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The ovulatory up-regulation of ACE2, a receptor for SARS-COV-2, in dominant follicles of the human ovary.

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Abstract

Objective: To determine the temporal expression of ACE2, a receptor for SARS-COV-2, in dominant follicles across the periovulatory period in women and the regulatory mechanisms underlying *ACE2* expression in human granulosa/lutein cells (hGLC).

Design: Experimental prospective clinical study and laboratory-based investigation.

Setting: University Medical Center and private IVF center.

Patients: Thirty premenopausal women undergoing surgery for tubal ligation and 16 premenopausal women undergoing IVF.

Intervention (s): Administration of hCG and harvesting of preovulatory/ovulatory follicles by timed laparoscopy and collection of granulosa/lutein cells and cumulus cells at the time of oocyte retrieval.

Main Outcome Measures: Expression and localization of ACE2 in granulosa cells and dominant follicles collected across the periovulatory period of the menstrual cycle and in hGLC using qPCR, immunoblotting, and immunohistochemistry.

Results: *ACE2* expression (mRNA and protein) is up-regulated in human ovulatory follicles after hCG administration. *ACE2* expression was higher in cumulus cells than granulosa cells. hCG increased the expression of *ACE2* in primary hGLC cultures, but the increase was inhibited by both RU486 (an antagonist for progesterone receptor and glucocorticoid receptor) and CORT125281 (a selective glucocorticoid receptor), but not by AG1478 (an EGF-receptor tyrosine kinase inhibitor) or by dexamethasone.

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The authors have nothing to disclose.

Conclusions: The hormone-regulated expression of *ACE2* in granulosa cells suggests a potential role of ACE2 in the ovulatory process. These data also implicate the possible impact of COVID-19 in a vital cyclic event of ovarian function, thus women's overall reproductive health. However, SAR-COV-2 infection in ovarian cells *in vivo* or *in vitro* has yet to be determined.

Keywords

ACE2; ovulation; ovary; follicle; humans

INTRODUCTION

Since the beginning of the COVID-19 pandemic, heightened attention has been given to angiotensin-converting enzyme 2 (ACE2) as it acts as an entry receptor for SARS-COV-2, a novel strain of the virus that causes COVID-19 (1). However, this role of ACE2 in the viral cellular entry is in contrast to its original reported function as a component of the renin-angiotensin system (RAS).

Initially, ACE2 was discovered as a zinc metalloprotease that is capable of catalyzing angiotensin I (Ang I) and angiotensin II (Ang II) to angiotensin-(1–9) and angiotensin-(1–7), respectively (2,3). ACE2 displays high homology to human angiotensin-converting enzyme (ACE)(2,4). ACE is well known for its ability to convert inactive angiotensin Ang I into the functionally active hormone Ang II (5). The best-elucidated actions of Ang II include vasoconstriction, stimulation of aldosterone and vasopressin secretion, vascular smooth muscle proliferation, and renal tubular sodium reuptake, thereby regulating body fluid volume, electrolyte balance, and arterial pressure (6,7). However, these effects of Ang II are tightly controlled by ACE2 whose role is to limit locally available Ang II concentration by rapidly hydrolyzing Ang II to Ang-(1–7)(8). Ang-(1–7) has also been shown to enhance vasodilation and exert anti-oxidative, anti-inflammatory, and pro-resolution effects, likely through the activation of the G protein-coupled receptor MAS1 (MAS1 Proto-Oncogene)(9). Therefore, ACE2 functions as a key counter-regulatory enzyme in the RAS. In addition to the role in the RAS, ACE2 can cleave other peptide substrates such as neurotensin, kinetensin, des-Arg bradykinin, and Apelins (−13, −17, and −36)(2,10).

Besides these enzymatic functions, ACE2 was identified as a binding site for human coronavirus HCoV-NL63 and the human severe acute respiratory syndrome coronaviruses, SARS-COV and SARS-COV-2 (11). The spike proteins on the surface of SARS coronaviruses bind to ACE2 on its target cells, initiating the virus-cell membrane fusion and ultimately resulting in viral replication inside the host cells (11).

ACE2 mRNA was detected in most human tissues examined, yet the detection of ACE2 protein was limited in several tissues, including the lung, kidney, heart, and intestine (12). Specifically, the abundant *ACE2* expression was localized to lung alveolar epithelial cells, enterocytes of the small intestine, and vascular endothelial cells of the heart and kidney (13). The testis was also among the tissues that expressed high levels of *ACE2* (mRNA and protein)(14). In contrast, little is known about the expression pattern of *ACE2* in the human ovary. Even two public databases for protein expression profiles (Human protein atlas and GeneCards) contradict each other; the latter suggests the high expression of ACE2,

whereas the former indicates little to no expression. Meanwhile, there were a limited number of studies documenting the expression of *ACE2* in the ovary of animals (15,16). Pereira et al. have reported that *ACE2* expression was increased in theca/interstitial layer of the immature rat ovary when administrated with eCG to stimulate follicle development (16). In the bovine ovary, the follicular levels of *ACE2* mRNA were transiently downregulated during the ovulatory period (15). As a proxy indicator of A*CE2* expression, Ang II and Ang (1–7) were reported to be present in the ovary of multiple species (15–18). Previous studies showed that Ang II and Ang-(1–7) stimulated steroid hormone production and enhanced ovulation and oocyte maturation in rats, rabbits, and cattle (17,19–22). Similarly, Ang-(1–7) was detected in the follicular fluid of women undergoing IVF procedure; this heptapeptide level was correlated clinically with the oocyte maturation rate (23).

The ovary undergoes constant remodeling and cyclic ovulation that requires precisely controlled angiogenesis and an acute inflammatory response {reviewed in (24)}. Given that ACE2 has been shown to play an important role in both angiogenesis and inflammatory responses, it is conceivable that this enzyme is expressed and involved in these processes in the human ovary. Moreover, accumulating evidence indicates that SARS-COV-2 can infect multiple organs including the ovary (25), but the long-term health impact of COVID-19 is yet to be determined. Therefore, it is critically important to assess the expression of *ACE2* in the human ovary. In the present study, we sought to: 1) characterize the expression pattern of *ACE2* (mRNA and protein) using dominant follicles collected before the LH surge and throughout the periovulatory period from naturally cycling women with ovulation induced by hCG and 2) dissect the cellular regulatory mechanism controlling *ACE2* expression using primary human granulosa/lutein cells.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all reagents were purchased from Sigma Chemical Co. or Thermo Fisher Scientific.

Human tissue collection

The protocol using human tissues was approved by the Human Ethics Committee of the Sahlgrenska Academy at the University of Gothenburg, and all patients had given their informed written consent before participating. Whole follicles were collected from patients across the periovulatory period as previously described (26). Women (age 30–38 yr) exhibiting regular menstrual cycles and had not taken hormonal contraceptives for at least 3 months prior to their enrollment in the study underwent laparoscopic sterilization. Women were monitored by transvaginal ultrasound for 2 to 3 menstrual cycles before surgery to ascertain cycle regularity and to monitor the growth of a dominant follicle during the follicular phase. These patients were divided into 4 groups: pre-, early-, late- and post-ovulatory phases. In the preovulatory group, surgery was performed when the follicle reached >14 mm and ≤17.5 mm in diameter prior to the endogenous LH surge. These patients were not given hCG. The remaining women were given recombinant hCG (Ovitrelle, 250 μg) and were divided into three groups; early-ovulatory (surgery between

12 to 18h post-hCG) and late-ovulatory (surgery between 18 to 34h post-hCG). To confirm that these patients followed a normal hormonal pattern before the LH surge or after hCG administration, blood samples were taken at surgery and measured for serum progesterone and estradiol (27). The whole intact follicle/early corpus luteum was removed using laparoscopic scissors and processed for either immunohistochemical or gene expression analysis. The follicle was bisected, and mural granulosa cells were gently scraped off from the interior of the follicle by small tissue forceps. For the gene expression study, the follicular fluid and cell suspension were combined and centrifuged at 500 × g to pellet and collect granulosa cells. For the immunohistochemistry study, dominant follicles were fixed as described below.

Human granulosa/lutein cell (hGLC) cultures

Human granulosa/lutein cells were obtained from aspirates of IVF patients. The collection protocol was approved by the Institutional Review Board of the University of Kentucky Office of Research Integrity. Ovarian hyper-stimulation was induced by the administration of recombinant human FSH in individualized doses to patients at the Bluegrass Fertility Center (Lexington, Kentucky). IVF patients were then given with hCG (10,000U) on Days 9 to 11 when one lead follicle reaches 18 mm or if there are 2 lead follicles of 16 mm or greater in mean diameter, and dominant follicles were aspirated 36 h later. The experiments with hGLC were carried out as described previously (26,28). Briefly, immediately after retrieval of cumulus oocyte complexes, the remaining cells in aspirates were subjected to percoll gradient centrifugations to remove red blood cells. The cells from patients aged 24–40 with non-ovarian etiologies (e.g., male factor and egg donor) were used. The isolated cells were first examined under the microscope for their morphology, counted, and resuspended with OptiMEM media supplemented with 10% fetal bovine serum and antibiotic-antimycotic and then seeded onto culture plates (2.5×10^5 cells/ml). The cells were acclimatized for 6 days, changing media every 24 h. At the end of acclimation, the hGLCs were treated with or without hCG (1 IU/ml) in the absence or presence of various reagents described in the results section and cultured in OptiMEM media supplemented with antibiotic-antimycotic cultured for stated hours.

Immunohistochemistry

Follicles were fixed in 4% formaldehyde, embedded in paraffin, and sectioned (7 μm). Immunostaining was conducted in the Markey Biospeciment Procurement and Translational Pathology Shared Resource Facility at the University of Kentucky as previously described (28). Briefly, heat-induced epitope retrieval was performed in a Biocare Medical Decloaking chamber utilizing Dako's low pH Target Retrieval Solution. Primary antibody incubation was carried out at 4°C overnight for ACE2 (Sigma-Aldrich, HPA000288, 1:200 dilution) and for 2 h at room temperature for PECAM1 (Roche Diagnostics, JC70 monoclonal antibody, pre-dilute), respectively. Rabbit IgG was used in place of primary antibodies as a negative control. The antibody staining was detected using an appropriate Immpress alkaline phosphatase kit and Vector Red AP chromogen (Vector Laboratories).

Gene expression analysis

The levels of mRNA for *ACE2* was measured using a methodology previously described (28). Briefly, total RNA was isolated from granulosa cells using an RNeasy mini kit (Qiagen). The synthesis of the first-strand cDNA was performed by reverse transcription of 500 ng total RNA using superscript III. The levels of mRNA for genes examined were measured by qPCR using Brilliant 3 Ultra-Fast SYBR green (Stratagene). Oligonucleotide primers corresponding to *ACE2* were designed using Primer3 software (5'-GGTGGGAGATGAAGCGAGAG-3', 5'-ACATGGAACAGAGATGCGGG-3'). The relative abundance of the target transcript was normalized to internal reference genes (*GAPDH* for *in vivo* sample and *RNA18S5* for *in vitro* sample) as previously described (26,28) and calculated according to the $2^{-\Delta\Delta CT}$ method (29).

Western blot analysis

Nuclear extracts were isolated from cultured cells, denatured, run on a 10% polyacrylamide gel, and then transferred onto a nitrocellulose membrane as described previously (28). The membrane was incubated overnight at 4°C in 5% skim milk in Tris-buffered saline including 0.1% Tween-20 solution containing primary antibodies against ACE2 (Sigma-Aldrich, HPA000288, 1:100 dilution) and ACTB (Santa Cruz Biotechnology, sc-47778, 1:1,000 dilution). The blots were incubated with the respective secondary HRP-conjugated antibody for 1 h at room temperature. Peroxidase activity was visualized using the SuperSignal®West Pico Chemiluminescent Substrate (Pierce Chemical).

Statistical analyses

All data are presented as means ± SEM. Data were tested for homogeneity of variance by Levene's test, and log transformations were performed as appropriate. Paired sample *t*-test or analysis of variance (ANOVA) were used to test differences in levels of mRNA for *ACE2* between cell types or across the time of tissue collection, time of culture, or among treatments *in vitro*, as appropriate. If the test revealed significant effects, the means were compared by Duncan's test, with $p < 0.05$ considered significant.

RESULTS

The expression of ACE2 in dominant follicles during the ovulatory period

To determine whether *ACE2* is expressed and regulated in human preovulatory follicles during the ovulatory period, the levels of mRNA for *ACE2* were assessed in granulosa cells isolated from dominant follicles collected during the pre-, early, late, and post-ovulatory period. The levels of mRNA for *ACE2* were markedly increased in granulosa cells during both early and late ovulatory phases (> 40-fold) compared to those obtained before hCG administration (Fig. 1). After ovulation, the levels of *ACE2* mRNA in granulosa cells of post-ovulatory follicles were similar to those of dominant follicles collected at the early and late ovulatory phases, but higher than those of dominant follicles collected before hCG administration (Fig.1A). Next, to determine whether *ACE2* is also expressed in cumulus cells of ovulatory follicles, we collected granulosa cells and cumulus cells from women undergoing IVF procedures at the time of oocyte retrieval. As shown in Fig. 1B, we found

the abundant expression of *ACE2* in cumulus cells of ovulatory follicles. The levels of mRNA and protein for *ACE2* were higher in cumulus cells compared to those in granulosa cells from all patients tested.

To determine whether the increase in *ACE2* mRNA levels translated to ACE2 protein and what types of cells expressed ACE2, the dominant follicles collected throughout the periovulatory phases were analyzed by immunohistochemistry analyses. The staining for ACE2 was negligible in granulosa cells and theca cell layer of dominant follicles obtained before the endogenous LH surge (Fig. 2A). In the dominant follicle obtained during the early ovulatory phase, positive staining for ACE2 became evident in granulosa cells and theca cell layer (Fig. 2B). During the late ovulatory period, intensive staining for ACE2 was localized to both granulosa cells and theca layer of dominant follicles (Fig. 2C&D). Interestingly, some cells in granulosa and thecal layer showed more robust staining (arrows) compared to the rest of cells in late ovulatory follicles. This concentrated immunopositive staining of ACE2 was more prominent in the late ovulatory follicle that displays the morphological change typical of follicles immediately prior to ovulation (e.g., dispersed granulosa cells, dissolution of basement membrane, and thinning of theca interna layer, Fig. 2D). Beside granulosa and theca cell layers, intense staining for ACE2 was detected throughout the stroma layer of late ovulatory follicles (Fig. 2D, wavy arrow). After ovulation, the staining for ACE2 was persistent in both granulosa- and theca-lutein cells of the post-ovulatory follicle as well as in the stroma layer, showing intense staining in some, but not all follicular cells (arrows and wavy arrows in Fig. 2E&F).

In the ovary, endothelial cells and leukocytes infiltrate into the granulosa and theca cell layer of ovulating follicles during follicle transformation into the corpus luteum (30). With the current finding that a subpopulation of cells in the granulosa and theca cell layer exhibited intense staining for ACE2, we questioned whether ACE2 was expressed in infiltrating endothelial cells and leukocytes. Therefore, the serial sections of late and post-ovulatory follicles used to detect ACE2 protein were evaluated for PECAM1 (Platelet And Endothelial Cell Adhesion Molecule 1) known to be expressed on the surface of endothelial cells, platelets, monocytes, neutrophils, and some types of T-cells (31). As expected, the immunopositive staining for PECAM1 was localized to endothelial cells lining various types of blood vessels and leukocytes inside blood vessels surrounding the ovulatory and postovulatory follicles (Fig. 3A & E). PECAM1 staining was also detected in endothelial cells located between the granulosa and theca interna layer throughout the late ovulatory follicle (Fig. 3B, arrows) and theca lutein layer of the post-ovulatory follicle (Fig. 3F, arrows). However, this endothelial cell and leukocyte expression of PECAM1 in the ovulatory and post-ovulatory follicles was not co-localized to the distribution of ACE2 (arrows in Fig. 3F and H point the same location).

Regulation of ACE2 expression in human granulosa cells

To investigate the cellular mechanisms underlying the marked up-regulation of *ACE2* expression in periovulatory follicles after hCG administration *in vivo*, we utilized primary human granulosa/lutein cells (hGLC) that were acclimated in cultures for 6 days without any hormone treatments to regain hCG responsiveness. As shown in Fig. 4A, we found that

hCG treatment increased the expression of *ACE2*, both at the levels of mRNA and protein compared to those in control samples, similar to those observed during the peri-ovulatory period of the menstrual cycle.

Next, to determine whether the hCG-induced increases in *ACE2* expression were mediated by key ovulatory mediators such as the EGF-receptor signaling, P4/PGR, or glucocorticoids/NR3C1, the hGLCs were treated with or without hCG in the absence or presence of AG1478 (an EGF-receptor inhibitor), RU486 (a dual antagonist for progesterone receptor and glucocorticoid receptor), CORT123567 (a selective NR3C1 inhibitor), or dexamethasone (a synthetic glucocorticoid). As expected, hCG increased *ACE2* expression, but the hCG-induced increases were completely abolished by RU486 at both 24 and 36 h (Fig. 4B). CORT125281 treatment also resulted in a partial reduction of hCG-induced *ACE2* expression at both time points. In contrast, neither AG1478 nor dexamethasone had effects on basal and hCG-induced *ACE2* expression.

DISCUSSION

In women throughout their reproductive age, the ovary undergoes a series of cyclic changes during the menstrual cycle, with the culmination being ovulation. The preovulatory LH surge initiates these cyclic changes by inducing the expression of specific genes. The encoded products of these ovulatory genes exert their biological actions to bring about necessary cellular and extracellular changes required for ovulation and corpus luteum (CL) formation. In the present study, we revealed that the expression of ACE2, an enzyme with carboxypeptidase activity and a primary receptor for SARS-COV-2, is rapidly and dramatically induced after hCG administration in dominant follicles obtained from naturally cycling women throughout the periovulatory period. Using the primary hGLC model that can recapitulate key ovulatory changes in gene expression, this study further provided experimental evidence that the ovulatory induction of *ACE2* expression was mediated by hCG and hCG-induced steroid hormones, progesterone and glucocorticoid, in ovulatory follicles. Noteworthy is also our finding of the higher expression of *ACE2* in cumulus cells than granulosa cells collected immediately prior to ovulation. Together, not only does this novel information suggest the potential involvement of ACE2 as a critical enzyme for the LH surge-induced cyclic events of ovulation, cumulus expansion, oocyte maturation, and luteal formation, but also implicate the possible impact of COVID-19 in vital cyclic ovarian functions, thus women's overall reproductive health.

Our comprehensive *in vivo* study is novel in that these data are: 1) the first report documenting the ovulatory induction of *ACE2* expression in naturally cycling women and 2) the only report showing the dramatic and rapid up-regulation after ovulatory induction among any species studied so far. In cattle, *ACE2* mRNA levels were initially down-regulated in granulosa cells after the LH surge induction, but then increased back to the preovulatory level at 24 h after GnRH injection, while no changes were observed in thecal levels of *ACE2* mRNA throughout the periovulatory period (15). No significant changes in *ACE2* expression pattern was observed in preovulatory follicles obtained before and throughout the ovulatory period in the non-human primate (Supplementary Fig. 1)(32). In the mouse ovary, *ACE2* mRNA profile showed low abundance without a clear pattern

throughout follicular development and the ovulatory period (http://okdb.appliedbioinfo.net, Supplementary Fig. 2). Therefore, our data indicate that the dramatic up-regulation of *ACE2* expression is specific to human ovulatory follicles and further points to the differences in specific mechanisms underlying the ovulatory process among different species. Of note, in the present study, hCG (rhCG for *in vivo* and urinary hCG for *in vitro* studies) was given to patients to mimic the endogenous LH surge. This was to control exactly when the ovulatory trigger was initiated in individual patients. Therefore, it remains to be determined whether the endogenous LH surge and hCG administration would exert the same effect on *ACE2* expression.

Another intriguing finding is the unique localization pattern for ACE2 protein observed in human ovulatory follicles. The initial induction of ACE2 staining was localized evenly throughout the granulosa and theca cell layer during the early ovulatory phase, and then, the staining became more intense and progressively more sporadic among cells when the dominant follicle progressed toward ovulation and transformed into the CL. The uneven, localized staining of ACE2 did not appear to be in non-follicular cells such as endothelial cells or leukocytes when compared to PECAM1 staining pattern in serial sections of the same follicle. Consistent with this finding, qPCR analysis of *ACE2* mRNA levels showed little to undetectable levels of *ACE2* mRNA in leukocytes compared to granulosa cells (Supplementary Fig.3). One possible explanation for this unique localization pattern might be that ACE2 exists in two different forms: the membrane-bound form and secreted form (33). During the early ovulatory phase when the level of *ACE2* mRNA was rapidly up-regulated, ACE2 would have been processed and expressed as a single-pass type I membrane protein. Then, with the progression toward the late and post-ovulatory phases, the enzymatically active extracellular domain of ACE2 could be cleaved, released, and aggregated onto specific cells or the area where the substrates for ACE2 are present. This possibility needs to be explored in future studies.

To explore the cellular mechanism underlying ovulatory *ACE2* expression, we have utilized granulosa cells pooled from multiple follicles of each patient obtained during IVF oocyte retrieval. Despite the potential limitations of pooling granulosa cells that may have come from different development stages of follicles, these cells (hGLC) increased the expression of genes involved in the ovulatory process, including PGR and EGF-like peptides and the production of progesterone and prostaglandins in response to hCG (28). Similarly, consistent with the *in vivo* expression pattern, hCG stimulated *ACE*2 expression in hGLC *in vitro*. Furthermore, this induction was completely inhibited by RU486, a dual antagonist for PGR and NR3C1 and partially by CORT125281, a selective antagonist for NR3C1. Our pilot study showed that hCG increased *NR3C1* expression and cortisol production in hGLCs (Supplementary Fig. 4). These data, taken together, indicate that the up-regulated expression of *ACE2* was mediated by the actions of progesterone and glucocorticoids in granulosa cells of the ovulatory follicle. Similar to our findings from granulosa cells, a recent study by Chadchan et al. reported that progesterone promoted *ACE2* expression in the endometrial stroma of both humans and mice (34). Meanwhile, the existing data on the regulation of *ACE2* expression by glucocorticoids or its receptor appear to be dependent on cell types and models tested (35,36). Therefore, our findings of the regulatory mechanisms involved in

ACE2 expression provide new insights into the ovulatory process and valuable data that may be useful in understanding SARS-COV-2 infection in reproductive-age women.

In normal physiology, ACE2 is best known for its ability to hydrolyze Ang II to Ang (1–7) (8,9). Therefore, the marked increases in *ACE2* expression could mean the shift in the balance between Ang II and Ang-(1–7), reducing Ang II levels while increasing Ang-(1–7). Both Ang II and Ang-(1–7) are present in the follicular fluid collected at the time of IVF in women (18). Studies using animal models have shown that Ang II and Ang-(1–7) stimulated steroid production, regulated blood flow, and promoted oocyte maturation and ovulation (20,37–39). Therefore, the current findings showing rapid induction of *ACE2* expression in human ovulatory follicles and high expression of *ACE2* in cumulus cells support the notion that Ang II/ACE2/Ang-(1–7) is likely involved in cumulus expansion, oocyte maturation, and the ovulatory process in the human ovary.

With the current global challenge with COVID-19, our findings take on another level of significance since ACE2 serves as a primary entry receptor for SARS-CO-2. A recent study has reported *ACE2* expression in oocytes, cumulus, and granulosa cells collected from patients at the time of IVF procedure (40). In that study, the expression of *TMPRSS2*, *CD147*, and *CTSL* was also documented in these ovarian cells as factors involved in SARS-COV-2 infection (40). Therefore, our current findings showing the rapid and dramatic induction of *ACE2* expression, together with the findings from the previous study (40), offer a compelling possibility that the ovary could be a target of SARS-COV-2, particularly vulnerable during the periovulatory period. In the scenario of SARS-COV-2 infection in the ovary, this virus could elicit negative impacts on female fertility, not only by blocking the physiological action of ACE2 necessary for oocyte maturation, ovulation, and CL formation but also destroying a mature oocyte and ovulatory follicle. In support of these possibilities, Orvieto et al. (41) reported that the proportion of top-quality embryos was significantly reduced in couples undergoing consecutive IVF cycles after recovering from COVID-19 infection compared to that obtained before the infection. Therefore, it will be vital to give adequate attention to the reproductive health to women who had COVID-19 and appropriate precautions and screening to women seeking IVF treatments. Once more data and information becomes available, it will be of great interest to assess the impact of COVID-19 on female fertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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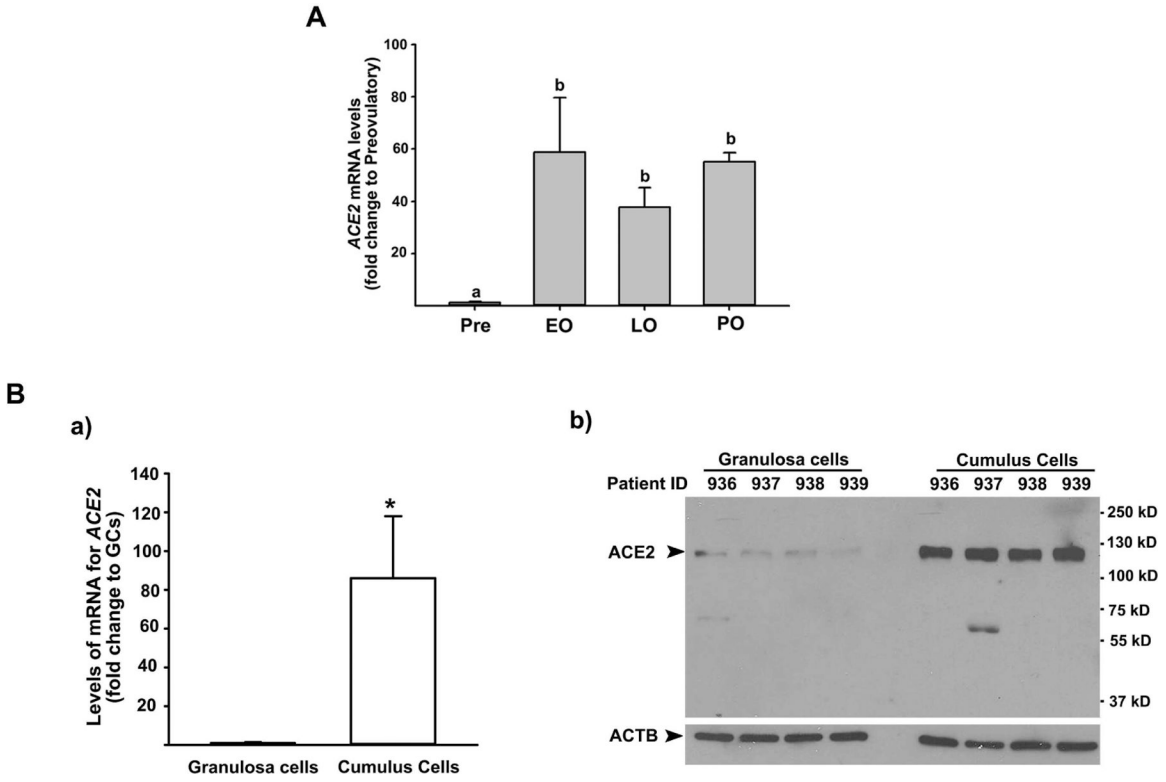

Figure 1. The levels of mRNA for *ACE2* in granulosa cells and cumulus cells of periovulatory follicles.

A) Dominant follicles were retrieved from the ovaries of women undergoing laparoscopic tubal sterilization before the LH surge or at various times after recombinant hCG administration and divided into four phases: pre- (Pre, n=6), early (EO, n=5), late (LO, n=6), and post- (PO, n=2) ovulatory phases. The levels of mRNA for *ACE2* were measured using qPCR in granulosa cells isolated from a dominant follicle collected at Pre, EO, and LO and whole follicles retrieved at PO and normalized to the levels of *GAPDH* mRNA in each sample. The levels were presented as fold change to Pre values. Bars with no common superscripts are significantly different ($p < 0.05$). B) Cumulus cells and granulosa cells were collected at the time of oocyte retrieval from women undergoing a standardized IVF procedure. a) The levels of mRNA for *ACE2* were measured by qPCR and normalized to the levels of *RNA18S5* in each sample (n = 5 independent samples). * $p < 0.05$. b) A representative Western blot image detecting ACE2 protein. Samples loaded were from independent patients indicated. ACTB detection in each lane was used as a protein loading control.

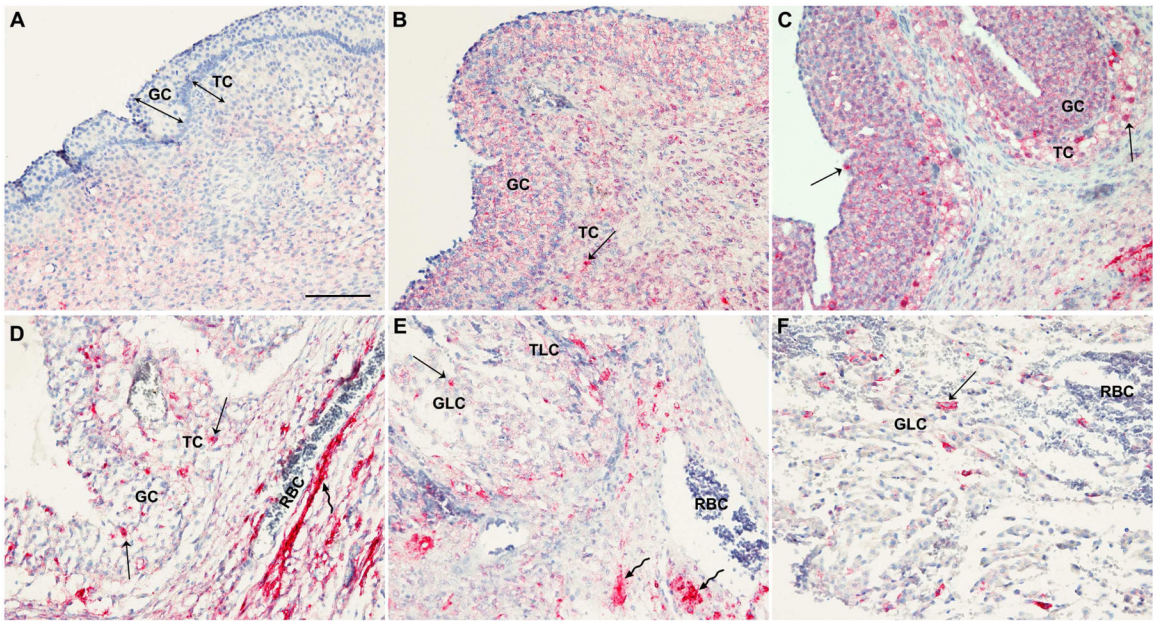

Figure 2. The expression pattern of ACE2 protein in periovulatory follicles

Dominant follicles were retrieved from the ovaries of women undergoing laparoscopic tubal sterilization before the LH surge or at various times after recombinant hCG administration. The paraffin embedded sections of dominant follicles obtained from various time points (A, preovulatory phase n=2; B, early ovulatory phase n=4; C & D, late ovulatory phase n=4; E, late ovulatory phase n=1) were subjected to immunohistochemical analyses to detect ACE2 protein. Sections in C & D are from different patients obtained from the late ovulatory period. F is an antrum region of the postovulatory follicle shown in the panel E. Pink/red staining indicates positive signals for ACE2. All sections were lightly stained with hematoxylin (blue staining) for nuclear counterstaining. Arrows point to granulosa and theca cells where the intense staining for ACE2 is detected. Wavy arrows point to ACE2 staining in the stroma layer. GC, granulosa cells; TC, theca cells; GLC, granulosa lutein cells; TLC, theca lutein cells; RBC, red blood cells. Scale bar in A; 100 μm

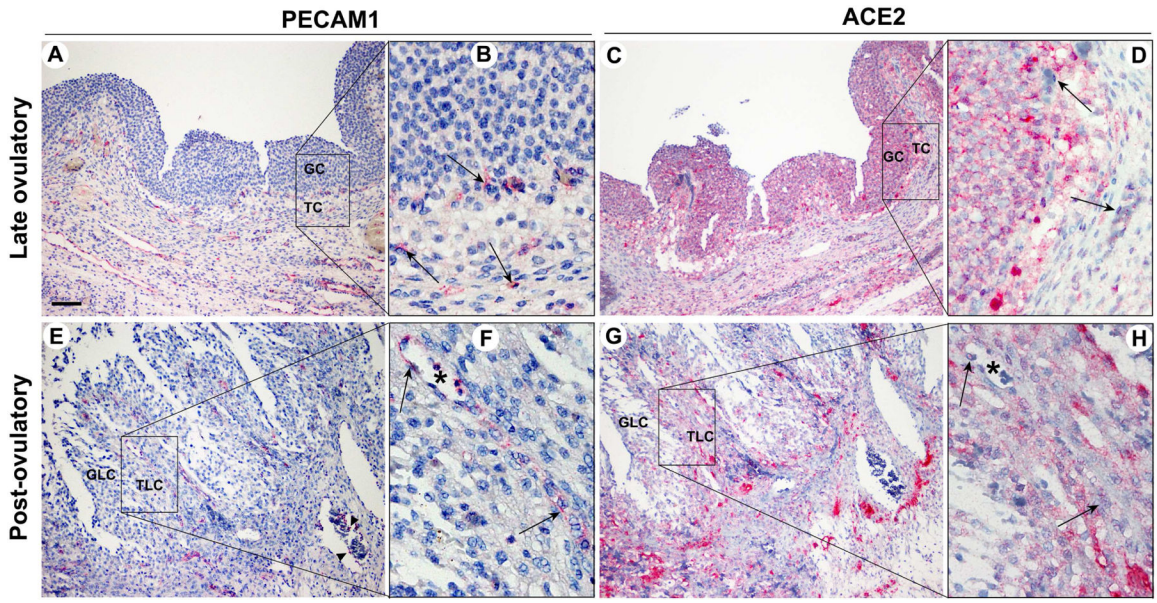

Figure 3. The comparison of the expression pattern between PECAM1 and ACE2 in late ovulatory and post-ovulatory follicles

Dominant follicles were retrieved from the ovaries of women undergoing laparoscopic tubal sterilization. The serial sections of dominant follicles obtained during the late (A & B) and post (C & D) ovulatory phase were subjected to immunohistochemical analyses to detect PECAM1 and ACE2. Square bars in A, C, E, and G are amplified in B, D, F, and H, respectively. PECAM1 was used to detect endothelial cells; it also stained a subpopulation of leukocytes. Asterisks in F and H were used to locate the same structure in the serial section of the same post-ovulatory follicle. Pink/red staining indicates positive signals for PECAM1 and ACE2. All sections were lightly stained with hematoxylin (blue staining) for nuclear counterstaining. Arrows (B, D, F, H) and arrowheads (E) point to endothelia cells and leukocytes stained with PECAM1, respectively. GC, granulosa cells; TC, theca cells; GLC, granulosa lutein cells; TLC, theca lutein cells. Scale bar in A; 100 μm

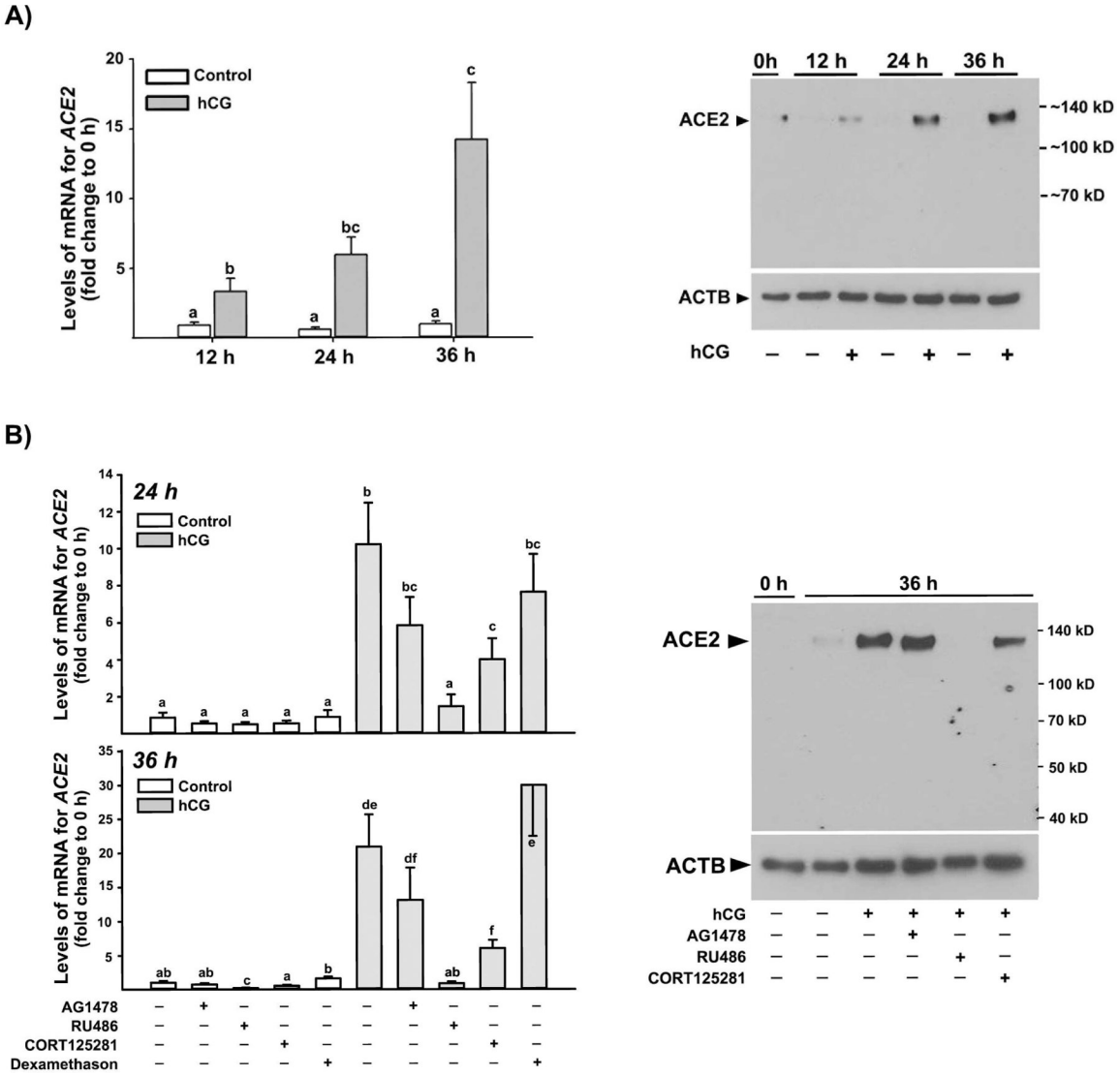

Figure 4. Regulation of *ACE2* expression in human granulosa cells

Human granulosa/lutein cells (hGLC) obtained from women undergoing a standardized IVF procedure were acclimated for 6 days. A) hGLCs were treated without (Control) or with hCG (1IU/ml) for 0, 12, 24, and 36 h. Right panel) the levels of mRNA for *ACE2* were measured by qPCR and normalized to the levels of *RNA18S5* in each sample (n ≥7 independent samples). Bars with no common superscripts are significantly different ($p < 0.05$). Left panel) a representative Western blot image detecting ACE2 protein. ACTB detection in each lane was used as a protein loading control. The experiments were repeated 3 times, each with independent samples. B) The cells were treated without (Control) or with hCG in the presence or absence of AG1478 (10μM), RU486 (20 μM), CORT123567 (50 μM) or dexamethasone (20 μM) for 24 and 36 h. Right panel) the levels of mRNA for *ACE2* were measured by qPCR and normalized to the levels of *RNA18S5* in each sample (n = 7 independent patient samples). Bars with no common superscripts are significantly different ($p < 0.05$). Left panel) a representative Western blot image detecting ACE2 protein. ACTB

detection in each lane was used as a protein loading control. The experiments were repeated three times, each with independent patient samples.