3′	201
COX1	5′-ACCCCCAGCCATAACACAGT-3′	5′-GGTGCCCAAAGAATCAGAAC-3′	206
COX2	5′-TCTCCCCTCTCTACGCATTCT-3′’	5′-GGCAGAACGACTCGGTTATC-3′	204
COX3	5′-AGGCCACCACACTCCTATTG-3′	5′-ATTCCTGTTGGAGGTCAGCA-3′	159
ATPase6	5′-CCGTCTCCATTCTTTCCAAC-3′	5′-AGTTTGTGTCGGAAGCCTGT-3′	243
ATPase8	5′-TGCCACAACTAGATACATCA-3′	5′-GGGGTTTTTACTTTTATGGT-3′	148
Polrmt	5′-TGCATTCTGGTGCCTACTTG-3′	5′-AGATCTGCAGCACCAAGTCC-3′	189
TFAM	5′-GTTTTTCCAGCATGGGTAGC-3′	5′-TCTGGTAGCTCCCTCCACAG-3′	154
TFB2M	5′-GCGGCTTCTCTGACTTCAAT-3′	5′-AAGTAATGCCCCAGTCAGGA-3′	218
Inha	5′-TTGGAGGCAGACGCCTTATT-3′	5′-CGGTCTCTGCTGCTCCTTTT-3′	175
Inhba	5′-GCTGCCCTCACAGTAGTTCC-3′	5′-GAGAGACGCTTAACCTGGA-3′	279
Ccnd2	5′-TCACCACGTGTTCCCAAGAC-3′	5′-AGCGAATTCCCTCCATCAGA-3′	243
Cyp19a1	5′-TTGCACCCAAATGAGGACAG-3′	5′-CTTCACTGGTCCCCAACACA-3′	290
Hsd17b1	5′-GAAGGTTTGTGCGAGAGTCTG-3′	5′-AAGCGGTTCGTGGAGAAGTAG-3′	287
Fshr	5′-TGGCCATTACTGGGAACACC-3′	5′-TGCCAAAGATGGGGAAGAGA-3′	261
Lhcgr	5′-ACTGGTGTGGTTTCAGGAATT-3′	5′-CCTAAGGAAGGCATAGCCCAT-3′	244
Internal control (Tcrd)	5′-CAATGTTGCTTGTCTGGTG-3′	5′-GTCAGTCGAGTGCACAGTTT-3′	

assessed by analyzing total DNA isolated from GCs with quantitative real-time PCR using SYBR Green chemistry according to the previous study [28]. The primer sets shown in Table 1 are also used for real-time PCR of DNA. DNA fragments encoding different mitochondrial ETC complex 1 (ND1), complex 3 (CYTB), complex 4 (COX1), and complex 5 (ATPase6) were normalized with the internal control.

H. Mitochondrial Activity

Mitochondrial activity in GCs was evaluated using MitoTracker Green FM (M-7514) according to the manufacturer’s protocol. Briefly, collected or cultured GCs were washed, centrifuged, and resuspended. The cell suspensions were then placed in 96-well plates and incubated with 20 nM MitoTracker Green for 5 minutes at 37°C in the dark. Analysis was performed using a multilabel reader (2030 ARVO X3 multilabel reader; PerkinElmer, Waltham, MA) at excitation of 485 nm and emission of 535 nm. Mitochondrial activity was calculated relative to total protein, and the average of triplicate samples was determined for each treatment.

I. Mitochondrial Membrane Potential

The mitochondrial membrane potential was analyzed using a MitoPT JC-1 assay kit (Immunochemistry Technologies) according to the manufacturer’s protocol. Briefly, GCs collected or cultured were washed, resuspended in media, and incubated with JC-1 (0.5 μM) at 37°C for 30 minutes. Cells were then washed and analyzed by flow cytometry using Attune NxT software (Thermo Fisher Scientific) at an excitation of 488 nm laser and emission at 597 and 590 to 600 nm. JC-1 monomers emit at 530/30 nm (BL1 channel) and J-aggregates emit at 574/26 nm (BL2 channel). Twenty thousand cells were acquired per sample. Dimethyl sulfoxide was used for compensation. The average of triplicate samples was determined for each treatment.

J. BrdU Cell Proliferation Assay

GCs collected from eCG-primed mouse ovaries were washed and dispensed according to treatments into a 96-well plate and incubated for 24 hours at 37°C in an atmosphere of 5% CO_2 and maximum humidity. Cell proliferation was assessed using a BrdU cell proliferation ELISA kit (ab126556; Abcam) according to the manufacturer's protocol. The absorbance of samples was measured using a 2030 ARVO X4 multilabel reader (PerkinElmer) at 450 nm. The average of triplicate samples was determined for each treatment.

K. Measurement of Cellular ATP Content in GCs

The EnzyLight ATP assay kit (EATP-100; Bioassay Systems, Hayward, CA) was used to detect GC ATP levels according to the manufacturer's instructions. Briefly, GCs were homogenized in 100 μL of cell lysate buffer, sonicated, and centrifuged at 13,500 rpm for 10 minutes. The supernatant was transferred to a separate well and assay buffer and substrates were mixed with the sample. Luminescence was detected with a luminometer (2030 ARVO X4 multilabel reader; PerkinElmer) within 1 minute of mixing. The ATP level was calculated relative to total protein content, and the average of triplicate samples was determined for each treatment.

L. Western Blotting

GCs were lysed with RIPA buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA, and 0.1% (w/v) SDS] containing complete protease inhibitors (Roche, Indianapolis, IN). Western blotting was performed according to our previous study [26]. Briefly, cell extracts (10 μg of protein) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose (polyvinylidene difluoride) membranes (GE Healthcare, Newark, NJ). Nonspecific binding sites were blocked by incubation in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) and 5% (w/v) nonfat dried milk (Nestle Co., Solon, OH). Membranes were immunoblotted with primary antibodies, including anti–MT-ND (Proteintech Group, catalog no. 19703-1-AP, RRID:AB_10637853 [29]), anti–MT-ND6 (Bioss, catalog no. bs-3955R, RRID:AB_10856632 [30]), and anti–β-actin (Cell Signaling Technology, catalog no. 4967, RRID: AB_330288 [31]) diluted in 2.5% BSA in TBST overnight at 4°C. Membranes were then incubated with HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, catalog no. 7074, RRID:AB_2099233 [32]) for mt-ND1 and mt-ND6 or HRP-conjugated anti-mouse IgG antibody (Cell Signaling Technology, catalog no. 7076, RRID:AB_330924 [33]) for β-actin. After washing in TBST, enhanced chemiluminescence detection was performed using the enhanced chemiluminescence system according to the manufacturer's specifications (GE Biosciences), and blots were exposed to Fuji x-ray film (Fujifilm, Tokyo, Japan) with appropriate exposure times. Band intensities were analyzed using a Gel-Pro analyzer (Media Cybernetics, Rockville, MD).

M. Statistical Analysis

All data from three replicates were analyzed by either a Student *t* test or one-way analysis of variance followed by a Tukey *post hoc* test (StatView; Abacus Concepts, Berkeley, CA). All values are presented as the mean ± SEM. Treatments were considered statistically different from one another at $P < 0.05$.

2. Results

A. Mitochondrial Activity, Membrane Potential, mt-DNA Copy Number, Gene Expression, Protein Synthesis, as Well as ATP Content Increase in GCs During Follicular Development In Vivo

To assess dynamic changes in mitochondrial DNA copy number, gene expression, and protein synthesis during follicular development, we collected GCs from antral follicles of immature

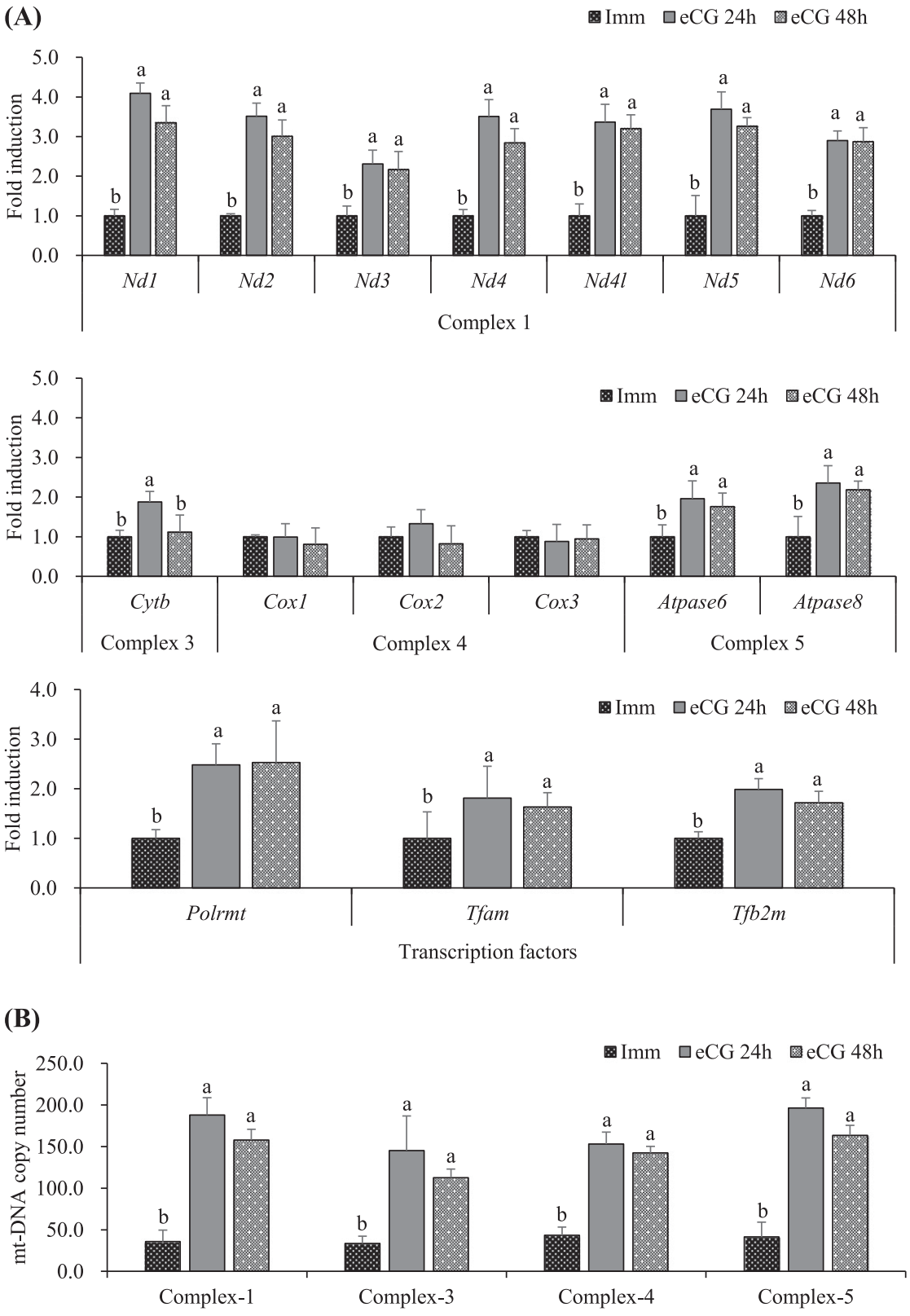

Figure 1. (A) Temporal changes in the expression of all protein-coding genes in mitochondrial ETC complex 1 (*Nd1, Nd2, Nd3, Nd4, Nd5, Nd6*), ETC complex 3 (*Cytb*), ETC complex 4 (*Cox1, Cox,2, Cox3*) and ETC complex 5 (*Atpase6, Atpase8*) and genes that regulate mitochondrial transcription (*Polrmt*, *Tfam*, *Tfb2m*) in GCs during follicular

development *in vivo*. Levels of mRNAs were normalized against *L19*. (B) Copy number of mt-DNA in complex 1 (ND1), complex 3 (CYTB), complex 4 (COX1), and complex 5 (ATPase6) where the levels of mt-DNA fragments were compared with the internal control (Tcrd). The immature (day 23) value was set as 1, and the data are expressed as fold induction. For all data, GCs were collected from immature (23-d-old) mice 24 or 48 h after injection with or without eCG. Values are the mean ± SEM of three replicates. Bars with different letters (a, b or c) differ significantly ($P < 0.05$).

(day 23) mouse ovaries before and at 24 or 48 hours after eCG stimulation. The expression of all protein-coding genes in mitochondrial ETC complex 1 (*Nd1* to *Nd6*), ETC complex 3 (*Cytb*), and ETC complex 5 (*Atpase6, Atpase8*) was significantly increased 24 hours after eCG stimulation [Fig. 1(A)]. However, after 48 hours, the expression of complex 1 and complex 5 genes was unchanged, but complex 3 gene expression was significantly decreased [Fig. 1(A)]. The expression of genes encoding complex 4 (*Cox1* to *Cox3*) was unchanged at both 24 and 48 hours after eCG treatment [Fig. 1(A)]. The expression of genes regulating mitochondrial transcription (*Plromt*, *Tfam*, *Tfb2m*) and copy number of mt-DNA at each complex were significantly increased 24 hours after eCG stimulation and was maintained at 48 hours [Fig. 1(A) and 1(B), respectively].

The protein levels of both mt-ND1 and mt-ND6 were increased at 24 hours compared with levels before eCG injection, and mt-ND1 was further significantly increased, but mt-ND6 was unchanged, at 48 hours [Fig. 2(A) and 2(B)]. Cellular ATP content, mitochondrial activity, and membrane potential were measured in GCs of immature mouse ovaries stimulated with or without eCG for 24 or 48 hours and observed to be significantly increased at 24 hours after eCG injection and remained elevated after 48 hours [Fig. 2(C)–2(E)].

B. Mitochondrial Gene Expression and Protein Synthesis Increases in FSH-Stimulated GCs In Vitro

FT supplementation significantly increased the expression of protein-coding genes of all mitochondrial ETC complexes (*Nd1*, *Nd6*, *Cytb*, *Cox1*, *Atpase6*), and also of genes regulating mitochondrial transcription (*Polrmt*, *Tfam*, *Tfb2m*) (Fig. 3). Western blotting showed that the levels of mitochondria proteins mt-ND1 and mt-ND6 were significantly increased in the GCs cultured in DMEM/F12 medium supplemented with FT [Fig. 4(A) and 4(B)].

C. CRP Suppresses FT-Induced Mitochondrial Protein Synthesis, Membrane Potential, Activity, as Well as ATP Content, Proliferation, and Differentiation of GCs In Vitro

To understand the role of mitochondria in the proliferation of GCs during follicular development, CRP was added to the hormone (FSH and T4)–supplemented DMEM/F12 medium. Mitochondrial membrane potential, activity, and, consequently, ATP content and BrdU incorporation (indicating proliferation) were significantly increased by FT treatment. These inductions were significantly suppressed when GCs were cultured in medium additionally supplemented with CRP (FT-CRP) [Figs. 4(C) and 5(A)–5(C)]. The levels of mt-ND1 and mt-ND6 increased through FT treatment were significantly reduced by CRP supplementation [Fig. 4(A) and 4(B)]. The expression of marker genes of GC differentiation was examined. The expression of *Fshr*, *Inha*, *Inhba*, *Cyp19a1*, *Hsd17β1*, and *Lhcgr* was significantly increased by FT treatment, whereas additional CRP supplementation significantly suppressed this induction [Fig. 5(D)].

D. ATP Production for GC Proliferation In Vitro *Is Independent of Medium Glucose Concentration*

To understand the source of ATP supply for the proliferation and differentiation of GCs, we cultured GCs collected from eCG-primed mouse ovaries for 24 hours in media containing different concentrations of glucose: (i) high-glucose DMEM/F12 (17.51 mM D-glucose), (ii)

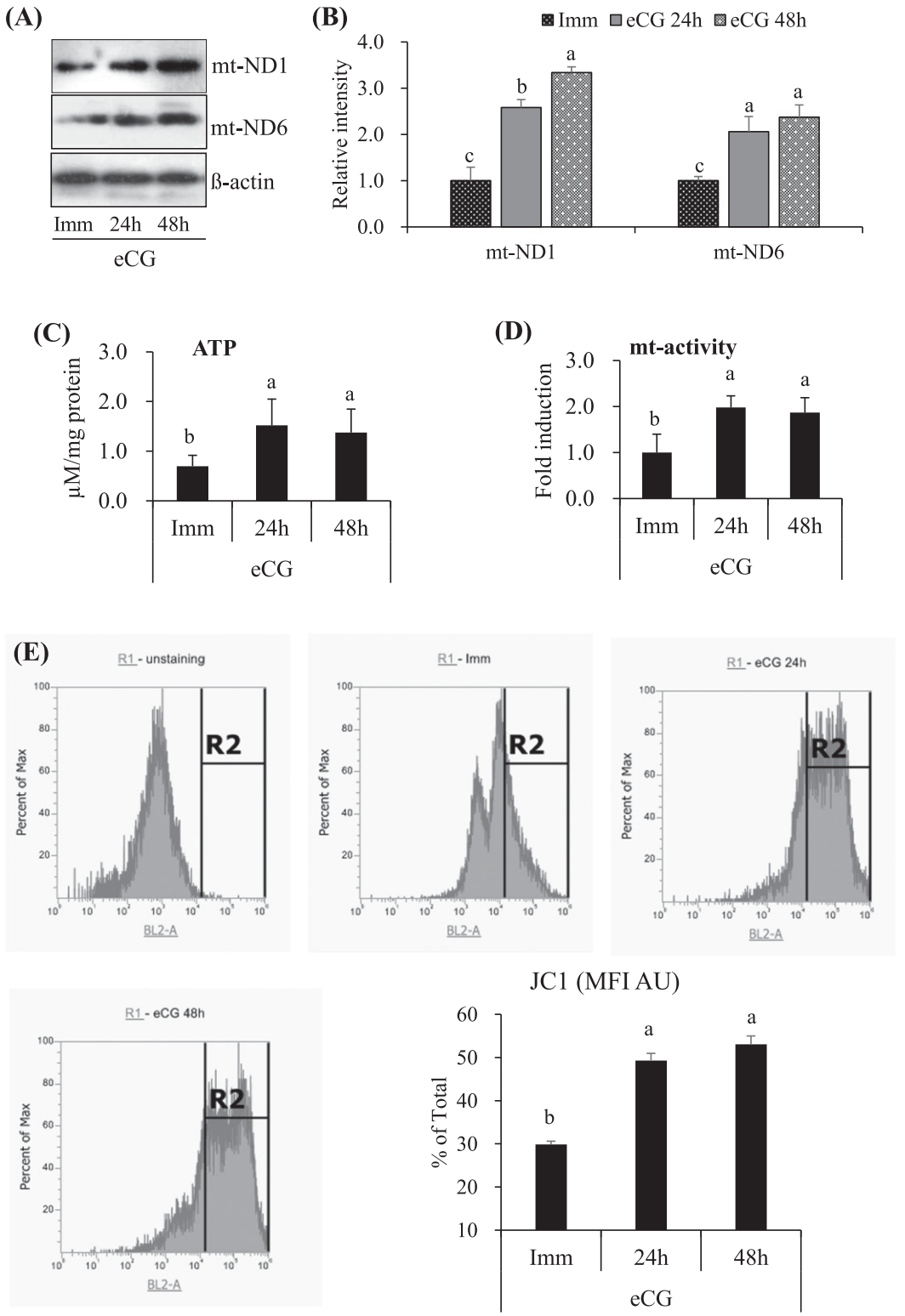

Figure 2. Changes of mitochondrial protein synthesis, cellular ATP content, mitochondrial activity, and membrane potential of GCs during follicular development *in vivo*. (A) Levels of mitochondrial proteins mt-ND1 and mt-ND6 were determined by western blotting where β-actin was used as control. (B) Quantitative expression of mitochondrial proteins mt-ND1 and mt-ND6 relative to β-actin in GCs. (C) Mitochondrial activity relative to total protein (mg). (D) ATP content (μM) relative to total protein content (mg) in GCs. (E) Mitochondrial

membrane potential by MitoPT JC-1 assay kit analyzed by flow cytometry using Attune NxT software at an excitation of 488 nm laser, and J-aggregates emit at 574/26 nm (BL2 channel). The immature (day 23) value was set as 1. For all data, GCs were collected from immature (23-d-old) mice 24 or 48 h after injection with or without eCG. Values are the mean ± SEM of three replicates. Bars with different letters (a, b, or c) differ significantly ($P < 0.05$).

DMEM/low glucose (5.56 mM D-glucose), and (iii) DMEM/no glucose. All media were supplemented with 1% (v/v) FCS and treated with or without FT or FT-CRP. There was no significant difference in proliferation and ATP content among GCs grown in the different media (Fig. 6), indicating that ATP production of GCs was not dependent on glucose content.

3. Discussion

An increase in cell number is dependent on intracellular proliferative activity, which requires a constant supply of energy [34, 35]. Although glycolysis and mitochondrial OXPHOS are two principal sources of cellular energy, numerous studies of the antral follicle indicate that glycolysis is the primary source of ATP in cumulus cells and that genes encoding glycolytic pathway proteins are poorly expressed in GCs [17–19]. Therefore, we hypothesized that mitochondria are the primary source of ATP and play a crucial role during proliferation and differentiation of GCs from antral follicles to preovulatory follicles. To test this hypothesis, we used both *in vivo* and *in vitro* models. Immature (23-day-old) mice ovaries contain a considerable number of antral follicles in addition to preantral follicles but no corpus luteum. Therefore, maximum follicles become responsive to FSH or eCG treatment and GCs can be harvested from the maximum number of follicles. In our *in vivo* study, the significant time-dependent increase in mitochondrial activity, membrane potential,

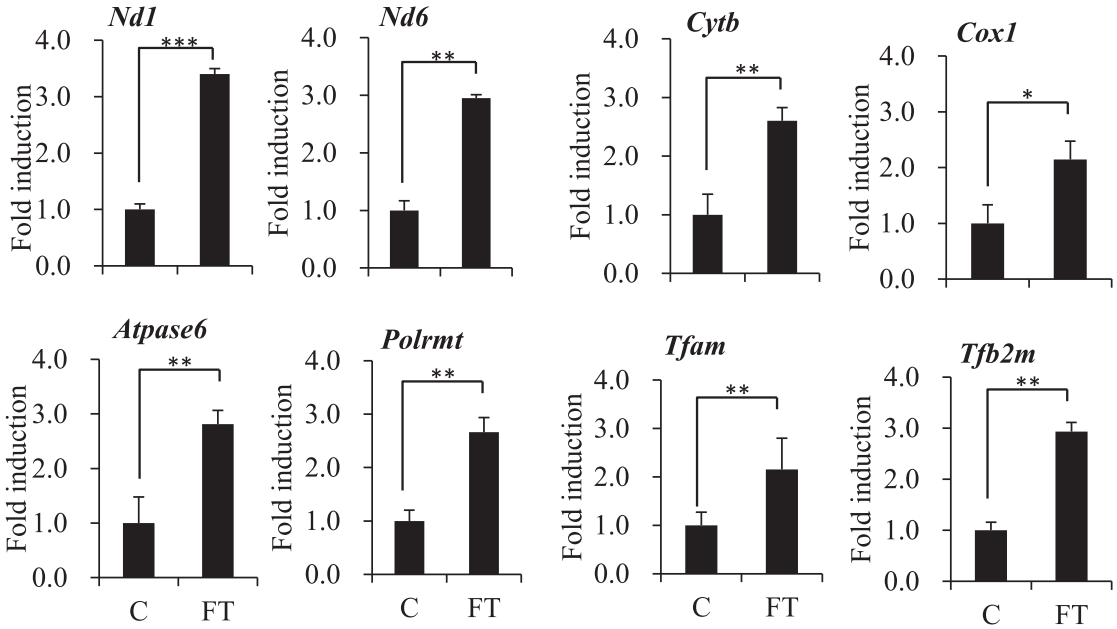

Figure 3. Changes in the expression of genes encoding mitochondrial proteins (*Nd1*, *Nd6*, *Cytb*, *Cox1*, *Atpase6*) and genes that regulate mitochondrial transcription (*Polrmt*, *Tfam*, *Tfb2m*) in GCs during follicular development *in vitro* using DMEM/F12 supplemented with serum plus FSH plus testosterone. GCs were collected from mouse ovaries 6 h after eCG priming and cultured for 24 h. The control value was set as 1, and the data are expressed as fold induction. Values are the mean ± SEM of three replicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

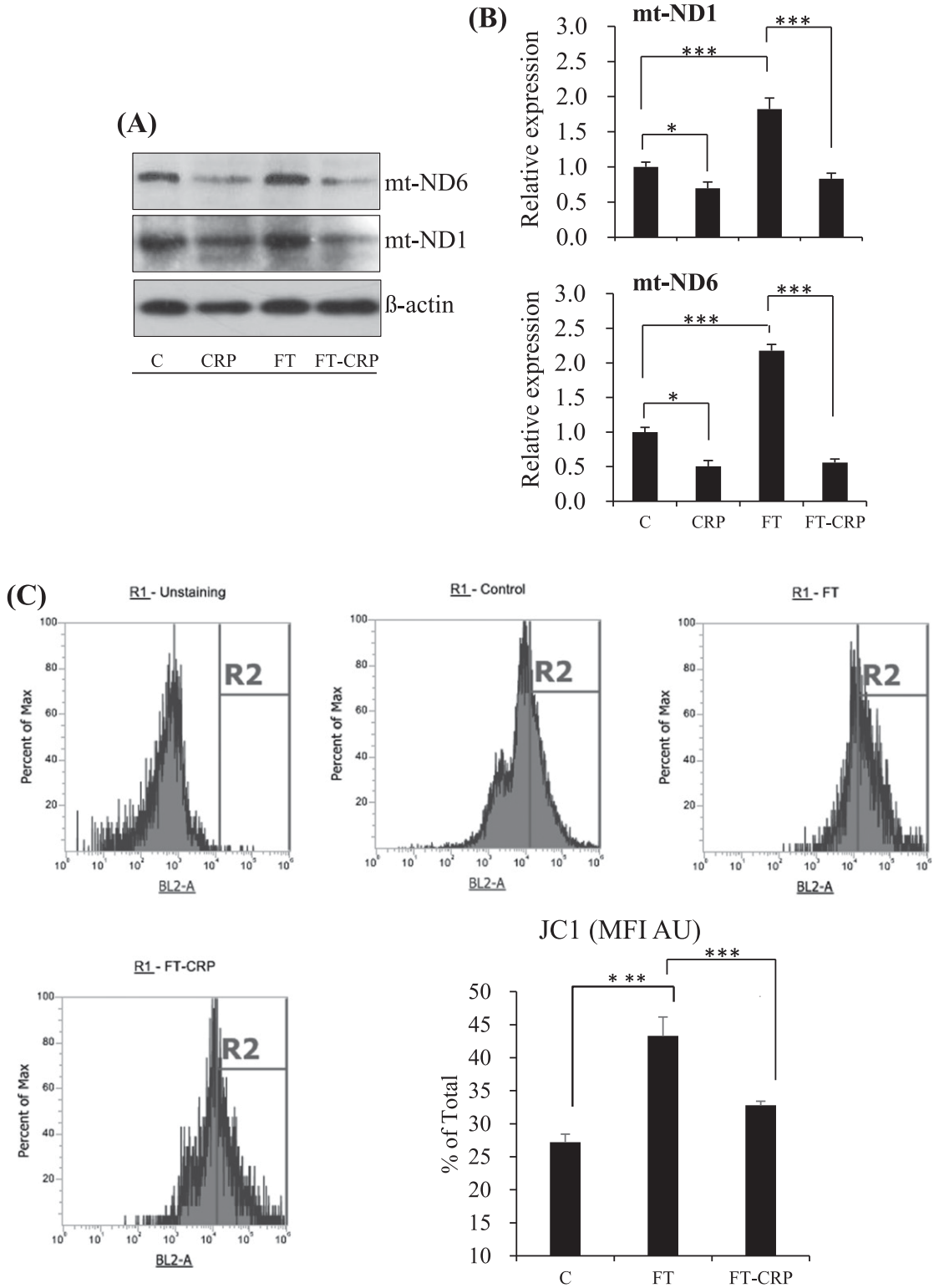

Figure 4. Changes in mitochondrial protein levels and membrane potential during *in vitro* culture of GCs. (A) Western blot image of mitochondrial proteins mt-ND1 and mt-ND6 where β-actin was used as control. (B) Quantitation of mt-ND1 and mt-ND6 relative to β-actin. (C) The mitochondrial membrane potential by MitoPT JC-1 assay kit analyzed by flow cytometry using Attune NxT software at an excitation of 488 nm laser, and J-aggregates emit at 574/26 nm (BL2 channel). GCs were collected from mouse ovaries 6 h after eCG priming and cultured for 24 h in 1% FCS-supplemented DMEM/F12 medium with or without CRP, FT, or

FT-CRP. The value of GCs without any treatment (C) was set as 1. All values are the mean ± SEM of three replicates. The following means were compared: C vs CRP, C vs FT, FT vs FT-CRP. *$P < 0.05$, ***$P < 0.001$.

expression of mitochondrial genes with the increase of copy number of mt-DNA, and levels of proteins indicate the importance of mitochondria during antral follicular development. Thus, the functions of mitochondria are dramatically increased in GCs during the follicular development process to preovulatory follicles owing to the increase of mitochondria number and/or the increase of activity in each mitochondria.

FSH is well known as the primary regulator of GC proliferation [36–38]. For a more clear understanding of the role of mitochondria, we induced GC proliferation *in vitro* by supplementing DMEM/F12 media with FSH. Interestingly, the mitochondrial activity, membrane potential, ATP content, gene expression, and protein synthesis were significantly increased in FSH-induced proliferating GCs. Therefore, mitochondrial activity, membrane potential, copy number, gene expression, and protein synthesis are important for ATP production during GC proliferation. Previously, mitochondrial functions and biogenesis were thought to be under the transcriptional regulation, but it is now thought that translation initiation is much more important for mitochondrial regulation [39, 40]. CRP is an ideal inhibitor of mitochondrial, but not cytoplasmic, protein synthesis, causing mitochondrial stress and decreased ATP biosynthesis [41–43]. We used CRP to clarify the importance of protein synthesis, and we observed that CRP not only significantly reduced protein levels but also drastically reduced mitochondrial activity, membrane potential, gene expression, and ATP content in GCs. Moreover, BrdU incorporation, indicating GC proliferation and expression of proliferation genes, was also significantly decreased by CRP treatment. Therefore, it is clear that activation of the translation process is crucial for mitochondrial activity and consequently for ATP production, which is required for GC proliferation. However, there has been insufficient information to clarify the role of mitochondria in GC proliferation and differentiation during follicular development.

To confirm the primary source of ATP during GC proliferation, we cultured GCs in DMEM media containing glucose at high or low concentrations or without glucose. Interestingly, ATP content and cell proliferation were independent of the glucose concentration. Mammalian follicular fluid contains a considerably lower level of glucose compared with that in blood serum. For example, the levels in mice are 0.05 to 0.10 vs 0.3 to 0.8 nM/μg protein [21], in humans are 3.0 to 3.39 vs 5.0 nM/L [22], and in cows are 2.01 to 3.75 vs 4.77 mM [23]. Therefore, we assume that mitochondria become active in proliferating cells during low glucose conditions and facilitate mitochondrial biogenesis and OXPHOS to produce ATP regardless of glycolysis. Several studies have also specified mitochondria as the predominant source of ATP despite active glycolysis for the proliferation of HeLa (human) and AS-30D (rodent) tumor cells [44–46].

Mitochondrial ATP is produced through a sequential redox reaction in the ETC complexes and reactive oxygen species are produced as byproducts that inhibit ATP synthesis [47] and induce oxidative damage of proteins [48]. Therefore, specific protein turnover mechanisms in mitochondria are required to minimize the effects of reactive oxygen species. Thirteen mt-DNA–encoded proteins, including several enzymes, such as reduced NAD dehydrogenase, succinate dehydrogenase, cytochrome *c* oxidase and reductase, and ATP synthase, are involved in the ETC complexes [45, 49], and mitochondria-specific transcription-related proteins POLRMT TFAM and TFB2M promote transcription of mitochondrial DNA [50]. Hence, it is suggested that mRNA expression and protein turnover in mitochondria are essential to maintain mitochondrial activity and ATP biosynthesis. However, mitochondrial dynamics and activities are regulated mainly by fusion and fission processes [51–53]. Unbalanced fusion and fission result in mitochondrial fragmentation, leading to improper transcription and translation. Consequently, mitochondrial activity is impaired and ATP biosynthesis is also reduced [54, 55]. Recently, Liu *et al.* [20] identified MIGA1/2 mitochondrial proteins and reported that *Miga1/2* knockout mice show disordered mitochondrial morphology and

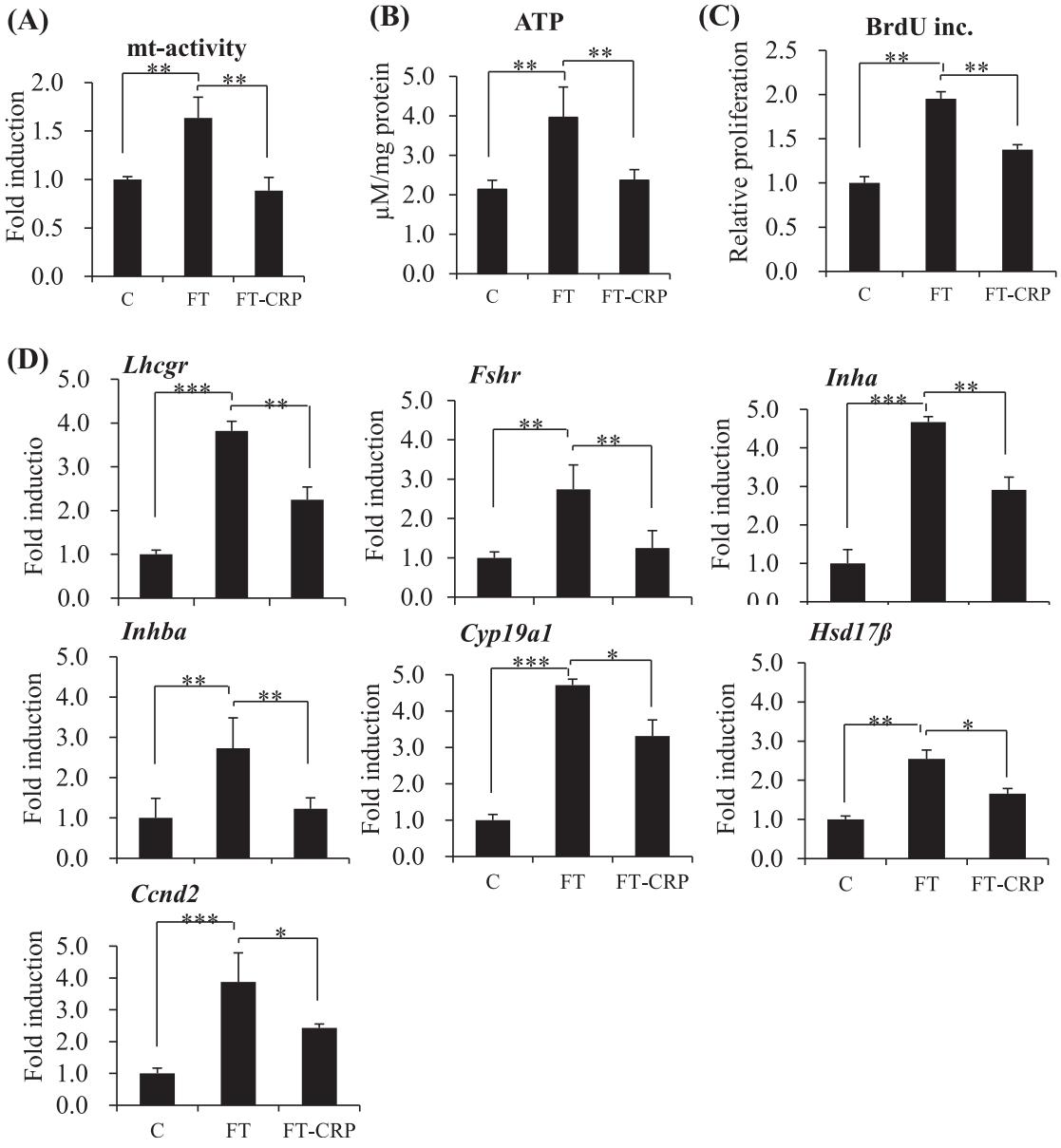

Figure 5. Effect of CRP on proliferation and differentiation of GCs *in vitro*. (A–C) Changes of (A) mitochondrial activity, (B) cellular ATP content (μM/mg protein), and (C) spontaneous proliferation (BrdU incorporation) by FT supplementation of DMEM/F12 medium with additional CRP treatment. (D) Temporal changes in the expression of genes involved in proliferation and differentiation (*Lhcgr*, *Fshr*, *Inha*, *Inhba*, *Cyp19a1*, *Hsd17β1*, *Ccnd2*) of GCs during *in vitro* culture. GCs were collected from mouse ovaries 6 h after eCG priming and cultured for 24 h in 1% FCS-supplemented DMEM/F12 medium with or without FT or FT-CRP. Levels of mRNAs were normalized against L19. Values are specified as the mean ± SEM of three replicates. In (A), (C), and (D), the values representing GCs without any treatment (C) were set as 1 and the data are expressed as fold induction/relative proliferation. The following means were compared: C vs CRP, C vs FT, and FT vs FT-CRP. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

functions in response to gonadotrophins. Their study supports a key finding of our study, that is, that mitochondrial protein synthesis is essential for normal mitochondrial activity and biogenesis.

Mitochondrial translation is important not only for proliferation but also for differentiation of GCs because CRP treatment not only significantly reduces proliferation but also the

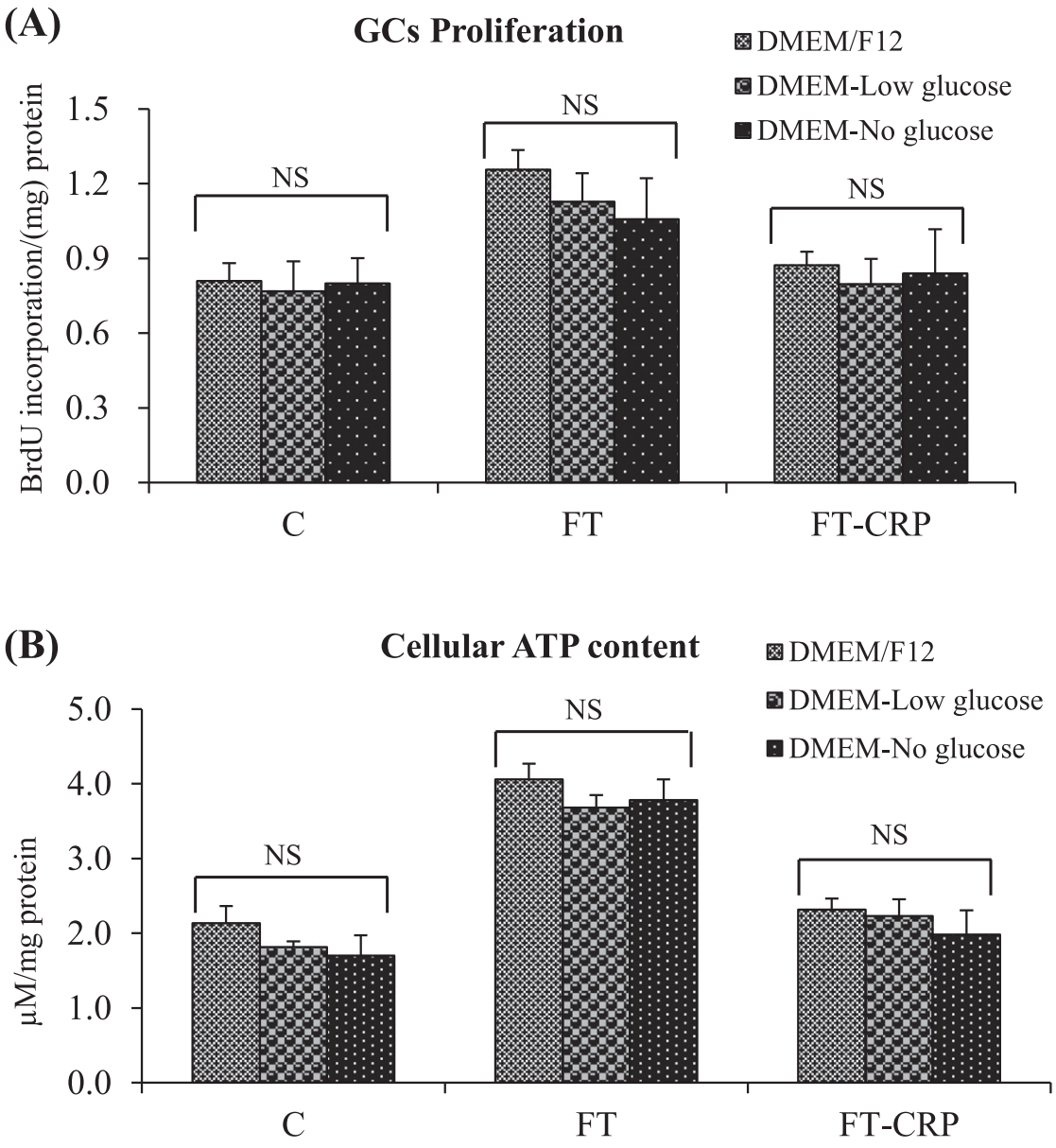

Figure 6. Mitochondria play a key role in ATP production and proliferation of GCs during follicular development. (A and B) Changes in (A) GC proliferation indicated by BrdU incorporation and (B) cellular ATP content in media containing different concentrations of glucose: (i) high-glucose DMEM/F12 (17.51 mM D-glucose), (ii) DMEM/low glucose (5.56 mM D-glucose), and (iii) DMEM/no glucose. GCs were collected from mouse ovaries 6 h after eCG priming and cultured for 24 h in different DMEM media supplemented with 1% FCS and treated with or without FT or FT-CRP. Results from the treated and untreated groups were compared among DMEM media. Values are the mean ± SEM of three replicates. NS, there is no significant difference among the different media.

expression of *Lhcgr*, a marker for differentiation. Several studies of estrogen receptor β mutant mice [5, 6] and mutant mice lacking *Ccnd2* [7, 8, 56] show a lower number of GCs and reduced expression of *Lhcgr* in GCs in the antral follicles, suggesting that proliferation might be necessary for differentiation of GCs. However, the underlying molecular mechanisms related to proliferation and differentiation in GCs are not clear. In activated B-cells, an S phase–synchronized differentiation switch is associated with extensive DNA demethylation and local acquisition of 5-hydroxymethylcytosine at enhancers and genes related to plasma

cell identity, suggesting that cell proliferation is involved in DNA methylation status during B-cell differentiation [57]. Kawai *et al.* [25] observed that eCG/FSH treatment significantly decreased the DNA methylation level in the *Lhcgr* promoter region with induction of *Lhcgr* expression *in vivo*. Our study shows that mitochondrial ATP production is necessary for eCG- or FSH-stimulated GC proliferation. Thus, we suggest that the GC proliferation that is dependent on mitochondrial ATP production is required for the reduced *Lhcgr* promoter methylation that is responsible for differentiation of GCs.

Therefore, we can conclude that mitochondria play a crucial role in GC proliferation and differentiation through essential ATP supply during antral follicular development to preovulatory follicles. In this developmental process, FSH plays a critical role in GC proliferation, but mitochondrial activity, membrane potential, and ATP production are critical for FSH-induced GC proliferation. Moreover, the activation of mitochondrial genes expression, increasing number of mtDNA, and protein synthesis are necessary for maintaining mitochondrial activity, membrane potential, and ATP synthesis. Nonetheless, the responsible ability of ovulation stimuli is led from the proliferation of granulosa cells. Thus, mitochondrial gene expression and protein synthesis are crucial for not only proliferation but also differentiation of GCs during antral follicular development to preovulatory follicles in mammalian ovaries. These findings highlight the physiological importance of mitochondria in GCs and contribute new insights into female fertility.

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