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Human eggs, zygotes, and embryos express the receptor ACE2 and protease TMPRSS2 protein necessary for SARS-CoV-2 infection

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PII: S2666-335X(20)30061-6
DOI: https://doi.org/10.1016/j.xfss.2020.12.005
Reference: XFSS 34

To appear in: F&S Science

Received Date: 13 October 2020
Revised Date: 1 December 2020
Accepted Date: 18 December 2020

Please cite this article as: Rajput SK, Logsdon DM, Kile B, Engelhorn HJ, Goheen B, Khan S, Swain J, McCormick S, Schoolcraft WB, Yuan Y, Krisher RL, Human eggs, zygotes, and embryos express the receptor ACE2 and protease TMPRSS2 protein necessary for SARS-CoV-2 infection, F&S Science (2021), doi: https://doi.org/10.1016/j.xfss.2020.12.005.

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Running title: Human oocytes and embryos express receptors to SARS-CoV-2

Human eggs, zygotes, and embryos express the receptor ACE2 and protease TMPRSS2 protein necessary for SARS-CoV-2 infection

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Abstract

Objective: To study mRNA and protein expression of SARS-CoV-2 entry receptors (ACE2, CD147) and proteases (TMPRSS2, CTSL) in human oocytes, embryos, cumulus and granulosa cells.

Design: Research Study.

Setting: Clinical IVF treatment center

Patient(s): IVF patients treated at the Colorado Center for Reproductive Medicine.

Intervention(s): Oocytes (GV, MII) and embryos (1 cell (1C); blastocyst (BL)) were donated to research at disposition by IVF patients. Follicular cells (cumulus (CC); granulosa (GC)) were collected from women undergoing egg retrieval after ovarian stimulation without an ovulatory trigger for IVM/IVF treatment cycles

Main Outcome Measure(s): The presence or absence of ACE2, CD147, TMPRSS2, and CTSL mRNA using RT-qPCR and protein using capillary Western blotting in human oocytes, embryos and ovarian follicular cells.

Result(s): RT-qPCR analysis revealed high abundance of ACE2 gene transcripts in GV and MII oocytes compared to CC, GC, and BL. ACE2 protein was only present in MII oocytes, 1C, and BL embryos, but other ACE2 protein variants were observed in all the samples. TMPRSS2 protein was present in all samples while mRNA was observed only at the blastocyst stage. All the samples were positive for CD147 and CTSL mRNA expression. However, cumulus and granulosa cells were the only samples that showed co-expression of both CD147 and CTSL proteins in low abundance.
Conclusion(s): Cumulus and granulosa cells are least susceptible to SARS-CoV-2 infection due to the lack of required receptors and proteases combination (ACE2/TMPRSS2 or CD147/CTSL) in high abundance. Co-expression of the ACE2 and TMPRSS2 proteins in MII oocytes, zygotes, and blastocysts demonstrate 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 yet know if infection occurs in vivo, or in vitro in an ART setting.

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

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 around the globe affecting 188 countries. Over 30 million people have been infected worldwide, and nearly 1 million people have died due to COVID19, the disease caused by SARS-CoV-2 (1). Initially, as countries rapidly shut down to control the spread of the novel coronavirus and avoid overburdening healthcare systems, assisted reproduction was also curtailed, being classified in most instances as non-essential. Very little was known about the potential for infection of sperm, eggs, or embryos, the potential for sexual transmission between partners, vertical transmission between a mother and conceptus, or possible effects of maternal infection on the early embryo and fetus. This lack of information led to great concern in the field about how the virus might potentially impact laboratory and pregnancy outcomes in IVF, in addition to speculation about potential changes in standard operating procedures in an IVF laboratory during a pandemic (2, 3).

As the pandemic continued, the impact on couples desiring treatment grew. Specific groups of infertile patients faced significantly reduced prognoses if their treatment continued to be delayed (4). However, the unknown effects of SARS-CoV-2 on reproduction made it difficult to determine best IVF laboratory practices necessary to mitigate the risks of contamination and infection to both patients and staff. For example, 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 preimplantation culture and cryostorage. It is therefore of great importance that we advance our understanding of the potential for 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 understanding of the molecular mechanism of viral entry continues to be refined, the described mechanism involves viral spike (S) protein mediated recognition and binding to the host cell receptor angiotensin 1 converting enzyme 2 (ACE2), after which the protease transmembrane serine protease 2 (TMPRSS2) cleaves the spike protein to facilitate cell entry (5). ACE2, a negative regulator of the renin-angiotensin system, has multiple physiological functions and is widely expressed throughout the body (6). The S protein cleavage site in SARS-CoV-2 is identical to SARS-CoV, which is also known to take advantage of the endosomal cysteine protease Cathepsin L (CTSL) to cleave specific peptide bonds and facilitate the fusion of viral and host cell membranes and the release of the viral genome into the host cell 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 co-expression of ACE2 and TMPRSS2, are more vulnerable to infection by SARS-CoV-2.

Scientifically, information has continued to emerge that impacts the clinical application of assisted reproductive technologies (ART). One popular method to provide needed information quickly has been to query existing transcriptomic databases for the presence of the receptors and proteases necessary for SARS-CoV-2 infection of host cells. 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, based upon gene expression studies (9, 10). Another study found that ACE2 was expressed in the ovary but only at low levels, and that TMPRSS2 was not expressed although CTSL was widely expressed; however, no cells co-expressed ACE2 and CTSL (11). Thus, existing transcriptomic information has not provided much clarity on the
potential for infection of the ovary and oocyte. The possibility remains that SARS-CoV-2 might affect female fertility by infecting ovarian granulosa cells and/or the oocyte directly, thereby negatively impacting ovarian function and reducing oocyte quality (12). Analysis of existing single cell RNA sequencing (scRNASeq) datasets also revealed the presence of transcripts for 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 have the potential to be infected by SARS-CoV-2 as well. The fallopian tube expresses ACE2 in a small number of cells, but the protease TMPRSS2 was widely expressed in different cell types although again there was no detectable co-expression 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 peri- and post-implantation stage human embryos, transcripts for ACE2 and TMPRSS2 are co-expressed in day 6 and day 7 trophoblast, day 12 and day 14 syncytiotrophoblast, 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). Again, this suggests the susceptibility of the human embryo to SARS-CoV-2 infection and potential implications for implantation and pregnancy, as well as the potential transmission of the virus from mother to fetus through the placenta. However, transcript levels often do not accurately reflect protein abundance, and this has been demonstrated for ACE2 as well (15). In fact, when comparing publically available proteomic and transcriptomic databases, ACE2 protein is abundant in the ovary despite its low mRNA levels (15). This is not terribly surprising, as processes including post-transcriptional, translational, and protein degradation occur after mRNA is transcribed, leading to differences between mRNA levels and protein abundance of as much as 15-70% (16, 17). Protein expression analyses in
oocytes, embryos and follicular cells has relied on immunohistochemistry (IHC) due to the scarcity of human sample availability. In addition to the potential for false positive results due to antibody cross-reactivity to similar proteins, IHC has been recognized for false negative results since its inception (18-21). A recent study (22) reported that IHC failed to detect ACE2 protein expression in either follicular or stromal ovarian cells, but high expression of ACE2 protein was found by mass spectrometry. Western blot is a gold standard technique to study protein expression, as it reveals the molecular mass of the protein along with its expression level. However, application of Western blot to the analysis of oocyte and embryo protein expression has been limited, as it requires a large number of samples (23, 24). In this study, we used a high sensitivity capillary Western blot approach (ProteinSimple™), 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 four proteins, ACE2, BSG (CD147), TMPRSS2, and CTSL, important for SARS-CoV-2 infection, in human follicular cells, mature oocytes, zygotes, and blastocysts. Our data demonstrates that these proteins are present in these tissues. Of interest, ACE2 and TMPRSS2 protein are both present in high abundance in mature oocytes, zygotes, and blastocysts. This work demonstrates that human oocytes and embryos have the necessary machinery to be infected with SARS-CoV-2 if they were to be exposed, either in vivo or in the laboratory. This information is important for IVF clinics as they work to safely provide infertility treatment during the global pandemic.

Materials and Methods
Sample collection

Oocyte, cumulus, and granulosa cell samples were collected from fertility patients undergoing treatment at the Colorado Center for Reproductive Medicine who had signed consents for biological materials discarded from their treatment cycles to be donated to research (WIRB #20142468). Surplus mature (metaphase II) eggs (n=69 eggs from 6 patients, average age = 36.4 years), zygotes (n=20 from 4 patients, average age 35.0 years), and embryos (n=80 from 14 patients, average age 28.6 years), were donated to research at disposition by patients. Granulosa cells were collected from fertility patients (n=5 patients, average age 34 years) undergoing egg retrieval after ovarian stimulation without an ovulatory trigger for IVM/IVF treatment. Cumulus cells were collected following transvaginal ultrasound guided oocyte retrieval and trimming of the expanded cumulus oocyte complexes prior to fertilization from standard IVF cycles (n=5 patients, average age 38 years). Oocytes that were recovered from 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). Immature and matured oocytes completely devoid of granulosa cells were washed three times with 1X PBS and stored at -80°C. Mural granulosa and cumulus cells were centrifuged at 1000g for 5 min to form a pellet, washed three times with 1X PBS, and stored at -80°C with a minimal amount of PBS. Mature eggs, zygotes and blastocysts were washed three times in 1X PBS and stored at -80°C.

RNA and Protein isolation

Granulosa cells and cumulus cells from individual patients (n=3 replicates/cell type) were used for RNA and protein isolation. Pools of 6 immature and 8 mature eggs or blastocysts (2 patients
per pool) were used for RNA isolation (n=3 replicates/sample type). Pools of 14-16 mature eggs (2-4 patients per pool), 10 zygotes (2 patients per pool) and 18 blastocysts (5 patients per pool) were used for protein isolation (n=3 replicates/sample type). Not enough zygotes were available for both RNA and protein analysis; therefore, protein expression data from zygotes were derived from two biological and one technical replicates. Total RNA from each sample was isolated using the RNeasy Micro Kit (Qiagen # 74004) according to the manufacturer’s protocol and eluted in 15 ul of nuclease free water. All the RNA isolated from oocytes and embryos was directly used in cDNA synthesis without quantification. RNA isolated from cumulus and granulosa cells was first quantified on a NanoDrop 2000 (Thermo Fisher), and then 200 ng of total RNA was used for cDNA synthesis. To isolate protein, RIPA buffer (Sigma # R0278) containing a mixture of protease (Sigma # 11836170001) and phosphatase (Thermo # 78440) inhibitors was used to lyse the samples. Oocyte and embryo lysates were directly used in western blot analysis by Jess (Protein Simple, San Jose, CA). Granulosa and cumulus cell lysates were subjected to ultrasonication followed by centrifugation at 14000g for 10 minutes at 4 °C to remove cellular debris. Supernatants collected from cell lysates were then quantified using a Bio-Rad DC kit assay, and 900 ng of protein was used for Jess analysis.

Reverse-transcription (RT) and Quantitative PCR (qPCR)

RNA obtained from each sample was converted into cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad # 1708840) following the manufacturer’s protocol. The subsequent cDNA was diluted 1:1 for oocytes and embryos and 1:3 for cumulus and granulosa cells in nuclease free water and stored at -20°C. For qPCR, primers for each target gene were designed against the conserved sequence of their protein coding mRNA isoforms published at
Ensemble genome browser using PerlPrimer software version 1.1.21. All the oligonucleotides used in this study were synthesized at Integrated DNA Technology (IDT) and are listed in Supplementary Table S1. For qPCR, a total of 2 ul of diluted cDNA was used in a reaction mixture (25ul) containing 2X Sso Advanced™ SYBR® green (Bio-Rad) master mix, 5 picomoles of each gene specific forward and reverse primers, and nuclease free water. Reaction mixtures were divided in half (12.5 ul) to prepare two technical replicates per sample and subjected for 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/extension step at 60°C for 1min. For each reaction product, a post amplification melt curve analysis was performed to ensure a single and specific amplicon. The relative abundance of endogenous GAPDH was used as an internal control for each sample and qPCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method.

**Jess Simple Western**

Protein expression analysis was performed by capillary Western blotting using the Jess system (ProteinSimple, product number 004-650). All the antibodies used in this study were produced against human peptides in rabbit and predicted (in silico) to cross react with mouse and rat homologous protein. A list of the antibodies used is provided in Supplementary Table S2. All antibodies were first validated using protein lysates from various human tissues and cell lines including liver, lung, colon, ovary, small intestine (OriGene), white blood cells (WBC), Hela and HEK293T (Santa Cruz). Based on the molecular weight (MW) of the target proteins and source of primary antibodies, the 12-230 kD separation module (ProteinSimple, SM-W004) and the Anti-Rabbit Detection Module (ProteinSimple DM-001) was used following the manufacturer’s protocol. Briefly, protein lysate from each sample was mixed with fluorescent master mix in a
4:1 ratio and heated at 95°C for 10 min, followed by a 5 minute incubation at room temperature. After brief vortexing and spin down, a total of 3 ul of a mixture of sample, protein normalization reagent, blocking reagent (antibody diluent 2), wash buffer, primary antibodies diluted 1:20, HRP-conjugated ready to use secondary antibodies (Protein Simple DM-001) and chemiluminescent substrate were pipetted into designated wells in the manufacturer provided microplate. After centrifugation at 1000Xg for 5 minutes, 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 target proteins. After completion of each run, electropherograms generated by Simple Western Compass™ software for each capillary were analyzed to determine if automatic peak detection required manual correction. Target and total protein quantity were determined by 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 (S/N) ratio of ≥10 for any target protein was considered significant and included in data analysis. All four proteins (ACE2, TMPRSS2, CTSL, CD147) were analyzed in each individual sample lysate, with 3 biological replicates. The blot image in the figures shows the chemiluminescence signal for each target protein generated by Compass™ software, and the bar graphs depict relative expression of the target protein normalized by total protein detected in the same sample.

**Statistical analysis**

All data were analyzed with one-way ANOVA using Prism V 8.2.1. Ct values obtained from RT-qPCR were used to perform statistical analysis. Western blot data were first checked for
assumptions of normality and log transformed before analysis if required. Differences between means were determined using one-way ANOVA followed by Tukey's test. Data are presented as untransformed mean ± SEM.

Results

Antibody validation

Protein lysate from human lung, liver, ovary, colon, small intestine, WBC, Hela cells, and HEK293T cells was used as positive controls to determine the specificity of all antibodies and their compatibility with capillary electrophoresis based immunodetection on Jess. We observed a specific signal for anti-ACE2 with lung and liver (100 kDa) (Figure S1A), anti-TMPRSS2 with HeLa, liver and small intestine (55kDa) (Figure S1B), and anti-CD147 with colon (43, 60 kDa), ovary (60 kDa) and lung (60 kDa) (Figure S1C). Antibody for Procathepsin L (38-42 kDa) + cathepsin L (25-35 kDa) (CTSL) were tested with HEK293T and Hela cell lysate as its’ well characterized positive control (25). We observed a higher (54kD) than expected MW (38 kDa) band with the positive controls in capillary western blot (Figure S1D) and therefore a 54 kD band observed in any target samples (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, 130 kDa) and CD147 (43 kDa) (Figure S1). ACE2 protein consists of 805 amino acids with a predicted molecular weight of 92.5 kD. However, a glycosylated form (105-130 kD) and two truncated forms (63.9 kD and 52.7 kD) of ACE2 have been previously reported to be expressed in human tissues (26-29) (Figure S2). Similarly, CD147 (30, 31) and CTSL (32) are also known to have
protein variants of different MWs resulting from alternative mRNA splicing or post-translational modification; we identified these bands as probable protein variants (V) based on their respective MWs compared to that of reported variants of these proteins (Table S3).

To confirm the origin of these bands are not the result of secondary antibody cross reactivity with target protein lysates, we used only secondary antibody (without primary antibody) with each sample type in Jess as a negative control. Results demonstrated that secondary antibody has no significant direct reactivity with any of the tissue, cell, oocyte or embryo lysates used in this study (Figure S1, S3). With each run on Jess, RIPA buffer is used as a negative control for all the antibodies to avoid any false positive chemiluminescent signal due to cross contamination (Figure S1).

**ACE2 receptors are present in human oocytes and preimplantation embryos**

RT-qPCR analysis showed that ACE2 transcript is present in both GV and MII stage human oocytes, and is reduced significantly (p<0.05) at the blastocyst stage. ACE2 mRNA is also preferentially (p<0.05) expressed in oocytes compared to cumulus and granulosa cells (Figure 2A). Analysis of ACE2 protein abundance in oocytes, embryos and follicular cells revealed the presence of the ACE2 protein, and/or varying variants, in all samples (Figure 2B, C). We observed a 100 kD MW band specific to the ACE2 protein in MII oocytes, 1C and BL stage embryos with no expression in CC and GC. A possible glycosylated form of ~130 kD ACE2 (ACE2-V1) was barely detected in blastocysts and was present in significantly higher (p<0.05) abundance in cumulus cells, but absent in MII oocytes, zygotes, and graulosa cells. Another 62 kD variant (ACE2-V2) was present in both GC and CC but was completely absent in MII stage
oocytes and embryos (1C and BL). Expression of ~53 kD variant (ACE2-V3) was observed in all the samples, except CC showed significantly lower abundance of ACE2-V3 compared to GC.

**TMPRSS2 protease is also present in human oocytes and preimplantation embryos**

Results demonstrated no mRNA expression of TMPRSS2 in cumulus or granulosa cells or in oocytes (GV, MII), but TMPRSS2 transcripts are present in human blastocysts (Figure 3A). In contrast, a specific band of TMPRSS2 protein (54kDa) was observed in MII oocytes, 1C and BL stage embryos, CC and GC. Expression of TMPRSS2 was significantly (p<0.05) higher in GC compared to CC (Figure 3B,C).

**Mature oocytes and embryos lack basigin (CD147) protein**

We observed significantly higher CD147 mRNA expression in immature oocytes compared to MII oocytes, BL embryos, CC, and GC (Figure 4A). CD147 is an N-linked glycosylated protein and contains multiple glycosylation sites, which results in its migration between 43 kD to 66 kD MW in Western blot analysis (30). Our results demonstrate the presence of a glycosylated CD147 (60 kDa) protein which was significantly higher (p<0.05) in granulosa cells compared to cumulus cells, and is completely undetected in oocytes or embryos (Figure 4 B, C).

**Procathepsin L+Cathepsin L (CTSL) protein is not present in human oocytes and 1C embryos**

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

Discussion

Our results demonstrate that the ACE2 receptor protein and the TMPRSS2 protease protein, both of which are crucial for SARS-CoV-2 infection of host cells, are present and co-expressed in mature human oocytes, zygotes, and blastocysts. This work demonstrates that human oocytes and embryos have the machinery necessary for infection in other tissues. We do not, however, know if infection could happen in vivo or in vitro; we only show that the machinery to support infection is present. The ACE2 independent receptor system, composed of the basigin (CD147) receptor protein and cathepsin-L (CTSL), are not expressed in oocytes, zygotes or blastocysts, although CTSL alone is found in blastocyst stage embryos. Granulosa and cumulus cells of the ovary do express all four of these proteins or their variants. However, both receptor and protease are not abundantly co-expressed in cumulus and granulosa cells, suggesting that the likelihood of infection of the oocyte via cumulus cells in the ovarian follicle is low.
We found that ACE2 mRNA was mainly expressed in oocytes, but protein was present in oocytes and embryos both suggesting the typical pattern of oocyte expressed transcripts which are translated into protein to support early embryo development and then degraded (33). ACE2 mRNA has multiple transcript variants which are predicted to encode a full length protein of 92.4 kD (Q9BYF1.1, NP_001358344.1) with observed molecular mass of 100-130 kD due to multiple sites of glycosylation (34), and truncated proteins of 90.3 kD (XP_011543851.1), 88.8 kD (XP_011543853.1), 79.4 kD (XP_011543854.1), and 63.9 kD (Q9BYF1.2) (35). Recently (36), a short 11 exon ACE2 novel isoform encoding 52.7 kD protein was also reported to be exclusively expressed in human airways, liver and kidney. We also observed this isoform in human liver, lung and small intestine when ACE2 antibody was tested with different human tissues. Our results demonstrated ~53 kD (ACE2-V3) protein in oocyte, embryo and cumulus cells, and ~62 kD (ACE2-V2) protein in cumulus and granulosa cells. However, ACE2-V1 lacks the signal peptide required for SARS-CoV2 spike binding (36), and ACE2-V2 is a soluble isoform which lacks the transmembrane domain necessary for membrane attachment and SARS-CoV2 entry (29, 37). Since both ACE2-V1 and ACE2-V2 are likely to be truncated isoforms lacking the key residues required for SARS-CoV2 entry, we hypothesize that the presence of these proteins is not a probable viral entry point in oocytes, embryos and ovarian somatic cells. Cumulus cells also had a 130 kD ACE2 isoform, which could be the result of hyper glycosylation of ACE2 (100 kDa) (28). However, further studies are required using PNGase mediated deglycosylation to confirm if the higher molecular weight protein observed in cumulus cells is due to glycosylation or cross reactivity of the antibody to similar protein. Oocytes, zygotes and blastocysts had a specific 100 kD molecular mass of ACE2 protein along with TMPRSS2, demonstrating the presence of an optimum environment for SARS-CoV-2 infection. The presence of TMPRSS2
protein without any detectable mRNA level in oocytes, zygotes, cumulus and granulosa cells suggests that the protein is translated from mRNA present in oocytes and ovarian somatic cells during early stages of follicular development (22).

Transcript and protein expression of these genes in various reproductive tissues has been an area of interest since the SARS-CoV-2 pandemic began. A published RNA Sequence database of non-human primate ovarian tissue described the expression of CD147 and CTSL in ovarian somatic cells, while ACE2 and TMPRSS2 expression was 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 ACE2 protein in ovarian cells (22). Since we did not detect a specific band of ACE2 protein (100 kDa) in either cumulus or granulosa cells in this study, protein expression reported in ovarian somatic cells at Human Protein Atlas/Proteome Map could be derived from truncated protein variants such as ACE2-V2 (62 kD), ACE2-V3 (53 kD) or glycosylated ACE2 (ACE2-130I) observed in the present study. Higher abundance of CD147 mRNA in immature oocyte compared to matured oocytes, embryos and ovarian cell suggest that CD147 transcript was degraded during end of oocyte maturation as no protein was detected in matured oocytes and early stage of embryos. Despite low mRNA abundance, protein expression of CD147 in cumulus and granulosa cells suggests that CD147 protein was translated during early stages of follicular development. Co-expression of CD147 receptor and CTSL proteases analyzed as an alternative pathway for SARS-CoV-02 host entry was absent in oocytes and embryos but was found in cumulus and granulosa cells with low abundance of Cathepsin-L. These results were in accordance to previously reported CD147 (22) and Cathepsin-L (38) protein expression in human ovarian cells.
In men, previously published single cell transcriptome analysis demonstrated low levels of ACE2 and TMPRSS2 expression in the testes with little co-expression, suggesting that SARS-CoV-2 infection is unlikely to occur in testicular cells (39). Other transcriptomic studies also found expression of ACE2 and TMPRSS2 in testicular cells including elongated spermatids and spermatogonial stem cells, but again co-expression was rare (22, 40, 41). Basigin was broadly 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 spermatagonia (22). Examining protein expression in testicular tissue using the two existing databases, ACE2 and BSG were detected in both databases while TMPRSS2 was not detected and CTSL was detected in one of the two 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 if the virus is present in semen and could potentially be sexually transmitted. In patients in the recovery phase following COVID-19, and in one man who died of the disease, no SARS-CoV-2 viral RNA was detected in semen (39, 42). In a separate study, 6 of 38 men (15.8%) in both the acute and recovery phase tested positive for SARS-CoV-2 in their semen (43).

There are several specific concerns in the ART laboratory while operating during the SARS-CoV-2 pandemic. Namely, can oocytes be infected at retrieval, can embryos be infected while in the laboratory, and can embryos infect other embryos during cryostorage? To minimize the risk of retrieving oocytes infected with the virus, most practices have implemented testing of patients prior to egg retrieval. However, viral RNA amplification tests can be influenced by viral load, sample site, collection method, and specimen shipment, resulting in the potential for false
negative results. Current antibody based testing lacks sensitivity and specificity until many days after symptom onset. These limitations create confusion about the utility of patient testing and increase the possibility of treating infected patients even if testing is in place (44). Coupled with existing delays in obtaining test results, it is difficult for clinics to guarantee that no infected patients undergo IVF. Given these limitations, the prevalence of asymptomatic people, as well as our findings that the mechanism necessary for SARS-CoV-2 infection is present, oocytes and embryos handled in the laboratory during the pandemic could be treated with special precautions, in the same manner as those from patients infected with other viruses. No guidelines currently exist for doing so with SARS-CoV-2 infertility patients, 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 should be in place for cleaning to prevent SARS-CoV-2 from entering the IVF laboratory. However, 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 oocytes. This suggests that zona pellucida can serve as a protective barrier for oocyte in vivo. This is particularly important in IVF procedures since the zona pellucida, which may serve as a viral barrier, is breached during treatment if ICSI and/or biopsy is performed. Laboratories are already equipped to safely store cryopreserved samples in such a way as to prevent cross contamination by previously existing pathogens (46). The risk of cross contamination between embryos with SARS-CoV-2 during cryostorage in modern closed cryodevices and liquid nitrogen vapor storage containers may be virtually negligible (47), even given the presence of the receptor and protease required for viral infection as we have
demonstrated. However, the possibility to re-infect the mother and re-spread the virus at a later date after warming and transfer of an infected embryo cannot be ignored. Thus, a robust patient and staff testing program in addition to rigorous laboratory protocols to prevent infection are critical pieces of practicing IVF during the SARS-CoV-2 pandemic. In the current climate, IVF laboratory directors must make all of these decisions without much information, as 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 for SARS-CoV-2 infection in oocytes and embryos that we have described, some patients may wish to postpone infertility treatment until the pandemic is better controlled. However, it is now widely recognized that postponement of treatment is detrimental for some patients, although a recent study 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 FET, offers an opportunity to postpone pregnancy to avoid the disease during gestation while respecting the importance of time for successful treatment (46). This approach does not completely avoid all potential risks, such as an active infection in the patient at egg retrieval or of the embryo in vitro or during cryostorage. This strategy may be a viable alternative, however, because it postpones pregnancy until we have more information about 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 mother and baby.

Acknowledgements:
The authors thanks Rachel West, Ph.D., for assistance with protein cell lysates to test the antibodies and helpful discussion. Funding for this work was provided by the Colorado Center for Reproductive Medicine.
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Figure Captions

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 spike (S) protein binding to ACE2 receptors which leads to either cell membrane fusion and release of the viral genome if activated by TMPRSS2, or endocytic uptake of virus. CD147 is
another host cell receptor that can be recognized by SARS-S protein and facilitate viral entry
through endocytosis. After viral entry into host cells via endosome, viral RNA is released into
the cytoplasm due to pH dependent cleavage by Cathepsin L (CTSL) and start encoding viral
protein. Figure adapted from Heurich et al., 2014 and Aguiar et al., 2020.

Figure. 2: ACE2 mRNA and protein expression in human oocytes, embryos and follicular
cells. (A) RT-qPCR analysis of human ACE2 mRNA expression in GV- and MII-stage oocytes,
blastocyst (BL) (n = 3 pools of oocytes or embryos), cumulus cells (CC), and granulosa cells
(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 ACE 2 protein expression and (C)
fold change in the abundance of ACE2 protein (ACE2-100 kD) and putative variants of 130 kD
(ACE2-V1), 62 kD (ACE2-V2), and 53 kD (ACE2-V3) in MII-stage oocytes, 1-cell (1C) and
BL stage embryos, and in CC and GC (n = 3 replicates). Arrow indicates the target protein.
ACE2 protein expression was normalized to the total protein present in each samples and data
was presented as average protein fold-change from 3 experiments. All bar graphs show the
mean ± SEM (standard error of the mean). Different letters above the bars in graph represent the
statistical difference (P < 0.05) determined by one-way ANOVA followed by Tukey’s test. ND
indicates “non-detectable”

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, blastocyst (BL), cumulus cells (CC), and granulosa cells (GC). Expression data
was normalized to GAPDH mRNA abundance and presented as a bar graph (n=3 replicates). (B)
Capillary western blot of total TMPRSS2 protein (54 kD) expression and (C) fold change in the TMPRSS2 protein abundance in MII-stage oocytes, 1-cell (1C) and BL stage embryos and in CC and GC (n = 3). Arrow indicates the target protein. TMPRSS2 protein expression was normalized to the total protein present in each samples and data was 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 < 0.05) determined by one-way ANOVA followed by Tukey’s test. ND indicates “non-detectable”.

Figure. 4: Expression of CD147 mRNA and protein in human oocytes, embryos and follicular cells. (A) RT-qPCR analysis of CD147 mRNA in GV- and MII-stage oocytes, blastocyst (BL) (n= 3 pools of 6 oocytes or embryos), cumulus cells (CC), and granulosa cells (GC) (n = 3). Expression data was normalized to GAPDH and presented as a bar graph. (B) Capillary western blot of total CD147 protein (60 kD) expression and (C) fold change in the CD147 protein abundance in MII-stage oocytes, 1-cell (1C) and BL stage embryos, and in CC and GC (n = 3 replicates). Arrow indicates the target protein. All bar graphs show the mean ± SEM. Different letters indicate statistical difference (P < 0.05), determined by one-way ANOVA followed by Tukey’s test. ND indicates “non-detectable”.

Figure. 5: Cathepsin-L (CTSL) mRNA and protein expression in human oocytes, embryos and follicular cells. (A) CTSL mRNA expression analyzed by RT-qPCR at GV- and MII-stage oocytes, blastocyst (BL) (n= 3 pools of 6 oocytes or embryos), cumulus cells (CC), and granulosa cells (GC) (n = 3). Expression data was 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 kD) and possible variants (60 kD) at MII-stage oocytes, 1-cell (1C) and BL stage embryos, and in CC and GC (n = 3 replicates). Arrow indicates the target protein. All bar graphs show the mean ± SEM. Significant differences in expression between samples were determined using one-way ANOVA followed by Tukey's test. No significant difference was observed in CTSL mRNA and protein expression present in the samples. ND indicates “non-detectable”.

Supplemental figure captions

Figure S1: Antibody validation for SARS-CoV-2 virus host entry receptors (ACE2, CD147) and proteases (TMPRSS2, CTSL) using human tissue and cell lysates. Liver, lung, small intestine, colon, and ovary tissues, and HEK293T and HeLa cells, were used as positive controls to test all the target antibodies. (A) ACE2 antibody showed a specific band of 100 kD in lung and liver, 130 KD variant in liver and 53, 62 kD variants in lung and small intestine. (B) HeLa cell, liver and small intestine protein lysate with TMPRSS2 antibody showed a specific band of 54 kDa. (C) Glycosylated forms of CD147 protein was observed with molecular mass of 43 and 60 kD in colon and 60 kD in ovary and lung. (D) HEK293T and HeLa cell protein lysate showed a 54 kD band using CTSL antibody. None of the protein lysates used as a positive control showed significant reactivity with only the secondary AB. RIPA buffer showed no chemiluminescent signal with any of the target antibodies. Red boxes indicate the target proteins.
Figure S2: Block diagram of angiotensin-converting enzyme 2 (ACE2) gene, and mRNA and protein variants. The ACE2 gene contains 18 exons (E) (yellow), three mRNA splice variants (E1-18; E1-12; E10-18) and downstream protein variants. Full-length ACE2 protein is comprised of N-terminal signal peptide (grey), ectodomain (green), transmembrane domain (orange), and the C-terminal cytoplasmic domain (blue) with predicted molecular weight of 92.5 kD. ACE2 protein has a total of 7 N-glycosylation sites (N53, 90, 103, 332, 432, 546) and 3 –O glycosylation sites (O155, 496, 730), which can result 105-130 kD molecular weight depending upon glycosylation level. The protein variant resulting from E1-12 lacks the transmembrane and cytoplasmic domain and is therefore referred to as soluble ACE2 (63.9 kD). Short ACE2 (52.7 kD) is another protein variant resulting from E10-18 that lacks the signal peptide and ectodomain part that is critical to interact with SARS-CoV-2.

Figure S3: Validation of oocyte, embryo, and ovarian follicular cell lysates for false positive signals. (A) MII oocyte, 1-cell (1C) and blastocyst (BL) stage embryo, and cumulus cell (CC) and granulosa cell (GC) protein lysates were analyzed on JESS in the absence of primary antibody. No false-positive chemiluminescent signal was observed as a result of cross reactivity of total protein lysates with secondary antibody (2AB). (B) Auto generated electropherogram by Compass software showing area under the blue line as total protein abundance of MII, 1C, BL, CC and GC samples.
SARS-CoV-2

RNA and N Protein
Spike (S) Protein
Membrane (M) Protein
Envelope (E) Protein

S protein binding to ACE2 or CD147 receptor
S protein activation by TMPRSS2 protease
CTSL mediated S protein activation
Infection

Endosomes

Figure 1
Figure 2

(A) Relative mRNA Expression

(B) ACE2 (100 kD)
ACE2-V1 (130 kD)
ACE2-V2 (62 kD)
ACE2-V3 (53 kD)

(C) Relative Protein Expression (Normalized to Total Protein)

ACE2 (100 kD)
ACE2-V1 (130 kD)
ACE2-V2 (63.9 kD)
ACE2-V3 (52.7 kD)
Figure 3

(A) 

```
|   | ND | ND | B  | B  |
|---|----|----|----|----|
| GV|    |    |    |    |
| MII|    |    |    |    |
| BL |    |    |    |    |
| CCsGCs|    |    |    |    |
```

Relative mRNA Expression

(B) 

```
KDa: 54 kD
```

(C) 

```
|   | MI| 1C | B | CCsGCs |
|---|---|----|---|--------|
| MI| AB| AB | AB|        |
```

Relative Protein Expression (Normalized to Total Protein)
Figure 4

(A) CD147

(B) Relative mRNA Expression

(C) Relative Protein Expression Normalized to Total Protein
Figure 5

(A) **CTSL**

(B) Relative Protein Expression (Normalized to Total Protein)

(C) Relative mRNA Expression