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Microphysiological systems of the placental barrier

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Abstract

Methods to evaluate maternal-fetal transport across the placental barrier have generally involved clinical observations after-the-fact, *ex vivo* perfused placenta studies, or *in vitro* Transwell assays. Given the ethical and technical limitations in these approaches, and the drive to understand fetal development through the lens of transport-induced injury, such as with the examples of thalidomide and Zika Virus, efforts to develop novel approaches to study these phenomena have expanded in recent years. Notably, within the past 10 years, placental barrier models have been developed using hydrogel, bioreactor, organ-on-a-chip, and bioprinting approaches. In this review, we discuss the biology of the placental barrier and endeavors to recapitulate this barrier *in vitro* using these approaches. We also provide analysis of current limitations to drug discovery in this context, and end with a future outlook.

Keywords

Placental biology; Transport; Metabolism; Microphysiological; Bioreactor; Hydrogel; Organ on a chip; Bioprinting

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Disclosures

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1. Introduction

As an important regulator of maternal and fetal health, the placenta is one of the most important organs in sustaining fetal life throughout pregnancy. Although research efforts have increased in recent years, in part due to the Human Placenta Project launched by the National Institutes of Health in 2014 [1,2], we still only have an elementary understanding of how the placenta coordinates signals in order to maintain a healthy intrauterine environment. Placental coordination begins with the cells that comprise the maternal-fetal interface, termed the placental barrier (PB), that receive signals from both maternal and fetal circulation [3]. The PB acts as a regulator, allocating, and even synthesizing vital nutrients, hormones, and growth factors needed for fetal development and pregnancy maintenance, removing waste from fetal circulation, and limiting fetal toxin exposure. In certain instances, PB dysfunction and placental insufficiency can lead to disease, such as intrauterine growth restriction (IUGR) [4,5]. Therefore, proper function of this barrier is necessary for healthy development [3,6,7]. Modeling the PB has been an active area of research for decades, with a particular focus on determining what substances are capable of crossing and reaching the fetus [8,9].Historical examples highlight the need to better understand how exogenous substances can interact with or cross this barrier, as exposure to medications [10] and infections [11,12] *in utero* impact fetal development and programming, leading to lifelong changes in health and disease risk. Thus, in recent years, significant advancements have been made to study the PB and aspects of placental transport through the use of tissue engineering and other microphysiological systems [13–17].

At a foundational level, the PB is a multilayered, multicellular interface that separates maternal and fetal blood. A large variety of specialized cells are present at this interface, the majority of which are trophoblast subtypes originating from the same blastocyst trophectoderm stem cell precursors [18]. These cell types include the villous cytotrophoblasts, syncytiotrophoblast, extravillous trophoblasts, and trophoblast giant cells. Additional cells that make up the placental milieu, but are not trophoblast in origin, are Hofbauer cells (placental macrophages), fetal endothelial cells, and decidual cells [19]. All of these cell types interact with one another, contributing to diverse placental functions ranging from immune tolerance and hormone production to serving as a chemical and physical barrier [8,20–23]. The syncytiotrophoblast serves as a predominant force in regulating placental transport [8,24], though the fetal endothelium also plays an important role [22]. In this review, we will discuss important biological features of the PB that should be taken into consideration when modeling transport *in vitro*.

In order to replicate this barrier, microphysiological models including hydrogel [13,15,25–28], bioreactor [29,30], organ-on-a-chip [14,16,31–35], and bioprinting [17] based models have been developed. We searched PubMed and Google Scholar for papers related to placental transport from the past 10 years, as well as searching for placental-related papers that utilized these techniques, whether it was in the context of transport or more generally placental biology. From these searches, 100s–1000s of search hits were identified, with the vast majority being discarded due to limited benefit in added discussion (for example, related to placental biology without a focus on transport, studying amino acids to elucidate transport mechanism rather than modeling, utilizing an animal model instead of a human

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model, similar studies already included in the discussion, amongst other related reasons). This left approximately 20–30 studies, all of which represent the array of techniques used to fabricate and model the PB and highlights ongoing areas of investigation related to placental transport. We also discuss challenges to drug discovery in the context of the placenta, namely its inclusion as a safety criterion for drug development and recent interest in targeted drug delivery to the placenta itself. The integration of *in vitro* placental transport approaches, along with interest in developing novel therapeutics for disease and imaging agents to probe placental and fetal development, provide a basis for a growing interest in studying the PB and placental transport.

2. Biology of the placental barrier

The human placenta is unlike that of many other animals [36–38]. Its hemochorial nature, meaning there is direct contact between maternal blood and the fetal chorion (*i.e.* syncytiotrophoblast, Fig. 1A), and discoid shape (Fig. 1C) are features that are only shared with non-human primates, rabbits, and rodents [37]. While rodent models have been a popular choice for *in vivo* studies, it is evident that the hemochorial placenta of both rats and mice differ from humans in gross structural morphology (hemotrichorial in rodents *vs* hemomonochorial in humans) and molecular features (differences in gene and protein expression) that make them somewhat limited in their translational comparison [24,36]. Understanding the unique biology of the human placenta requires the use of *in vivo* (for biological relevance) and *in vitro* (for targeted questions) modeling systems. Using what is already known about the PB in order to generate a biologically relevant *in vitro* system will assist in modeling molecular transport across the maternal-fetal interface.

2.1. Maternal-fetal interface

The interface between maternal and fetal blood is defined here as the PB. Passage across this barrier can occur through simple diffusion, as is the case for the transfer of oxygen to the fetus, or through protein dependent transport [39]. The majority of substances, including biological small molecules, antibodies, and drugs [9,40] require membrane proteins and therefore passage is limited by this barrier. Protein-dependent transport occurs *via* facilitated diffusion or active transport [41] and is bidirectional, allowing communication between the maternal and fetal compartments [24]. As is the case for most biological barriers, including the blood brain barrier, the apical side and the basal side are structurally and biochemically distinct and meant to facilitate the uptake of what is necessary while restricting access of potentially harmful and biologically active compounds. The apical side of the PB is a brush border membrane that increases its surface area and contact with maternal blood. Transport proteins are highly abundant on the apical membrane, including those positioned to increase the concentration of nutrients in the cell (influx transporters) and decrease the concentration of unwanted materials (efflux transporters) [42]. Although the basal membrane that is in close proximity to fetal capillaries lacks microvilli, it does house a large variety of transporters (Fig. 1B). Fetal health and development rely on proper functioning of this barrier designed to ensure entry of important nutrients from maternal circulation and removal of potentially harmful substances from the placenta and fetal circulation. A number of studies have highlighted the role of placental transporters in fetal health,

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demonstrating that changes in the expression or function of nutrient transport can have detrimental impacts. For example, changes in the expression and function of glucose, fatty acid, and amino acid transporters have been implicated in fetal IUGR [43]. Unfortunately, innate transport mechanisms can be hijacked by exogenous substrates which may lead to unwanted placental or fetal exposure. For instance, the solute carrier SLC22A5 is an apical membrane transporter responsible for the influx of lactate, folate, and L-arginine, essential nutrients needed by the developing fetus. However, this transporter also imports common drugs, including antibiotics and antidepressants [44,45], which may compete with nutrient transfer and/or result in unwanted placental and fetal drug exposure. Once these drugs have entered into the cells of the PB, there are several potential outcomes, icluding transport into fetal circulation or back into maternal circulation, retension in the PB, and/or metabolism within the PB's cells. Certain substances are not able to pass through the placenta at all (Fig. 1B), including drugs like heparin and insulin, which may serve to protect the fetus but is also important to consider when thinking about drug delivery to the placenta or fetus [46].

The cells within the PB contain the enzymatic machinery necessary for both Phase I and Phase II metabolism. This means that drugs can be modified (Phase I; *i.e.* oxidized, hydrolyzed) and conjugated with chemical groups (Phase II). Data on placental biotransformation is limited and metabolic activity in the placenta, compared to the liver, is relatively low and not expected to limit the passage of xenobiotics [47]. Further, within the placenta, expression of metabolic enzymes, such as the cytochrome P450 family, fluctuates over the course of pregnancy [48] suggesting that the placenta's metabolic capacity also changes throughout gestation. This potential for the placenta to transform drugs should not be ignored, especially as products of Phase I metabolism are often more bioreactive, and therefore potentially more toxic, than their parent compounds. Accordingly, both the parent drug and its potential metabolite(s) should be considered in the context of placental transfer, as either of these chemical compounds could cross into fetal circulation. Moreover, a thorough appreciation of placental transport mechanisms will likely require a better understanding of placental metabolism and is ultimately paramount to developing targeted therapies and prevention of unwanted placental and fetal exposures.

While multiple cell types are important for proper barrier function, the syncytiotrophoblast is the dominant cell regulating placental transport [24,49]. The remaining cell types and components, including villous cytotrophoblast [23,49], extravillous trophoblasts, trophoblast giant cells, Hofbauer cells [21,49], fetal endothelial cell [22,49–51], decidual cells, and proteins found in the basement membrane [37], all play a significant but supporting role in placental transport (Fig. 2). The biology of these cell types and the tissue environment are discussed in more detail below.

2.2. Biology of the syncytiotrophoblast

The syncytiotrophoblast functions as an arbitrator, negotiating for nutrient resources while providing a protective barrier on behalf of the fetus. Moreover, the syncytiotrophoblast serves a critical role as the primary source of placental hormones. Arising from the fusion of rapidly dividing villous cytotrophoblasts, the syncytiotrophoblast is a multinucleated cell layer that lacks lateral cell borders (Fig. 1A). This results in the formation of a true

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syncytium lining the outermost surface of the villous tree, an important protective feature providing a continuous physical barrier [52]. At a molecular level, the syncytiotrophoblast also serves as a chemical barrier regulating the absorption of endogenous and exogenous substances [24,49]. The major transporter super-families found in the human placenta are ATP-binding cassette (ABC) and solute carrier protein (SLC) transporters. While these two super-families are responsible for the influx of physiologically relevant compounds into the syncytiotrophoblast that can be dispatched to the fetus or used as substrates to generate other biological molecules, drugs and other xenobiotics also act as substrates for these transporters [44]. In fact, for some transporters drug and xenobiotic efflux is their primary function. ABC transporters in particular govern fetal protection through their predominant role as efflux transporters moving drugs and other xenobiotics away from fetal circulation. One primary example is ABCB1, which encodes for a protein known as P-glycoprotein, a transporter that is highly expressed on the apical membrane and has a wide range of substrates including chemotherapeutics, steroids, drugs of abuse, and antidepressants [45]. Conversely, SLC transporters are largely responsible for the influx of hydrophilic and charged molecules including glucose, amino acids, vitamins, fatty acids, and sulfated steroids. Therefore, biologically active exogenous substrates that are capable of utilizing SLC transporters may pose a threat to fetal health and development. Other known substrates of ABC and SLC transporters include cardiac drugs, antibiotics, and nonsteroidal anti-inflammatory drugs (NSAIDs) [44]. Competition between endogenous and exogenous substrates may alter nutrient availability or result in harmful drug delivery to the fetus [45,53]. For a more thorough description of ABC and SLC transporters in the human placenta see the recent review by Walker, *et al* [44].

In addition to its regulatory role, hormone production by the syncytiotrophoblast is essential for maintaining a healthy pregnancy [19,54]. The syncytiotrophoblast houses the enzymatic tools necessary for the biosynthesis of a number of hormones that are needed for placental development, angiogenesis, embryo implantation, fetal development, and several other processes that are critical for the establishment and maintenance of pregnancy [19]. Hormones produced by the syncytiotrophoblast include human chorionic gonadotropin (hCG), progesterone, oestrogens, and placental lactogen, amongst others [19,54]. The production of these hormones relies, in part, on transporter activity. For example, the uptake of the sulfated steroid dehydroepiandrosterone (DHEAS) by SLC transporters is necessary for the placental synthesis of oestrogens [44]. Early in pregnancy, placental lactogen and progesterone initiate signals that alter maternal metabolism and allocation of maternal resources to the developing placenta and fetus [55]. Further, expression of placental transporters is regulated by hormones [44]. Therefore, hormone activity and its effect on placental transport should be considered when modeling the PB.

2.3. Modeling the syncytiotrophoblast in vitro

Recapitulating the phenotype of the syncytiotrophoblast is critically important to being able to effectively produce a model of the PB. In cell culture models, the phenotypic characteristics most commonly used to validate barrier ‘maturity’ include biochemical and morphological differentiation. This phenotype allows the cultured barrier to mimic syncytiotrophoblast function, including the presence of a continuous physical barrier

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through syncytialization (*i.e.* cellular fusion) and hormone production (hCG) [16,56]. There are multiple methods used to induce trophoblast cell fusion, a process that is thought to be mediated through cyclic AMP signaling/protein kinase A pathway [57–60]. Chemical induction of syncytialization by exposing cells to chemicals such as forskolin is a common technique used in *in vitro* models [16,59]. However, the application of such chemicals can change the abundance and function of transporters, and may confound studies of placental transport [61]. Hormone production, specifically hCG production, is also commonly assessed. Generally, both primary and non-primary trophoblast cell lines secrete this hormone, however, most studies report higher levels of hCG following the induction of syncytialization [52,56,61].

There are a number of cell lines to consider when modeling the syncytiotrophoblast, including primary human trophoblasts (PHT) and clones of human choriocarcinoma cell lines. While PHTs may appear to be the most physiologically relevant choice, there are some important limitations to consider. For ethical reasons, PHTs can only be collected from early elective terminations (12–20 weeks, with upper fetal age limit depending on the country) or at term, at which point the physical and biochemical properties of the syncytiotrophoblast is likely very different from its state at mid-gestation [45]. Due to the complexity and large number of cell types within the placenta isolating a pure population of PHTs is near impossible. Often times these cultures end up contaminated by mesenchymal cells, such as fibroblast and smooth muscle cells, as well as endothelial cells which can disrupt the growth and viability of the trophoblast cells. Furthermore, difficulty in handling and propagating, as well as innate variability between samples (due to gestational age, area of placenta sampled, contamination, *etc.*) hinder reproducibility when using PHTs [62]. Nonetheless, recent advancements in sequencing technologies have enabled researchers to derive human trophoblast stem cells from PHTs, a powerful new tool to study molecular and functional features of human trophoblasts with potential application in transport and studies of placental pathologies [63–65]. While immortalized human trophoblast cell lines exist, HTR-8 (non-malignant) and ACH-3P (malignant) for example, they still suffer from issues of contamination and reproducibility [62,66]. For these reasons, the human choriocarcinoma cell lines remain a popular choice, with BeWo, Jeg-3 and JAR representing the current well-established placental cell models in the field. Although these cell lines represent a cancer cell model, they share key features with human trophoblasts including their barrier capacity, hormone release, and expression of nutrient transporters [52]. Several studies have demonstrated that the BeWo cell line, including the b30 clone [67] that is more suited for monolayer formation, is an appropriate model for transport studies as they have significant overlap in transporter expression [45,67] and hormone profiles with primary trophoblasts [68], and they can form a confluent monolayer. However, there are limitations with all the cell lines mentioned, including differences in transporter expression and molecular transport [24,52] that should be carefully considered when modeling the PB and conducting transport studies. Therefore, use of these cancer cell lines to identify specific transport mechanisms may warrant a comparison with PHTs. A recent comparative study has already been conducted by Rothbauer, et al. and provides some great insight about four different human trophoblast-derived cell lines and their relevance in placental barrier model studies [52].

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2.4. Biology of supporting cells and extracellular environment

In addition to the syncytiotrophoblast, cell types within the PB include villous cytotrophoblast, extravillous trophoblasts, trophoblast giant cells, Hofbauer cells, fetal endothelial cells, and decidual cells (Fig. 1A). Additionally, the microenvironment within the PB, including the basement membrane (or extracellular matrix, ECM), and blood flow, contribute to molecular transport across the PB. The villous cytotrophoblasts, which arise from the differentiation of cytotrophoblast stem cells (Fig. 2), can have one of two fates. They can fuse together to form the multinucleated syncytiotrophoblast or, in the absence of a maternal environment, they can take on a more invasive role as extravillous trophoblasts. Within the PB, villous cytotrophoblasts are mononuclear cells that help to maintain the syncytiotrophoblast layer and provide structural support for the villous tree [49]. As a result, these cytotrophoblasts help regulate aspects of syncytiotrophoblast physiology, such as cell fusion and trophoblast turnover *via* the apoptotic cascade [69,70]. Generally, villous cytotrophoblasts are not thought to play an active role in the PB transport regulation. However, a recent study found that at term, these cells are more metabolically active than the syncytiotrophoblast [71], suggesting that they may play an important role in biotransformation and altering the transport and/or reactivity of compounds within the PB. Further consideration of their inclusion in a barrier model is warranted.

When villous cytotrophoblasts take on an invasive phenotype, they begin to form cytotrophoblast cell columns aiding in anchoring the placenta to the uterus. Some of the cytotrophoblasts that make up cell columns will develop into extravillous trophoblasts. The extravillous trophoblasts break through the overlying syncytiotrophoblast cell layer, invading and remodeling maternal tissue (interstitial extravillous trophoblasts), uterine spiral arteries (endovascular extravillous trophoblasts), and uterine glands (endoglandular extravillous trophoblasts) (Fig. 1A) [19,72,73]. Interstitial extravillous trophoblast cells aid in anchoring the placenta to the maternal decidua and can fuse to form multinucleated trophoblast giant cells which lose their ability to migrate and invade, potentially preventing deeper penetration into the uterine wall. Furthermore, trophoblast giant cells may play an important role in regulating blood flow through their release of vasoactive and angiogenic factors [74]. This invasion and remodeling results in increased maternal blood flow to the placenta (interstitial and endovascular) and access to histiotrophic nutrients (endoglandular) [19,73,75].

Decidualization is the process by which endometrial cells undergo functional and morphological changes in preparation for pregnancy. Changes include an influx of leukocytes to the area as well as development of a secretory lining that secretes cytokines, growth factors, and proteins. There is some evidence that decidual stromal cells and leukocytes that are maternal in origin initiate remodeling of maternal vasculature prior to the invasion of extravillous trophoblasts. However, colonization of the decidua by extravillous trophoblasts is required for thorough remodeling of the spiral arteries [76]. Specialized extravillous trophoblasts, referred to as endovascular extravillous trophoblasts, invade through the walls of uterine spiral arteries, migrating along the lumen, and dramatically widening these vessels to modify blood flow to the placenta (Fig. 1A). During the first trimester the endovascular extravillous trophoblasts form plugs (not shown in Fig. 1) that partially block maternal blood flow to the placenta, leading to a transient state of hypoxia,

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a physiological state that is believed to be important for early placental development and trophoblast differentiation [77,78]. Similarly, endoglandular extravillous trophoblasts, invade the walls of uterine glands and can even replace glandular epithelial cells (Fig. 1A) providing the developing fetus with early access to nutrients during a time that maternal blood flow to the placenta is restricted [79]. Together, these specialized trophoblasts and decidual cells aid in placental transport by creating an environment that supports early placental development, providing early access to nutrients for fetal growth and development, and ensuring sufficient perfusion of the placenta with maternal blood [73].

Hofbauer cells are placental resident macrophages, though like villous cytotrophoblasts, their role in regulating transport is relatively unknown [21,49]. These cells are thought to be more related to the M2 macrophage, which functions in repair processes including ECM construction, rather than an M1 macrophage that triggers an inflammatory response in order to fight off microbes [80]. Further, these cells may play a role in regulating growth factor and cytokine expression [49,80] that can directly affect trophoblast function. Ultimately, the exact role of these cells still remains largely unknown and needs clarification, particularly if they may play a role in immunologic transfer or protection of the fetus.

Fetal endothelial cells play a very important role within the PB. Fetal blood enters the placenta through the umbilical arteries picking up oxygen, nutrients, and hormones as it flows through a labyrinth of fetal capillaries, and flows back to the fetus through the umbilical vein (Fig. 1C). Fetal endothelial cells that line these capillaries exhibit differential arterial and venous phenotypes [50,51]. Beyond their phenotype as vascular cells, they also present an additional barrier to regulate transport, one that is often neglected in placental transport studies. Similar to the syncytiotrophoblast, fetal endothelial cells express transporters that allow substrates taken up by the syncytiotrophoblast layer to enter fetal circulation, and waste and potentially harmful substances to be removed (Fig. 1B) [22]. Recently, *in vitro* studies have begun using human umbilical vein endothelial cells (HUVECs) in co-culture systems when modeling placental transport. These studies found that these cells do contribute to the overall phenotype, by aiding in the replication of physiologically relevant architecture and permeability of the PB model [13,16,27], and thus merit continued inclusion for transport studies. However, whether these are the most physiologically relevant cell type for the PB remains to be determined. Some studies have suggested differential phenotypes between macrovascular cells, such as HUVEC, and microvascular cells, such as those derived from placental microvilli, including how these cell types interact with biological substances, such as insulin [81,82]. Thus, it is important to consider the type of endothelial cell utilized as they may impact PB function differently, leading to different results.

Lastly, the PB environment should be considered in developing placental transport models. The PB contains fibroblasts and basement membrane proteins, including fibronectin, laminin, and collagen, that provide numerous biochemical and biomechanical cues that help regulate trophoblast behavior [49,83,84]. Changes within the basement membrane, such as thickening and stiffness, have been associated with certain pathologies, such as IUGR [83,85]. Additionally, changes in trophoblast cell organization, differentiation, and gene expression profiles have been observed, depending upon the composition or thickness of the

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basement membrane [70,83,86]. Similarly, fluid flow directly influences molecular transport through shear stress on cells within the barrier and turnover of nutrients and waste present in a local area [87–89]. Reduction in fluid flow has been implicated in fetal growth restriction [5], and thus, the biological impact of fluid flow should not be overlooked.

Collectively, these cell types and the environment around them all play an important role in the physiology of the PB, thereby influencing aspects of placental transport. No models, to our knowledge, have recapitulated all of these components in a single system. Full replication of this complex tissue is near impossible, in part due to our lack of understanding and ability to regulate trophoblast differentiation *in vitro* to include all of the relevant subtypes [90]. Though there have been recent efforts to generate trophoblast stem cells [18,63], the biology of trophoblast differentiation is not straightforward, with our knowledge continually evolving [23,72,90–92]. For example, whether extravillous cytotrophoblast and villous cytotrophoblast originate from a common progenitor [23], or whether extravillous cytotrophoblast differentiate from villous cytotrophoblast [72], remains unclear, though to our knowledge, recent findings have suggested the latter. Regardless, steps in the right direction are being taken with co-culture systems, including cell lines that mimic undifferentiated cytotrophoblasts and fetal endothelial cells seeing increased use and validation.

3. Microphysiological models

Microphysiological models have been developed with the intent of recapitulating the native biology of a tissue system. In recreating a tissue, there are a number of approaches that can be taken, ranging from traditional scaffold or hydrogel fabrication techniques to more modern organ-on-a-chip, bioreactor, and bioprinting approaches (Fig. 3). Here, we discuss a variety of techniques to recreate the PB. While all of the models discussed below emphasize the importance of replicating specific structural and functional features of the syncytiotrophoblast, namely barrier formation and hormone production, there are additional features that warrant further discussion for their relevance in modeling the PB (Table 1). For additional context, we begin with a brief section on traditional approaches to studying placental transport.

3.1. Traditional approaches – transwell and perfused placenta

In the field of placental transport, two of the most prominent and time-tested approaches are (1) the use of Transwell inserts with trophoblast cells (Fig. 3A), and (2) the use of the *ex vivo* perfused placenta model (Fig. 3B) [8,95]. The latter is considered the gold standard, with both Transwell and newer approaches often performed as comparative studies against the perfused placenta model [16,96]. In the perfusion setup, both the maternal and fetal vasculature are connected to catheters and perfused such that bidirectional molecular transport can be studied by introducing a substance into one side of the vascular system and analyzing the perfusate from the other [8]. One recent example from Stirrat, *et al*, utilized a deuterated tracer in order to study placental transfer and metabolism of cortisol [97], the primary circulating glucocorticoid hormone in humans. Understanding the pharmacokinetics (where it is) and pharmacodynamics (what it is doing) of glucocorticoids

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during pregnancy has been an intense area of research for several key reasons, including their role in fetal development and tissue maturation and use of synthetic glucocorticoids in reproductive medicine [98]. Furthermore, overexposure to glucocorticoids as a result of maternal stress is associated with alterations in development and fetal programming, including fetal growth restriction and increased risk of neurodevelopmental disorders [99]. Here the authors showed that transfer of maternal cortisol to fetal circulation was surprisingly low (3%) and highlighted the enzyme 11-*β*-hydroxysteroid dehydrogenase-type 2 (11*β-HSD2*), which converts cortisol to its inactive form corticosterone and is highly abundant in the placenta, as the rate limiting step in maternal transfer [97]. While this provides the most physiologically relevant model to evaluate placental transport, there are some significant limitations, including technical limitations in maintaining tissue viability following delivery of the placenta and for applicability in longer studies. For instance, mRNA and activity levels of 11*β-HSD2* is reduced by maternal stress [100], which may render the fetus more vulnerable to the deleterious effects of excess glucocorticoid exposure. Findings from the study by Stirrat, *et al,* support reduced 11*β-HSD2* activity, through the use of a potent inhibitor, can lead to increased fetal glucocorticoid exposure. However, they were unable to fully test regulation of this enzyme by glucocorticoids, as seems to be the case in models of prenatal maternal stress, for a more prolonged period due to issues with tissue viability [97]. Additionally, due to limitations in tissue availability there are broader concerns about the lack of ability to replicate earlier stages of pregnancy using *ex vivo* placental perfusion. More recently, rodent models have been used for *ex vivo* perfusion studies, allowing assessment of placental transport at different stages of development [101]. However, these studies are also technically very limited due to time, equipment, and tissue viability. Findings from *ex vivo* perfusion studies like the one described here would be significantly complimented by the mechanistic insight that can be obtained using the *in vitro* models described below.

The Transwell approach has become more common as trophoblast cell lines form monolayers *in vitro* and do not suffer from the constraints in viability and technical difficulty experienced with the *ex vivo* approach [8,95,96]. In this approach, cells are seeded into a Transwell insert, creating apical and basolateral compartments within the Transwell-well plate setup [96,102–107]. BeWo cells, and the b30 clone, are commonly used in this approach, with the vast majority of studies utilizing these cells [95,96,103–106]. Moreover, in these studies, cells are often seeded at 100,000 cells/cm^2, a density that is high to enable monolayer formation [102,104]. This has enabled robust study of transport and *in vitro* effects of exposure to chemicals, such as bisphenol A [104] and drug-loaded nanoparticles [105,106]. These models have also been utilized to study bi-directional (*i.e.* maternal to fetal and fetal to maternal) transfer of compounds [104], which could be beneficial for understanding potential compound-induced injury to the fetus. However, as articulated above, utilizing a trophoblast cell line in a Transwell, as has often been done [20,95,96,102–107], does not recapitulate the complexity of the PB. In particular, these studies often lack an endothelial cell population needed to recreate the second major cell-regulated transport barrier within the PB, though some Transwell models do include endothelial cells [107,108]. Thus, tissue engineering approaches are addressing some of the concerns arising from Transwell studies.

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3.2. Bioreactor-based models

Bioreactors provide one tissue engineering approach to producing PB models. The key advantage with bioreactors is being able to perfuse the model, in turn producing fluid flow and shear stress that can be modulated through various input parameters. One of the earliest PB studies using a tissue engineering approach was a bioreactor based model, demonstrated nearly two decades ago in 1999 [29]. More recent examples of bioreactors include the use of rotating wall bioreactors [30,109], based on a design by the US National Aeronautics and Space Administration (NASA), and examples of custom-built bioreactor systems (Fig. 3C) [27,28].

One of the earliest examples, to our knowledge, is by Ma, *et al* [29]. In this study, the authors utilized a poly(ethylene teraphthalate) (PET) matrix as a scaffold where modifications were made to reduce the hydrophobicity of the material and to place hydroxyl groups on the surface. Dual compartments were designed to mimic the maternal and fetal side of the PB, and first trimester trophoblast cells were seeded onto the scaffold. Microscopy images showed cells appeared to attach onto the scaffold and form a barrier, though the data presented are not compelling in showing the temporal changes in barrier formation. Further, expression of intercellular barrier markers, such as tight junction proteins, was not assessed, nor were transport studies performed for any biologically relevant (*i.e.* glucose) or synthetic (*i.e.* nanoparticles) substances, thus raising concerns as to whether this barrier model is truly sufficient for studying drug transport across the PB.

More recently, rotating wall bioreactors were utilized to induce syncytialization of trophoblast cells *in vitro*, and to develop 3D-based cultures to study microbial resistance and Zika virus infection [30,109]. In one study by McConkey, *et al*, beads were utilized to grow a 3D co-culture of microvascular endothelial and trophoblast cells [30]. Microvascular cells were initially seeded onto Cytodex®-3 beads and grown for 3–5 days, at which point trophoblast cell lines were seeded onto the beads and grown for an additional 21 days. JEG-3 cells were found to have approximately 75% coverage of the beads, along with protein and gene expression profiles comparable to primary human syncytiotrophoblasts. Beta-human chorionic gonadotropin (βhCG) was the only protein evaluated, while gene expression included human placental lactogen, hCG, syncytin, major facilitator superfamily domain-containing protein 2 (MFDS2), and placental protein 13 (PP13). Further, within this system, the authors showed formation of syncytia and a brush border, indicative of an advanced barrier formation beyond simple inclusion of a high density of cells. In a second related study by Corry, *et al*, these organotypic models were utilized for investigating Zika virus infection of the maternal-fetal barrier [109]. While both of these studies did not explicitly utilize the models formed from the bioreactor for transport studies, they demonstrated promise in recapitulating an advanced PB, with appropriate phenotype and tissue-level features such as syncytialization and brush border formation. As presented, these models could prove useful for understanding cellular uptake of compounds by trophoblast cells, though not transport across trophoblast, with a more advanced barrier model, compared to other techniques with less complexity in the model. Adapting these techniques for barrier formation to techniques more amenable to transport studies could provide an advantage of fabricating a PB with better established biological validation. One

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approach could be through the use of beads made from engineered materials that would enable uptake and release of molecules [110,111], and also facilitate removal of cells from the beads without damaging the beads or the molecules [111], though this hypothesis would need to be tested.

In another series of studies by Levkovitz, *et al*, the authors developed a custom well and bioreactor, enabling the use of an amniotic membrane with trophoblast and endothelial cells in co-culture on opposing sides of the membrane [27,28]. In the first study, the authors developed a custom well chamber to hold the thin amniotic membrane derived from term placenta in place for transport experiments [27]. They utilized HTR8 cells trophoblast cells and HUVECs endothelial cells within the model, showing that the cells remained on the membrane over time, maintained their unique phenotype, and formed tight junctions. Further, they calculated the permeability for the amniotic membrane alone, for cells in monoculture on the membrane, and for cells in co-culture on the membrane, finding that the permeability when using only trophoblast within the model differed from the permeability when using the co-culture with both cell types. Endothelial cells on their own were a closer approximation to the co-culture (within 16–18%), perhaps due to their enhanced tight junction formation, and thus maintained a tighter and more regulated barrier compared to the trophoblast. In the second study, the authors further evaluated transport within the bioreactor model, focusing on glucose transfer [28]. As before, glucose transfer was higher for the amniotic membrane alone (60% + transfer at 24 h), but was slightly reduced with trophoblast cells in monoculture (50–55% transfer at 24 h), endothelial cells in monoculture (40–45% transfer at 24 h), and both cell types in co-culture (30–35% transfer at 24 h). This pattern carried over to calculated permeability, indicating that trophoblast and endothelial cells in co-culture lead to reduced rates of molecular transport across the barrier compared to either cell type in monoculture. In summary, these studies provided a method for developing an *in vitro* PB model with appropriate cell types present in co-culture, as well as providing a perfused flow that may better mimic the PB environment compared to static culture.

3.3. Hydrogel-based models

Hydrogel-based tissue engineering is one of the oldest and most common methods for fabricating artificial tissues, dating back multiple decades (Fig. 3D) [112,113]. However, given the general lack of studies related to the placenta [2], it was only in the past few years that placental tissue models have been fabricated and studied using hydrogels [13,15,25,26], introducing an element of 3D tissue modeling to the field largely dominated by Transwell-based studies [8,20].

In a recent example, Nishiguchi, *et al*, developed a PB model with connective tissue and vasculature to mimic the multilayer complexity of the barrier [15]. This model utilized fibronectin, gelatin, collagen, and laminin as the biomaterials within the hydrogel, fabricating a multilayer construct that contained biologically relevant components of the ECM. In this study, the authors performed three sets of experiments: (1) to show formation of the PB model with primary cytotrophoblast and the BeWo cell lines; (2) to incorporate a biomimetic vascular bed with connective tissue within the PB model and investigate the role of this addition to the model; and, [114] to assess how the conditioned media from this PB

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raised under hypoxic conditions (2% oxygenation) can impact neuronal signaling. The first set of experiments confirmed that this model displays two key features of a PB, including formation of a barrier by confirming expression of cadherins and hormone production with levels of hCG up to 1500 pg/mL after 4–5 days. The second set of experiments indicated that a vascular-like bed could form within the hydrogel, with positive cell adhesion molecule staining between endothelial cells and formation of tubular-like structures, though it is unclear whether these are truly perfusable-tubules comparable to blood vessels *in vivo*. Finally, the authors demonstrated that changes in the environment in which this PB model is cultured, including hypoxic conditions and direct vs. indirect contact with the vascular-like bed, can have significant impacts on dendrite outgrowth of cultured cortical neurons. These findings suggest suboptimal function of the PB under hypoxic conditions, potentially through the secretion of biologically active molecules that may disrupt fetal development, and highlights the importance of the vascular endothelium in transplacental maternal-fetal communication.

This study presented a method for 3D co-culture of trophoblast and endothelial cells that allows for study of molecular transport across the PB. However, it is not without limitations. One concern relates to viability of the primary cytotrophoblast and the reported transepithelial electrical resistance (TEER) values, which both differ significantly from a previous study by Huang, *et al*, that also utilized primary cytotrophoblast within a Transwell setup [56]. Perhaps this can be attributed to a difference in 2D and 3D culture, though it seems unlikely that 3D culture, as in Nishiguchi, *et al*, would lead to TEER values reduced by two orders of magnitude compared to 2D culture (Huang, *et al*, reported nearly 1500 $\Omega\cdot cm^2$ in their 2D study, compared to approximately 30 $\Omega\cdot cm^2$ reported by Nishiguchi, *et al,* in their 3D study). Notably, very few studies have utilized primary trophoblast cells, and thus there is no definitive reference for expected TEER values using these cells. One set of experiments that would be of interest would be evaluating the PB model in a co-culture system with neuronal cells to evaluate whether this indirect culture method results in different observations compared to the conditioned media.

A series of studies by Arumugasaamy, *et al*, developed a biomimetic PB model and studied interactions between the PB and fetal-like cells using a hydrogel based barrier and an indirect co-culture system [13,25,26]. Here, the authors utilized gelatin methacrylate as the biomaterial, and two layers of BeWo b30 cells to mimic the syncytio- and cytotrphoblasts of the PB as well as a single layer of HUVECs as endothelial cells [13]. The authors demonstrated expression of cadherin and zonula occludins-1 (ZO-1), indicative of adherens and tight junctions, respectively, as well as progressive barrier formation through cell growth and TEER testing, reporting TEER values near 90 $\Omega\cdot cm^2$, falling below the threshold value suggested in Table 1. Relevant bioactivity was also confirmed in this model with the production of hCG, progesterone, and VEGF observed. Interestingly, this model was then utilized in a co-culture setup with neural progenitor cells to study Zika virus infection, where the authors demonstrated the virus crossed the PB and suggested, based upon neural progenitor cell viability, that the PB modulated Zika virus infection.

Subsequent studies, using a modified version of this PB model, investigated selective serotonin reuptake inhibitors (SSRIs), examining effects on the cells within the PB [25]

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and on iPSC-derived cardiomyocytes, intended to mimic fetal cardiomyocytes, downstream of the barrier [26]. In the first of these studies, the authors incorporated placenta-derived extracellular matrix (pECM) into the PB model, observing that this pECM impacted trophoblast secretion of transforming growth factor-beta (TGFβ) [25]. The bulk of this study evaluated two common SSRIs, fluoxetine and sertraline, and showed that these drugs were removed from the PB by the drug efflux pumps P-glyocoprotein and breast cancer resistance protein (BCRP), that both SSRIs influenced CAM secretions of the endothelial cells in the model, and that sertraline influenced TGFβ secretion. In the second study, the authors demonstrated that both SSRIs influenced cardiomyocyte calcium handling, whether the drug was presented directly to the cardiomyocytes or it passed through the PB [26]. Notably, the authors observed differences in outcome severity, with effects on calcium handling being amplified as a result of indirect exposure through the PB model compared to direct drug exposure. The authors recognize that this may in part be due to the trophoblast cell line used in this model. Though the authors investigated the impact of multiple endothelial cell types within these placental transport studies [25], they did not investigate multiple trophoblast cell types. Therefore, a comparison of PHTs or other human choriocarcinoma cell lines in these studies would be of interest and enhance our understanding of the role of the trophoblast in co-culture models. Ultimately, both of these hydrogel-based approaches are simple enough to enable some moderate throughput scalability and provide an *in vitro* platform for studying how substances that influence or cross the PB are able to impact development of fetal-mimicking cells.

3.4. Organ-on-a-chip models

Much like bioreactors, organ-on-a-chip models provide the advantage of perfusion within the system (Fig. 3E). However, the system is designed at a much smaller scale, allowing for reduced consumption of resources in these studies. Surprisingly, though fewer resources are used in these studies, the application of organ-on-a-chip models remains to be seen in a high throughput manner (*i.e.* greater than 96-samples per plate). This has not, however, impeded progress in developing a placenta-on-a-chip, with a series of studies led by Huh and Blundell [14,16,34], as well as others [31,33,35]. Further, as this work has been discussed more thoroughly in a recent review [32], the discussion in this section is brief and intended as a more 'high-level' overview related to the organ-on-a-chip approach for PB transport studies.

The first example, to our knowledge, of the placenta-on-a-chip was presented in a paper by Lee, *et al* [14]. In this study, the authors used the JEG-3 cell line as trophoblast and HUVECs as endothelial cells to generate a two-compartment co-culture model that allowed for perfused flow across both compartments. Blundell, *et al,* built on this by using the BeWo cell line as trophoblast and primary microvillous endothelial cells isolated from placenta as endothelial cells, demonstrating trophoblast syncytialization, brush border formation, and glucose transport across the barrier [16]. Following this work, studies utilized a placenta-on-a-chip approach to study transport of glyburide [34], caffeine [35], nanoparticles [31], and the inflammatory response to bacterial infection [33].

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The rapid uptake of this approach to studying interactions at, and transport across, the PB indicate its utility as a readily available method for investigating biological questions. Indeed, organ-on-a-chip approaches have gained tremendous interest from the scientific community as they provide a small-scale tool for studying biology. However, as noted above, throughput remains an issue for being able to utilize this approach for large-scale studies. Additionally, though some studies induce syncytialization of the trophoblast [34], this is not a widespread approach in the field. Given the relative ease by which cells are seeded into the model, it would be prudent for all placenta-on-a-chip studies to induce syncytialization of the trophoblast layer. Interestingly, only one placenta-on-a-chip study [115], to our knowledge, utilizes primary cells, despite their utilization in other approaches. Notably, this study is unrelated to placental transport. It is not clear why primary cells are not utilized in more placenta-on-a-chip studies, though it would be of considerable interest to utilize these cells.

3.5. Bioprinting-based models

Bioprinting, described here as 3D printing utilizing biological components, including biomaterials, growth factors, and cells, has grown in tremendous interest across tissue engineering, with multiple examples of placental tissue bioprinting (Fig. 3F). Mandt, *et al*, utilized high-resolution two-photon polymerization to create a PB on a chip [17]. BeWo b30 cells were co-cultured with HUVECs within a methacrylamide- and methacrylate-functionalized gelatin (15 wt% final concentration) to produce 3D structuring at a microscale, and enabling precise control over cell positioning within their model. Their model was further designed to function as a microfluidic device, allowing for fluid flow (50–70 μL/h) within their barrier system. They showed proof-of-concept results through cell metabolic activity and permeability of example molecules dextran and riboflavin. Further, through this study, the authors demonstrated that they are able to produce microcurvature with high control over cell positioning using bioprinting approaches. However, they do not demonstrate tissue-specific functionality or biologically relevant transport beyond the example molecules. Thus, it remains to be determined whether the advantages that bioprinting provides in precise control over location of cells and materials truly provides an added advantage to the PB model as a whole.

Other studies have utilized bioprinting to investigate placental biology more generally, demonstrating that bioprinting as a technique can be useful to placental models when the appropriate rationale is used in model design. A series of studies by Kuo, *et al*, utilized bioprinting to produce a placental model for investigating trophoblast cell migration [86,116,117]. The first study utilized bioprinting to generate concentric circles of growth factor, empty hydrogel, and trophoblast cells, demonstrating that trophoblast cells migrated through the 3D bioprinted hydrogel in response to the chemotactic gradient, and that this migration could be measured [116]. Subsequent work investigated the role of biochemical factors from extracellular matrix and how these factors influenced trophoblast response within the matrix [86]. The last study incorporated additional 3D printed components to build a bioreactor, demonstrating measurable shear stress impacts the trophoblast cells depending on their distance from the fluid flow in the system [117]. Together, these studies

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further demonstrated potential utility of bioprinting in controlled fabrication of placental models, whereby placental biology can be perturbed and investigated.

3.6. Minimum requirements for model design and validation

From the discussion above, it becomes clear that numerous approaches are able to yield a PB model effective for the intended studies. However, as the field continues to progress, consistency is needed to develop suitable models that builds upon the knowledge we have already gained. As such, we propose that certain criteria are necessary, at a minimum, towards building effective and useful PB models, regardless of the modeling approach used. This includes criteria around the model design, as well as criteria around validation of the model.

In model design, cell types and environment are critical and should be selected based on the experimental question. At a minimum a cell line modeling the syncytiotrophoblast should be included, whether this is through the utilization of malignant or non-malignat cell lines or primary cells. Cell lines enable a higher number of samples, given the ease of culturing the cells, though primary cells are often thought of as being more relevant to the *in vivo* scenario, despite the fact that they are often collected at term. Through these ethical collection procedures, primary cells are thus not representative of earlier stages of pregnancy. In either case, syncytialization of these cells should be considered to recapitulate the major phenotype in the PB, keeping in mind that some of the techniques used to promote syncytialization may confound mechanistic interpretations of placental transport. Moreover, with the number of methods for co-culture increasing, endothelial cells should be included, regardless of model utilized. These two cells provide the critical barrier cells and the field, as a whole, is at a point where standarization of trophoblast-endothelial cell co-culture is readily feasible [13,16,27]. There are also several important environmental factors that can be included in these models: fluid flow with shear stress, a 3D microenvironment with biomaterial considerations, and architectural complexity. Fluid flow can be applied through bioreactor and organ-on-a-chip approaches, though this experimental setup can quickly become complex, and may only provide limited additional information compared to the added complexity of the system. Similarly, a 3D microenvironment can be considered, where the biomaterials utilized may likely impact cell phenotype [83,84,86]. Additionally, the size of the 3D environment should be considered, as this will impact diffusion across the PB and there is a temporal component to size *in vivo*, with the PB thinning over time. 2D environments, such as Transwell or organ-on-a-chip approaches, are generally simpler in the technical aspects of cell culture, but are likely to yield phenotypic differences compared to 3D cell culture. Architectural complexity can also be considered through the use of bioprinting techniques, where modeling topographical features and structure to the PB may be of interest. However, size also needs to be considered here, both in the scope of transport across the PB and technical limitations of the bioprinter utilized in model development. Though these environmental factors are important, there is a lack of standardization around these approaches and thus, though recommended for advanced model design, they should not be considered a minimum requirement.

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In model validation, factors related to phenotypic outcomes are considered. Here, it becomes much easier to standardize a minimum assessment of the model as functional outcomes. Details are listed in Table 1, and can be broken down into two tiers. The first tier has been consistently demonstrated across various models and includes characterization of: junction proteins, permeability (for both permeable and nonpermeable compounds), microvilli, syncytialization, and hormone production. At minimum, this first tier should be demonstrated for any new PB model as this provides a phenotypic characterization of the model that can then be more easily compared to existing models and previously published data. The second tier includes characterization of membrane transporters and metabolic activity. This second tier is not needed for every model, but it should be considered in the context of the study. Studies related to drug transport could easily be misinterpreted if there is a lack of understanding what membrane transporters the drug is a substrate for or what proteins might metabolize the drug, and whether that metabolite then further interacts with the same or different transporters or metabolic proteins. Thus, these two tiers provide differential levels of functional characterization of the model, and can provide improved consistency as the field progresses.

4. Challenges towards tissue modeling and drug discovery

4.1. Physiology of the human placenta

The placenta is a highly dynamic tissue showing dramatic structural, morphological, and biochemical changes over the course of pregnancy. This inherent plasticity makes accurately predicting and modeling drug interactions and transport across this barrier difficult. There are several key factors worth mentioning and should be taken into consideration when examining drug safety, gestational age, sex of the fetus, and the presence of pregnancy complications.

Most of our insight regarding human placental biology comes from imperfect animal models or human first trimester or term placenta samples. Therefore, we are severely lacking in our understanding of the human PB between 20 and 38 weeks of gestation. We have been able to gain some insight using rodent models, however species differences in lengths of gestation and transporter expression require caution when assuming the mechanism of maternal-fetal transport will be similar in humans [68]. One clear example where rodent models overlap with humans is the thinning of the syncytial membrane over the course of pregnancy [118]. In humans, the thickness of the PB over which drugs and other biological molecules diffuse decreases from 50 μm in the first trimester to 5 μm by the third trimester [45], highlighting the potential increase in maternal-fetal transport as the barrier thickness decreases over the course of pregnancy.

As the majority of the placenta is made up of trophoblast cells that are fetal in origin, the placenta expresses fetal sex (XX *vs* XY). Although current human data that shows differences in transporter function as a result of fetal sex is limited, we do have evidence from animal and human studies showing that transcript levels in male and female placentas differ suggesting potential functional differences between the sexes [99,114,119–122]. Furthermore, transporter expression is influenced by hormone exposure and the intrauterine hormonal environment is established in part by fetal sex, making it is reasonable to assume

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that placental transport may be different between male and female placentas over the course of gestation.

Finally, pregnancy complications and environmental risk factors, such as maternal stress, that impact the overall function of the placenta have the potential to significantly affect or result from changes in the integrity of the PB. The initial steps of placentation and pregnancy involve trophoblast invasion into the spiral arteries of the maternal uterus, with remodeling of the maternal uterine tissue occuring prior to establishment of placental blood flow [123]. These spiral arteries are unique to the uterus, and their remodeling is necessary to ensure a healthy pregnancy, with sufficient nutrient transport between mother and fetus [123]. Failure to undergo this remodeling has been linked to pregnancy complications including preeclampsia and fetal growth restriction. Thus, understanding how spiral artery remodeling and changes to the earliest steps in pregnancy impact fetal nutrition is critical. For example, preeclampsia is a pregnancy complication that is normally characterized by high blood pressure and the presence of protein in the urine. While it is unclear if preeclampsia leads to changes in placental function, or *vice versa*, placental pathologies commonly associated with preeclampsia include a proinflammatory state, increased apoptosis, and alterations in the expression of transporters, such as reduced expression of the SLC glucose transporter GLUT-1 (SLC2A1) [124,125]. The transport of glucose from maternal circulation to the fetus is necessary for fetal development and survival, as the fetus can only produce minimal amounts of glucose itself. Therefore, reduced expression GLUT-1 may drive phenotypes associated with preeclampsia, such as fetal growth restriction. Maternal stress can also disrupt normal PB functions. Changes in transporter expression, including the multispecific drug transporter p-glycoprotein 1 and GLUT-1, have been observed in PHTs and other trophoblast cell-lines exposed to glucocorticoids as well as in animal models of maternal stress [99,125,126]. Therefore, alterations in maternal mental and physical health have the potential to compromise PB functions.

4.2. The placental barrier as a safety consideration

Perhaps the most obvious concern relating the PB and drug discovery is the desire to confirm that the PB does indeed limit fetal exposure to toxins and potential therapeutic agents. Unfortunately, there is at least one historical example where a prescription medication, thalidomide, was able to pass through the PB, leading to congenital abnormalities in a large number of people [10]. Thalidomide was marketed as a drug intended to help alleviate nausea and morning sickness, leading to the drug being prescribed to pregnant women. It took a few years before physicians and scientists were able to identify that thalidomide crosses the PB and alters fetal development, including severe limb malformations to the extent that, effectively, there was no medical recourse. Thus, ensuring that a novel therapeutic does not cross the PB and/or impact fetal development is, in our opinion, critically important.

To that end, there has been a push in recent years to include pregnant women in clinical trials [127]. This stems from pregnant women generally being excluded from clinical trials to minimize risk to the mother and the developing fetus. As a result, pregnant women

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are prescribed medications that are often not studied in a pregnant population, where 80% of pregnant women are estimated to use medications over the course of pregnancy [128]. However, as the techniques and studies above have clearly demonstrated, there are *in vitro* methods now emerging that can provide platforms to test drug safety in a way that is relevant to *in utero* transport and exposure [13,15,26]. Adoption of these techniques could prove useful for drug prioritization in the drug development process, by helping to rule out the use of potentially harmful drugs that are able to reach the fetus. Whether that will occur though remains to be seen.

4.3. Delivery to the placental barrier

One area of research that has gained interest in recent years is drug delivery to the placenta itself. As there is continued investment in placenta related studies for the sake of rapidly increasing our knowledge of placental function throughout the course of pregnancy [2], there is also interest in developing therapeutics and interventional agents that can help probe the placenta [129], and/or overcome diseases thought to begin with the placenta, such as preeclampsia [130]. For additional details in designing nanomaterials for maternal-fetal medicine, the authors suggest a recent review on the subject [131].

One recent study of interest, by Zhang, *et al*, developed a strategy to specifically target the placenta through delivery of trophoblast-specific nanoparticles [129]. Based on the accumulation of erythrocytes in the placenta during placental malaria infection, the authors utilized a strategy of peptide binding to chondroitin sulfate A (CSA) on the surface of trophoblasts, building nanoparticles with a synthetic placental CSA-binding peptide. Studies were performed in pregnant mice, where tissue distribution and localization were studied. The authors found that their nanoparticles with the targeting moiety localized to the placenta and specifically targeted trophoblast cells. Moreover, they were able to demonstrate delivery of methotrexate to the placenta, impairing placental and fetal development, as is anticipated for methotrexate use during pregnancy. This proof-of-concept study provides a new tool to better probe the placenta and deliver therapeutics specifically to the tissue.

Other nanoparticles actively being studied for their use as a targeted drug delivery system are extracellular vesicles (EVs). EVs are membrane bound vesicles that contain proteins, lipids, and small non-coding RNAs. These vesicles are produced naturally by most tissues in the body, including the placenta, and contain intrinsic cell targeting properties thereby acting as an important form of inter- and intracellular communication through the specific delivery of bioactive cargo [132]. Interest in decoding the messages delivered by EVs and identifying the mechanisms by which they target specific cells has been an intense area of research due to their potential use as disease biomarkers [133], role in intergenerational transmission [134], communication between the feto-placental unit and the mother [135], and use as a mode of drug delivery[136]. However scalable production of EVs and lack of understanding of targeting mechanisms has limited the application as potential drug delivery vehicles. Recent studies showed significant antiviral properties of EVs derived from PHTs and serum from pregnant women. The authors illustrated that the miRNAs packaged in PHT derived EVs are one mechanism by which viral resistance is transferrable to placental and non-placental cells, a pathway that likely exists to protect the fetus from viral infections

[137,138]. The *in vitro* models discussed here provide a platform to start probing EVs as a potential mode of placenta-specific drug delivery and enhance our understanding of placental EV production.

The placental transport models discussed throughout this review provide tools to assess whether the targeted cargo does cross the PB and how it may impact fetal-mimicking cells. However, these models also present an opportunity to assess uptake into the placenta and potential changes in placental function associated with drug exposure. A variety of xenobiotics including environmental contaminants [139–144] as well as drugs [145] have been found to accumulate in placental tissue. Since the placenta plays such an active role in fetal development and programming [41], through the transfer and synthesis of nutrients, hormones, growth factors, and neurotransmitters, placental dysfunction as a result of drug exposure is of critical importance to fetal health. Therefore, when used in a drug discovery platform it would be highly beneficial to assess overt functional changes in the cells used to mimic the PB through the use of the engineered systems described herein.

5. Conclusions

5.1. Summary

Through the discussion herein, the current state of tissue engineering the PB for molecular transport studies should be clear. The cellular and structural complexity requires techniques beyond the traditional Transwell and *ex vivo* perfused placenta models that have long dominated the field of placental transport. Moreover, the present time represents a dramatic shift in the field. PB models are being fabricated using hydrogel, bioreactor, organ-on-a-chip, and bioprinting approaches that are enabling the field to improve the physiological relevance of *in vitro* PB models. These models will help further our understanding of therapeutics in the context of pregnancy and may provide methods to assess maternal-fetal transport without the need to include pregnant women in clinical trials, ultimately making medications safer for use during pregnancy.

5.2. Future outlook

Building upon existing models, through the inclusion of different cell types to mimic the barrier's natural complexity and the addition of other key environmental factors such as fluid flow, will provide enhanced techniques for engineering a more biologically relevant PB to effectively model the usage, transport, and potential impact biologically active compounds have on fetal development. While some substances, such as alcohol, are known to affect fetal development, employing these barrier models as a form of high throughput risk assessment and identification of novel therapeutics, particularly in the applications of treating pregnancy-related diseases such as preeclampsia, would be transformative. Moreover, studies to understand how environmental factors, such as maternal stress, and upstream processess, such as spiral artery remodeling, impact the PB and nutrient transport are critical to a more thorough appreciation for the complex biology of this tissue. With newer technologies, such as bioprinting, being employed in this space, the field is at a critical juncture towards enhanced, complex *in vitro* tissue models advancing our understanding of placental biology and how placental transport is impacted by the broader

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physiology of the tissue. As these models are developed, we encourage the utilization of the minimum requirements for model design and validation, as we've discussed in section 3.6 and Table 1, above.

In addition to the *in vitro* systems described throughout this review, placenta organoids have recently been developed. In the last two years, Turco, *et al*, developed a human trophoblast organoid system that may provide a new model with enhanced complexity to study maternal-fetal transmission. This model showed structural features similar to that of placental villi *in vivo*, with the basement membrane located on the outside and syncytiotrophoblast lining the central cavity, secretion of placental-peptides and hormones, and a diversity of trophoblast subtypes including villous cytotrophoblasts, syncytiotrophoblast, and extravillous trophoblasts [65]. While not discussed in greater detail in this review due to their limited applications in the context of placental transport [64,65], the field is likely to see an increase in their use to study maternal-fetal interactions in the placenta. With the continued ethical questions around inclusion of pregnant women in clinical trials, having *in vitro* methods to study placental drug transfer and/or biodistribution in a 'mother-fetus' model can help improve knowledge of medications before clinical application.

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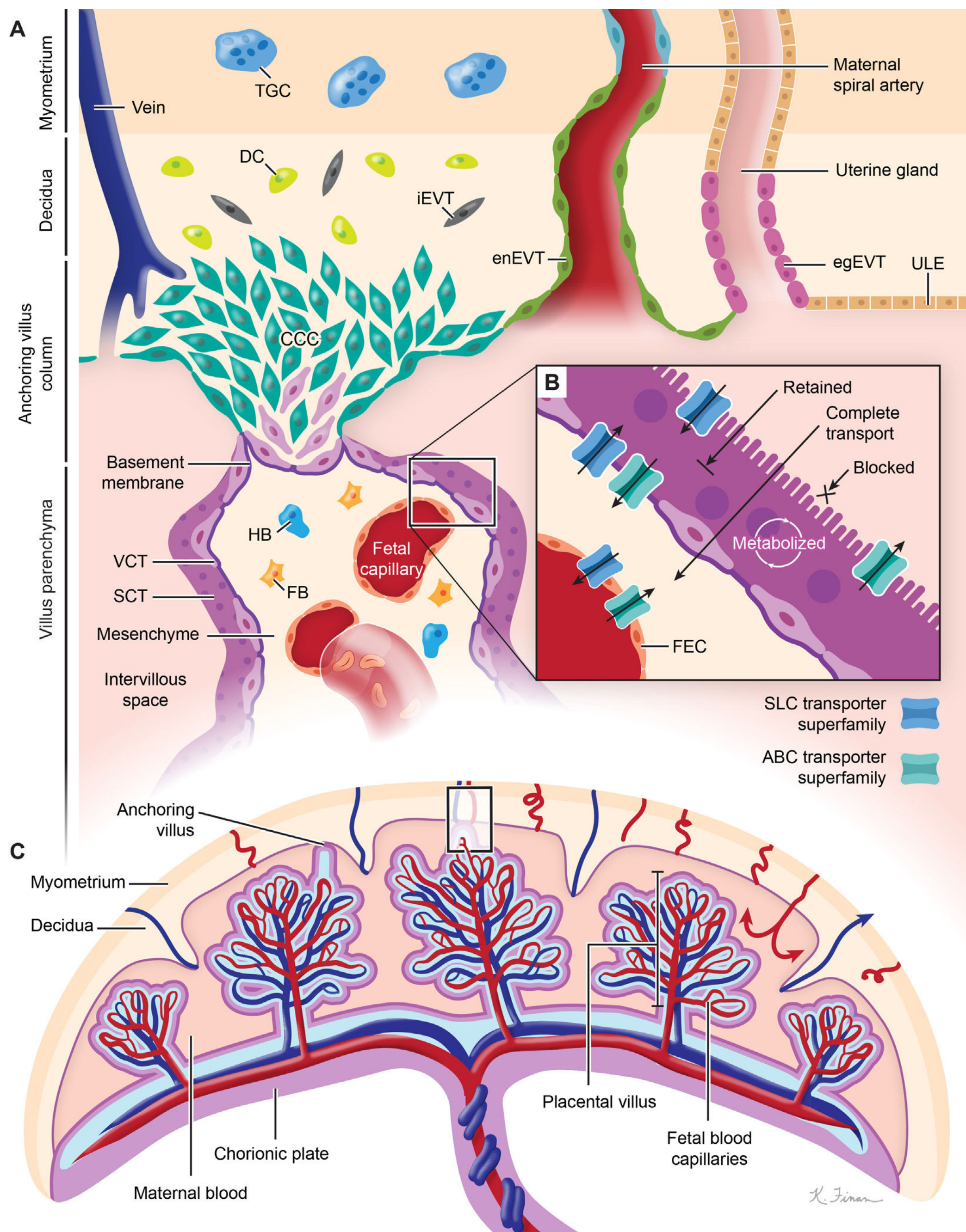

Fig. 1.

Graphical representation of the human placenta characterized by its (A) hemochorial nature (direct contact between maternal blood and the fetal chorion, *i.e*. syncytiotrophoblasts (SCT)) and (C) discoid shape. A) Modified spiral arteries enable sufficient perfusion of the placenta with maternal blood that bathes the intervillous space and makes direct contact with the SCT. (B) The SCT serves as the dominant regulator of placental transport expressing ATP-binding Cassette (ABC, ATP dependent efflux) and Solute Carrier protein (SLC, exchange, coupled, or passive influx) transporters on both the apical and basal membranes. Biological molecules that are taken up into the SCT may be retained, metabolized, and/or

transported all the way through where they can then be taken up by fetal endothelial cells (FEC). Other molecules are blocked completely and not taken up by the placenta. (C) Fetal blood enters the placenta through the umbilical arties (blue) and flows into the capillaries in the placental villus where it picks up nutrients, oxygen, and hormones before returning to the fetus *via* the umbilical vein (red). (VCT: Villous Cytotrophoblast, TGC: Trophoblast Giant Cells, HB: Hofbauer Cells, FB: Fibroblast, CCC: Cytotrophoblast Cell Column, DC: Decidual Cells, iEVT: Interstitial Extravillous Trophoblast, enEVT: Endovascular Extravillous Trophoblast, egEVT: Endoglandular Extravillous Trophoblast, ULE: Uterine Luminal Epithelium.

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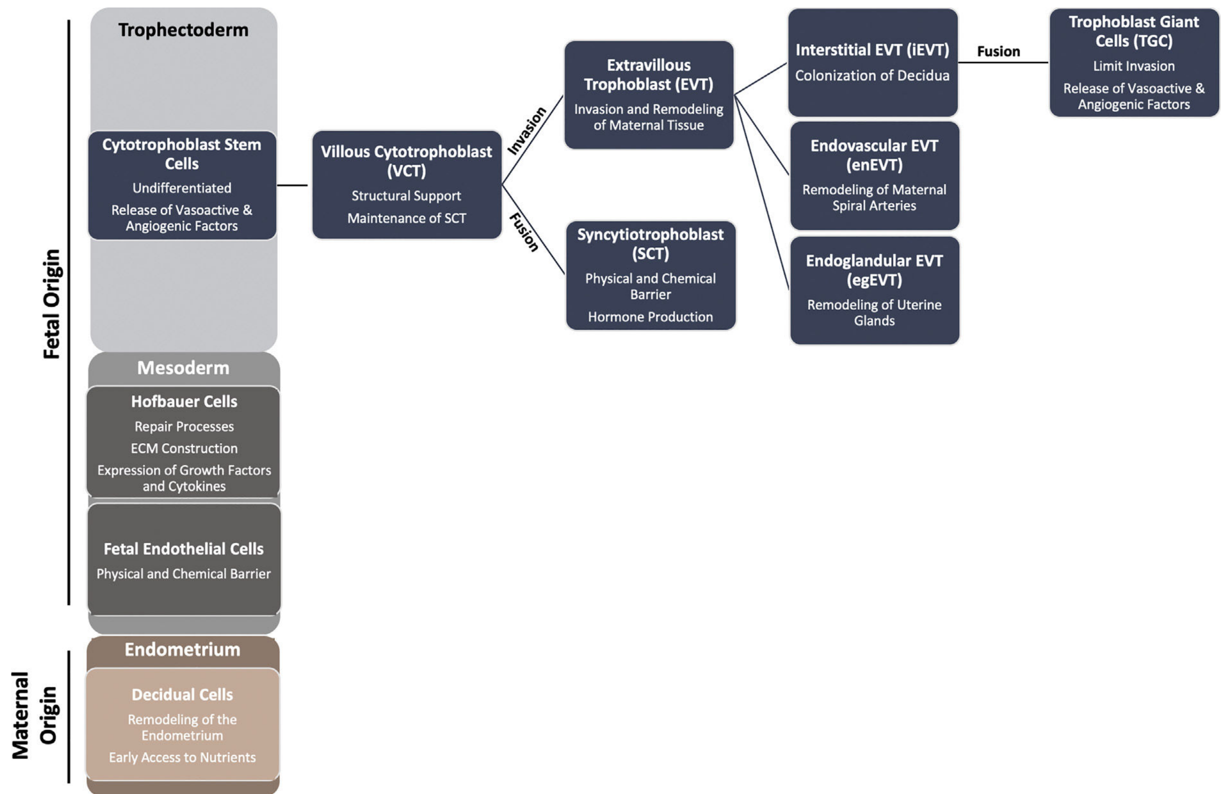

Fig. 2.

Numerous cell-types, of both maternal and fetal origin, are required for proper development and function of the placenta barrier. Trophoblast subtypes, derived from the fetal trophectoderm, make up majority of these cells. Originating from a population of cytotrophoblast stem cells the VCT follow one of two paths, VCT can fuse together to form the SCT that serves as the predominant regulator of placental transport and hormone production, or they can acquire invasive properties and differentiate into EVTs. EVT are required for remodeling of maternal tissue (iEVT), maternal vasculature (enEVT), and uterine glands (egEVT). Invasion of EVT into maternal tissue is limited by the fusion of iEVTs and formation of TGC. Other important cells of fetal origin and derived from the mesoderm include HB and FEC that play important roles in regulating trophoblast function and placental transport, respectively. Maternal DC originating from the endometrium are important for early access to nutrients during placental and fetal development.

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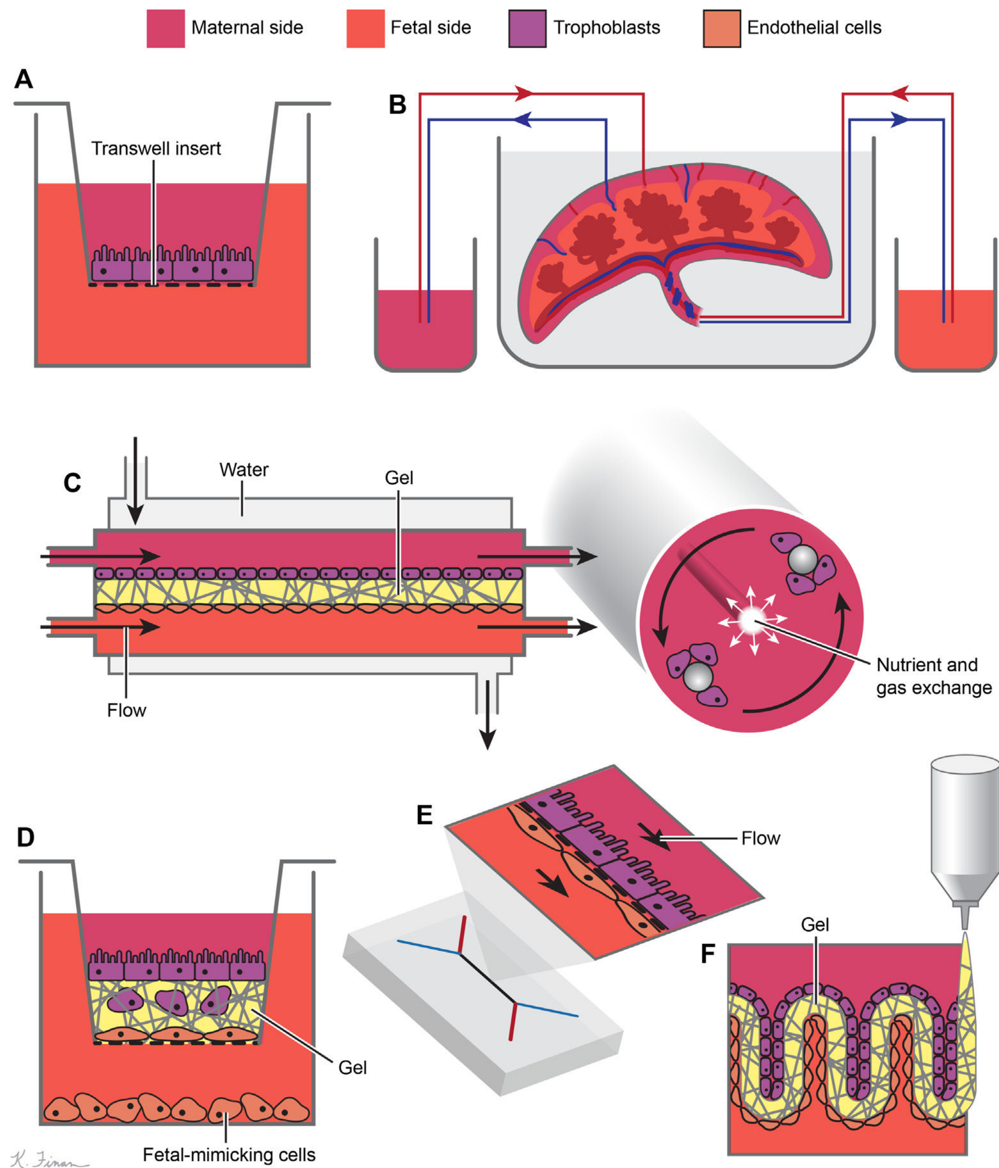

Fig. 3.

Schematics of *in vitro* models of the PB. (A) The Transwell model, with trophoblast cells seeded within a Transwell insert, creating both maternal (apical) and fetal (basal) sides of the PB. (B) The perfused placenta model, where the maternal and fetal vasculature of the placenta are connected to tubing, and separate fluid reservoirs for the materanl and fetal sides. (C) Two bioreactor models, using a perfusion setup (left, adapted from Ma, et al. [29]) or a rotating wall setup (right, adapted from McConkey, et al. [30]). The perfusion setup involves trophoblast and endothelial cells on either a hydrogel (Gel) or a semi-permeable membrane, such as a Transwell insert-style mesh, and fluid flow through separated compartments, with potential external stimuli (Water) to help maintain physiologic temperature at the interface. The rotating wall setup involves cells attaching onto beads as the walls rotate, and an inner source for gas and nutrient exchange for cell culture. (D) The hydrogel model, where trophoblast and endothelial cells are seeded onto a hydrogel

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to create a 3D architecture. Published studies have also utilized fetal-mimicking cells, including cardiovascular and neural cell types, when the hydrogel model is utilized within a Transwell insert setup (see references [13, 15, 26] for additional details), though these are separate from the PB model. (E) The organ-on-a-chip model, whereby trophoblast and endothelial cells are seeded on opposite sides of a semi-permeable membrane, and fluid flow is utilized to create a closed, microfluidic perfusion system. Note that the zoom in portion of this panel is indicative of the cross-section of the inner channel, such that the cells and membrane are stacked vertically with the chip laying flat. (F) The bioprinting model, where a bioprinter creates a custom-designed pattern utilizing a hydrogel bio-ink, and cells are seeded on opposing sides of the gel (adapted from Mandt, et al. [17]). Of note, cells can also be included within the bio-ink and, depending upon the bioprinter utilized, multiple bio-inks can be utilized (for example, three bio-inks could be used, with one containing trophoblast, one containing endothelial cells, and one being acellular). Note that the gel depicted in panels C, D, and F, shows fibrillar architecture that is only visible at micro- and sub-micron scale, not at the macroscopic scale as depicted here. Similarly, the scale of microfluidics shown in panel E is on the micron scale. Schematics are not drawn to size.

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Table 1

Biological functions of the PB, with the significance of each function, analytical tests and biomarkers that can be assessed to measure these functions within an engineered model, the relevant cell types or models, and related references for further details.

Biological Function	Biological Significance	Analytical Tests & Biomarkers	Relevant Models/Cell Types	References
Regulated Barrier				
• Junction Proteins	• Intercellular barrier to prevent leakage and maintain cellular polarity (apical vs basal surface)	• Immunostaining o Adherens junction: *E*-Cadherin, VE-Cadherin o Tight junction: Occludin, Zonula Occludens-1 (ZO-1)	• Primary and choriocarcinoma cell lines • HUVECs and Microvascular ECs	• Comparative study of BeWo, JAR, Jeg-3, ACH\ \3P, and PHT functional parameters relevant to placental barrier model [52]
• Membrane Transporters	• Facilitate and regulate nutrient transport • Protection from harmful, biologically active compounds	• mRNA and Protein Expression: o SLC Transporters (P-glycoprotein, MDR1) o ABC Transporters (Breast Cancer Resistance Protein, ABCG2)	• Primary and choriocarcinoma cell lines o Expression and function may be confounded by Forskolin treatment • HUVECs and Microvascular ECs	• Review of drug transporters in BeWo, Jeg-3, JAR, and PHTs [45]
• Metabolic Activity	• Synthesis and catabolism of hormones • Metabolism of vitamins, fatty acids, and drugs	• mRNA and Protein Expression: o Phase I Enzymes: CYP450s o Phase II Enzymes: UGTs, GSTs, SULTs	• Primary and choriocarcinoma cell lines	• Major CYP450 forms present in human placenta are present and inducible in BeWo [93] and Jeg-3 [94]
• Permeability	• Maternal-fetal exchange of biological and non-biological substances • Barrier integrity	• TEER (>100 $\Omega\cdot cm^2$) • Diffusion studies: glucose (permeable), fluorescently conjugated heparin (nonpermeable) o Calculated Diffusion Coefficient and % Rate of Transfer	• Primary and choriocarcinoma cell lines • HUVECs and Microvascular ECs	• Comparative study of BeWo, JAR, Jeg-3, ACH\ \3P, and PHT functional parameters relevant to placental barrier model [52] • Diffusion coefficient calculations [13] • % rate of transfer calculations [16]
• Presence of Microvilli	• Barrier maturity & increased surface area for molecular transport	• Immunostaining: Ezrin • Scanning Electron Microscopy	• Primary and choriocarcinoma cell lines o Facilitated by fluid flow	• Fluid shear induced microvilli formation in BeWo cells [89]
Cell & Organ Function				
• Syncytialization	• Differentiation towards syncytiotrophoblast and presence of a true syncytium	• Immunostaining: Junction Proteins & Nuclei o Nuclear Aggregation o % of Multinucleated Cells o Cytoplasmic Fusion	• Primary and choriocarcinoma cell lines o Facilitated by Forskolin treatment and ECM proteins	• Forskolin treatment induced syncytialization BeWo b30 clone [34] • ECM regulates trophoblast organization, function, and expression profiles [83]
• Hormone production	• Endocrine function for pregnancy maintenance and fetal development	• ELISA: hCG, Progesterone • mRNA: hCG, placental lactogen	• Primary and choriocarcinoma cell lines o Amplified by Forskolin treatment	• Comparative study of BeWo, JAR, Jeg-3, ACH\ \3P, and PHT functional parameters relevant to placental barrier model [52]
Environmental Factors				
• Fluid flow	• Shear stress from blood flow	• Design feature: Perfusion/ syringe pump	• Perfused Placenta • Bioreactor • Placenta-on-a-chip	• Bioreactor [30] • Placenta-on-a-chip [16,32,89]
• Extracellular Matrix (ECM)	• 3D microenvironment provides biochemical and biomechanical cues that regulate cell behavior	• Design feature: Incorporation of ECM proteins in culture matrix (ex: collagen, laminin, fibronectin)	• Perfused Placenta • Hydrogel • Bioreactor • Bioprinted	• Use of ECM films to create a 3D-vascularized primary placental barrier model [15] • ECM regulates trophoblast

Biological Function	Biological Significance	Analytical Tests & Biomarkers	Relevant Models/Cell Types	References
				organization, function, and expression profiles [83,86]

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