Expression of SARS-CoV-2 receptor ACE2 and the spike protein processing enzymes in developing human embryos

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Abstract

The novel coronavirus disease, COVID-19 caused by Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2), has resulted in thousands of critically ill patients and poses a serious threat globally. To investigate if SARS-CoV-2 can affect early human embryos, we analysed scRNA-seq datasets of human embryos for the SARS-CoV-2 receptors ACE2 and BSG (CD147), the serine protease TMPRSS2 for viral spike protein priming, and the endosomal protease CTSL for spike protein activation via the endosomal route. The results reveal that ACE2 and TMPRSS2 are co-expressed in a proportion of epiblast cells, and both BSG and CTSL are expressed in all the stages of embryonic development. The cells of the blastocysts express genes encoding for other coronavirus receptors such as DPP4 and ANPEP as well. Interestingly, the cells of the epiblast also express genes involved in viral endocytosis and replication. We further identified 194 genes that are differentially expressed in ACE2- and TMPRSS2-positive cells as compared to ACE2- and TMPRSS2-negative cells of the epiblast. Our results show that developing human embryos express the receptors for SARS-CoV-2 and other coronaviruses; embryos also harbour the necessary machinery for viral internalization and replication. We suggest that couples be advised to avoid conceiving during the pandemic and that IVF procedures be kept to a minimum to prevent any possible hazard to the developing embryos.

Keywords: SARS-CoV-2, COVID-19, ACE2, BSG, TMPRSS2, CTSL, Embryo, coronaviruses, viruses
Introduction

In December 2019, a cluster of atypical pneumonia associated with a novel coronavirus was first reported from Wuhan, Hubei province, in China (Zhu et al., 2020). The disease, termed COVID-19, is caused by coronavirus Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2), previously termed 2019-nCoV (Singhal, 2020). The virus has since spread worldwide and is a serious global health concern as it has high human-to-human transmission (Ralph et al. 2020).

SARS-CoV-2 has sequence similarity to the bat-SL-CoVZC45 and bat-SL-CoVZXC21; the external subdomain of the SARS-CoV-2 receptor binding domain is similar to that of the SARS-CoV identified in 2002 (Yadav et al., 2020; Zhu et al., 2020). Like most viruses, the coronaviruses enter the host cell by receptor binding followed by endocytosis, genome replication, exocytosis, and budding (Fehr et al. 2015). Recent studies have shown that the human Angiotensin-Converting Enzyme II (ACE2) is the host receptor for SARS-CoV-2 (Letko, Marzi and Munster, 2020). Furthermore, the SARS-CoV-2 does not use other coronavirus receptors viz; alanine aminopeptidase N (ANPEP) and dipeptidyl peptidase 4 (DPP4), which are used by CoV-229E and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), respectively, for entry into host cells (Li, 2015; Letko, Marzi and Munster, 2020).

ACE2 is necessary for entry of SARS-CoV-2 into host cells, and studies have shown that the spike (S) protein of SARS-CoV-2 is processed by transmembrane serine protease 2 (TMPRSS2) (Matsuyama et al. 2020, Hoffmann and Pöhmann 2020) for enhanced viral entry. Another endosomal protease, cathepsin L (CTSL), is also involved in processing of the SARS-CoV-2 S protein via the endosomal route (Ou et al., 2020). In addition, the SARS-CoV-2 utilizes Basigin (BSG), also known as CD147, as a receptor for viral entry (Wang et al., 2020). These findings imply that, in a given tissue, the cells co-expressing these molecules would be the key determinants of susceptibility to SARS-CoV-2 infection.

Respiratory distress is one of the common clinical presentations of SARS-CoV-2 infection, and the lung airway epithelium is a primary target for SARS-CoV-2 action. Indeed, ACE2 and TMPRSS2 co-expressing cells are reported amongst human airway alveolar epithelial cells (Lukassen et al., 2020; Qi et al., 2020). Beyond the lung, ACE2 is expressed in other human organs such as the oral and nasal mucosa, nasopharynx, stomach, small intestine, colon, skin, lymph nodes, thymus, bone marrow, spleen, liver, and kidney (Hamming et al., 2004; Qi et al., 2020; Seow et al., 2020; Xu et al., 2020), suggesting that SARS-CoV-2 may have more widespread effects than presently thought based on clinical presentations.

Beyond the adult tissues, certain viruses can also infect the gametes and the developing embryos of the host (Racicot and Mor 2017). However, the main focus of the studies on SARS-CoV-2 and host interactions is on adult tissues, little is known about the effects of the virus on embryos. Studies on preimplantation mammalian embryos are important because maternal viral infections during early pregnancy can induce embryonic death or abnormal embryonic development (Hardy, 1974; Silasi et al., 2015; Racicot and Mor, 2017). With the high population estimates of SARS-CoV-2 spread (Li et al., 2020) and the observation that a large proportion of individuals with SARS-CoV-2 infection may be asymptomatic, (Ling et al. 2020, Pan et al. 2020, Wu et al. 2020) it is likely that many women may have conceived during the course of subclinical infection. Secondly, many assisted reproduction
clinics may have handled human gametes and embryos from subclinically infected couples or inadvertently exposed them to infected laboratory personnel. Given the fact that SARS-CoV-2 has a long shelf life on various surfaces (Ong et al., 2020; van Doremalen et al., 2020), there are many potential routes by which gametes/embryos could be exposed to SARS-CoV-2. However, whether the gametes or early human embryos can be affected by SARS-CoV-2 or other coronaviruses remains unknown.

To explore this possibility, in the present study, we analysed single-cell RNA-Seq datasets of human embryos (Yan et al., 2013; Stirparo et al., 2018) to determine the expression of the SARS-CoV-2 receptors ACE2 and BSG as well as S protein primers TMPRSS2 and CTSL in zygote to late-blastocyst-stage human embryos. In addition, we determined whether the cells of early embryos also express endocytotic and viral replication machinery.

**Methods**

Single-cell RNA-Seq data were obtained (Yan et al., 2013; Stirparo et al., 2018) and levels of ACE2, BSG, TMPRSS2, CTSL, DPP4, and ANPEP (in terms of fragments per kilobase of transcript per million mapped reads, FPKM) were obtained for each cell at each stage. The numbers and percentage of cells co-expressing ACE2 and BSG, ACE2 and TMPRSS2, ACE2 and CTSL, and BSG and CTSL were also calculated. Supplementary Table 1 gives the number of embryos and total number of cells tested at each time point of embryonic development.

The list of genes involved in the Endosomal Sorting Complex Required for Transport (ESCRT I, II, and III) and the list of genes involved in SARS virus replication in host cells were obtained (de Wilde et al., 2018; Ahmed et al., 2019). A recent study identified 332 human proteins that physically interact with SARS-CoV-2 (Gordon et al., 2020). Mean FPKM values for these genes were extracted and datasets were visualized using Morpheus (https://software.broadinstitute.org/morpheus) and R Studio version 3.6.2.

To determine the phenotypes of ACE2 in epiblast cells, the numbers of ACE2- and TMPRSS2-positive and ACE2- and TMPRSS2-negative cells were identified, and the mean FPKM values of all the genes expressed in both the datasets were computed. The data were filtered for genes whose mean values were ≥0.5 FPKM. This was followed by obtaining the ratio of the mean value in ACE2- and TMPRSS2-positive cells / ACE2- and TMPRSS2-negative cells. Only those genes that had a ratio of ≥1.5 or ≤0.5 were filtered. Student’s t test was applied. Only those genes that had significantly different mean values (p<0.05) were filtered.

The biological processes associated with the differentially expressed genes in ACE2- and TMPRSS2-positive cells were obtained using the DAVID Knowledgebase version 6.8 (https://david.ncifcrf.gov) using the GOTERM_BP_FAT filters with highest stringency. Categories with enrichment score >0.5 were considered for analysis.
**Results**

mRNA levels of coronavirus-associated genes *ACE2, TMPRSS2, BSG, CTSL, DPP4*, and *ANPEP* in early stages of human embryonic development

Table 1 summarizes the receptors used by the various coronaviruses known to infect humans. We explored publicly available online datasets of human preimplantation embryos at different stages of development to determine the mRNA level of these proteins. As evident from Figure 1., high amounts of *ACE2* mRNA was detected in the zygotic stage of development followed by a decrease until morula stage with a subsequent sudden increment in the inner cell mass (ICM), epiblast, and the primitive endoderm. *BSG* mRNA was consistently detected in all stages of embryonic development with an increase in levels from zygotic stage to blastocyst stage. *TMPRSS2* was not detected in the zygotes, but a low abundance of transcripts was detected at early stages of embryonic development that further increased during later stages. The highest mRNA levels of *TMPRSS2* were observed in epiblast cells. *DPP4* and *ANPEP* mRNA was not detected in the zygote or 4-cell embryos; however, abundant *DPP4* and *ANPEP* transcripts were abundantly detected at all stages of embryonic development (Figure 1.). The pattern of expression for all the genes remained roughly consistent in another independent dataset of single-cell RNA-Seq of human embryos (Supplementary Fig. 1.).

**Table 1: Summary of host receptors of coronaviruses that infect humans**

| Virus      | Human host receptor          |
|------------|------------------------------|
| SARS-CoV-2 | ACE2, CD147 (BSG)           |
| SARS-CoV   | ACE2, CD147 (BSG)           |
| MERS-CoV   | DPP4                        |
| HCoV-OC43  | 9-O-acetylsialic acids      |
| HCoV-HKU1  | 9-O-acetylsialic acids      |
| HCoV-229E  | ANPEP                       |
| CoV-NL63   | ACE2                        |

Co-expression of transcripts of SARS-CoV-2 receptor *ACE2* and *TMPRSS2*, *BSG*, and *CTSL* in early stages of human embryonic development
We next determined the numbers of cells at different stages of embryonic development that co-express transcripts of ACE2 and TMPRSS2. In addition, we determined the co-expression of ACE2 and CTSL, ACE2 and BSG, and BSG and CTSL. The results reveal that at the zygote and 4-cell stage, all cells co-express ACE2 and BSG (Figure 2). Nearly 86% (37/43) of cells co-expressed ACE2 and BSG in the ICM, 39% (25/64) in the epiblast, and 39% (11/28) in the primitive endoderm (Supplementary Table 1). Maximum numbers of ACE2 and TMPRSS2 co-expressing cells were detected in the epiblast (Figure 2). All the ACE2- and TMPRSS2-positive cells in the epiblast also co-expressed BSG and CTSL. In general, BSG and CTSL were abundantly detected in almost all cells of the developing embryos from the morula stage to the blastocyst stage (Figure 2).

Fig. 1. mRNA expression of coronavirus receptors and spike protein processing enzymes in early human embryos. Data was extracted from single-cell RNA-Seq of developing human embryos (Stirparo et al., 2018). The X axis represents various stages of human embryonic development and Y axis represents FPKM values. Each dot represents data of a single cell at that developmental stage. ACE2 and BSG are the receptors for SARS-CoV-2 and SARS-CoV, DPP4 is the receptor for MERS-CoV, and ANPEP is the receptor for HCoV-229E. The viral spike protein is processed by host enzymes TMPRSS2 or CTSL.
Fig. 2. Co-expression of mRNA of SARS-CoV-2 receptors and spike protein processing enzymes in early human embryos.
Co-expression of ACE2 and TMPRSS2, ACE2 and BSG, ACE2 and CTSL, and BSG and CTSL in single cells of human embryos at zygote, 4-cell, 8-cell, compact morula, early inner cell mass (ICM) epiblast and primitive endoderm. Each dot represents data of a single cell; double positive co-expressing cells are shown in blue, single positive cells are shown in red and purple. Data was extracted from single-cell RNA-Seq of developing human embryos (Stirparo et al., 2018).
mRNA levels of genes involved in viral endocytosis and replication in human embryos at different stages of development.

We next hypothesized that if the virus can bind to the embryonic cells, the cells may contain the necessary machinery for viral endocytosis and viral replication. Towards this, we analysed the average FPKM values of the 33 genes involved in human ESCRT of viruses. We observed that from zygote to morula stage, most of the ESCRT genes are absent or present in low abundance. However, the transcripts of almost 90% of these genes dramatically increased in the ICM, epiblast, and primitive endoderm (Figure.3A.).

SARS-CoV viruses enter the host cells and utilize the host cell machinery for replication. We analysed the mRNA levels for host genes involved in SARS-CoV replication and observed that the genes involved in viral replication were absent or present at low abundance from zygote to morula stages of embryonic development. However, the mRNA levels of most of these genes surged in the ICM, epiblast, and the primitive endoderm (Figure.3B.).

Analysis of individual cells of the epiblast revealed that almost all the genes whose protein products play a role in ESCRT were expressed in most cells of the ACE2- and TMPRSS2-positive and negative cells with minimal cell-to-cell heterogeneity in level of expression (Figure.3C.). Almost all the host genes involved in viral replication are expressed in most of the cells with little heterogeneity (Figure.3D.).

![Fig.3](image-url)

**Fig.3. Heat maps of the genes involved in viral endocytosis and replication in human embryos at different stages of development.** All data were extracted from single-cell RNA-Seq of developing human embryos (Stirparo et al., 2018). In all heat maps, each row depicts a gene and each column depicts a stage of embryonic development (in A and B) or each cell in the epiblast (in C and D). A) Genes involved in endosomal sorting complexes required for transport (ESCRT) in different stages of embryonic development. B) Genes involved in coronavirus replication in different stages of embryonic development. C) Genes involved in ESCRT in ACE2- and TMPRSS2-negative (ACE2–TMPRSS2–) and ACE2- and TMPRSS2-positive (ACE2+TMPRSS2+) cells of the epiblast D) Genes involved in coronavirus replication in in ACE2- and TMPRSS2-negative (ACE2–TMPRSS2–) and ACE2- and TMPRSS2-positive (ACE2+TMPRSS2+) cells of the epiblast.
SARS-CoV-2 interacts with 332 proteins in human cells. In the epiblast of human embryos, mRNA for 320/332 SARS-CoV-2 interacting proteins was detected. However, some of the mRNA transcripts were highly abundant and others were weakly expressed (Figure 4). Although the heterogeneity in mRNA levels of SARS-CoV-2 interacting proteins between cells was not high, 54/320 genes were overexpressed (ratio > 1.5) in ACE2- and TMPRSS2-positive cells as compared to ACE2- and TMPRSS2-negative cells of the epiblast.

Fig. 4. mRNA levels of genes in the epiblast of human embryos whose protein products are known to interact with SARS-CoV-2.

Information for 320 genes whose protein products are reported to interact with SARS-CoV-2 was extracted from single-cell RNA-Seq of the epiblast of developing human embryos (Stirparo et al., 2018). Each row is a gene and each column is a single cell of the epiblast. The data is a relative color scheme in which the minimum and maximum values in each row are used to convert values to colors.
Differentially expressed genes and the biological processes enriched in ACE2 and TMPRSS2 co-expressing cells of the epiblast

In the epiblast, 19 cells expressed ACE2 and TMPRSS2 while 23 cells did not express both ACE2 and TMPRSS2 (Figure.2.). We identified 194 genes that were differentially expressed (fold change >1.5 or <0.5) with statistical significance (p<0.05) between ACE2- and TMPRSS2-positive cells and ACE2- and TMPRSS2-negative cells. Figure.5A. depicts the distribution of these 194 differentially expressed genes in each cell of the epiblast. As evident from Figure.5A., as compared to ACE2- and TMPRSS2-negative cells, most (189/194) of these genes were overexpressed in the ACE2- and TMPRSS2-positive cells of the epiblast; only 5 genes were downregulated. While there was extensive cell-to-cell heterogeneity in expression of the 194 differentially abundant genes in the ACE2- and TMPRSS2-negative cells, most of these genes were uniformly upregulated in most ACE2- and TMPRSS2-positive cells. Analysis of the differentially expressed genes revealed that they belong to diverse biological processes including genes involved in various metabolic processes, development, and viral entry into host cells (Figure.5B.).
Fig. 5. Differentially expressed genes and the biological processes enriched in ACE2- and TMPRSS2-positive cells in epiblast of developing human blastocysts. Data were extracted from single-cell RNA-Seq of the epiblast of developing human embryos (Stirparo et al 2018). A) Distribution of 194 differentially expressed genes in ACE2- and TMPRSS2-negative (ACE2–TMPRSS2–) and ACE2- and TMPRSS2-positive (ACE2+TMPRSS2+) cells of the epiblast. Rows represent genes and columns represent individual cells, presented on a relative colour scale. B) Biological processes enriched in the ACE2+TMPRSS2+ cells of the epiblast showing differential expression of 194 genes. The Y axis indicates the enriched biological processes and X axis is the enrichment score.
Discussion

The results of the present study demonstrate that early human embryos express coronavirus entry receptors and S protein proteases. Embryonic cells also express the genes for proteins that are involved in viral endocytosis and replication.

Considering the scale at which the SARS-CoV-2 virus has spread globally and the fact that a proportion of individuals harbouring the virus are initially asymptomatic, it is likely that some of the infected individuals may have conceived or are trying to conceive during the duration of the pandemic. Further, with nearly 1 in 6 couples worldwide facing infertility, many couples resort to in vitro fertilization (IVF) techniques for achieving biological parenthood. In both these scenarios, it is imperative to understand whether the developing embryo is at risk of SARS-CoV-2 infection. To address this question, we analysed single-cell RNA-Seq datasets of developing human embryos for SARS-CoV-2 receptors ACE2 and BSG along with the spike protein processing enzymes TMPRSS2 and CTSL in zygotes to hatched blastocysts. Our results reveal that gametes, zygotes, and 4-cell embryos express abundant amounts of ACE2 and BSG along with CTSL but not TMPRSS2. While the levels of ACE2 decline in the compact morula, the expression of ACE2 and the processing enzymes increases in the ICM and epiblast. While ACE2 is essential, the processing of the spike protein by the membrane-bound serine protease TMPRSS2 promotes viral infectivity. We observed that although more than 80% of cells of the ICM express ACE2, none of these cells express TMPRSS2, indicating that the early ICM may not be highly susceptible to viral infection. However, in the epiblast (but not the primitive endoderm) a significant proportion of cells co-express ACE2 and TMPRSS2 suggesting that these cells would be targets for viral entry. Beyond ACE2, a study has shown that the extracellular matrix metalloproteinase enhancer CD147 encoded by the gene BSG binds to both SARS-CoV-2 and SARS-CoV and promotes viral entry independent of ACE2 and TMPRSS2 (Chen et al., 2005; Wang et al., 2020). We observed that BSG transcripts were abundantly expressed in all the cells of the developing embryos from zygote stage to blastocyst stage. With regard to S protein priming, cathepsins are a class of endosomal proteases, and of these, cathepsin L encoded by CTSL is required for endosomal cleavage of SARS-CoV and SARS-CoV-2 spike proteins (Ou et al., 2020). Interestingly, along with BSG, CTSL was co-expressed in most of the cells of the developing embryo. Presently it is unclear if CD147-mediated viral entry requires cathepsin L; the fact that both these proteins are co-expressed in most embryonic cells implies that developing embryos may be susceptible to coronavirus infections by the ACE2-independent mode of action.

Two other members of the coronavirus family that are highly infectious and cause significant mortality are MERS and HCoV-NL63. Unlike SARS-CoV and SARS-CoV-2, MERS and HCoV-229E utilize DPP4 and ANPEP as receptors to infect the human host cells (Li, 2015). Interestingly, we found that DPP4 and ANPEP transcripts are not expressed in the zygotes until the morula stage, but both receptors are expressed in almost all the cells of the ICM, epiblast, and early endoderm. Together our data for the first time suggest that early human embryos could be susceptible to infection by coronaviruses in general.

Once the virus binds to the receptor and the spike protein undergoes cleavage, SARS-CoV-2 releases its content into the host cells and initiates replication. In this course, most enveloped viruses recruit the ESCRT machinery in which the viral structural protein engages the tumour susceptibility gene protein (TSG101) in ESCRT-I, which ultimately delivers ESCRT-III to sites of viral budding where
membrane scission releases the viral particles (Votteler and Sundquist, 2013). Several proteins are involved in the ESCRT pathway, and the blocking of any of these enzymes can prevent viral endocytosis including that of the coronavirus family (Wang et al., 2017; Mazzon and Marsh, 2019). For SARS-CoV-2 to be pathogenic to human embryos, the early embryonic cell must possess the components of the ESCRT machinery for viral endocytosis and release. Interestingly, we observed that genes for many members of the ESCRT machinery are not expressed in zygote and morula stages, but a surge in the expression of these select genes occurs in the cells of the blastocyst including the ICM, epiblast, and primitive endoderm. These results indicate that the human blastocysts expressing SARS-CoV-2 receptors not only have the necessary machinery for the virus to bind, but also facilitate endocytosis and viral budding.

Once the virus enters the cells, the next step in the infection cycle is viral replication via the interaction of cellular host proteins with the viral proteins (de Wilde et al., 2018). Our results reveal that as compared to the zygote, 4- to 8-cell stages, and morula stage embryos, the cells of the blastocyst show a surge in mRNA expression of most host proteins involved in viral replication. Almost all of the proteins involved in viral replication were abundantly expressed in the ICM, epiblast, and primitive endoderm. Together our data show that early developing human embryos not only have the necessary machinery to bind the virus and permit its entry and exit, but also facilitate viral replication.

Our analysis, so far, suggests that several genes involved in endocytosis and viral replication are expressed in the cells of developing embryos. However, it remains imperative to determine if all or a subset of the cells express these genes. Since most ACE2- and TMPRSS2-positive cells were present in the epiblast, we analysed the data of individual cells of the epiblast and observed that irrespective of the expression of ACE2 and TMPRSS2, cells of the epiblast express transcripts of genes involved in viral endocytosis and replication, albeit with varying degrees of expression. These genes were also uniformly expressed in cells of the early ICM and the primitive endoderm (not shown). These results imply that the epiblast and plausibly other cells of the blastocyst are susceptible to SARS-CoV-2 infection. Since the developing embryos lack a functional immune system, the viruses from these embryonic cells may not be cleared, allowing the viral replication cycle to proceed soon after infection.

Once the viral RNA enters the host cells, it translates several of its non-structural proteins, which in turn physically interact with various host proteins to regulate host cell machinery. A recent study has identified 332 human proteins that physically interact with SARS-CoV-2 in HEK293T cells (Gordon et al., 2020). The proteins that interact with SARS-CoV-2 have a wide range of functional roles including DNA replication, vesicle trafficking, lipid modification, RNA processing and regulation, nuclear transport machinery, cytoskeletal organization, mitochondrial functions, and extracellular matrix modelling (Gordon et al., 2020). We observed that mRNA encoding for 320 of the 332 SARS-CoV-2 interacting proteins are expressed in the epiblast of the developing embryo. Almost all of these 320 genes were uniformly expressed in all the cells of the epiblast and also in the ICM and primitive endoderm (not shown). Interestingly, as compared to ACE2- and TMPRSS2-negative cells, 54/320 genes whose protein products physically interact with SARS-CoV-2 are overexpressed in ACE2- and TMPRSS2-positive cells of the epiblast. Together our observations imply that in human embryos, a proportion of cells in the epiblast abundantly expresses the mRNA for proteins that interact with SARS-CoV-2 to regulate host cellular processes.
In developing embryos, the health of the cells of the epiblast is crucial, since these cells subsequently undergo organogenesis. Any damage to these cells, physically or functionally, may lead to embryo lethality or dysfunctions in the organs of the foetus/adults. To understand the potential damage that may occur to the embryos by SARS-CoV-2 infection, we analysed the gene signature of ACE2- and TMPRSS2-positive cells of the epiblast. We identified mRNA for 194 genes that were differentially expressed between the ACE2- and TMPRSS2-positive cells and ACE2- and TMPRSS2-negative cells of the epiblast. Unique gene signatures of ACE2-positive cells have also been reported for other tissues (Seow et al., 2020; Xu et al., 2020). Interestingly, as compared to ACE2- and TMPRSS2-negative cells, 189/194 genes were overexpressed in the ACE2- and TMPRSS2-positive cells, and only 5/194 genes were present in lower abundance in the ACE2- and TMPRSS2-positive cells. Amongst the overabundant genes were cathepsins (including CTSL), prostaglandin synthetase, and glutamyl aminopeptidase, which have been associated with SARS-CoV-2 infection or enriched with ACE2 in different tissues (Ou et al., 2020; Qi et al., 2020). In the adult human liver, the highest expression of ACE2 and TMPRSS2 is detected in TROP2-positive cells (Seow et al., 2020). In the present study, high expression of TROP2 (also known as TACSTD2) was also detected in the cells of the epiblast. These results imply that the ACE2- and TMPRSS2-positive cells may have unique functions in developing embryos. Interestingly, functional annotation revealed that the 194 differentially abundant genes play important roles in many aspects of development such as cell migration and metabolism. Furthermore, the gene signature also included genes facilitating viral entry into host cells and vacuole acidification. In this context it is imperative to note that pH reduction inside endosomes is essential for CTSL activation, which in turn is required to cleave the spike protein of SARS-CoV and SARS-CoV-2 (Ou et al., 2020). Thus, our analyses reveal that the ACE2- and TMPRSS2-positive cells in the epiblast have key roles in embryonic development and may be particularly susceptible to SARS-CoV infection. Damage to these cells may cause embryo lethality at early stages of development or damage the process of tissue specification.

Beyond ACE2 for SARS-CoV, DPP4 and ANPEP are other known coronavirus receptors (Li, 2015). We herein show the expression profiles of both DPP4 and ANPEP at the blastocyst stage, indicating that different coronaviruses can target similar cell types in the human host. Future studies should investigate these proteins and their genetic networks in embryonic cells to experimentally confirm the effects of coronaviruses on early human development.

With regard to other viral infections in pregnancy, miscarriages and stillbirths are more common with influenza, and the mosquito-borne Zika virus adversely affects pregnancy and foetal development (Adams Waldorf et al., 2018). Currently, little is known about the impact of COVID-19 on pregnancy. While coronaviruses are unrelated to the influenza and Zika viruses, our data imply potential embryotoxic effects of SARS-CoV-2 and other coronaviruses. Presently, the American Society of Reproductive Medicine recommends that individuals with confirmed or presumed COVID-19 should avoid pregnancy. However, there is no clarity on the course of action for women/couples seeking IVF treatment, and several clinics continue to service patients seeking IVF, excluding only those who are symptomatic or who have recent travel history. The European Society of Human Reproduction and Embryology (ESHRE) has released its report of the ESHRE COVID-19 working group to monitor scientific reports relevant to reproductive medicine. The committee recommends that assisted reproduction treatments should not be started during the COVID-19 pandemic mainly for social reasons, as the report indicates that there is no specific data available; instead, it states an assumption
that sperm, oocytes, and embryos do not have receptors for SARS-CoV-2 and are unlikely to be infected. Contradicting this assumption, we herein show that the receptors for coronaviruses are expressed in developing embryos. While we believe that the zona pellucida will act as a barrier to viral entry in the zygotes until the morula stages, receptors for coronaviruses are abundantly expressed in the cells of hatched blastocysts that can potentially get exposed to contaminated media and surfaces. We believe that our data will aid clinicians and societies in decision making regarding the management of patients seeking IVF or reporting miscarriages during the pandemic.

In the light of our data and the possibility that the SARS-CoV-2 infected patients/clinic and laboratory personnel may be asymptomatic, we do not suggest performing IVF procedures during the COVID-19 pandemic. Procedures, if any, carried out during the pandemic should be executed with utmost precaution; patients, embryologists, and doctors should be aware of the potential risks; and couples must be appropriately counselled.

**Conclusion**

Our single-cell RNA-Seq analysis shows that many cells of developing human embryos express the receptors for coronaviruses and also contain the necessary machinery for viral internalization and replication. We suggest that couples should be advised to avoid conception and pregnancy during the pandemic and IVF procedures should be kept to a minimum. Asymptomatic patients and laboratory/clinical personnel should be frequently screened for SARS-CoV-2 and other coronaviruses to prevent potential threats to the developing embryos.

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References

Adams Waldorf, K. M. et al. (2018) ‘Congenital Zika virus infection as a silent pathology with loss of neurogenic output in the fetal brain’, Nature Medicine. Nature Publishing Group, 24(3), pp. 368–374. doi: 10.1038/nm.4485.

Ahmed, I. et al. (2019) ‘The regulation of Endosomal Sorting Complex Required for Transport and accessory proteins in multivesicular body sorting and enveloped viral budding - An overview’, International Journal of Biological Macromolecules, 127, pp. 1–11. doi: 10.1016/j.ijbiomac.2019.01.015.

Chen, Z. et al. (2005) ‘Function of HAb18G/CD147 in Invasion of Host Cells by Severe Acute Respiratory Syndrome Coronavirus’, The Journal of Infectious Diseases. Oxford University Press (OUP), 191(5), pp. 755–760. doi: 10.1086/427811.

van Doremalen, N. et al. (2020) ‘Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1.’, The New England journal of medicine. doi: 10.1056/NEJMc2004973.

Gordon, D. E. et al. (2020) ‘A SARS-CoV-2-Human Protein-Protein Interaction Map Reveals Drug Targets and Potential Drug-Repurposing’, bioRxiv. Cold Spring Harbor Laboratory, p. 2020.03.22.002386. doi: 10.1101/2020.03.22.002386.

Hamming, I. et al. (2004) ‘Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis’, Journal of Pathology, 203(2), pp. 631–637. doi: 10.1002/path.1570.

Hardy, J. B. (1974) ‘Fetal consequences of maternal viral infections in pregnancy’, Obstetrical and Gynecological Survey, 29(4), pp. 265–267. doi: 10.1097/00006254-197404000-00011.

Letko, M., Marzi, A. and Munster, V. (2020) ‘Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses’, Nature Microbiology. Nature Research, 5(4), pp. 562–569. doi: 10.1038/s41564-020-0688-y.

Li, F. (2015) ‘Receptor Recognition Mechanisms of Coronaviruses: a Decade of Structural Studies’, Journal of Virology, 89(4), pp. 1954–1964. doi: 10.1128/jvi.02615-14.

Li, R. et al. (2020) ‘Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV2).’, Science (New York, N.Y.). doi: 10.1126/science.abb3221.

Lukassen, S. et al. (2020) ‘SARS-CoV-2 receptor ACE2 and TMPRSS2 are predominantly expressed in a transient secretory cell type in subsegmental bronchial branches’, bioRxiv. Cold Spring Harbor Laboratory, p. 2020.03.13.991455. doi: 10.1101/2020.03.13.991455.

Mazzon, M. and Marsh, M. (2019) ‘Targeting viral entry as a strategy for broad-spectrum antivirals [version 1; peer review: 3 approved]’, F1000Research, 8. doi: 10.12688/f1000research.19694.1.

Ong, S. W. X. et al. (2020) ‘Air, Surface Environmental, and Personal Protective Equipment Contamination by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) From a Symptomatic Patient.’, Jama, pp. 3–5. doi: 10.1001/jama.2020.3227.

Ou, X. et al. (2020) ‘Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV.’, Nature communications. Springer US, 11(1), p. 1620. doi: 10.1038/s41467-020-15562-9.

Qi, F. et al. (2020) ‘Single cell RNA sequencing of 13 human tissues identify cell types and receptors of human coronaviruses’, Biochemical and Biophysical Research Communications. doi: 10.1016/j.bbrc.2020.03.044.

Racicot, K. and Mor, G. (2017) ‘Risks associated with viral infections during pregnancy The Journal of Clinical Investigation’, J Clin Invest, 127(5), pp. 1591–1599. doi: 10.1172/JCI87490.
Seow, J. J. W. et al. (2020) ‘scRNA-seq reveals ACE2 and TMPRSS2 expression in TROP2+ Liver Progenitor Cells: Implications in COVID-19 associated Liver Dysfunction’, bioRxiv, p. 2020.03.23.002832. doi: 10.1101/2020.03.23.002832.

Silasi, M. et al. (2015) ‘Viral Infections During Pregnancy’, American Journal of Reproductive Immunology, 73(3), pp. 199–213. doi: 10.1111/aji.12355.

Singhal, T. (2020) ‘A Review of Coronavirus Disease-2019 (COVID-19)’, Indian Journal of Pediatrics. Springer. doi: 10.1007/s12098-020-03263-6.

Stirparo, G. G. et al. (2018) ‘Integrated analysis of single-cell embryo data yields a unified transcriptome signature for the human pre-implantation epiblast’, Development (Cambridge), 145(3). doi: 10.1242/dev.158501.

Votteler, J. and Sundquist, W. I. (2013) ‘Virus budding and the ESCRT pathway’, Cell Host and Microbe, 14(3), pp. 232–241. doi: 10.1016/j.chom.2013.08.012.

Wang, K. et al. (2020) ‘SARS-CoV-2 invades host cells via a novel route: CD147-spike protein’, bioRxiv. Cold Spring Harbor Laboratory, p. 2020.03.14.988345. doi: 10.1101/2020.03.14.988345.

Wang, X. et al. (2017) ‘Development of small-molecule viral inhibitors targeting various stages of the life cycle of emerging and re-emerging viruses’, Frontiers of Medicine, 11(4), pp. 449–461. doi: 10.1007/s11684-017-0589-5.

de Wilde, A. H. et al. (2018) ‘Host factors in coronavirus replication’, in Current Topics in Microbiology and Immunology. Springer Verlag, pp. 1–42. doi: 10.1007/82_2017_25.

Xu, H. et al. (2020) ‘High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa’, International Journal of Oral Science. Springer US, 12(1), pp. 1–5. doi: 10.1038/s41368-020-0074-x.

Yadav, P. et al. (2020) ‘Full-genome sequences of the first two SARS-CoV-2 viruses from India’, Indian Journal of Medical Research, 0(0), p. 0. doi: 10.4103/ijmr.IJMR_663_20.

Yan, L. et al. (2013) ‘Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells’, Nature Structural and Molecular Biology. Nature Publishing Group, 20(9), pp. 1131–1139. doi: 10.1038/nsmb.2660.

Zhu, N. et al. (2020) ‘A novel coronavirus from patients with pneumonia in China, 2019’, New England Journal of Medicine. Massachussetts Medical Society, 382(8), pp. 727–733. doi: 10.1056/NEJMoa2001017.
|                   | ACE2+ TMPRSS2+ | ACE2+ BSG+ | ACE2+ CTSL+ | BSG+ CTSL+ | Total Cells Analysed | Total Embryos Analysed |
|-------------------|----------------|------------|-------------|-------------|----------------------|------------------------|
| Zygote            | 0 (0%)         | 3 (100%)   | 3 (100%)    | 3 (100%)    | 3                    | 3                      |
| 4-Cell Stage      | 0 (0%)         | 10 (100%)  | 10 (100%)   | 10 (100%)   | 10                   | 3                      |
| 8-Cell Stage      | 0 (0%)         | 5 (31%)    | 5 (31%)     | 16 (100%)   | 16                   | 3                      |
| Morula            | 0 (0%)         | 3 (10%)    | 3 (10%)     | 29 (100%)   | 29                   | 3                      |
| Inner Cell Mass   | 5 (12%)        | 37 (86%)   | 37 (86%)    | 43 (100%)   | 43                   | 3                      |
| Epiblast          | 22 (34%)       | 25 (39%)   | 25 (39%)    | 64 (100%)   | 64                   | 12                     |
| Primitive Endoderm| 6 (21%)        | 11 (39%)   | 11 (39%)    | 28 (100%)   | 28                   | 12                     |

**Supplementary Table 1:** Numbers and percentage of cells expressing ACE2+TMPRSS2+, ACE2+BSG+, ACE2+CTSL+, BSG+CTSL+ in human embryos at different stages. Data was extracted from single-cell RNA-Seq of the epiblast of developing human embryos (Stirparo et al 2018). The total cells and the numbers of embryos analysed are also given.
Supplementary Fig.1. mRNA expression of coronavirus receptors and spike protein processing enzymes in early human embryos. Data was extracted from single-cell RNA-Seq of developing human embryos (Yan et al., 2013). The X axis represents various stages of human embryonic development and Y axis represents FPKM values.