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Human eggs, zygotes, and embryos express the receptor angiotensin 1-converting enzyme 2 and transmembrane serine protease 2 protein necessary for severe acute respiratory syndrome coronavirus 2 infection

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Objective: To study messenger ribonucleic acid (mRNA) and protein expressions of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry receptors (angiotensin 1-converting enzyme 2 [ACE2] and CD147) and proteases (transmembrane serine protease 2 [TMPRSS2] and cathepsin L [CTSL]) in human oocytes, embryos, and cumulus (CCs) and granulosa cells (GCs).

Design: Research study.

Setting: Clinical in vitro fertilization (IVF) treatment center.

Patients: Patients undergoing IVF were treated at the Colorado Center for Reproductive Medicine.

Interventions: Oocytes (germinal vesicle and metaphase II [MII]) and embryos (1-cell [1C] and blastocyst [BL]) were donated for research at the disposition by the patients undergoing IVF. Follicular cells (CC and GC) were collected from women undergoing egg retrieval after ovarian stimulation without an ovulatory trigger for in vitro maturation/IVF treatment cycles.

Main Outcome Measures: Presence or absence of ACE2, CD147, TMPRSS2, and CTSL mRNAs detected using quantitative reverse transcription polymerase chain reaction and proteins detected using capillary Western blotting in human oocytes, embryos, and ovarian follicular cells.

Results: The quantitative reverse transcription polymerase chain reaction analysis revealed high abundance of *ACE2* gene transcripts in germinal vesicle and MII oocytes than in CC, GC, and BL. ACE2 protein was present only in the MII oocytes, and 1C and BL embryos, but other ACE2 protein variants were observed in all the samples. TMPRSS2 protein was present in all the samples, whereas mRNA was observed only in the BL stage. All the samples were positive for CD147 and CTSL mRNA expressions. However, CCs and GCs were the only samples that showed coexpression of both CD147 and CTSL proteins in low abundance.

Conclusions: CCs and GCs are the least susceptible to SARS-CoV-2 infection because of lack of the required combination of receptors and proteases (ACE2/TMPRSS2 or CD147/CTSL) in high abundance. The coexpression of ACE2 and TMPRSS2 proteins in the MII oocytes, zygotes, and BLs demonstrated that these gametes and embryos have the cellular machinery required and, thus, are potentially susceptible to SARS-CoV-2 infection if exposed to the virus. However, we do not know whether the infection occurs in vivo or in vitro in an assisted reproductive technology setting yet. (Fertil Steril Sci® 2021;2:33–42. ©2021 by American Society for Reproductive Medicine.)

Key Words: SARS-CoV-2, human IVF, oocytes and embryos, ovarian cells

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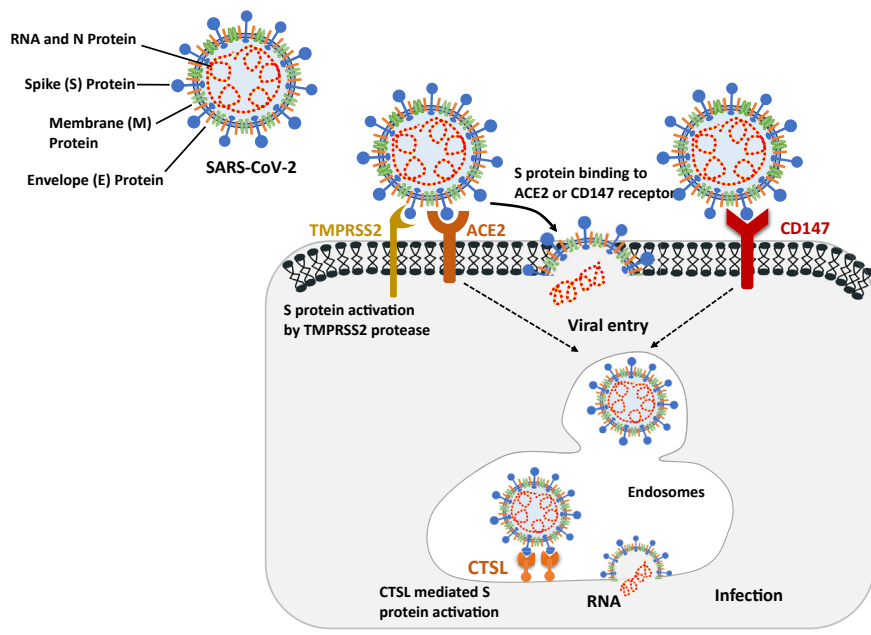
Since the description of a novel coronavirus in Wuhan, China, in late 2019, the virus, now called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread worldwide, affecting 188 countries. More than 30 million people have been infected worldwide, and nearly 1 million people have died because of coronavirus disease 2019 (COVID-19), the disease caused by SARS-CoV-2 (1). Initially, assisted reproduction was also curtailed, being classified as nonessential in most instances, because countries rapidly shut down to control the spread of the novel coronavirus and avoid overburdening healthcare systems. Very little was known about potential of infection of the sperm, eggs, or embryos; potential of sexual transmission between partners; vertical transmission between a mother and conceptus; or possible effects of maternal infection on an early embryo and fetus. This lack of information raised an important concern in the field about how the virus might potentially affect laboratory and pregnancy outcomes in in vitro fertilization (IVF), in addition to speculation about potential changes in standard operating procedures in an IVF laboratory during the pandemic (2, 3).

As the pandemic continued, impact on couples desiring treatment increased. If their treatment continued to be delayed, specific groups of infertile patients faced significantly reduced prognoses (4). However, the unknown effects of

SARS-CoV-2 on reproduction made it difficult to determine the best IVF laboratory practices necessary to mitigate the risks of contamination and infection to both patients and staff. For example, the current IVF practice often involves breaching the zona pellucida for biopsy and genetic testing, presenting a potential opportunity for the virus to gain access to embryonic cells both during culture before implantation and cryostorage. Therefore, it is of great importance that we advance our understanding of the potential of infection by SARS-CoV-2 in gametes and embryos.

The role of proteins associated with SARS-CoV-2 host cell entry is outlined in Figure 1. Although the understanding of the molecular mechanism of viral entry continues to get refined, the described mechanism involves viral spike (S) protein-mediated recognition and binding to the host cell's receptor angiotensin 1-converting enzyme 2 (ACE2), after which the protease transmembrane serine protease 2 (TMPRSS2) cleaves S protein to facilitate cell entry (5). ACE2, a negative regulator of the renin-angiotensin system, has multiple physiologic functions and is widely expressed throughout the body (6). The S protein cleavage site in SARS-CoV-2 is identical to that in SARS-CoV, which is also known to take advantage of the endosomal cysteine protease cathepsin L (CTSL) to cleave specific peptide bonds and facilitate fusion of viral and host cell membranes and release of

FIGURE 1



Role of ACE2 and CD147 receptors and TMPRSS2 and CTSL proteases in SARS-CoV-2 entry into host cells. The primary pathway of SARS-CoV-2 to enter host cells is by S protein binding to the ACE2 receptors, which leads to either cell membrane fusion and release of the viral genome if activated by TMPRSS2 or endocytic uptake of the virus. CD147 is another host cell receptor that can be recognized by the SARS-S protein, which facilitates viral entry through endocytosis. After viral entry into the host cells via an endosome, viral RNA is released into the cytoplasm because of pH-dependent cleavage by CTSL and start encoding viral protein. Figure adapted from Heurich et al (2014) and Aguiar et al (2020). ACE2 = angiotensin 1-converting enzyme 2; CTSL = cathepsin L; S = spike; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; TMPRSS2 = transmembrane serine protease 2.

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the viral genome into the host cell's cytoplasm (7, 8). An alternative ACE2-independent mechanism, with basigin (BSG or CD147) as the cellular receptor and CTSL as the protease, may also mediate SARS-CoV-2 cell entry. Theoretically, tissues and cells that contain these receptors and proteases, particularly high coexpression of ACE2 and TMPRSS2, are more vulnerable to infection by SARS-CoV-2.

Scientifically, information on the effects of clinical application of assisted reproductive technologies (ARTs) continues to emerge. A popular method that quickly provides the needed information involves querying existing transcriptomic databases for the presence of the receptors and proteases necessary for SARS-CoV-2 infection of host cells. Gene expression studies have shown that the SARS-CoV-2 receptor *ACE2* gene is transcribed in the ovary, where it is thought to regulate follicle development, hormone secretion, and oocyte maturation (9, 10). Another study has found that *ACE2* is expressed in the ovary but only at low levels and that *TMPRSS2* is not expressed, although *CTSL* is widely expressed; however, no cells coexpressed *ACE2* and *CTSL* (11). Thus, the existing transcriptomic information does not provide much clarity on the potential of infection of the ovary and oocyte. The possibility remains that SARS-CoV-2 infection might affect female fertility by directly infecting ovarian granulosa cells (GCs) and/or oocytes, thereby negatively affecting ovarian function and reducing oocyte quality (12). Analysis of existing single-cell ribonucleic acid (RNA) sequencing datasets has also revealed the presence of transcripts of SARS-CoV-2-related genes, including the receptors *ACE2* and *BSG* and the proteases *TMPRSS2* and *CTSL*, in human embryos (13), suggesting that they may also have the potential to be infected by SARS-CoV-2. The fallopian tube expresses *ACE2* in a small number of cells, but the protease *TMPRSS2* is widely expressed in different cell types, although there was no detectable coexpression of *ACE2* and *TMPRSS2* (11), suggesting that the site of fertilization and early embryonic development is unlikely to be infected. In the uterus, *ACE2* is expressed at a low level, but *TMPRSS2* expression is absent, again suggesting that infection is unlikely (11). In periimplantation- and postimplantation-stage human embryos, the transcripts of *ACE2* and *TMPRSS2* are coexpressed in 6- and 7-day trophoblasts, 12- and 14-day syncytiotrophoblasts, and some hypoblast cells; *TMPRSS2* alone is expressed in the epiblast (14). *BSG* and *CTSL* transcripts are expressed in all cell types at this stage (14). This again suggests the susceptibility of the human embryo to SARS-CoV-2 infection and its potential implications on implantation and pregnancy and the potential transmission of the virus from a mother to the fetus through the placenta. However, the transcript levels often do not accurately reflect protein abundance, and this has also been demonstrated for *ACE2* (15). In fact, an analysis comparing publically available proteomic and transcriptomic databases has shown that despite its low messenger RNA (mRNA) level, *ACE2* protein is abundant in the ovary (15). This is not terribly surprising because processes such as post-transcriptional, translational, and protein degradation processes occur after the mRNA is transcribed, leading to differences of as much as 15%–70% between mRNA levels and protein abundance (16, 17). Because of scarcity of human

sample availability, protein expression analyses in oocytes, embryos, and follicular cells rely on immunohistochemistry (IHC). In addition to potential false-positive results due to antibody cross reactivity with similar proteins, IHC has been recognized for its false-negative results since its inception (18–21). A recent study (22) has reported that IHC fails to detect *ACE2* protein expression in either follicular or stromal ovarian cells, but high expression of *ACE2* protein is detected by mass spectrometry. Western blot is a gold-standard technique to study protein expression because it reveals molecular mass of proteins along with their expression levels. However, the application of Western blot to the analysis of oocyte and embryo protein expressions has been limited because it requires a large number of samples (23, 24). In this study, we used a high-sensitivity capillary Western blot approach (ProteinSimple; Jess, ProteinSimple, San Jose, CA), which was further optimized in our laboratory to detect up to 10 proteins from a pool of 10 oocytes or embryos.

The objective of this work was to characterize the presence or absence of 4 proteins, *ACE2*, *BSG* (CD147), *TMPRSS2*, and *CTSL*, important for SARS-CoV-2 infection, in human follicular cells, mature oocytes, zygotes, and blastocysts (BLs). Our data demonstrated that these proteins are present in these tissues. Of interest, *ACE2* and *TMPRSS2* proteins are both present in high abundance in mature oocytes, zygotes, and BLs. This work demonstrated that human oocytes and embryos have the necessary machinery to be infected by SARS-CoV-2 if they are exposed, either in vivo or in vitro. This information is important for IVF clinics because they are working to provide safe infertility treatment during the global pandemic.

MATERIALS AND METHODS

Sample Collection

Oocyte, cumulus cell (CC), and GC samples were collected from fertility patients undergoing treatment at the Colorado Center for Reproductive Medicine who signed consent forms for biological materials discarded from their treatment cycles to be donated for research (WIRB #20142468). Surplus mature (metaphase II [MII]) eggs ($n = 69$ eggs from 6 patients, average age = 36.4 years), zygotes ($n = 20$ zygotes from 4 patients, average age = 35.0 years), and embryos ($n = 80$ embryos from 14 patients, average age = 28.6 years) were donated for research at the discretion by the patients. GCs were collected from fertility patients ($n = 5$ patients, average age = 34 years) undergoing egg retrieval after ovarian stimulation without an ovulatory trigger for in vitro maturation/IVF treatment. CCs were collected from standard IVF cycles after transvaginal ultrasound-guided oocyte retrieval and trimming of expanded CC-oocyte complexes before fertilization ($n = 5$ patients, average age = 38 years). Oocytes that were recovered from the standard IVF cycles, denuded, found to be in an immature state, and subsequently discarded from the treatment cycle were collected immediately at the germinal vesicle (GV) stage ($n = 57$ oocytes from 49 patients, average age = 37 years). The immature and mature oocytes completely devoid of GCs were washed 3 times with 1X phosphate-

buffered saline (PBS) and stored at -80°C . Mural GCs and CCs were centrifuged at $1000 \times g$ for 5 minutes to form a pellet, washed 3 times with 1X PBS, and stored at -80°C with a minimal amount of PBS. Mature eggs, zygotes, and BLs were washed 3 times in 1X PBS and stored at -80°C .

RNA and Protein Isolation

GCs and CCs from individual patients ($n = 3$ replicates per cell type) were used for RNA and protein isolations. Pools of 6 immature and 8 mature eggs or BLs (2 patients per pool) were used for RNA isolation ($n = 3$ replicates per sample type). Pools of 14–16 mature eggs (2–4 patients per pool), 10 zygotes (2 patients per pool), and 18 BLs (5 patients per pool) were used for protein isolation ($n = 3$ replicates per sample type). Not enough zygotes were available for both RNA and protein analyses; therefore, protein expression data for zygotes were derived from 2 biologic and 1 technical replicates. Total RNA from each sample was isolated using the RNeasy Micro Kit (QIAGEN #74004, Valencia, CA) according to the manufacturer's protocol and eluted in 15 μL of nuclease-free water. All the RNA isolated from the oocytes and embryos was directly used for cDNA synthesis without quantification. The RNA isolated from CCs and GCs was first quantified using NanoDrop 2000 (Thermo Fisher, Waltham, MA), and then, 200 ng of the total RNA was used for cDNA synthesis. To isolate protein, a radioimmunoprecipitation assay buffer (Sigma #R0278) containing a mixture of protease (Sigma #11836170001, St. Louis, MO) and phosphatase (Thermo Fisher #78440) inhibitors was used to lyse the samples. The oocyte and embryo lysates were directly used for Western blot analysis by Jess (product number 004-650; ProteinSimple, San Jose, CA). GC and CC lysates were subjected to ultrasonication, followed by centrifugation at $14,000 \times g$ for 10 minutes at 4°C to remove cellular debris. Supernatants collected from the cell lysates were then quantified using a Bio-Rad DC kit assay, and 900 ng of the protein was used for the Jess analysis.

Reverse Transcription and Quantitative Polymerase Chain Reaction

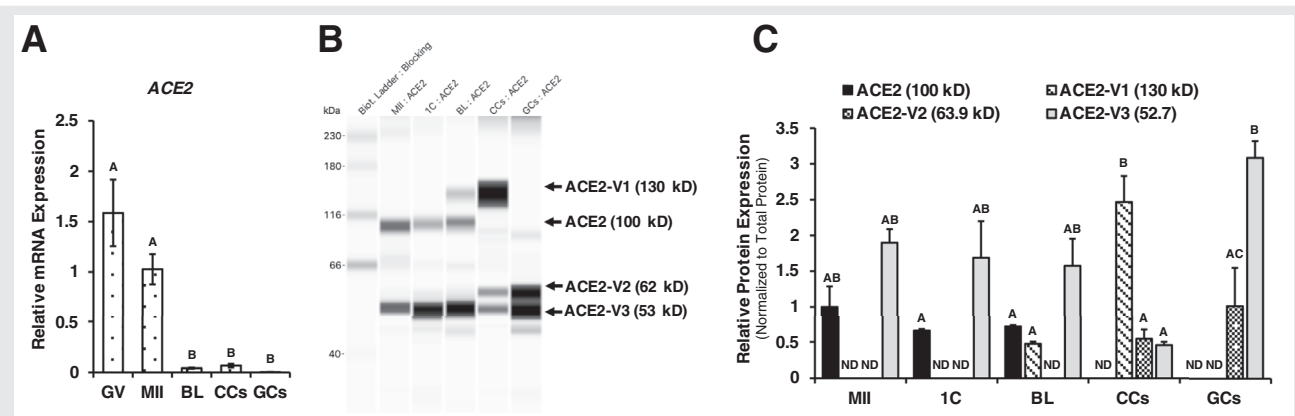
RNA obtained from each sample was converted into cDNA using iScriptReverse Transcription Supermix (Bio-Rad #1708840) based on the manufacturer's protocol. The resulting cDNA was diluted in nuclease-free water in 1:1 for the oocytes and embryos and 1:3 for CCs and GCs and stored at -20°C . For quantitative polymerase chain reaction (qPCR), primers for each target gene were designed for the conserved sequence of their protein-coding mRNA isoforms published in Ensemble genome browser using the PerlPrimer software, version 1.1.21. All the oligonucleotides used in this study were synthesized using integrated DNA technology and are listed in [Supplementary Table 1](#) (available online). For qPCR, a total of 2 μL of the diluted cDNA was used in a reaction mixture (25 μL) containing 2X Sso Advanced SYBR green (Bio-Rad) master mix, 5 pM each of the gene-specific forward and reverse primers, and nuclease-free water. The reaction mixtures were divided in half (12.5 μL each) to prepare 2

technical replicates per sample and subjected to qPCR amplification at 95°C for 10 minutes for initial denaturation, followed by 45 cycles of 95°C denaturation for 15 seconds, and an annealing or extension step at 60°C for 1 minute. For each reaction product, a melt-curve analysis after the amplification was performed to ensure a single and specific amplicon. Relative abundance of endogenous *GAPDH* was used as an internal control for each sample, and qPCR data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method.

Jess Simple Western Blotting

Protein expression analysis was performed by capillary Western blotting using the Jess system. All antibodies used in this study were produced against human peptides in rabbit and predicated (in silico) to cross react with mouse and rat homologous proteins. A list of the antibodies used is presented in [Supplementary Table 2](#) (available online). All the antibodies were first validated using protein lysates from various human tissues, including the liver, lung, colon, ovary, small intestine (OriGene, Rockville, MD), and cell lines, including white blood cells, HeLa, and HEK293T (Santa Cruz, CA). Based on molecular weight (MW) of the target proteins and source of primary antibodies, the 12–230 kDa separation module (ProteinSimple, SM-W004) and anti-rabbit detection module (ProteinSimple DM-001) were used according to the manufacturer's protocol. Briefly, the protein lysate from each sample was mixed with a fluorescent master mix in a 4:1 ratio and heated at 95°C for 10 minutes, followed by 5-minute incubation at room temperature. After a brief period of vortexing and spin down, a total of 3 μL of a mixture of the sample, protein-normalization reagent, blocking reagent (antibody diluent 2), wash buffer, primary antibodies diluted at 1:20, horseradish peroxidase-conjugated ready-to-use secondary antibodies (ProteinSimple DM-001), and chemiluminescent substrate were pipetted into designated wells in the manufacturer-provided microplate. After centrifugation at $1,000 \times g$ for 5 minutes, the plates were loaded onto the Jess instrument, and the default setting was used to run the chemiluminescent and protein normalization-based Jess program for immunodetection of the target proteins. After completion of each run, electropherograms generated by Simple Western Compass software for each capillary were analyzed to determine whether automatic peak detection required manual correction. The target and total protein quantities were determined based on area-under-the-peak analysis by the Compass software. Expression of each target protein was normalized by total protein signal in the same capillary. To discriminate the target protein signal from background, a peak signal-to-noise ratio of ≥ 10 for any target protein was considered significant and included in the data analysis. All 4 proteins (ACE2, TMPRSS2, CTSL, and CD147) were analyzed in each individual sample lysate using 3 biologic replicates. The blot image in the figures ([Figs. 2–5](#)) shows the chemiluminescence signal for each target protein generated by the Compass software, and the bar graphs depict the relative expression

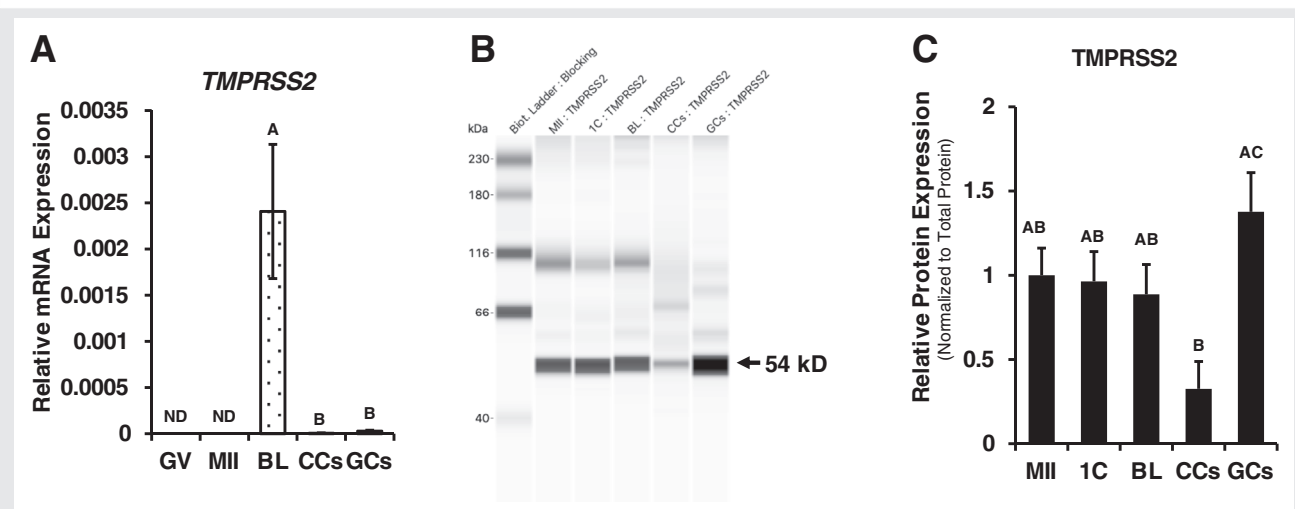
FIGURE 2



ACE2 mRNA and protein expressions in human oocytes, embryos, and follicular cells. **(A)** RT-qPCR analysis of human *ACE2* mRNA expression in GV- and MII-stage oocytes, BL (n = 3 pools of oocytes or embryos), CC, and GC (n = 3). Relative expression of *ACE2* mRNA was normalized to *GAPDH* expression and presented as a bar graph. **(B)** Capillary Western blot of total *ACE2* protein expression and **(C)** fold change in the abundance of *ACE2* protein (100 kDa) and putative variants of 130 kDa (*ACE2*-V1), 62 kDa (*ACE2*-V2), and 53 kDa (*ACE2*-V3) in MII-stage oocytes, 1C- and BL-stage embryos, CC, and GC (n = 3 replicates). Arrow indicates the target protein. *ACE2* protein expression was normalized to the total protein present in each sample, and data were presented as average protein fold change from 3 experiments. All bar graphs show the mean ± SEM. Different letters above the bars in graph represent the statistical difference ($P < .05$) determined by 1-way ANOVA followed by Tukey's test. 1C = 1-cell; *ACE2* = angiotensin 1-converting enzyme 2; ANOVA = analysis of variance; BL = blastocyst; CC = cumulus cells; GC = granulosa cells; GV = germinal vesicle; MII = metaphase II; mRNA = messenger ribonucleic acid; ND = nondetectable; RT-qPCR = quantitative reverse transcription polymerase chain reaction; SEM = standard error of the mean.

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FIGURE 3



Expression of *TMPRSS2* mRNA and protein in human oocytes, embryos, and follicular cells. **(A)** *TMPRSS2* mRNA level measured by RT-qPCR analysis in GV- and MII-stage oocytes, BL, CC, and GC. The expression data were normalized to *GAPDH* mRNA abundance and presented as a bar graph (n = 3 replicates). **(B)** Capillary Western blot of total *TMPRSS2* protein (54 kDa) expression and **(C)** fold change in the *TMPRSS2* protein abundance in MII-stage oocytes, 1C- and BL-stage embryos, CC, and GC (n = 3). Arrow indicates the target protein. *TMPRSS2* protein expression was normalized to the total protein present in each sample, and data were presented as average protein fold change from 3 experiments. All bar graphs show the mean ± SEM. Different letters above the bars in graph indicate statistical difference ($P < .05$) determined by 1-way ANOVA followed by Tukey's test. 1C = 1-cell; ANOVA = analysis of variance; BL = blastocyst; CC = cumulus cells; GC = granulosa cells; GV = germinal vesicle; MII = metaphase II; mRNA = messenger ribonucleic acid; ND = nondetectable; RT-qPCR = quantitative reverse transcription polymerase chain reaction; SEM = standard error of the mean; *TMPRSS2* = transmembrane serine protease 2.

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of the target protein normalized by total protein detected in the same sample.

Statistical Analysis

All data were analyzed with 1-way analysis of variance (ANOVA) using Prism software, version 8.2.1. Ct values obtained from quantitative reverse transcription polymerase chain reaction (RT-qPCR) were used to perform statistical analysis. The Western blot data were first checked for assumptions of normality and log-transformed before the analysis if required. Differences between means were determined using 1-way ANOVA followed by Tukey's test. Data are presented as untransformed mean \pm standard error of the mean.

RESULTS

Antibody Validation

The protein lysates from the human lung, liver, ovary, colon, small intestine, white blood cells, HeLa cells, and HEK293T cells were used as positive controls to determine the specificity of all the antibodies and their compatibility with capillary electrophoresis-based immunodetection on the Jess system. We observed a specific signal for anti-ACE2 with lung and liver (100 kDa) (Supplemental Fig. 1A, available online), anti-TMPRSS2 with HeLa cells, liver, and small intestine (55 kDa) (Supplemental Fig. 1B), and anti-CD147 with colon (43, 60 kDa), ovary (60 kDa), and lung (60 kDa) (Supplemental Fig. 1C). Antibody for procathepsin L (38–42 kDa) and CTSL (25–35 kDa) was tested with HEK293T and HeLa cell lysates because it is a well-characterized positive control (25). We observed a higher-than-expected (54 kDa) MW (38 kDa) band with the positive controls in the capillary Western blot (Supplemental Fig. 1D), and therefore, a 54-kDa band observed in any target sample (oocyte, embryos, and follicular cells) was considered CTSL-positive and included in the analysis. In addition to the specific band, some of the cell and tissue lysates showed additional bands when probed with the antibody against ACE2 (53, 62, and 130 kDa) and CD147 (43 kDa) (Supplemental Fig. 1). ACE2 protein consists of 805 amino acids and a predicted MW of 92.5 kDa. However, a glycosylated form (105–130 kDa) and 2 truncated forms (63.9 kDa and 52.7 kDa) of ACE2 have been previously reported to be expressed in human tissues (26–29) (Supplemental Fig. 2, available online). Similarly, CD147 (30, 31) and CTSL (32) are also known to have protein variants of different MWs resulting from alternative mRNA splicing or posttranslational modification; we identified these bands as probable protein variants (V) based on their respective MWs compared with those of reported variants of these proteins (Supplemental Table 3, available online).

To confirm that the origin of these bands was not a result of secondary antibody cross reactivity with target protein lysates, we used only a secondary antibody (without primary antibody) with each sample type as a negative control in the Jess analysis. The results demonstrated that the secondary antibody had no significant direct reactivity with any of the tissue, cell, oocyte, or embryo lysates used in this study (Supplemental Figs. 1 and 3, available online). With each

run on the Jess system, the radioimmunoprecipitation assay buffer was used as a negative control for all the antibodies to avoid any false-positive chemiluminescent signal due to cross contamination (Supplemental Fig. 1).

ACE2 Receptors are Present in Human Oocytes and Preimplantation Embryos

The RT-qPCR analysis showed that the *ACE2* transcript was present in both GV- and MII-stage human oocytes and was significantly reduced ($P < .05$) in the BL stage. *ACE2* mRNA was also preferentially ($P < .05$) expressed in the oocytes than in CCs and GCs (Fig. 2A). Analysis of ACE2 protein abundance in the oocytes, embryos, and follicular cells revealed the presence of ACE2 protein and/or varying variants in all the samples (Fig. 2B and C). We observed a 100-kDa MW band specific to the ACE2 protein in the MII oocytes, 1-cell (1C)-stage embryos, and BL-stage embryos, with no expression in CCs and GCs. A possible glycosylated form of approximately 130-kDa ACE2 (ACE2-V1) was barely detected in BLs and was present in significantly higher ($P < .05$) abundance in CCs but absent in the MII oocytes, zygotes, and GCs. Another 62-kDa variant (ACE2-V2) was present in both GCs and CCs but was completely absent in the MII-stage oocytes and embryos (1C and BL). Expression of an approximately 53-kDa variant (ACE2-V3) was observed in all the samples, but CC showed significantly lower abundance of ACE2-V3 compared with GC.

TMPRSS2 Protease is Also Present in Human Oocytes and Preimplantation Embryos

The results demonstrated no mRNA expression of *TMPRSS2* in CCs, GCs, or oocytes (GV and MII), but *TMPRSS2* transcripts were present in human BLs (Fig. 3A). In contrast, a specific band of TMPRSS2 protein (54 kDa) was observed in the MII oocytes, 1C- and BL-stage embryos, CC, and GC. The expression of TMPRSS2 was significantly ($P < .05$) higher in GC compared with that in CC (Fig. 3B and C).

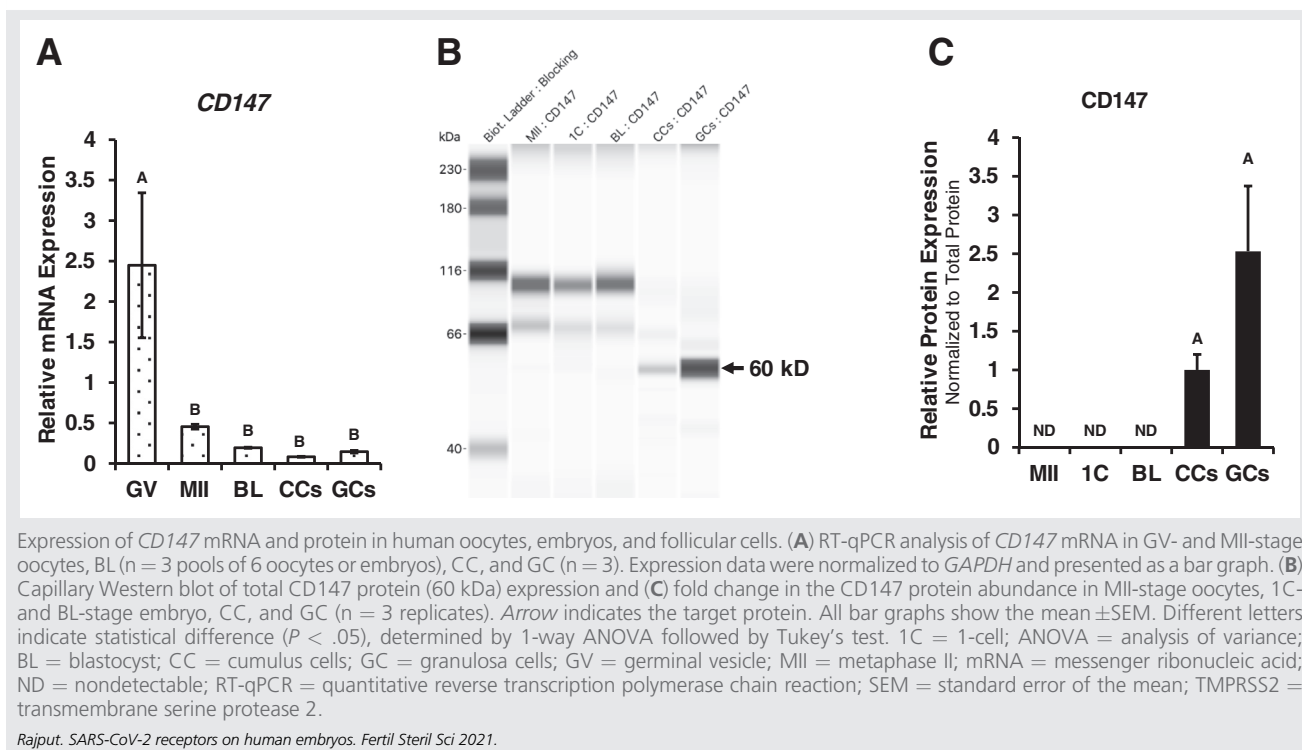
Mature Oocytes and Embryos Lack Basigin (CD147) Protein

We observed significantly higher *CD147* mRNA expression in the immature oocytes than in the MII oocytes, BL embryos, CC, and GC (Fig. 4A). CD147 is an N-linked glycosylated protein and contains multiple glycosylation sites, which result in its migration between 43-kDa and 66-kDa MW in Western blot analysis (30). Our results demonstrated the presence of glycosylated CD147 (60 kDa) protein, the expression of which was significantly higher ($P < .05$) in GCs than in CCs and was completely undetected in the oocytes or embryos (Fig. 4B and C).

Procathepsin L and Cathepsin L Proteins are not Present in Human Oocytes and 1-cell Embryos

The RT-qPCR analysis revealed the expression of the *CTSL* transcript in the oocytes, embryos, and follicular cells (Fig. 5A). The CTSL antibody used to analyze protein

FIGURE 4



expression in all the target samples was designed to recognize both procathepsin L and CTSL proteins. Procathepsin L is an inactive zymogen of 38–42 kDa that undergoes autolysis to give mature forms (CTSL) of a 25–35-kDa enzyme. CTSL is a lysosomal endopeptidase expressed in most eukaryotic cells but has also been reported in the nucleus, with 34-, 55-, and 60-kDa molecular mass variants (32). Since the CTSL antibody used in this study resulted in a 55-kDa band with the HEK293T and HeLa cells used as positive controls in the capillary Western blot, we considered this molecular mass specific to the CTSL protein. Our results demonstrated that CTSL (55 kDa) was completely absent in the MII-stage oocytes and 1C embryos, but its expression was observed in the BL-stage embryos, CC, and GC. In addition, CC and GC also showed a 60-kDa variant (CTSL-V1) (Fig. 5B and C).

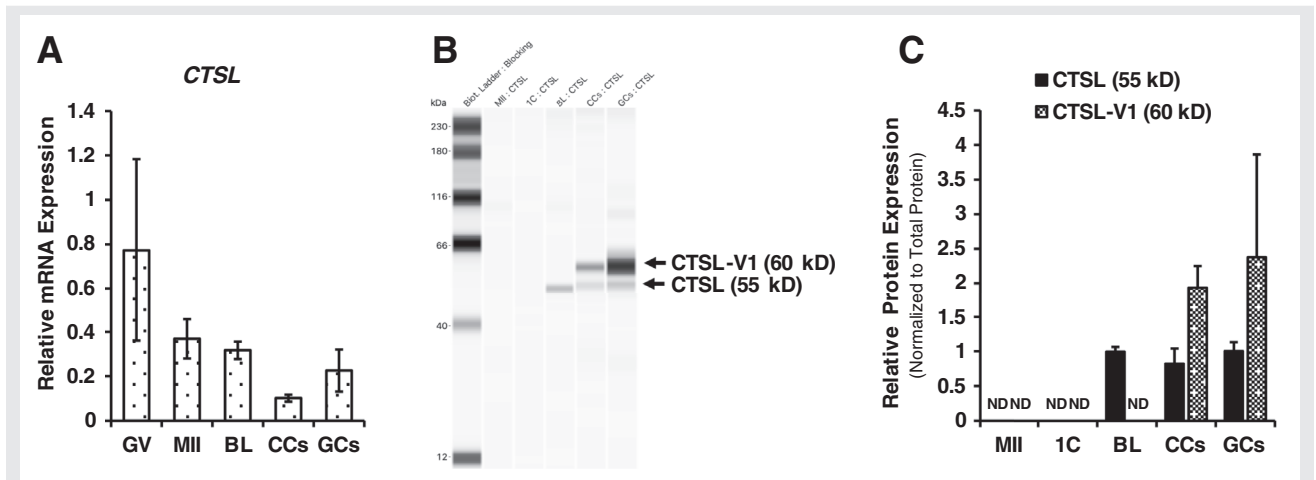
DISCUSSION

Our results demonstrated that the ACE2 receptor protein and TMPRSS2 protease protein, both of which are crucial for SARS-CoV-2 infection of host cells, are present and coexpressed in mature human oocytes, zygotes, and BLs. This work demonstrated that human oocytes and embryos have the machinery necessary for infection in other tissues. However, we do not know whether the infection happens in vivo or in vitro; we only showed that the machinery to support the infection is present. An ACE2-independent receptor system, composed of the BSG (CD147) receptor protein and CTSL, was not expressed in oocytes, zygotes, or BLs, although CTSL alone was found in the BL-stage embryos. GCs and CCs

of the ovary expressed all 4 of these proteins or their variants. However, both the receptor and protease were not abundantly coexpressed in CCs and GCs, suggesting that the likelihood of infection of the oocyte via CCs in the ovarian follicle is low.

We found that the ACE2 mRNA was mainly expressed in the oocytes, but the protein was present in the oocytes and embryos, suggesting the typical pattern of oocyte-expressed transcripts that are translated into protein to support early embryonic development and then degraded (33). The ACE2 mRNA has multiple transcript variants that have been predicted to encode a full-length protein of 92.4 kDa (Q9BYF1.1, NP_001358344.1), with an observed molecular mass of 100–130 kDa because of multiple sites of glycosylation (34) and truncated proteins of 90.3 kDa (XP_011543851.1), 88.8 kDa (XP_011543853.1), 79.4 kDa (XP_011543854.1), and 63.9 kDa (Q9BYF1.2) (35). Recently (36), a short 11-exon ACE2 novel isoform encoding a 52.7-kDa protein has also been reported to be exclusively expressed in the human airways, liver, and kidney. When the ACE2 antibody was tested with the different human tissues, we also observed this isoform in the human liver, lung, and small intestine. Our results demonstrated an approximately 53-kDa (ACE2-V3) protein in the oocytes, embryos, and CCs and approximately 62-kDa (ACE2-V2) protein in CCs and GCs. However, ACE2-V1 lacks the signal peptide required for SARS-CoV2 spike binding (36), and ACE2-V2 is a soluble isoform that lacks the transmembrane domain necessary for membrane attachment and SARS-CoV-2 entry (29, 37). Because both ACE2-V1 and ACE2-V2 are likely to be truncated isoforms lacking the key residues required for SARS-

FIGURE 5



CTSL mRNA and protein expressions in human oocytes, embryos, and follicular cells. **(A)** *CTSL* mRNA expression analyzed by RT-qPCR in GV- and MI-stage oocytes, BL ($n = 3$ pools of 6 oocytes or embryos), CC, and GC ($n = 3$). Expression data were normalized to *GAPDH* and presented as a bar graph. **(B)** Capillary Western blot of total *CTSL* protein expression and **(C)** fold change in the abundance of *CTSL* protein (55 kDa) and possible variants (60 kDa) in MI-stage oocytes, 1C- and BL-stage embryos, CC, and GC ($n = 3$ replicates). Arrow indicates the target protein. All bar graphs show the mean \pm SEM. Significant differences in expression between samples were determined using 1-way ANOVA followed by Tukey's test. No significant difference was observed in *CTSL* mRNA and protein expressions present in the samples. 1C = 1-cell; ANOVA = analysis of variance; BL = blastocyst; CC = cumulus cells; *CTSL* = cathepsin L; GC = granulosa cells; GV = germinal vesicle; MI = metaphase II; mRNA = messenger ribonucleic acid; ND = nondetectable; RT-qPCR = quantitative reverse transcription polymerase chain reaction; SEM = standard error of the mean; *TMPRSS2* = transmembrane serine protease 2.

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CoV-2 entry, we hypothesized that the presence of these proteins is not a probable viral entry point in oocytes, embryos, and ovarian somatic cells. CCs also had a 130-kDa ACE2 isoform, which might be the result of hyperglycosylation of ACE2 (100 kDa) (28). However, further studies using PNGase-mediated deglycosylation are required to confirm whether the higher-MW protein observed in CCs was because of glycosylation or cross reactivity of the antibody with a similar protein. The oocytes, zygotes, and BLs had a specific 100-kDa molecular mass of ACE2 protein along with *TMPRSS2*, demonstrating the presence of an optimum environment for SARS-CoV-2 infection. The presence of the *TMPRSS2* protein without any detectable mRNA level in oocytes, zygotes, CCs, and GCs suggests that the protein is translated from mRNA present in the oocytes and ovarian somatic cells during early stages of follicular development (22).

Transcript and protein expressions of these genes in various reproductive tissues have been an area of interest since the COVID-19 pandemic began. A published RNA sequence database of a nonhuman primate ovarian tissue has described the expression of *CD147* and *CTSL* in ovarian somatic cells, whereas *ACE2* and *TMPRSS2* expressions were restricted to germ cells (22). This is in agreement with our protein findings. Protein expression from 2 public databases, Human Protein Atlas and Human Proteome Map, found the presence of ACE2 protein in ovarian cells (22). Because we did not detect a specific band of ACE2 protein (100 kDa) in either CCs or GCs in this study, the protein expression reported in the ovarian somatic cells in the Human

Protein Atlas and Proteome Map databases might have been derived from truncated protein variants, such as ACE2-V2 (62 kDa), ACE2-V3 (53 kDa), or glycosylated ACE2 (ACE2-130I) observed in this study. The higher abundance of *CD147* mRNA in the immature oocytes than in the matured oocytes, embryos, and ovarian cell suggests that the *CD147* transcript was degraded during the end of oocyte maturation because no protein was detected in the matured oocytes and early stage of the embryos. Despite the low mRNA abundance, the protein expressions of *CD147* in CCs and GCs suggest that *CD147* protein was translated during early stages of follicular development. The coexpression of *CD147* receptor and *CTSL* proteases, analyzed as an alternative pathway for SARS-CoV-2 host entry, was absent in the oocytes and embryos but was found in CCs and GCs, with low abundance of *CTSL*. These results were in accordance with previously reported *CD147* (22) and *CTSL* (38) protein expressions in human ovarian cells.

In men, a previously published single-cell transcriptome analysis has demonstrated low levels of *ACE2* and *TMPRSS2* expression in the testes, with little coexpression, suggesting that SARS-CoV-2 infection is unlikely to occur in testicular cells (39). Other transcriptomic studies have also found expression of *ACE2* and *TMPRSS2* in testicular cells, including elongated spermatids and spermatogonial stem cells, but again, coexpression was rare (22, 40, 41). BSG was widely expressed across testicular cell types and was coexpressed with *CTSL* in early and late primary spermatocytes and to a lesser degree in Leydig cells, myoid cells, endothelial

cells, and differentiating spermatogonia (22). An analysis of protein expression in the testicular tissue using the 2 existing databases showed the presence of ACE2 and BSG in both the databases, whereas TMPRSS2 was not detected, and CTSL was detected in 1 of the 2 databases (22). This information suggests that SARS-CoV-2 is unlikely to infect testicular cells using the traditional ACE2/TMPRSS2 mechanism but that the testes may be susceptible to the ACE2-independent mechanism of infection. It remains unclear whether the virus is present in the semen and whether it can potentially be sexually transmitted. In patients in the recovery phase after COVID-19 and in a man who died of the disease, no SARS-CoV-2 viral RNA was detected in the semen (39, 42). In a separate study, 6 of 38 men (15.8%) in both acute and recovery phases tested positive for SARS-CoV-2 in their semen (43).

There are several specific concerns while operating in an ART laboratory during the SARS-CoV-2 pandemic, namely, whether oocytes can be infected at retrieval, whether embryos can be infected while in the laboratory, and whether embryos can infect other embryos during cryostorage. To minimize the risk of viral infection while retrieving oocytes, most practices have implemented testing patients before egg retrieval. However, viral RNA amplification tests can be influenced by viral load, sample site, collection method, and specimen shipment, resulting in potential false-negative results. The current antibody-based test lacks sensitivity and specificity until many days after symptom onset. These limitations create confusion about the utility of the patient test and increase the possibility of treating infected patients even if the test is in place (44). Coupled with the existing delays in obtaining test results, it is difficult for clinics to guarantee that no infected patients undergo IVF. Given these limitations and based on the prevalence of asymptomatic people and our findings that the mechanism necessary for SARS-CoV-2 infection is present, oocytes and embryos handled in laboratories during the pandemic should be treated with special precautions in the same manner as those for oocytes and embryos from patients infected with other viruses. Currently, no guidelines exist for doing so for patients infected with SARS-CoV-2 undergoing infertility treatment or with oocyte or sperm donors (45), although this may be a relevant safety precaution to implement. To avoid the infection of embryos during their time in the laboratory, a staff-testing program may be implemented, although it carries the same limitations as that for patients. Certainly, a rigorous protocol over and above normal safety measures for cleaning should be in place to prevent SARS-CoV-2 from entering IVF laboratories. However, the SARS-CoV-2 genome was not detected in oocytes analyzed from infected women (38) despite the cellular machinery required for SARS-CoV2 infection being present in the oocytes. This suggests that the zona pellucida can serve as a protective barrier for oocytes in vivo. This is particularly important in IVF procedures because the zona pellucida, which may serve as a viral barrier, is breached during treatment if intracytoplasmic sperm injection and/or biopsy are performed. Laboratories are already equipped to safely store cryopreserved samples in a way that prevents cross contamination by previously existing pathogens (46). The risk of cross contamination with SARS-CoV-2 between embryos during

cryostorage in modern, closed cryodevices and liquid nitrogen-vapor storage containers may be virtually negligible (47) despite the presence of the receptor and protease required for the viral infection, as we have demonstrated. However, the possibility to reinfect a mother and respread the virus at a later date after warming and transfer of an infected embryo cannot be ignored. Thus, robust patient- and staff-testing programs in addition to rigorous laboratory protocols to prevent the infection are critical pieces of practicing IVF during the SARS-CoV-2 pandemic. In the current scenario, IVF laboratory directors must make all of these decisions without much information because we are still in the process of learning about this virus and how it may affect reproduction. In fact, it is this lack of information that has prevented professional societies from establishing their own SARS-CoV-2-specific recommendations (48).

Given the presence of the mechanism of SARS-CoV-2 infection in oocytes and embryos that we have described, some patients might wish to postpone infertility treatment until the pandemic is better controlled. However, it has now been widely recognized that the postponement of treatment is detrimental for some patients, although a recent study has found no decrease in live birth rate among patients with diminished ovarian reserve who initiated treatment immediately or delayed treatment for up to 6 months (49). Assisted reproduction, in the form of IVF and frozen embryo transfer, offers an opportunity to postpone pregnancy to avoid the disease during gestation while respecting the importance of time in successful treatment (46). This approach does not completely avoid all potential risks, such as active infection in the patient during egg retrieval or infection of the embryo in vitro or during cryostorage. However, this strategy may be a viable alternative because it postpones pregnancy until we have more information about the effects of the disease during gestation on the fetus, until the pandemic is less of a threat to pregnant women, or until there is a safe and effective vaccine for both mothers and babies.

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