



Modeling the Human Placenta to Investigate Viral Infections During Pregnancy

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Throughout gestation, the placenta is vital for proper development of the fetus. Disruptions in trophoblast, the main functional cell type of the placenta, stress the pregnancy, with potential adverse outcomes for both mother and baby. While the placenta typically functions as an effective pathogen barrier to protect the fetus, there are scenarios in which viral infections either cross the placenta or disturb its function. Here, we briefly review placental morphologic and functional changes across pregnancy and how these relate to routes for and protection from pathogens. We then explore the potential advantages and limitations of the current primary trophoblast models (primary cultures/explants, cell lines, trophoblast stem cells and trophoblast organoids) and stem cell-derived trophoblast models (naïve and primed embryonic stem cells [ESCs], and blastoids) and discuss these in the context of what is already known about (1) how viruses cross the placenta and the mechanisms that are used for its protection and (2) how these protective mechanisms change across gestation.

Keywords: trophoblast, placenta, virus, maternal infections, models, stem cells, organoids

INTRODUCTION

The success of human pregnancy relies on a healthy and functional placenta. The placenta is responsible for gas, nutrient, and waste exchange while concurrently functioning to protect the fetus from dangerous substances or microorganisms originating in the mother. While the placenta protects against many pathogens, there are multiple examples of maternal infections that cause adverse pregnancy outcomes or congenital deficits in the fetus. The most well-known are complications associated with the “TORCH” pathogens, which include *Toxoplasma gondii*, other [including Zika virus (ZIKV; *Flaviviridae*; *Flavivirus*) and human immunodeficiency virus (HIV; *Retroviridae*; *Lentivirus*)], rubella virus (RuV; *Matonaviridae*; *Rubivirus*), human cytomegalovirus (HCMV; *Herpesviridae*; *Cytomegalovirus*), and herpes simplex virus 1 and 2 (HSV-1, HSV-2; *Herpesviridae*; *Simplexvirus*) (1). Researchers are currently investigating whether severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; *Coronaviridae*; *Betacoronavirus*) can cross the placenta as well. Given the current pandemic and threats of future viral outbreaks, it is important to understand how the placenta can succeed or fail in protecting the fetus from maternal infections. In this review, we focus on how viruses can cross the placenta throughout gestation and envision how

researchers can utilize newly derived human placental models to further our understanding of these processes.

PLACENTAL STRUCTURES ACROSS GESTATION AND ROUTES OF PATHOGEN TRANSMISSION

The placenta originates from the trophoblast, the outermost layer of the human blastocyst (**Figure 1A**). Initial implantation of the embryo into the maternal endometrium (decidua) begins with the formation of a highly invasive primary syncytiotrophoblast (SCT) layer migrating ahead of a zone of proliferating cytotrophoblast (**Figure 1B**). This early, pre-villous placenta has been defined as primitive trophoblast. During this stage of pregnancy, crossing the SCT is the only placental route for vertical (mother-to-fetus) transmission of viruses to the fetus. Little is known about the human primitive placenta and what is known stems from rare, archived hysterectomy samples and non-human primate research (2–4). At this early time in its development, the placenta is essentially nourished entirely by glandular secretions in the maternal decidua (5). While studying this peri-implantation phase in humans is nearly impossible, murine models suggest that the placenta can enable vertical virus transmission during this time window, i.e., prior to mature hemochorial placental formation (6–8). A human cohort study estimated congenital HCMV infection occurred at a rate of 45% after maternal periconceptual infection (defined as one week before and five weeks after the last menstrual period) providing further evidence of placental susceptibility during this time window (9).

As pregnancy progresses, the proliferative cytotrophoblast cells eventually break through the primitive syncytium and begin forming the primary chorionic villous structures. Villous cytotrophoblast cells (VCT) fuse to form a continuous layer of non-invasive multi-nucleated SCT (**Figure 1C**). At the distal ends of some of these villi (anchoring villi), the SCT layer is absent and instead, the VCT form cytotrophoblast cell columns (CCC) that anchor the placenta to the basal plate. In early pregnancy, this external layer of cytotrophoblast cells is known as the cytotrophoblastic shell, which spreads laterally to form a continuous layer between the anchoring villi and the decidua basalis (10, 11). The cytotrophoblast cells within the cell columns subsequently differentiate into extravillous trophoblast cells (EVT), which invade deeply into the decidua (**Figure 1C**). Initially, these EVT also form plugs in the maternal spiral arteries to prevent blood flow to the developing villous structures. The CCC and EVT are in contact with cells residing in the maternal decidua and serve as a potential route for viral transmission to the fetus *via* the villous placenta prior to onset of maternal blood flow into the intervillous space.

Around the 10th week of pregnancy, the EVT plugs dissipate and the EVT continue a process that remodels the spiral arteries by replacing the endothelial layer with endovascular trophoblast (EndoVT) to allow relatively unfettered maternal blood supply to the intervillous space (12) (**Figure 1D**). Once the plugs are dissolved, the SCT layer is bathed directly in maternal blood. This provides an additional route for viral particles present in maternal blood to infect the growing villous placenta (across the SCT layer). To infect a fetus, the virus must then traverse the underlying layer of VCT to enter the villous core that contains fetal blood vessels, fibroblasts, and fetal macrophages known as Hofbauer cells. These Hofbauer cells have the potential to

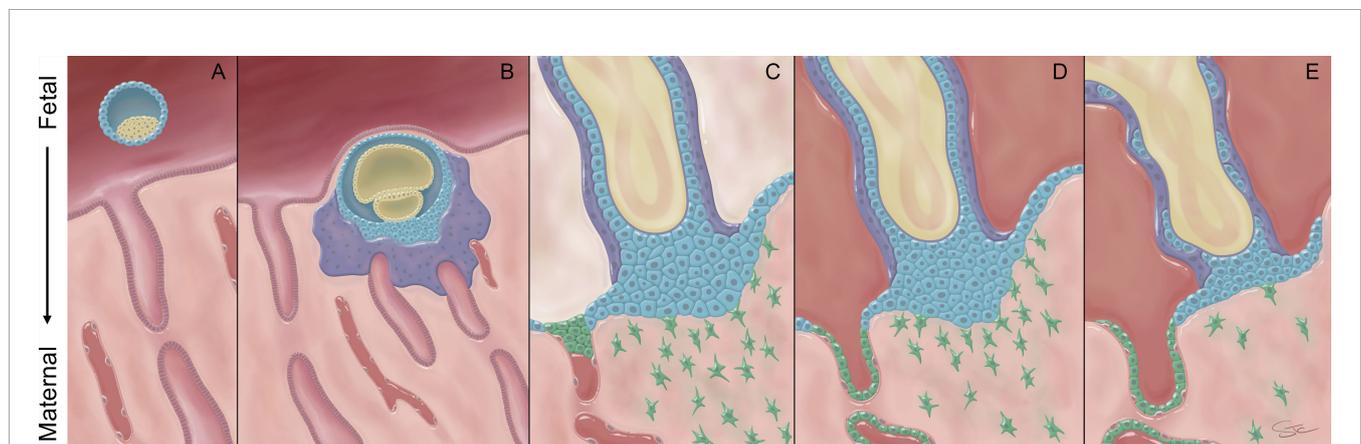


FIGURE 1 | Morphology of the human placenta across gestation. **(A)** Just prior to implantation, the blastocyst is comprised of an inner cell mass (yellow) that is surrounded by a single layer of trophoblast, termed the trophoblast, (blue). **(B)** Once the embryo contacts the maternal decidua, the trophoblast forms a highly invasive primitive syncytium (purple). This multi-nucleated syncytium contacts maternal glands that provide histotroph to nourish the growing embryo. Beneath the primitive syncytium, the villous cytotrophoblast cells (VCT; blue) proliferate and eventually break through the primitive syncytium to generate tree-like primary and, ultimately, more mature villous structures. **(C)** Villi that reach and anchor to the maternal decidua are the source of the cytotrophoblast cells that will form the cytotrophoblastic shell (blue). Extravillous cytotrophoblast cells (EVT, green) invade into the decidua and interact with maternal cells and plug the maternal spiral arteries to prevent onset of maternal blood flow into the space between the placental villi, called the intervillous space. **(D)** Near the end of the first trimester, the EVT plugs dissolve and the EVT continue to remodel the spiral arteries to establish robust maternal blood flow into the intervillous space. **(E)** By the end of pregnancy, the VCT layer becomes sparse and the cytotrophoblastic shell discontinuous.

provide an additional layer of defense through their response to Toll-like receptor stimulation (13, 14). Studies have indicated that Hofbauer cells are susceptible to viral infections, including TORCH pathogens, HIV (15) and ZIKV (16, 17), but respond differentially to these infections.

As the primary villi mature and pregnancy progresses, the number of migratory and spindle-like EVT decreases and the cell columns become more compact (18, 19) (**Figure 1D**). In addition, the VCT become sparse and infrequent underneath the continuous layer of SCT lining the villi (20, 21) (**Figure 1E**). While the focus of this review is on the placenta's role in vertical transmission, the maternal decidua also contributes to infection risk and maternal response to infection during pregnancy (22). The fetal membranes, the decidua parietalis and the decidua basalis all have distinct immune cell phenotypes and may play roles in vertical transmission that differ based on the pathogen involved (23, 24). For instance, the best studied of these, the decidua basalis, contains multiple types of immune cells, including natural killer cells, dendritic cells, macrophages and T cells (24) indicating a robust potential to respond to viral insults. In contrast, there is also evidence that the decidual tissue can host active infections of TORCH pathogens, like HCMV and ZIKV, implying a role as a reservoir for infection during pregnancy (25–28). The balance among these decidual responses, and those of “para-placental” routes involving the parietal decidua and fetal membranes, likely differ by pathogen and level and timing of infectious load and, like the placenta, contribute to the ultimate risk for vertical transmission.

PLACENTAL PROTECTION FROM AND SUSCEPTIBILITY TO INFECTION

The SCT serves as the primary trophoblast barrier between fetal and maternal blood. The structure of the syncytial layer in and of itself serves as a protective boundary. The multinucleated and continuous layer of SCT lacks cell junctions, which are often hijacked by viral particles as entry points in other tissues (1). The dense actin structure underneath the brush boarder of the SCT surface may also play a role in preventing pathogen invasion and attachment (29, 30).

The chromosome 19 miRNA cluster (C19MC) has been demonstrated to have antiviral properties, specifically at term (31, 32). This miRNA cluster is located on a maternally imprinted chromosome and is predominantly expressed by trophoblast. However, less is known about the functional role and potential of C19MC miRNAs to provide viral protection during early gestation. One study assessed release of C19MC miRNAs into maternal circulation following successful *in vitro* fertilization (IVF) procedures (33). The investigators in this study were able to detect C19MCs in maternal blood as early as 2 weeks after blastocyst implantation, with a substantial increase during the first trimester of the resultant pregnancy. It is hard to deduce whether this initial low level of C19MC miRNA expression is due to the small size of the embryo (and thus the

placenta) at this earliest stage of pregnancy or if there are indeed changes in C19MC miRNA expression across gestation that outpace placental growth and villous maturation. With the establishment of new first-trimester trophoblast models, discussed in the following sections, (34, 35), it is now possible for researchers to assess the role of these C19miRNAs in early gestation.

Generally speaking, trophoblast constitutively produces interferons (IFNs) which may aid in placental protection from viral insults (36–38). In mice, loss of type I IFN signaling leads to increased placental viral load upon exposure to murine herpesvirus-68 and increased vertical transmission to the fetus (39). Most viruses have developed a variety of strategies to avert immune recognition and allow for unencumbered propagation within the infected cell with subsequent transmission. For instance, many flaviviruses are capable of antagonizing type I IFN responses (40, 41). ZIKV NS5, the viral RNA-dependent RNA polymerase which is the most conserved protein amongst flaviviruses, binds to and subsequently targets signal transducer and activator of transcription 2 (STAT2) for degradation (42). STAT proteins are part of the signaling cascade that converts cytokine signals into immune cell responses, including proliferation and differentiation, to fight viral invasion (43). This same mechanism is conserved for another flavivirus, dengue virus (DENV), although here STAT2 degradation relies on successful binding of ubiquitin protein ligase E3 component N-recogin 4 (UBR) to the NS5 protein (44). NS5 has also been shown to bind cellular heat shock protein 90 leading to instability and subsequent degradation of janus kinase (JAK), another member of the signaling pathways involved in antiviral responses, (45). Type III interferon responses also rely on JAK/STAT signalling cascades and therefore may be similarly antagonized by flaviviruses (46). However, we expect that this possibility of antagonism would be reduced in trophoblast cells, considering that their expression and release of type III IFNs is constitutive and can therefore be independent of viral insults (47, 48).

Transport of maternal IgG across the SCT layer of the placenta occurs through the neonatal Fc receptor (FcRn) (49). The Fc region of IgG only binds to FcRn at acidic pHs and therefore must be taken up by endosomes within the SCT. The mechanism underlying the crossing of the stroma or fetal endothelium by IgG after SCT uptake, however, remains elusive (50). In humans, onset of transfer occurs around 13 weeks of gestation and continually rises during pregnancy (51). Maternal transfer of IgG can offer passive immunity to the developing fetus. This has been demonstrated in infants born to mothers who were vaccinated against Influenza virus (*Orthomyxoviridae*) and tetanus-diphtheria-pertussis (Tdap) during pregnancy (52–54). Recently, researchers discovered that in the third trimester neonates can acquire SARS-CoV-2 antibodies *via* placenta transfer (55). However, there is the potential for a virus to hijack FcRn to facilitate its own entry and vertical transmission across the SCT layer. Both HIV and HCMV have been shown to utilize this IgG-virion transcytosis mechanism to gain access to the placenta (56, 57).

The adverse fetal outcomes first reported among pregnant women in South America who were exposed to Asian strains of ZIKV were the first strong evidence for vertical transmission of this virus (58–60), which had caused previous outbreaks in Africa and Asia without reported fetal effects. Antibody-dependent enhancement (ADE) (61) is just one of the suggested mechanisms for this change in transmission dynamics. ADE occurs when a prior virus infection generates (cross-reacting/heterotypic) antibodies that are poorly neutralizing against a subsequent infection by a separate but similar virus (62). Instead, these antibodies can lead to increased pathogenicity for the subsequent infection. Sequence alignments indicate that various ZIKV isolates share approximately 99% amino acid sequence identity and that similar levels of homology can be found between the four DENV serotypes (DENV1-4, 98–99% amino acid sequence identity) (63). Of all *Flavivirus* species, DENV1-4 and ZIKV share the most similar amino acid sequences. This has led researchers to investigate whether the cross-reacting antibodies produced following a primary infection with DENV might influence disease outcome upon subsequent infection with ZIKV. One case report has suggested ADE in a converse combination of flavivirus infections. Here, prior ZIKV infection enhanced infection by DENV1, which led to severe and fatal consequences (64).

ADE of ZIKV transmission has been demonstrated in mice previously infected with DENV, suggesting that DENV-specific antibodies are able to increase the incidence of ZIKV vertical transmission and associated fetal microcephaly (65, 66). This process likely involves FcRn and transcytosis of ZIKV attached to a non-neutralizing, cross-reactive, DENV-recognizing antibody (65, 67, 68). The human populations exposed to ZIKV during the outbreaks in South America in 2015/2016 also exhibited high levels of DENV immunity (i.e., in DENV-endemic regions) (69), suggesting that ADE caused by subsequent ZIKV infection in DENV-exposed women might be involved. There are, however, conflicting observations. One study using type I interferon receptor-deficient mice indicated that anti-DENV monoclonal antibodies (mAbs) may act to neutralize ZIKV and thereby protect the fetus of a ZIKV-infected mother from vertical transmission of the virus (70). Another group reported that DENV immunity provided enhancement of ZIKV infection *in vitro* (as observed in a K562 human erythroleukemic cell line, an otherwise nonpermissive cell line but one that bears an Fcγ receptor) but not *in vivo* (i.e., AG129 mice), suggesting that the variation in results may be an artifact of the exploited *in vitro* model systems (71). Differences in stimulated antibody titers and variations in the pathogenicity of specific viral strains could also be responsible for these discrepant outcomes.

MODELING PLACENTAL INFECTIONS IN VITRO

Understanding the potential and the limitations of existing and emerging *in vitro* model systems for early human placental development is of the utmost importance (72–74). Recent

advancements in available human trophoblast models provide a platform to further investigate how viruses infect the placenta and to explore the possible consequences of such infection on placental function and fetal health. Animal models do provide some insight into placental susceptibility to infections; however, all have significant structural and functional differences that must be considered (75, 76). For this reason, we have chosen to only discuss human models here. For those interested in further exploration, we suggest extensive reviews of these models by others (77–79). While many studies have elucidated human trophoblast susceptibility or resistance to different viruses, the savvy reader must carefully account for discrepancies among the gestational ages represented by the selected trophoblast models, the specific trophoblast lineages assessed by these models, and the plausibility of vertical transmission *via* the route that is being mimicked *in vitro*.

Primary Cultures: Explants and Cells

Primary trophoblast cells and placental explant cultures have been used to demonstrate placental susceptibility to several viruses associated with adverse pregnancy outcomes (Figure 2A). Primary cultures are restricted by their relatively short lifespan, the limited range of gestational ages from which they can be obtained and the relative frequency of sample availability. Primary cultures from term pregnancies rapidly differentiate to SCT and lack a robust migratory set of EVT's (19, 80). This essentially limits the infectious disease researcher to the study of SCT susceptibility to pathogens, including viruses, at term. First trimester explants can capture all subtypes of trophoblast but are inaccessible to many researchers. Further, sample availability can be infrequent, making replication of studies challenging when using primary first trimester samples.

Trophoblast lineage susceptibility to a multitude of viruses has been demonstrated using first-trimester placental explants. For example, it was shown that the specific site of HCMV replication resides in first-trimester VCT and that the overlaying SCT is relatively resistant to HCMV infection (81, 82). SCT express the Epidermal Growth Factor Receptor (EGFR) but lack integrin coreceptors, both of which are critical for efficient HCMV attachment and subsequent cell entry and intracellular replication (83). Using first trimester explants, integrin co-receptor expression in VCT led to HCMV entry and replication, whereas SCT and proximal cytotrophoblast columns, which do not exhibit such co-receptor expression, supported viral attachment but not cell entry or replication. Interestingly, HCMV may also interfere with syncytialization, as primary human term cytotrophoblast differentiation was suppressed following HCMV infection (84). Evidence regarding placental susceptibility to HCMV in early versus late pregnancy is conflicting, but it is generally observed that SCT are infected less frequently and do not allow for productive viral replication when compared with VCT (81, 82, 85, 86). These discrepancies may be in part due to the use of the high-passage HCMV strain's, AD169 and Toledo, in most prior studies (87). Glycoprotein mutations, gained through viral propagation in fibroblast cells, are now characterized in the HCMV strains,

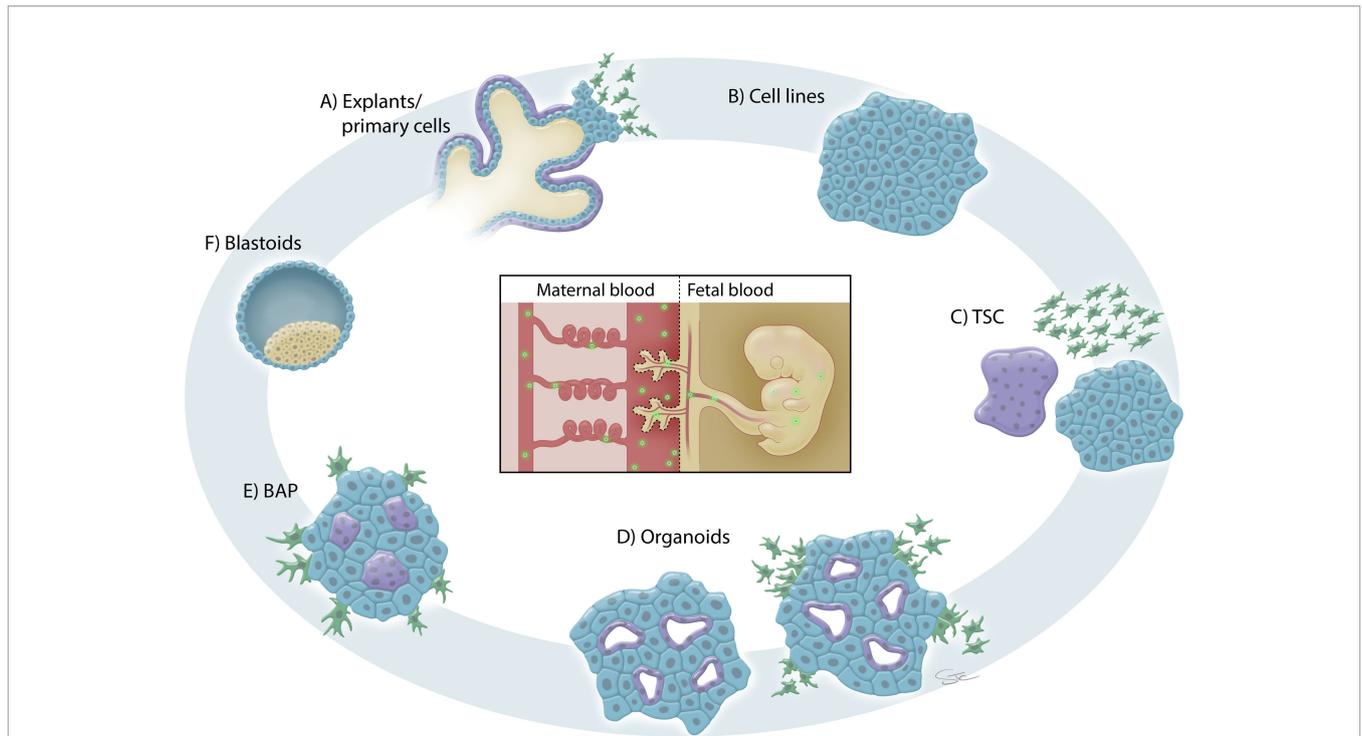


FIGURE 2 | *In vitro* trophoblast models for placental infection studies. **(A)** Primary cells and explant cultures from the first, second and third trimesters of human pregnancy have been used to study placental susceptibility to infection. These explant cultures contain all the trophoblast lineages (VCT, blue; SCT, purple; EVT; green) albeit at different proportions depending on the gestational age of the sampled tissues. **(B)** Various immortalized and cancer-derived trophoblast cell lines have also been used to study placental susceptibility to infection. The cartoon is simplified to demonstrate that typically only a single cell type is represented in these models. **(C)** Trophoblast stem cells (TSC) provide a new model for the study of lineage-specific effects of viral infections during pregnancy since all trophoblast lineages can be induced. TSC can be generated from embryonic/induced pluripotent stem cells or isolated from blastocyst outgrowths and first trimester placental tissue. **(D)** Trophoblast organoids can be generated from first trimester placentas. Organoids more closely mimic the villous structures of the placenta than two dimensional models and can be induced to differentiate into EVT. Trophoblast organoids can be utilized to study vertical transmission of virus as the layers of the placental villi are present in three-dimensions (albeit “inside-out”). **(E)** Stem cell-derived trophoblast (BAP) have been used to study placental susceptibility to several viral infections. This model most closely represents early placentation and contains a heterogeneous population of trophoblast cells. **(F)** Blastoids, generated from embryonic/induced pluripotent stem cells, provide a new model of early human embryo structures. Blastoids could be used to investigate placental susceptibility to infection during the peri-implantation phase of pregnancy.

AD169 (88–90) and Toledo (91), and contribute to the loss of tropism for epithelial and endothelial cells. One study found that SCT from first and third-trimester placentas can be infected with HCMV although the third-trimester samples required higher viral doses (85). Similar to HCMV, experiments using primary cells and explants from first and second trimester placental samples indicated that ZIKV infects VCT, proximal cell columns and EVT but SCT were once again spared (17, 92). There are conflicting observations concerning the susceptibility of human term SCT to ZIKV infection (28, 47, 93–95). Given the limitations of primary cell cultures, these discrepancies could be related to variation in cell culture methods and/or infection protocol variations and differences in the specific ZIKV-strains and viral titers used among studies. Human first, second, and third trimester placental explant cultures have also been used to demonstrate the role of DENV-related ADE in ZIKV vertical transmission (67, 96, 97). Exploitation of anti-DENV antibodies by ZIKV to aid transport across the placental barrier could explain how the latter could cross what is typically an otherwise non-permissive barrier for many viruses during the

third trimester of human pregnancy (47). This same enhancement was not evident with sera from yellow fever virus (YFV; *Flaviviridae*; *Flavivirus*) or chikungunya virus (*Togaviridae*; *Alphavirus*) infected subjects (96). Notably, YFV is not as closely related to ZIKV as is DENV.

Primary cultures (of term SCT) have been used to investigate the packaging and release of C19MC miRNAs into exosomes. These exosomes have been demonstrated to confer resistance against numerous viral insults to recipient cells (31, 47, 98–100). These same studies also demonstrated that SCT were relatively resistant to infections caused by picornaviruses (Coxsackie B virus and Poliovirus), rhabdoviruses (Vesicular stomatitis virus), herpesviruses (HCMV, HSV-1), and flaviviruses (ZIKV, DENV) (31, 47). While primary human term SCT are relatively resistant to ZIKV, inhibition of JAK signaling increased viral replication in these cells (101). This inhibition of JAK-STAT signalling, i.e., interferon signalling, is likely necessary for productive infection as these term SCT are known to constitutively mount an interferon response (47, 102). SCT isolated and cultured from first-trimester placentas expresses the innate immune recognition receptor, toll-

like receptor 3 and mounts an antiviral IFN β response upon activation of this receptor (38). Mid-to-late gestation SCT constitutively releases type III IFN λ (47, 48), which has been reported to prevent viral transmission at skin and mucosal surfaces (103). It is notable that infection of primary human term SCT was minimal and only led to production of what the authors describe as “thin coated” virions when these term SCT were exposed to a clinical isolate of ZIKV (PRVABC59 strain from Puerto Rico, 2015), suggesting release of immature, infection-deficient viral particles (101). In contrast, upon interruption of JAK-STAT signaling, ZIKV infection of these same cells produced mature, “thick coated”, and likely more virulent virions. Interestingly, in human macrophages, ZIKV infection does not antagonize STAT2 phosphorylation and this leads to restricted viral replication (104).

Cell Lines

Trophoblast-like cell lines have been frequently used to study placental infections. Unlike primary cultures, these cells are permissive to genetic manipulations and provide a better tool for mechanistic studies. These cell lines can be grouped into two main types: lines derived from choriocarcinomas (BeWo, JAR, JEG-3) and lines derived from first trimester placental cells that were subsequently immortalized (Swan71, ACH-3P, HTR8/SVneo) (**Figure 2B**). Since they are widely available, immortal, simple to propagate, and relatively stable in culture, these cell types are often chosen for initial experiments, which can be easily replicated and expanded in scale. Further, these cells can grow in simple media cocktails and on plastic culture dishes, making them a cost-effective choice. They have been used to assess treatments to prevent or limit infections. For example, treatment with palmitoleate reduced virus-induced apoptosis in ZIKV-infected HTR-8/SVneo, JEG-3 and JAR cells (105) and treatment with a non-nucleoside RNA polymerase inhibitor reduced virus replication in ZIKV-infected HTR8-SVneo cells (106). HTR8-SVneo cells were used to characterize pathogenicity of a ZIKV glycan-deficient mutant, which was shown to be less neuroinvasive in mice, yet the mutant virus displayed similar growth characteristics in this trophoblast cell line as did the wild-type (107).

These cell lines, however, have several limitations that can affect experimental design and interpretation. First, aside from BeWo cells, which are typically used to model SCT fusion, other immortalized trophoblast cell lines have a limited to non-existent capacity to differentiate into other trophoblast subtypes in culture. This characteristic restricts the utility of immortalized trophoblast cell lines in assessing viral effects on trophoblast differentiation. Further, there are crucial differences in the extent to which diverse trophoblast cell lines are able to form tight cellular junctions (108) and express and respond to Toll-like receptor activation (109, 110) when compared to primary trophoblast cells. Thus, experiments aiming at elucidating viral infection patterns across the placental barrier and immune responses to infections at this site become difficult to interpret when using immortalized or cancer-derived trophoblast-like cell lines. HTR8/SVneo cells (an extravillous-like trophoblast cell

line) have the capacity to mount an antiviral response. Following infections with the flaviviruses ZIKV, DENV4, and YFV, infection patterns and immune responses were assessed in this cell line (111). All three viruses exhibited similar tropisms, yet ZIKV induced a stronger inflammatory response (involving IL-6, IL-8, CCL2, CLL3, and CCL5) but a lower IFN response when compared to DENV and YFV. HTR8/SVneo cells also responded to the presence of viral ssRNA in a TLR8-dependent manner (112). JEG-3 cells, which are typically cultured in two-dimensions, demonstrated an increased capacity to form syncytium, resist ZIKV infection, and basally expressed higher levels of IFN-stimulated genes when cultured in a three-dimensional system (48). Knocking down TLR7 and/or TLR8 by using siRNA also increased ZIKV replication in JEG-3 cells (113). Researchers often utilize one of these trophoblast cell lines alongside primary cultures to evaluate consistencies. For example, ZIKV-infected Swan71 and first-trimester primary cells (VCT) showed a similar induction of IFN β and interferon stimulated genes (114).

Trophoblast Stem Cells (TSC) and Trophoblast Organoids

Recently, new models of first-trimester trophoblast have been developed that will greatly enhance our knowledge of early pregnancy susceptibility to a variety of insults, including infection. Human trophoblast stem cells (TSC) can be cultured long-term without differentiation in two dimensions (2D) or stimulated to differentiate into SCT or EVT (34) (**Figure 2C**). These TSC can be established from blastocyst outgrowths or isolated from first trimester placental digests (34) and are permissive to genetic manipulations (115–117). Similarly, human trophoblast organoids have been derived from first trimester placentas and provide a three-dimensional (3D) representation of the villous structures of the placenta that contains both SCT and VCT (35, 118, 119) (**Figure 2D**). Upon stimulation, these organoids will differentiate to form outgrowths of EVT that are highly invasive and move through a Matrigel substratum (35, 119). One caveat, however, is that these organoids routinely grow “inside-out”, with the SCT structures forming cavities in the center of the organoid, when compared to the *in vivo* structure of the placental villi. This poses logistical hurdles when planning infection-based studies. To truly model vertical transmission during pregnancy, the SCT should be the first trophoblast subtype to contact virus, which would typically be introduced *via* maternal blood *in vivo*. It is therefore imperative that virus microinjection studies be optimized to allow direct exposure of “internal” SCT to virus using existing “inside-out” trophoblast organoids or that suspension and scaffold-based techniques be developed to invert the polarity of these organoids (120, 121).

Interestingly, even though both TSC and trophoblast organoids are derived from first-trimester placenta, each has a bias in terms of the specific *in vivo* trophoblast lineage counterpart it most closely resembles (122). Using distinct proliferation media, multiple groups have demonstrated that TSC's are nearly all positive for ITGA2 (> 90%) (122, 123), a

marker of the proliferative cells at the base of the cytotrophoblast cell columns (124), yet trophoblast organoids contain large areas of syncytium, numerous VCT (TP63+) and a small portion of ITGA2+ cells (< 23%) (35, 122). Further, there is also conflicting evidence about how well these TSC form multicellular organoid structures. When directly comparing TSC-derived organoids (TSC-organoids) and trophoblast organoids derived directly from first trimester tissue, the TSC-organoids formed significantly fewer SCT and secreted lower levels of HCG into the spent culture media (122). Other groups have demonstrated the ability of TSC-organoids to form SCT (115, 125–127) but it should be noted that none of these groups directly compared them to trophoblast organoids. It is still unclear why these results differ; however, we suspect that distinct methodologies might play a role. Sheridan et al. (122) passaged the TSC's in organoid form for more than five passages, whereas the other groups analyzed only directly converted 3D cultures. Perhaps, once these TSC-organoids are passaged, they might lose the ability to spontaneously form SCT to the same degree as *bonafide* trophoblast organoids.

Due to ethical concerns and legal restrictions, not all investigators have access to early placental tissues. However, several research groups have recently described the ability to generate first trimester-like trophoblast stem cells from embryonic or induced pluripotent stem cells (123, 126, 128–130). These technologies increase world-wide access to approaches that model first trimester human placental development but do not require primary tissues. Most recently, it has been reported that trophoblast stem cells can be isolated from term placentas (131, 132; [Preprint](#): 133), an accomplishment that was previously thought to be impossible. That said, the exact identity and function of these proliferative trophoblast cells needs to be further defined. Do these proliferative trophoblast cells represent the same proliferative cells found in first trimester human placental tissues or are they an entirely different stem cell population only found in later pregnancy? Such questions need further clarification, but the models may provide an exciting platform for disease-based studies given that some diseases of human pregnancy only manifest clinically in late pregnancy and the pathology of the placenta at the time of isolation of these term trophoblast stem cells is known. Lack of information on pregnancy outcomes remains a seemingly insurmountable limitation of stem cells isolated from many first trimester specimens.

BMP4 Induced Trophoblast

Exposing pluripotent stem cells to bone morphogenetic protein 4 (BMP4) has also been employed as a useful model to study trophoblast differentiation (**Figure 2E**). The complete differentiation cocktail includes BMP4, an ACTIVIN/transforming growth factor (TGF) β inhibitor (A83-01) and a fibroblast growth factor (FGF)2 inhibitor (PD173074) and has been named BAP treatment and hence BAP-derived trophoblast (134). This model has been utilized by our group to investigate infections of the human placenta with ZIKV (102, 135) and SARS-CoV-2 (136), and by other groups with protocol modifications (137, 138). We found that these BAP-derived

trophoblast were highly susceptible to ZIKV infection and that the African-lineage strains induced more severe cell lysis when compared to the contemporary Asian-lineage strains commonly associated with fetal microcephaly (102, 135). These findings led us to speculate that a maternal infection in early pregnancy with an African-lineage strain would actually result in a preclinical, early pregnancy loss which would abrogate the possibility of vertical transmission and potential fetal abnormalities. Interestingly, the BAP-derived trophoblast cultures also express reduced levels of C19MC miRNAs when compared to other trophoblast models, including trophoblast cell lines and term placenta (99, 102, 139) which could be a potential reason for their increased susceptibility to ZIKV. Another study suggests that stem-cell-derived trophoblast are equally lysed by Asian and African lineage-strains of ZIKV; however, in this study BMP4 was used in the absence of the other BAP cocktail ingredients and for only a single day to induce differentiation prior to infection (137), whereas our studies induced differentiation for four days with the complete BAP cocktail (102, 135). In fact, the ability of the African-lineage strains of ZIKV (in comparison to strains of the Asian-lineage) to induce massive cell death has been reported in other models, including stem cell-derived neural progenitor cells (140) and mouse embryos (141).

Important limitations of the BAP-derived trophoblast model are the short-lived nature of the cultures when grown in two dimensions and the heterogenous nature of the trophoblast generated during differentiation. Over the years, groups have questioned the validity of this model based upon the idea that stem cells from the epiblast might not have the ability to give rise to trophoblast (126, 130, 139, 142). However, numerous studies and stringent comparisons with *in vivo* trophoblast indicate that these cells certainly represent trophoblast (143–146). A lingering question for BAP-derived trophoblast surrounds the precise trophoblast lineages and the developmental stage of placentation recapitulated by the model. It is clear that the transcriptome of SCT generated from BAP treatment is distinct from *in vitro*-derived SCT collected from term placentas (144). Recently, BAP-derived trophoblast were subjected to single nuclei RNA sequencing and demonstrated the presence of multiple transcriptomic cell clusters representing those seen for SCT in first-trimester placenta (24, 146). This same analysis revealed that only a few of the identified transcriptomic cell clusters exhibited cellular markers that were weakly associated with the EVT-like population isolated from primary first trimester placentas, suggesting the possibility that BAP treatment perhaps may not generate *bona fide* EVT (146). This finding would support the postulated identity of BAP-derived trophoblast as primitive syncytium, i.e., the invasive placenta prior to villous formation, since SCT would be highly represented at this early point in peri-implantation placentation whereas migratory EVT-like cells would be rare (102, 147, 148).

Blastoids

While it is impossible to validate the expression profile of the primary SCT (a.k.a., primitive trophoblast) formed in early pregnancy *in vivo*, advancements in extended embryo culture

(149, 150) and in the culture of blastoids generated from pluripotent stem cells (151–154) could provide important insights into this stage of human placental development (**Figure 2F**). Blastoid structures can also be directly generated by reprogramming fibroblast cells (155) or through the use of extended-potential stem cells (156). Very little is known about blastocyst implantation and very early placentation in humans and these models offer a unique opportunity to study the impact of maternal infections during the peri-implantation phase of pregnancy. They also allow improved comparisons to previous works on peri-implantation rodent and non-human primate placentation. The establishment of reproducible and efficient blastoid protocols are highly important as work with human embryos is challenging due to the rarity of samples and their restricted access for many research groups.

THE EFFECT OF GESTATIONAL AGE

The placenta's many functions include nutrient exchange between mother and fetus, physical support for the fetus, immune protection, and maintenance of a maternal physiology that advances fetal development *via* production of placental hormones. The placenta must simultaneously respond to continuous changes in fetal growth and development, maternal physiology, as well as to pathogenic and environmental stressors across gestation (157–159). The available routes for viral breaching of the placental barrier and the ability of virus to infect and be transported across this barrier also change throughout gestation as does the degree of insult to the developing fetus infected by a pathogen *via* vertical transmission.

RuV, the causal agent of Rubella (German measles), was the first reported example of a teratogenic virus (160). Although no longer prevalent in the United States due to the development and widespread use of an effective vaccine (MMR, effective against measles, mumps, and rubella), there are still many Rubella cases worldwide in places where vaccination programs are not as common (161). The fetal effects of maternal RuV infection during pregnancy are closely linked to the timing of infection and thereby the risk for vertical transmission. The risk of congenital infection is significantly higher when the mother is infected with the virus within the first 12 weeks of pregnancy and this risk decreases dramatically by the third trimester (162). Very little is known about how RuV crosses the placenta. In one case study, viral antigen was detected in the basal plate and in the endothelial cells within the chorionic villi of the placenta at week 35 of gestation following primary maternal infection in the 13th week of pregnancy (163). This is indicative of a persistent mode of infection, which would allow for prolonged exposure of the placenta/fetus to the virus when infection occurs early in pregnancy.

Prospective cohort studies of primary HCMV infections indicate that the risk for intrauterine vertical transmission is the highest when maternal infection occurs in the third trimester of pregnancy (164, 165). This risk of transmission correlates with the proposed mechanism that HCMV traverses the SCT *via*

FcRn-facilitated transcytosis, a potential transmission pathway that becomes increasingly robust throughout later pregnancy (56). Even so, the risk for congenital defects is highest when the mother is infected in her first trimester (166).

The risk for vertical transmission of ZIKV and associated fetal abnormalities is also strongly and positively associated with first trimester infections (167–171). Although several initial case studies reported fetal abnormalities associated with third-trimester infections (69, 172), larger cohort studies indicated that the risk for microcephaly and severe brain defects in the fetus is limited to pregnancies in which maternal infection occurred in the first trimester (171). *In vitro* models also suggest that ZIKV infection is enhanced in placental trophoblast from the first trimester when compared to that from term pregnancy. Our group demonstrated that the stem-cell derived trophoblast (BAP) that most closely resembles the primitive placenta of post-implantation human pregnancy are highly susceptible to ZIKV infection (102, 135). One study suggests that cross-talk between maternal decidual and fetal trophoblast cells may also help to determine susceptibility to ZIKV infection throughout gestation (28). To this point, decidual cells from first trimester pregnancies were infected by ZIKV at much higher rates than equivalent cells collected at term. Further, conditioned media from ZIKV-infected first trimester decidual cells enhanced ZIKV infection of primary term SCT, which had previously been shown to be resistant to ZIKV infection (28, 47). VCT isolated from term placentas also showed the presence of actively replicating ZIKV, suggesting that vertical transmission of the virus could occur in the third trimester if ZIKV has been able to cross the SCT barrier by then (16).

As discussed above, there are examples of stark, gestational-age-dependent differences in the risk for placental infection and vertical transmission after maternal exposure to viruses. Therefore, studies indicating that trophoblast generated/collected from term placentas are relatively resistant or susceptible to a particular virus may not directly translate to similar levels of susceptibility in the first trimester.

CONCLUSION

The placenta is critical for protecting and nourishing the developing fetus. While there are many mechanisms in place to prevent harmful substances and pathogens from entering and traversing the placenta, there are several examples of viruses that can do just that and thereby disrupt placental and fetal development. Advancements in *in vitro* trophoblast modeling and a burgeoning understanding of placental development and function will promote an improved capacity to study placental infections from a more critical viewpoint. A long history of experimental inconsistencies and a diverse set of *in vitro* placental models that may not recapitulate *in vivo* events have led to contradictions concerning how (and to what degree) the human placenta is infected with different viruses. Future studies using more robust and standardized model systems will

hopefully generate more unified results. More consistent results, in turn, should allow for the development of new diagnostic tests and strategies to prevent and/or treat viral infections in pregnancy.

AUTHOR CONTRIBUTIONS

MS wrote the manuscript. JZ, AF, and DS provided critical feedback and review and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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