



Expression map of entry receptors and infectivity factors for pan-coronaviruses in preimplantation and implantation stage human embryos

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Abstract

Purpose To predict if developing human embryos are permissive to multiple coronaviruses.

Method We analyzed publicly available single-cell RNA-seq datasets of human embryos for the known canonical and non-canonical receptors and spike protein cleavage enzymes for multiple coronaviruses like SARS-CoV, SARS-CoV-2, MERS-CoV, hCoV-229E, and hCoV-NL63. We also analyzed the expression of host genes involved in viral replication, host proteins involved in viral endosomal sorting complexes required for transport (ESCRT), genes of host proteins that physically interact with proteins of SARS-CoV-2, and the host genes essential for coronavirus infectivity.

Results Of the known receptors of SARS viruses, *ACE2*, *BSG*, *GOLGA7*, and *ZDHHC5* were expressed in different proportions in the zygote, 4-cell, 8-cell, morula, and blastocysts including the trophectoderm. The MERS-CoV receptor, *DPP4*, and hCoV-229E receptor, *ANPEP*, were expressed mainly from the compact morula to the blastocyst stages. Transcripts of the MERS-CoV alternate receptor *LGALS1* were detected in most cells at all stages of development. *TMPRSS2* transcripts were detected in the epiblast, primitive endoderm, and trophectoderm, while transcripts of the endosomal proteases *CTSL*, *CTSB*, and *FURIN* were expressed in most cells at all stages of development. *ACE2* and *TMPRSS2* were co-expressed in a proportion of epiblast and trophectoderm cells. The embryonic cells expressed genes involved in ESCRT, viral replication, SARS-CoV-2 interactions, and coronavirus infectivity. The *ACE2* and *TMPRSS2* co-expressing cells were enriched in genes associated with lipid metabolism, lysosome, peroxisome, and oxidative phosphorylation pathways.

Conclusion Preimplantation and implantation stage human embryos could be permissive to multiple hCoVs.

Keywords Virus · Coronavirus · SARS-CoV-2 · COVID-19 · ACE2 · TMPRSS2 · Replication · Interaction · *In vitro* fertilization · scRNAseq

Introduction

Human coronaviruses (hCoVs) are zoonotic pathogens that cause respiratory tract illnesses ranging from mild common cold to serious virus-associated pneumonia. The seasonal

hCoVs including hCoV-OC43, hCoV-NL63, and hCoV-229E cause mild to moderate upper respiratory tract infections; the Severe Acute Respiratory Syndrome (SARS) family of hCoVs such as SARS-CoV, Middle East Respiratory Syndrome (MERS-CoV), and SARS-CoV-2 are highly pathogenic and cause serious diseases. With multiple spill-over events in recent history, it is clear that the hCoVs have the potential to cross species, spread rapidly, and cause significant morbidity and mortality [1].

Epidemiologic and clinical evidence suggests that pregnant women with hCoV infection are at a higher risk for severe illness and death, as well as adverse pregnancy outcomes, mother-to-child transmission, and congenital viral syndromes [2–6]. While the placenta protects the developing fetus from maternal infections, we and others have shown that the human

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placenta expresses the SARS-CoV-2 receptors and the virus has been detected in placental trophoblasts obtained from infected women, leading to vertical transmission [7–12]. Developmentally, the placenta originates from the cells of the trophoblastic layer of the blastocyst and could be potentially permissive to SARS-CoV-2 infection. Indeed, the SARS receptor, angiotensin-converting enzyme 2 (ACE2), and the viral spike (S) protein priming protease, transmembrane protease serine 2 (TMPRSS2), are expressed in early embryos, mainly in the trophoblastic layer [13–17] while the pseudotyped SARS-CoV-2 virus also enters the cells of the human blastocysts [18]. These observations indicate that human embryos are permissive to SARS-CoV-2 infection.

Considering the spread and pathogenicity of SARS-CoV-2, the current endeavors focused on SARS-CoV-2 are appropriate. However, judging by the rate at which highly virulent coronavirus strains are emerging, we must expand our focus to gain an insight into the permissiveness of human embryos not just to SARS-CoV-2 but other coronaviruses as well. For a complete understanding of the tropism of multiple hCoV, it is imperative to document the expression profiles of the canonical and non-canonical receptors, co-receptors, and other intracellular factors that are determinants of pan hCoV infectivity. However, if the human embryos are permissive to other hCoV infections is still unclear.

ACE2 and TMPRSS2 are the well-established receptor-enzyme pair required for entry of SARS and SARS-CoV-2 and hCoV-NL63 in host cells; MERS, on the other hand, utilizes dipeptidyl peptidase 4 (DPP4) while CoV-229E utilizes aminopeptidase N (ANPEP) as a host receptor [19, 20]. The expression of these receptors in the host cells is a determinant of viral entry. However, it is now apparent that most of the hCoVs are promiscuous and utilize a plethora of other host proteins that either, independently or synergistically with ACE2, aid in hCoV entry. These include basigin (BSG), neuropilin-1 (NRP1), zinc finger DHHC-type palmitoyltransferase 5 (ZDHHC5), golgin A7 (GOLGA7), and galectin 1 (LGALS1) which interact with the S protein of coronaviruses and potentially act as the non-canonical mechanism of entry [21–25]. Akin to the receptors, along with TMPRSS2, other members of the TMPRSS family such as the TMPRSS4 and TMPRSS11A aid in S protein priming [26, 27]. Furthermore, in the endosomal route of hCoV infection, cysteine proteases called cathepsins (CTSL and CTSB) and a paired basic amino acid cleaving enzyme called FURIN process the S proteins of multiple coronaviruses including that of SARS-CoV-2 [26–29]. The cathepsins and FURIN independently and cooperatively with TMPRSS2 aid in viral entry both in vivo and in vitro [30, 31]. However, limited information exists on the expression of such non-canonical factors and other endosomal proteases in human embryos.

For efficient endocytosis, replication, and budding, the hCoVs use an assortment of intracellular factors that are also considered to be determinants of infectivity. While this

information for SARS-CoV-2 and other coronaviruses is beginning to accumulate, studies have identified an array of host proteins that control the infectivity of multiple coronaviruses [7, 25, 32, 33]. Whether these factors are expressed in human embryos is currently not known.

In the present study, we developed an expression map of the canonical and non-canonical hCoV receptors, co-receptors, and the other intracellular factors that are determinants of hCoV entry, replication, and infectivity in the zygote to late-blastocyst-stage human embryos. We also characterized the molecular signature of the ACE2 and TMPRSS2 co-expressing cells to gain an insight into how the embryos might augment viral infectivity and survival. Our results show that the cells of developing embryos express the necessary machinery to aid in the infectivity of hCoVs. The ACE2 and TMPRSS2 co-expressing cells of the embryos are enriched for genes in metabolic pathways that promote viral survival and replication.

Materials and methods

To determine the mRNA levels of the factors that are determinants of hCoV entry, two single-cell RNA-Seq (scRNAseq) datasets [34, 35] were analyzed. The dataset by Stirparo et al. 2018 was chosen as it contained uniformly analyzed samples from three published studies [35–37] and recapitulated the known lineage markers of the zygote to late blastocysts. It also allowed an in-depth exploration of the transcriptome of multiple cells in the inner cell mass (ICM), epiblast and primitive endoderm. The dataset from Petropoulos et al. 2016 (accession number: E-MTAB-3929) was chosen as it permitted exploration of a large number of trophoblastic cells from day 5 to day 7 blastocysts. Supplementary Table 1 gives the number of embryos and the total number of cells analyzed in this study.

The host receptors utilized by various hCoVs were identified by systematic literature review and the list is given in Table 1. The genes encoding protein products involved in the endosomal sorting complex required for transport

Table 1 Summary of host receptors of coronaviruses that infect humans

Receptor	Viral tropism
ACE2	SARS-CoV-2, SARS-CoV, hCoV-NL63
ANPEP	hCoV-229E
BSG	SARS-CoV-2, SARS-CoV
DPP4	MERS-CoV
GOLGA7	SARS-CoV-2, SARS-CoV
LGALS1	MERS-CoV
NRP1	SARS-CoV-2, SARS-CoV
ZDHHC5	SARS-CoV-2, SARS-CoV

(ESCRT) and SARS virus replication in host cells were obtained [32, 38] and the list provided in Supplementary Tables 2 and 3 respectively. The mRNA levels of 332 human proteins that physically interact with proteins of SARS-CoV-2 [33] are listed in Supplementary Table 4. The expression of 67 host genes that are essential for infectivity of multiple coronaviruses [25] is given in Supplementary Table 5.

The mRNA levels of selected factors in individual cells of embryos at each stage of development were extracted from the datasets. Psuedo-bulk analysis was carried out for each gene as described before [9]. To define the gene signatures of *ACE2*+*TMPRSS2*+ cells, pseudo-bulk analysis of single-cell data of the epiblast and trophectoderm cells of day 7 blastocyst was carried out. The epiblast and day 7 trophectoderm were chosen as they had a reasonable proportion of cells co-expressing *ACE2*+*TMPRSS2*+ versus those that did not express either. This allowed us to have enough cells in both groups to reasonably apply statistical testing. For pseudo-bulk analysis, the mean values of all the genes expressed in the *ACE2* and *TMPRSS2* co-expressing cells (*ACE2*+*TMPRSS2*+) and *ACE2* and *TMPRSS2* negative cells (*ACE2*-*TMPRSS2*-) were computed. Genes that had a ratio of ≥ 1.5 or ≤ 0.5 with a significantly different mean value ($p \leq 0.05$) using Welch's *t*-test were filtered as described previously [9].

To assess the permissiveness of human embryos to different hCoV, we noted the expression of the canonical receptors along with *TMPRSS2*. Those embryonic stages which co-expressed the canonical receptor along with *TMPRSS2* were considered permissive to hCoV. Besides, we scored the embryonic stages for the percentage of expressed genes in ESCRT, viral replication,

and SARS-CoV-2 infectivity. When 1–10% of the total genes for the above processes were expressed in the embryos, a score of +1 was assigned, a score of +2 was assigned when 11–50% of genes were expressed, and a score of +3 was assigned when >50% of genes were expressed.

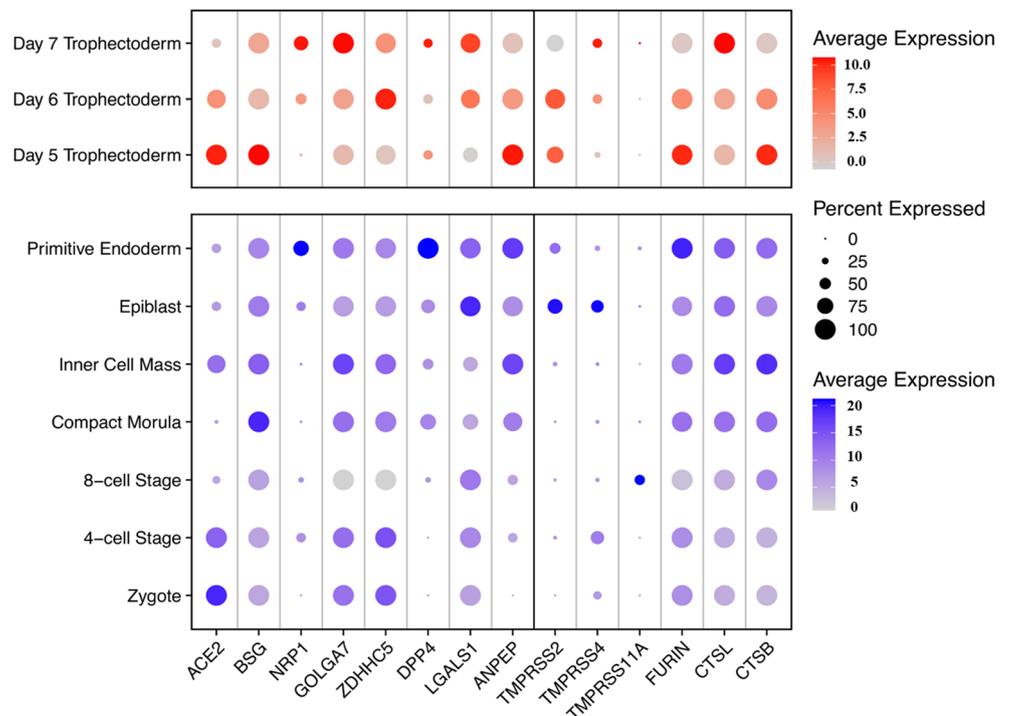
The Gene Ontologies (GO) were obtained using gProfiler (<https://biit.cs.ut.ee/gprofiler/gost>). Categories enriched with Benjamini-Hochberg False Discovery Rate [FDR] < 0.05 were considered. Pseudo-bulk and deconvoluted single-cell data were visualized using Morpheus (<https://software.broadinstitute.org/morpheus>) and R Studio version 3.6.2 with the Seurat and ggplot2 packages (RStudio Team, Boston, MA <http://www.rstudio.com/>). Figure 6 was created using BioRender (<https://biorender.com/>).

Results

Embryos express receptors and S protein processing enzymes for multiple coronaviruses

Of the SARS receptors, *ACE2* was abundantly present in the zygotes after which its level progressively reduced until the compact morula stage. The numbers of cells and the transcript levels of *ACE2* increased in the ICM and again reduced in the epiblast and primitive endoderm. Transcripts for *BSG*, *ZDHHC5*, and *GOLGA7* were detected in most cells of the developing embryo. *NRP1* mRNA was detected primarily in a proportion of cells of the epiblast and primitive endoderm with little or no expression in the cells at other developing stages (Fig. 1).

Fig. 1 mRNA expression of coronavirus receptors and spike protein processing enzymes in human embryos. Bubble plots showing relative expression and proportion of cells expressing *ACE2* (receptors for SARS-CoV, SARS-CoV-2, and CoV-NL63), *BSG*, *NRP1*, *ZDHHC5* and *GOLGA7* (SARS-CoV, SARS-CoV-2 alternate receptors), *DPP4* and *LGALS1* (receptor/interacting partners of MERS-CoV), and *ANPEP* (receptors for hCoV-229E). scRNAseq data for embryonic cells was obtained from [34] while that of trophectodermal cells on different days of embryonic development was obtained from [35]



DPP4 (the MERS-CoV receptor) mRNA was detected in a proportion of cells of the compact morula and the expression was highest in the primitive endoderm. *LGALS1* is a MERS S protein-interacting partner and its transcripts were detected in most cells of the embryos at all stages (Fig. 1).

ANPEP (the hCoV-229E receptor) transcripts were present in low abundance in the zygote to 8-cell stage embryos. The transcript levels increased in the compact morula, ICM, epiblast, and the primitive endoderm (Fig. 1).

TMPRSS2 mRNA was detected in a proportion of cells of the epiblast and primitive endoderm, while *TMPRSS4* transcripts were detected in the zygote, 4-cell stage, and the epiblast. *TMPRSS11A* was expressed weakly only in the 8-cell embryo (Fig. 1). *FURIN*, *CTSL*, and *CTSB* transcripts were detected in most of the cells of the developing embryos at all stages (Fig. 1).

Dynamic changes in expression of coronavirus receptors and S protein processing enzymes in the trophoctoderm

High levels of *ACE2* mRNA were detected in most trophoctoderm cells of the day 5 and 6 blastocysts which were reduced on day 7. *BSG*, *GOLGA7*, and *ZDHHC5* mRNA was expressed in all the cells of the trophoctoderm of day 5–7 blastocysts (Fig. 1). *NRP1* on the other hand was expressed only in a subset of trophoctoderm cells of day 6 blastocysts which increased on day 7.

Of the MERS receptors, *DPP4* was expressed in a small proportion of trophoctoderm cells of day 5–7 blastocysts. *LGALS1* mRNA levels progressively increased in the trophoctoderm of day 5 to day 7 blastocysts. *ANPEP* mRNA was abundantly expressed in all cells of the trophoctoderm of day 5 blastocysts which progressively reduced by day 7 (Fig. 1).

Of the S protein processing enzymes, *TMPRSS2* was abundantly expressed in most trophoctoderm cells of day 5 and day 6 blastocysts which reduced on day 7. In contrast, *TMPRSS4* mRNA levels progressively increased in a small proportion of trophoctoderm cells from day 5–7 blastocysts. *TMPRSS11A* however was weakly expressed in a very small proportion of trophoctoderm cells at all stages. *FURIN*, *CTSL*, and *CTSB* mRNA were expressed in varying proportions in most trophoctoderm cells of the day 5–7 blastocysts (Fig. 1).

Co-expression of *ACE2* and *TMPRSS2* in cells of developing embryos

Since the entry of SARS-CoV, SARS-CoV-2, and hCoV-NL63 in host cells requires the processing of the S protein on the cell surface, we determined the numbers of cells co-expressing *ACE2* and *TMPRSS2*. As evident from the tSNE plots in Fig. 2, zygote to morula stage embryos did not have any cells co-expressing *ACE2* and *TMPRSS2*. A small proportion of cells in the ICM (12%) and primitive endoderm (16%) co-expressed *ACE2* and *TMPRSS2*; 41% of the cells of the

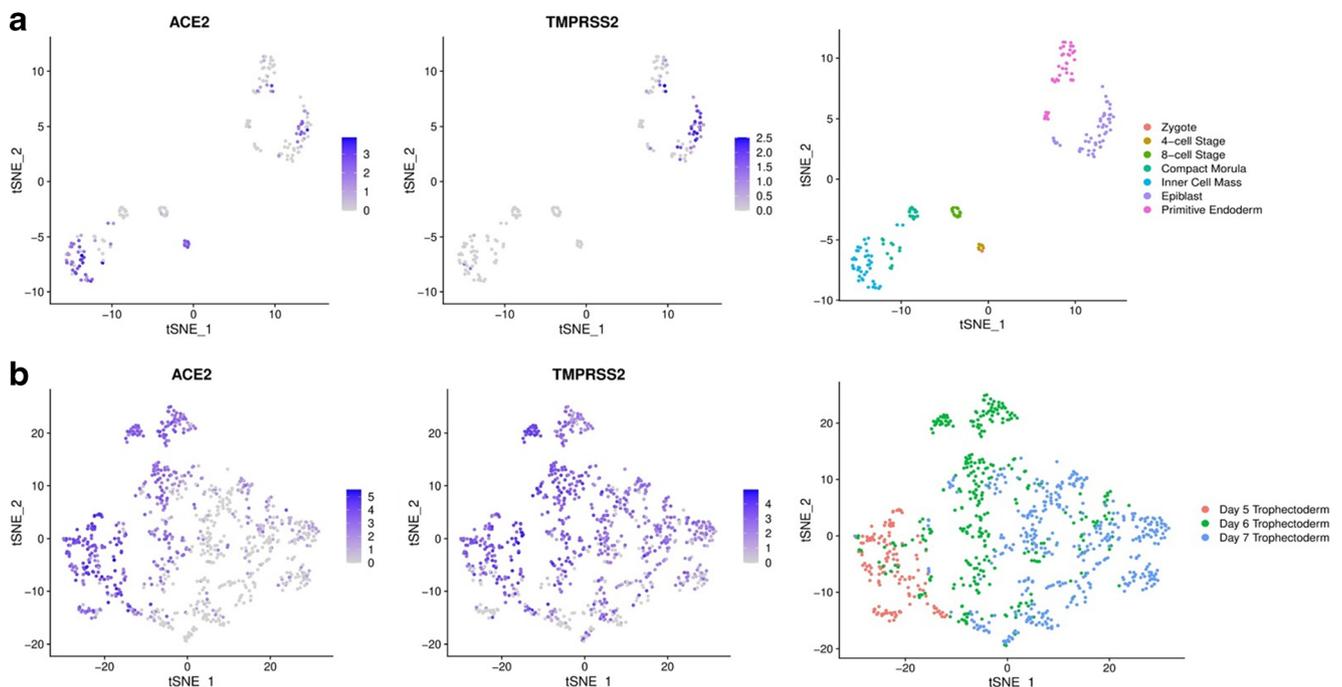


Fig. 2 *ACE2* and *TMPRSS2* expressing cells in the developing human embryos. tSNE plots for *ACE2* and *TMPRSS2* in cells of the developing embryos (a) and trophoctoderm of day 5–7 blastocysts (b). Data was obtained from scRNAseq of developing human embryos [34, 35]

epiblast co-expressed both these genes (Fig. 2, Supplementary Table 1).

In the trophectodermal lineage, 77% of day 5 blastocysts and 83% of day 6 blastocysts co-expressed *ACE2* and *TMPRSS2*. The numbers of *ACE2* and *TMPRSS2* co-expressing trophectoderm cells of the day 7 blastocyst declined to 33% (Fig. 2, Supplementary Table 1).

mRNA levels of proteins involved in viral endocytosis, replication, and SARS-CoV-2 interaction

We analyzed mRNA levels of the genes involved in ESCRT for viruses. Pseudo-bulk analysis revealed that very few ESCRT genes were expressed from the zygote to the 8-cell stage. The expression levels of these genes dramatically increased in the ICM, epiblast, primitive endoderm, and trophectoderm of day 5-7 blastocysts (Fig. 3, Supplementary Table 2).

Pseudo-bulk analysis revealed that mRNA of host genes involved in coronavirus replication were barely expressed in the zygote, 4- and 8-cell stages. Their levels surged in the compact morula, ICM, epiblast, and the primitive endoderm. Expression of most of these genes was observed in the trophectoderm cells of day 5-7 blastocysts (Fig. 3, Supplementary Table 3).

SARS-CoV-2 proteins interact with 332 proteins in human cells. As evident by pseudo-bulk analysis, only few of these genes were expressed in the zygote and 4-cell stage embryos. Their levels increased from the 8-cell stage onwards until the blastocyst stage. Most of these genes were also expressed in the trophectoderm of day 5-7 blastocysts (Fig. 3, Supplementary Table 4).

At single-cell resolution, the mRNA levels of proteins involved in ESCRT, viral replication, and SARS-CoV-2 interaction were found to have minimal heterogeneity in most cells of the epiblast and the trophectoderm, irrespective of their *ACE2* and *TMPRSS2* status (Supplementary Fig. 1).

Host genes essential for coronaviruses infectivity are expressed in the developing embryos

We next studied the mRNA levels of 67 host proteins that are experimentally shown to interact with proteins of multiple coronaviruses and are necessary for SARS-CoV-2 replication. Pseudo-bulk analysis revealed that most of these genes were expressed in low abundance in the cells of the zygote, 4- and 8-cell embryos. However, their expression increased in the compact morula, ICM, epiblast, primitive endoderm, and the trophectoderm (Fig. 3, Supplementary Table 5).

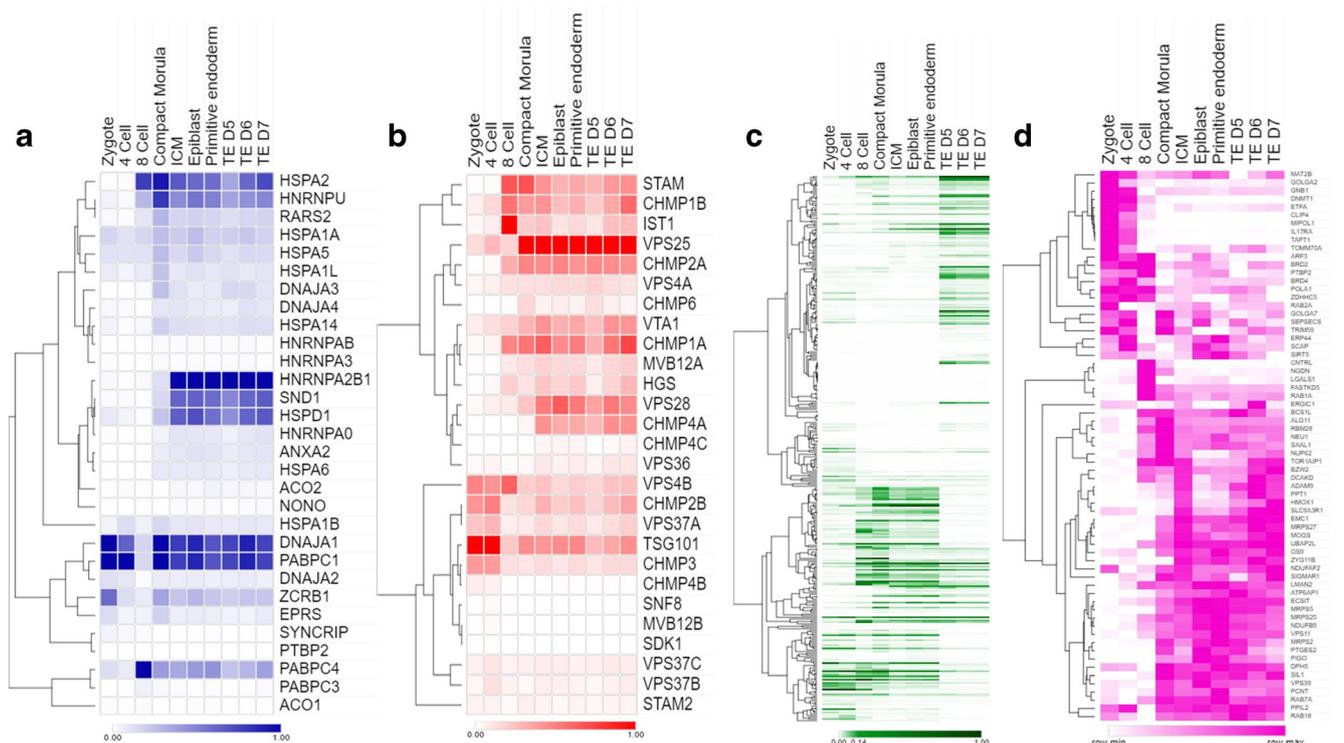


Fig. 3 mRNA levels of genes involved in endosomal complexes required for transport (ESCRT), viral replication, host-SARS-CoV-2 protein-protein interactions, and essential genes involved in coronavirus infectivity in human embryos. Pseudo-bulk data for the mRNA levels of genes involved in (a) ESCRT, (b) viral replication, (c) SARS-CoV-2

interactions, and (d) coronavirus infectivity. All data were extracted from scRNAseq of developing human embryos [34] and from trophectoderm cells from [35]. In all heat maps, each row depicts a gene and each column depicts a stage of embryonic development. Data is presented in the relative color scale

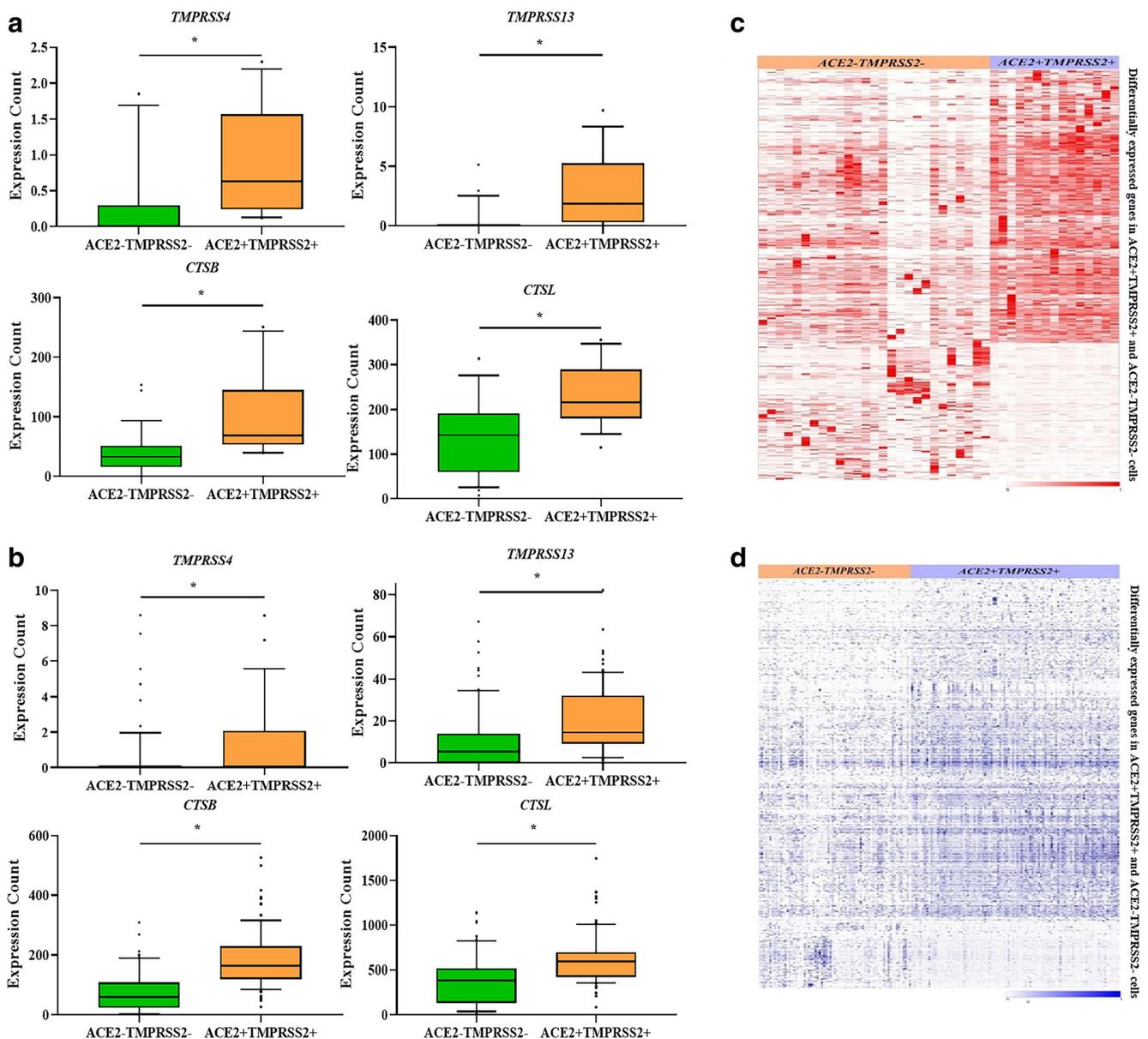


Fig. 4 Differentially expressed genes in *ACE2* and *TMPRSS2* co-expressing cells in epiblast and trophectoderm cells. **a** Expression of *TMPRSS4*, *TMPRSS13*, *CTSB*, and *CTSL* in *ACE2*–*TMPRSS2*– and *ACE2*+*TMPRSS2*+ cells of epiblast. **b** Expression of *TMPRSS4*, *TMPRSS13*, *CTSB* and *CTSL* in *ACE2*–*TMPRSS2*– and *ACE2*+*TMPRSS2*+ cells of trophectoderm. Significantly different ($p < 0.05$) mean values are marked as *. **c** Heat map of the distribution of differentially expressed 1985 genes in *ACE2*– and *TMPRSS2*–negative (*ACE2*–

TMPRSS2–) and *ACE2* and *TMPRSS2* co-expressing (*ACE2*+*TMPRSS2*+) cells of the epiblast. **d** Heat map of the distribution of 1453 differentially expressed genes in *ACE2*–*TMPRSS2*– and *ACE2*+*TMPRSS2*+ trophectoderm cells. In **c** and **d**, rows represent genes and columns represent individual cells, presented on a relative color scale. Data were extracted from scRNAseq of the epiblast of developing human embryos [34]; trophectoderm data was extracted from [35]

***ACE2* and *TMPRSS2* co-expressing cells of the epiblast and trophectoderm are enriched for genes that aid in viral pathogenesis**

In the epiblast and trophectoderm, the *ACE2* and *TMPRSS2* co-expressing cells (*ACE2*+*TMPRSS2*+ cells) were enriched for several S protein cleavage enzymes (Fig. 4). In the *ACE2*+*TMPRSS2*+ cells of the epiblast and day 7 trophectoderm,

TMPRSS4, *TMPRSS13*, *CTSB*, and *CTSL* transcripts were significantly overexpressed as compared to the cells not co-expressing both these genes (p -value ≤ 0.05). Other members of the *TMPRSS* and *Cathepsin* family were also enriched in the *ACE2*+*TMPRSS2*+ cells, although they did not reach statistical significance (Supplementary Fig. 2).

We identified 1985 genes that were differentially expressed between the *ACE2*+*TMPRSS2*+ and *ACE2*–*TMPRSS2*– cells

of the epiblast (Fig. 4, Supplementary Table 6). Of 1985 differentially expressed genes in the epiblast, nearly 67% of genes were overexpressed in the *ACE2*⁺*TMPRSS2*⁺ cells as compared to *ACE2*⁻*TMPRSS2*⁻ cells. The majority of these genes were uniformly expressed in the *ACE2* and *TMPRSS2* co-expressing cells but their expression was highly heterogeneous in the *ACE2*⁻*TMPRSS2*⁻ cells (Fig. 4).

In the trophoctodermal lineage, pseudo-bulk analysis identified 1453 differentially expressed genes between *ACE2*⁺*TMPRSS2*⁺ and *ACE2*⁻*TMPRSS2*⁻ cells (Fig. 4, Supplementary Table 7). Of these, 83.5% were overexpressed in the *ACE2*⁺*TMPRSS2*⁺ cells as compared to *ACE2*⁻*TMPRSS2*⁻ trophoctoderm cells (Supplementary Table 7).

Comparative analysis of the differentially expressed transcripts identified 216 genes that were common in both the epiblast and day 7 trophoctodermal lineage; 1768 genes were unique to the epiblast, while 1236 genes were unique to the trophoctoderm (Fig. 5, Supplementary Table 8). The biological processes associated with the 216 common genes were steroid metabolism and biosynthesis of sterol, alcohol, and cholesterol. The enriched Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathways included lysosome and steroid biosynthesis (Fig. 5). The biological processes associated with genes unique to epiblast cells were associated with embryonic development, small molecule and energy metabolism, and cholesterol biosynthesis. The enriched KEGG pathways were oxidative phosphorylation and metabolic pathways (Fig. 5). The biological processes associated with the genes unique to the trophoctoderm were associated with glycolipid metabolism, lipopolysaccharide metabolism, and leukocyte activation. The KEGG pathways associated with these genes were lysosome, peroxisome, glycan degradation, and herpes simplex virus infection (Fig. 5).

Our results show that different stages of the developing human embryos may be permissive to multiple hCoVs (Fig. 6). Based on the expression map of *ACE2*, *ANPEP*, and *DPP4* along with *TMPRSS2*, the early stages (zygote until 8-cell stage) may not be permissive to hCoVs as they lack *TMPRSS2*. However, the cells of these stages do express the alternate receptors and abundant amounts of endosomal proteases. The zygote and 4-cell stage embryos express almost 10% of genes involved in viral endocytosis, replication, and

Fig. 5 Biological processes enriched in *ACE2* and *TMPRSS2* co-expressing epiblast and trophoctoderm cells. Venn diagram showing numbers of differentially abundant genes unique and common in *ACE2* and *TMPRSS2* co-expressing day 7 trophoctoderm (D7 TE) cells and epiblast cells. The hatched bar are terms of the KEGG pathways, the solid bars represent the biological processes. Values on X-axis are $-\text{Log}_{10}$ adjusted *p*-value. Data were obtained from scRNAseq of the epiblast [34] and trophoctoderm cells of day 7 blastocyst [35]

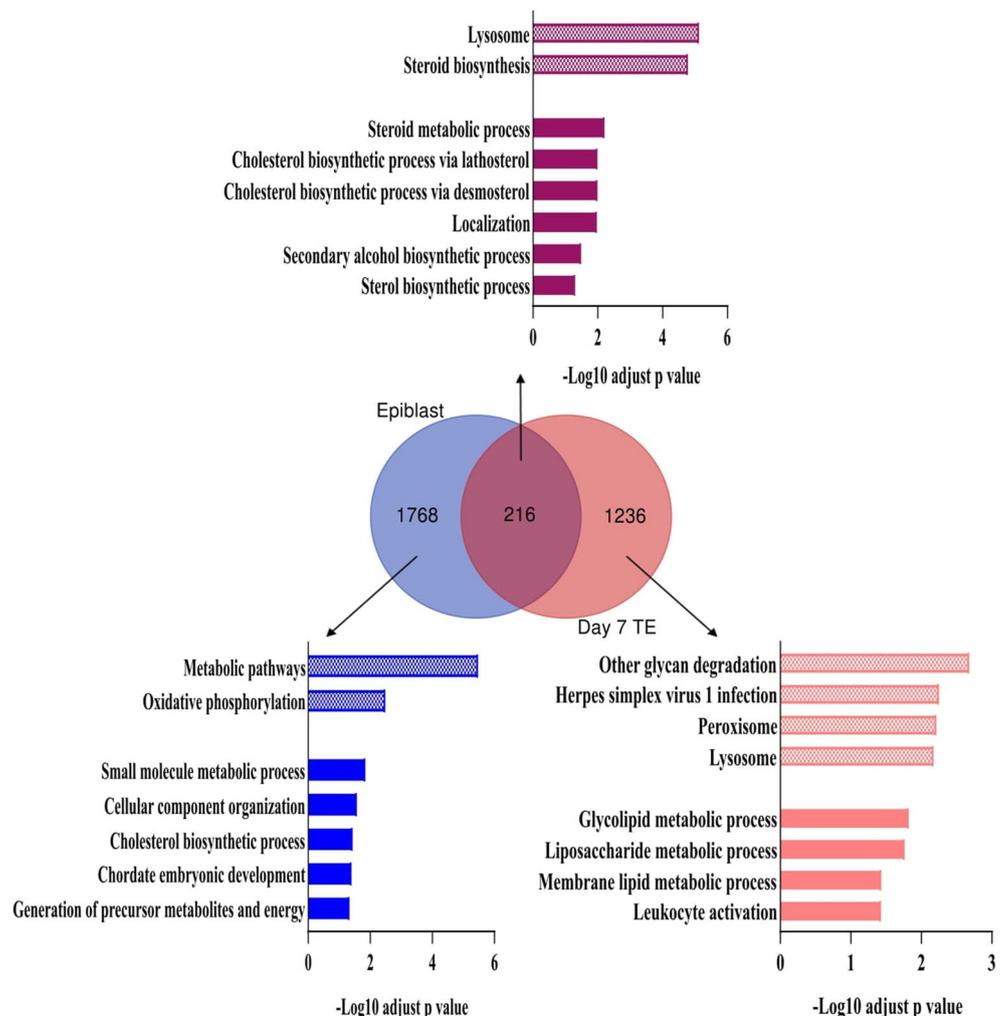


Fig. 6 Permissiveness of human embryos to multiple coronaviruses. Different colors represent different cell types in the blastocyst stage. Blue, green, and yellow cells represent trophoblast, epiblast, and primitive endoderm respectively. The permissiveness of human embryos to different coronavirus was estimated based on the presence of the mRNA for canonical receptors (*ACE2*, *DPP4*, and *ANPEP*) and *TMPRSS2* and the numbers of expressed genes involved in viral ESCRT, viral replication, and viral infectivity. If 1–10% of the total genes for the above processes were expressed in the embryos, a score of +1 was assigned, a score of +2 was assigned when 11–50% of genes were expressed, and a score of +3 was assigned when > 50% of genes were expressed

ESCRT genes	Viral Replication genes	Corona Virus Infectivity genes	ACE2	ANPEP	DPP4	TMPRSS2	Tropism for corona viruses
+1	+1	+3					Zygote ?
+2	+1	+3					4 Cell stage ?
+2	+2	+2					8 Cell stage ?
+2	+2	+3					Morula hCoV-NL63 hCoV-299E SARS-CoV SARS-CoV2 MERS-CoV
+3	+3	+3					Early Blastocyst hCoV-NL63 hCoV-299E SARS-CoV SARS-CoV2 MERS-CoV
+2	+2	+3					Late Blastocyst hCoV-NL63 hCoV-299E SARS-CoV SARS-CoV2 MERS-CoV
+3	+3	+3					Late Blastocyst hCoV-NL63 hCoV-299E SARS-CoV SARS-CoV2 MERS-CoV

coronavirus infectivity. From the 8-cell stage onwards, the cells of the embryos consistently express receptors for multiple hCoVs and most of the genes involved in ESCRT, viral replication, and SARS infectivity. The trophoblast cells are most enriched for mRNA of the receptors, ESCRT, viral replication, and coronavirus infectivity-related proteins.

Discussion

The results of the present study demonstrate that the developing preimplantation and implantation stage human embryos dynamically express the mRNA of receptors and the S protein processing enzymes for multiple coronaviruses. They also express the factors that aid in endocytosis, replication, and infectivity of multiple hCoVs.

The pandemic of SARS-CoV-2 causing COVID-19 has drawn tremendous attention of researchers globally and it is now clear that COVID-19 during pregnancy is associated with multiple adverse outcomes including mother-to-child transmission [3, 4, 6, 8, 10]. Considering the high rate of human-to-human transmission and viral shedding from both symptomatic and asymptomatic individuals [39], high asymptomatic to symptomatic patient ratio [40] and ability of the viruses to survive on inanimate objects for extended periods and at sub-zero temperatures [41, 42], the clinics offering assisted reproduction

services are at risk of spreading COVID-19 [43]. With the resumption of services for assisted reproduction in many parts of the world [44, 45], the clinics must be aware of the risks that the virus poses on gametes and early embryos. Indeed, we and others have demonstrated that ACE2 is expressed in the different types of embryonic and trophoblast cells [14–18]. Herein, we observed that nearly 12% of ICM, 16% of primitive endoderm, 41% of the epiblast cells, and 70–80% of the trophoblast cells (of day 5–6 blastocysts) and 33% of day 7 blastocysts co-expressed both ACE2 and TMPRSS2. These numbers are comparable to those reported in previous studies that analyzed ACE2 and TMPRSS2 co-expressing cells in human embryos using different scRNAseq datasets [14, 16]. These results reflect the robustness of our analysis and further reinforce the notion that human embryos could be permissive to SARS-CoV-2.

Considering the severity and urgency of the COVID-19 pandemic, the current endeavors focused on SARS-CoV-2 are appropriate. However, due to the high frequency at which virulent coronavirus strains emerge, there is a need to identify the tropism of multiple hCoVs to human embryos. Towards this, we profiled the cells of the developing human embryos for expression of the canonical and non-canonical receptors and S protein cleavage enzymes for multiple hCoVs. Our results show that the canonical SARS receptor ACE2 is expressed from the zygote to blastocyst stages. The MERS

receptor *DPP4* and the hCoV-299E receptor *ANPEP* are expressed from the compact morula stage and continue its expression in a proportion of ICM and the trophectoderm. Interestingly, the cells of the epiblast and primitive endoderm also express mRNA for the S protein cleavage enzyme *TMPRSS2*. Besides, a proportion of the *ACE2*, *ANPEP* and *DPP4* expressing cells co-express *TMPRSS2* (data not shown) indicating that the canonical mode for multiple hCoVs entry exist in the morula and blastocysts. Intriguingly, the *ACE2* and *TMPRSS2* co-expressing cells are enriched for the mRNA of several members of *TMPRSS* and cathepsin family of proteases that aid in S protein processing of different hCoVs that may potentiate infectivity. Along with the canonical receptors, many cells of developing human embryos right from the zygotic stages express the mRNA for the non-canonical hCoV entry receptors *BSG*, *ZDHHC5*, *GOLGA7* and *LGALS1*. These results imply that the human preimplantation and implantation stage embryos could be permissive to multiple hCoVs through both the canonical and non-canonical entry routes.

Along with *ACE2*-*TMPRSS2*-facilitated entry, many hCoVs can also enter the host cells via the endosomal route [28, 29, 46]. Intriguingly, some coronaviruses like the HCoV-229E, HCoV-OC43, and hCoV-HKU1 prefer the endosomal mode of entry over receptor-mediated pathways [29]. In the endosomal mode, the spike proteins need to be proteolytically activated, which is achieved by CTSL, CSTB, and FURIN [28, 29]. These enzymes cooperatively and independently promote SARS-CoV-2 entry in host cells both in vitro and in vivo [29–31]. Herein, we observed that all these three enzymes are expressed in most cells of the preimplantation and implantation stage embryos including the trophectoderm. Interestingly, the cells of the embryos are also enriched for other cathepsin family members implying that along with receptor-mediated entry, the endosomal mechanisms are operative and may also potentiate viral entry in embryonic cells. Indeed, a neutralizing antibody against *ACE2* reduced but did not completely abolish reporter signals in the trophectoderm of human blastocyst challenged with pseudotyped SARS-CoV and SARS-CoV-2 [18]. These results indicate that human embryos could be permissible to multiple coronaviruses by both receptor and non-receptor mediated pathways.

Once the virus binds to the receptor and the S protein undergoes priming, most enveloped viruses recruit the ESCRT machinery of the host cell for endocytosis [32]. Current evidence suggests proteins of hCoVs also interact with members of the ESCRT pathway and blocking some of these hinders their replication [7, 25, 33]. Interestingly, we observed that the transcripts of proteins involved in ESCRT are expressed in the zygote and 4-cell embryos; a surge in the expression of these genes occurs in the 8-cell stage onwards. Notably, these genes were expressed in most cells irrespective of their *ACE2* status. Given the fact that the coronavirus can also enter the cells by

the endosomal route, the abundance of ESCRT genes may facilitate the entry of hCoVs in the early stages of human embryos even in absence of the *ACE2*-*TMPRSS2* facilitated entry machinery.

Once the virus enters the cells, the next step in the infectious cycle is replication, which involves a distinct set of host proteins [25, 38]. Our results reveal that as compared to the earlier stages, the cells of the compact morula show a surge in mRNA expression of most host proteins involved in viral replication. Notably, at the single-cell resolution, we observed that irrespective of whether the cells expressed the necessary receptors and the proteases, the genes of the ESCRT and the viral replication pathways are expressed in most cells of the blastocysts. These results indicate that the human blastocysts not only have the necessary machinery for viral attachment and entry but also readily express the infrastructure to facilitate viral replication.

Inside the host cells, coronaviruses translate proteins that physically interact with a plethora of host proteins. These host proteins have roles in a wide range of cellular functions including DNA replication, lipid modification, RNA processing, cytoskeletal organization, and mitochondrial functions [33]. We observed that mRNA for SARS-CoV-2 interacting proteins are expressed in most cells from the 8-cell stage embryos and onwards. Notably, within this human and SARS-CoV-2 protein interactome, there are 67 proteins that also interact with proteins of other hCoVs. Further, depleting these 67 proteins individually causes a significant reduction in SARS-CoV-2 replication in human cells [25]. We observed that the mRNA of the 67 coronavirus infectivity-related genes are abundantly expressed in the cells of the developing embryos specifically from the compact morula stage onwards. Taken together, our analysis provides evidence for the presence of the regulatory hubs involved in coronavirus infectivity in preimplantation and implantation stage human embryos.

To understand how the cells of the embryos augment viral infectivity and survival, we scanned the cells of the epiblast and trophectoderm that co-express *ACE2* and *TMPRSS2*. The results revealed that the *ACE2* and *TMPRSS2* co-expressing cells of the epiblast and trophectoderm are enriched for genes involved in lipid metabolism. Host cell fatty acid synthesis machinery is known to aid in viral replication and to shield the viral nucleic acids from immune surveillance [47]. In the context of coronaviruses, a fine-tuned host lipid profile is essential to achieve optimal viral replication [48]. In addition to sterol metabolism, the *ACE2* and *TMPRSS2* co-expressing trophectodermal cells are enriched for genes involved in herpes simplex viral infection, glycan modifications, and peroxisomes. Host glycans and peroxisomes are known to support the replication and spread of some viruses [49]. The *ACE2* and *TMPRSS2* co-expressing epiblast cells are enriched for genes in oxidative phosphorylation. A higher expression of genes involved in metabolic pathways including oxidative

phosphorylation is reported in neutrophils of COVID-19 patients [50, 51] suggesting a possible role of mitochondrial activity in SARS-CoV-2 infection. Thus, our analyses reveal that the *ACE2* and *TMPRSS2* co-expressing cells of the developing blastocysts specifically are augmented for a plethora of factors that aid in viral survival and replication.

Human embryos could be potentially exposed to the hCoVs via systemic infection in vivo or during in vitro embryo manipulative procedures in patients undergoing assisted reproduction. While the zona pellucida is thought to protect the early embryos from viral infections, exposure of zona-intact embryos to the Zika virus compromises their development [52, 53]. Further, in a proportion of embryos that were exposed to Zika viruses at preimplantation stages, the resulting fetuses had altered brain development [52]. These results indicate that the zona pellucida may not always protect the embryos from viral infection. While similar information for hCoVs is not yet available, considering our results that show that the preimplantation embryos have an abundance of genes required for viral endocytosis and infectivity, it is plausible that if the hCoVs cross the zona barrier, they may be embryo-toxic and may have long-term sequelae. Indeed, human embryos exposed to pseudotyped SARS-CoVs have compromised development, cell fragmentation, and even embryonic demise [18]. Thus, clinicians and patients seeking fertility options via in vitro fertilization must be aware of the potential negative impact of hCoVs on early developing embryos.

Overall, this study provides insight into the permissiveness of developing human embryos for multiple hCoVs and suggests that the morula and blastocyst stage embryos are most likely to get infected (Fig. 6). While this is the first in-depth survey of pan-coronavirus entry and infectivity factors in developing human embryos, it is limited by the constraints of scRNAseq. These include under-representation of rare cell types, isolation biases, and statistical cut-offs. Furthermore, RNA expression levels do not always accurately reflect protein abundance; therefore, our observations must be corroborated for protein expression in situ. Additionally, the expression of these factors may be modulated by coronaviruses themselves, resulting in completely diverse consequences. Nevertheless, our study provides valuable baseline data which will be an important resource for clinics performing in vitro fertilization procedures and to develop evidence-based guidelines for patient management and counseling during viral pandemics.

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Author contribution DM conceived the study and analyzed the results. SC, KC and DS took the lead role, executed the study, and analyzed the data. AS, AH, RS, and AM carried out the literature survey and collected the information. NS, AB, DS, and NA helped in data assembly, analysis, and manuscript preparation. AB, DS, and KC prepared the figures. All the authors agreed to the final version of the manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

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