Co-culture assay method design to model antibody transport across the placenta

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Technical Report

Co-culture assay method design to model antibody transport across the placenta

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Abstract

The CDC recommends pregnant women to receive the Tdap vaccine in the third trimester of pregnancy to protect the newborn from pertussis toxin (PT)[6]. The optimal timing can be further optimized such that maternal vaccine dynamics synergize with the kinetics of transplacental transfer. This timing optimization requires understanding of the underlying biological mechanisms of transfer. Additionally, this recommendation does not take premature birth into account. Accurate *in vitro* models of placental antibody transfer are needed to elucidate rules of transfer. The placenta has two major barriers to entry for antibodies: syncytiotrophoblasts (STB) and microvascular endothelial cells (mEC). In most *in vitro* models, these layers are characterized in separate cultures which fail to capture the interplay between these two cell types. Here, we attempt to create a co-culture model with cell lines HMEC-1 and BeWo b30 representing the mECs and the STBs, respectively. By using immunofluorescence (IF) staining we confirm the presence of the neonatal Fc receptor (FcRn) that is the key receptor involved in the transport of antibodies from mother to fetus. The permeability of these cellular layers to a small molecule, FITC-dextran, and trans-epithelial electrical resistance (TEER) tests were performed to optimize parameters in single-culture such as seeding densities and time to reach confluence, both of which are iterated through in the results of this paper. Despite intermediate successes, due to time constraints, the co-culture model were not fully achieved but protocols established and the lessons learned have paved the way for future success.

Keywords: Placenta, co-culture, IgG, pertussis, HMEC-1, BeWo b30

Introduction

The Center for Disease Control recommends pregnant women get the Tdap vaccine at some point between the 27th and the 36th week of each pregnancy to protect their newborns from whooping cough[6]. Unfortunately, this recommendation does not account for best practice in the context of premature birth, and the underlying mechanisms of antibody transport from mother to fetus remain unknown.

Various animal models of the placenta have been attempted in the past. The placenta, however, is a highly species-specific organ, and consequently there are substantial limitations in attempting to model the placenta in this way. These limitations have been well documented for many commonly used animal models such as mouse, guinea pig, rabbit, and sheep models[5,16,17,19]. As a result, *in vitro* methods for modeling the placenta are necessary.

Antibody transport across the placenta is regulated by two cell types. These are microvascular endothelial cells (mECs) and syncytiotrophoblasts (STBs). These two cell types both express the neonatal Fc receptor (FcRn), which transports the antibody Immunoglobulin-G (IgG). STB's role in maternal IgG transfer has been characterized in the past, but the role of mECs is largely unknown[8,17]. Models with different strains of the BeWo cell line have been used as a model for STBs, and IgG transcytosis characterized in those

conditions[8]. But the role of mECs in antibody transport remains largely unexplored. To better understand the role of mECs, their ability to transfer IgG antibody must be characterized. Furthermore, to understand the underlying mechanisms of IgG transfer as it normally exists within the placenta, it is necessary to observe both cell types together in the context of IgG antibody transfer[14,21]. This double-layered nature of the placenta is where the difficulty lies with *in vitro* modeling.

To address this difficulty, we work towards creating a co-culture model to represent the placenta. The intention of this co-culture is to see how antibody transfer differs when both cells are present versus when each cell type is alone. We use the cell line BeWo b30 to represent the syncytiotrophoblasts and the cell line HMEC-1 to represent the microvascular endothelial cells. BeWo b30 was chosen because of its ability to form a confluent monolayer when grown on membranes, and it has been used in previous research to model the placenta. It should be noted that this research has been in the context of the transport or diffusion of certain molecules across the placental barrier, not antibody transport[4,11,15,19]. The HMEC-1 cell line was also chosen because of its use in past placental research. As with the BeWo b30 cell line, this research was not aimed at understanding antibody transport[1,9,20]. To work towards achieving this co-culture we optimize the growth conditions of each cell type individually by changing seeding densities and culture time. Only at the conclusion of these tests can antibody transcytosis assays be run with confidence in the proper techniques.

Materials and Methods

Immunofluorescence Staining and Microscopy

To ensure presence of FcRn, immunofluorescence (IF) staining was performed on both BeWo b30 and HMEC-1. Cells were seeded on a microscope cover slip and allowed to adhere and grow for approximately two days. Experimental groups were first incubated with rabbit anti-FCGRT primary antibody then incubated with AF647 anti-rabbit secondary antibody. Experimental groups include dilutions of 1:100 and 1:200 for the primary antibody and 1:500 and 1:1000 for the secondary antibody. Samples were counter stained with 0.5 ug/mL DAPI in PBS. Control groups include unstained and no primary antibody samples.

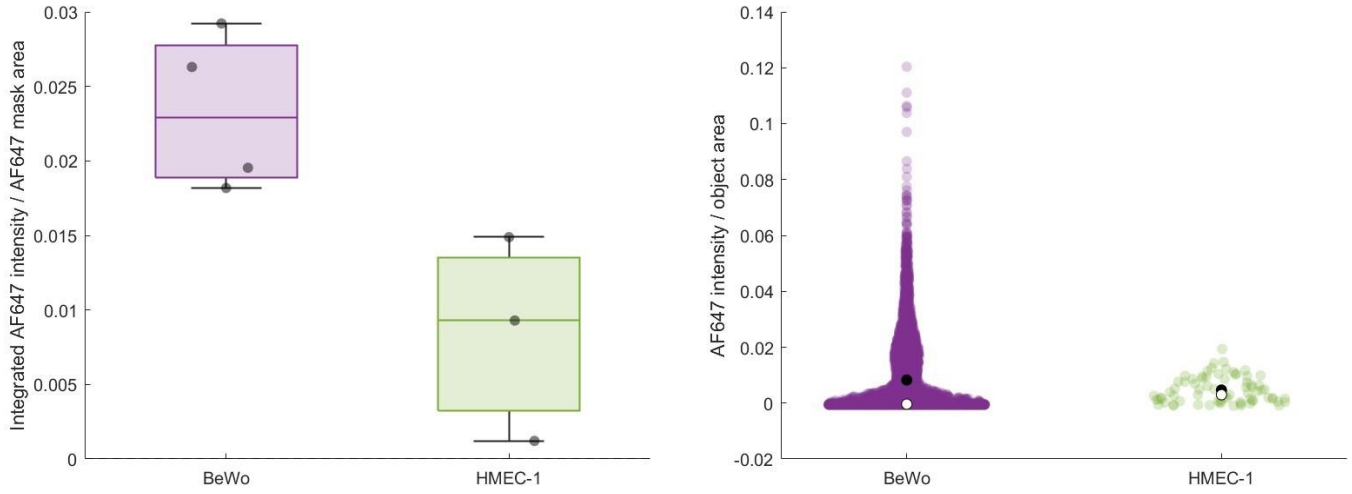
Images were taken on a Leica THUNDER microscope and run through a CellProfiler pipeline. The BeWo b30 cells were run through a pipeline to first identify nuclei from DAPI staining, then identify cells based on nucleus. For the HMEC-1 cells, there was an error in DAPI staining, so the same pipeline could not be used. Instead, cells were differentiated by space between them because they were adequately separated. After running images through CellProfiler, MATLAB was used to create Figures 1A and 1B. A two-sample t-test was used to test for statistical significance between BeWo b30 and HMEC-1 expression of FcRn.

BeWo b30 and HMEC-1 Cell Passaging

BeWo b30 cells were seeded at 5 x 10^5 cells/cm^2 and passaged every five days when the cells were approximately 80% confluent. Media changes were performed every other day with F12K media. HMEC-1 cells were seeded at 3 x 10^5 cells/cm^2 on a 0.12% gelatin coating and passaged every five days when the cells were approximately 80% confluent. MCDB131 media was used, and no media changes were performed between passages.

BeWo b30 FITC-dextran Confluence Tests

The first confluence test using FITC-dextran was only performed on BeWo b30 cells. 24 well, or 0.33 cm^2, Transwell inserts were used in all experiments. All Transwell inserts were first coated with 1 mg/mL collagen dissolved in PBS. After drying overnight, BeWo b30 cells were then seeded on the apical side of the Transwell inserts at three different seeding densities: 0.5 x 105 cells/cm^2, 1.0 x 105 cells/cm^2, and 1.5 x 105 cells/cm^2. There was also a control group with collagen coating on a Transwell insert but no cells. Measurements were taken on days four through seven post-seeding. Each experimental group had three biological replicates, and different wells were used for each day of data collection. To collect measurements the media was removed from the apical and basolateral sides of the membrane. PBS was



inserted into the basolateral side, and 0.5 mg/mL suspension of FITC-dextran was inserted into the apical side of the membrane. The cells were then incubated with the FITC-dextran. After one hour, samples were taken from the basolateral side of each Transwell insert and put into a 96 well plate. The fluorescence was then measured with a fluorescence plate reader. All readings were corrected by a blank measurement. Data normalization and visualization was performed in MATLAB. The absolute value of the experimental fluorescence minus the control fluorescence was divided by the control fluorescence to get the percent of the negative control.

BeWo b30 and HMEC-1 FITC-dextran Confluence Tests

The second confluence tests using FITC-dextran were performed on both BeWo b30 and HMEC-1 cells. The same collagen coating was used for the BeWo b30 cells as was used for the previous FITC-dextran experiment, and 0.12% gelatin was used as a coating for the HMEC-1 experimental groups' membranes. Both cell types had three seeding densities. For BeWo b30 cells the seeding densities were 0.1 x 10^5 cells/cm^2, 1.0 x 10^5 cells/cm^2, and 2.4 x 10^5 cells/cm^2. For the HMEC-1 cells the seeding densities were 0.5 x 10^5 cells/cm^2, 2.5 x 10^5 cells/cm^2, 7.5 x 10^5 cells/cm^2. The same procedure was followed to collect measurements as in the previous FITC-dextran experiment except that the cells were only incubated for 30 minutes with the FITC-dextran. Three biological replicates were used for each seeding densities, but the same cells were reused for each day's FITC-dextran measurement. Equation 1 was used to calculate the percent of the negative control. Data from the FITC-dextran component of this experiment is not presented in this paper because it is better visualized with the trans-epithelial electrical resistance (TEER) measurements taken in parallel.

TEER Confluence Experiments

Trans-epithelial electrical resistance (TEER) measurements were taken on both HMEC-1 and BeWo b30 cells. All BeWo b30 experimental groups had a 1 mg/mL collagen coating on the membranes of the Transwell inserts, and all HMEC-1 experimental groups had a 0.12% gelatin coating on the membrane of the Transwell inserts. An EVOM2 device was used to collect resistance measurements. To take a measurement, the media was removed from the apical and basolateral compartments of the Transwell insert and replaced with PBS. The prongs were inserted so that the longer prong was in the basolateral compartment and the shorter prong was in the apical compartment. The prongs were held at a 90-degree angle until the reading leveled out on the EVOM2. The control (Transwell insert with the specified coating for each cell type but no cells) value was subtracted from the experimental values. All biological replicate

Figure 1: Integrated intensity of fluorescence over area. (Left) Intensity per image divided by the area over which fluorescence is present in an image (the mask). (Right) Intensity per cell divided by the cell's area (object area).

readings were averaged and multiplied by 0.33 to convert the data to Ω*cm^2. One-way ANOVAs were used to test statistical significance between seeding densities. Visualizations and statistical tests were performed in MATLAB.

Results

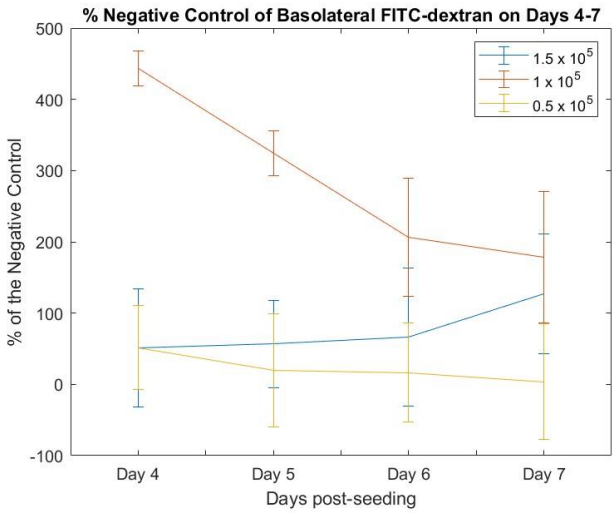
Immunofluorescence Staining to Verify Presence of FcRn

To ensure the cell lines we had chosen expressed the neonatal Fc receptor (FcRn), we performed immunofluorescence (IF) staining. We found that both BeWo b30 and HMEC-1 cells expressed FcRn. Interestingly, we found that BeWo b30 cells express a significantly higher amount of FcRn than HMEC-1. The p-value or FcRn analyzed over entire images was 0.0227 (Figure 1 Left), and the p-value when looking at the FcRn in each individual cell was 0.0291 (Figure 1 Right). This is as we expected based on the previous belief that endothelial cells did not express FcRn at all, and the research later released implicating endothelial cells in antibody transport[2,17].

FITC-dextran Confluence Test with BeWo b30 Cells

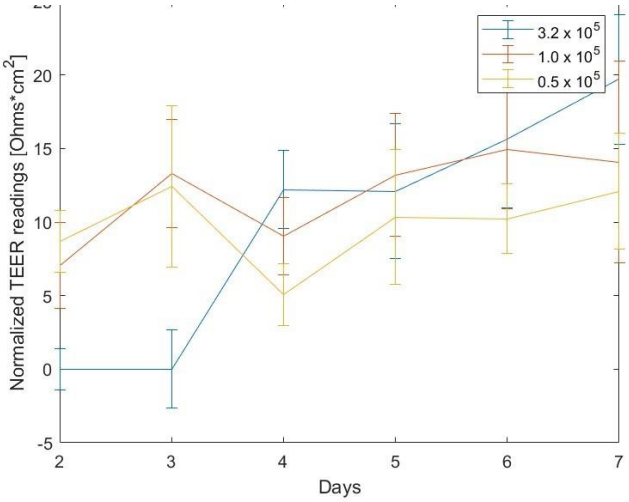
A small, fluorescent molecule, FITC-dextran, was used to detect the confluence of cells. FITC-dextran was small enough to diffuse across the semipermeable Transwell membrane and was assumed to be impermeable in the presence of a confluent layer of BeWo b30 cells. As a result, basolateral FITC-dextran concentration was expected to be lower for the BeWo b30 experimental groups than in the control sample, with the lowest values occurring when cells were fully confluent. The results, however, did not support that hypothesis.

It was found that, on all days post seeding, the seeding density of 1.0 x 10^5 cells/cm^2 had a higher concentration of FITC-dextran in the basolateral PBS than in the basolateral compartment of the control sample. The seeding density of 1.5 x 10^5 cells/cm^2 seemed to block the movement of FITC-dextran across the membrane for days four through six post seeding, there was a higher concentration than the control on day seven post seeding (Figure 2). The lowest seeding density, 0.5 x 10^5 cells/cm^2, had similar basolateral FITC-dextran concentrations to the control.



Due to this unexpected finding, we developed a new hypothesis relating to the movement of FITC-dextran across the membrane in the presence of BeWo b30 cells. We believe that when BeWo b30 cells are growing well they transport FITC-dextran across the membrane as they did for the 1.0 x 10^5 cells/cm^2 seeding density. When there is an excess number of cells, the BeWo b30 cells cannot transport FITC-dextran which explains why the higher seeding density of 1.5 x 10^5 cells/cm^2 did not initially transport it. Once cell growth had stabilized in this seeding density (after excess cells died off and were removed in the media changes) the cells began transporting FITC-dextran. Finally, it seems that the seeding density of 0.5 x 10^5 cells/cm^2 is too low for proper growth of BeWo b30 cells based on that experimental group's similarity to the control sample. Further experimentation needs to be performed to confirm that these results are accurate, and not the result of possible human error or damaged membranes.

Figure 2: Percent of the negative control for FITC-dextran concentrations. FITC-dextran was inserted into the apical compartment of the Transwell insert and incubated for an hour. Samples were then taken from the basolateral compartment.

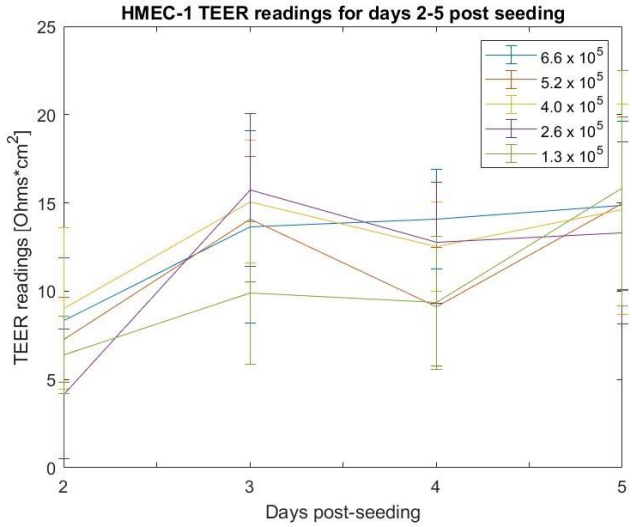


Source	Seeding Density (cells/cm^2)	Time to Confluence (hrs)
[3]	4.5 x 10^5	48
[7]	6.1 x 10^5	48 to 96
[13]	3.0 x 10^5	Not Stated
[18]	1.5 x 10^5	48
[22]	9.1 x 10^5	48

TEER Confluence Tests with BeWo b30 and HMEC-1 Cells

To replace FITC-dextran as the method for optimizing seeding densities and time to confluence, trans-epithelial electrical resistance (TEER) measurements were performed on both BeWo b30 and HMEC-1 cells.

Figure 3: HMEC-1 and BeWo b30 resistance measurements. (Left) BeWo b30 resistance values on days 2-7 post seeding. (Right) HMEC-1 resistance tracted from the resistance values, making the control reference always equal



<u>BeWo b30 results</u>

A general upward trend can be observed over time as was expected, however, expected resistance values – between 30 and 60 **Ω***cm^2 – for confidence in a confluent layer were never reached[19]. Additionally, standard deviation values were very high, reducing confidence in overall results (Supplemental Figure 2). It can be noted, though, that values were continuing to rise by day seven in seeding densities 3.2 x 10^5 cells/cm^2 and 0.5 x 10^5 cells/cm^2 but had plateaued in the 1.0 x 10^5 cells/cm^2 seeding density. This implies that the middle seeding density might be the preferable density for reaching confluence within our timeframe which is five to seven days (Figure 3).

Table 1: HMEC-1 literature review. Seeding densities and time to confluence from each paper is listed with the source reference in the left-most column.

HMEC-1 results

An upward trend can again be observed in HMEC-1 and expected confluence values – between 15-30 Ω*cm^2 – were achieved in approximately three days for two of the seeding densities, 2.6 x 10^5 cells/cm^2 and 4.0 x 10^5 cells/cm^2, and five days for the lowest seeding density, 1.3 x 10^5 cells/cm^2 (Figure 3)[12]. The difference between seeding densities was only statistically significant on day three post seeding (Supplemental Figure 1). To understand this, a literature review was performed on normal seeding densities and time to confluence for HMEC-1 cells (Table 1). A wide range of seeding densities were seen in the literature review, with the cells in most papers reaching confluence within two days of seeding. Therefore, we hypothesize that HMEC-1 cell seeding density is flexible due to their fast growth rate.

Drawback of Experiment

Partway through the experiment the resistance probe was discovered to be broken (Supplemental Figure 3). This could be why the standard deviations were so high. There is also a chance it threw off the measurements overall, so further confluence tests were performed after replacing the probe.

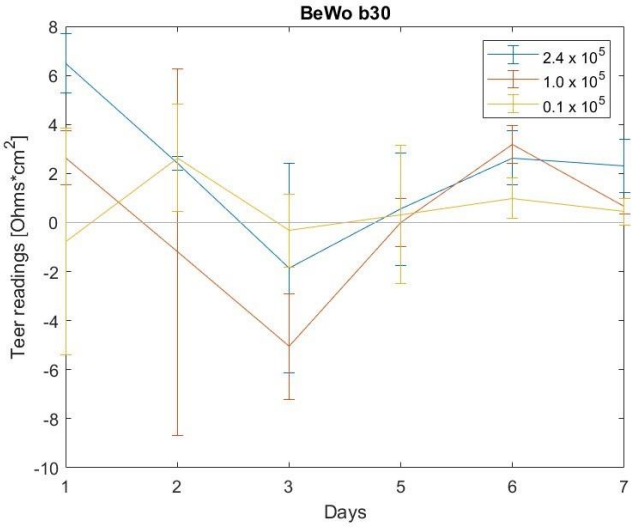
TEER and FITC-dextran Confluence Tests with Both Cell Lines

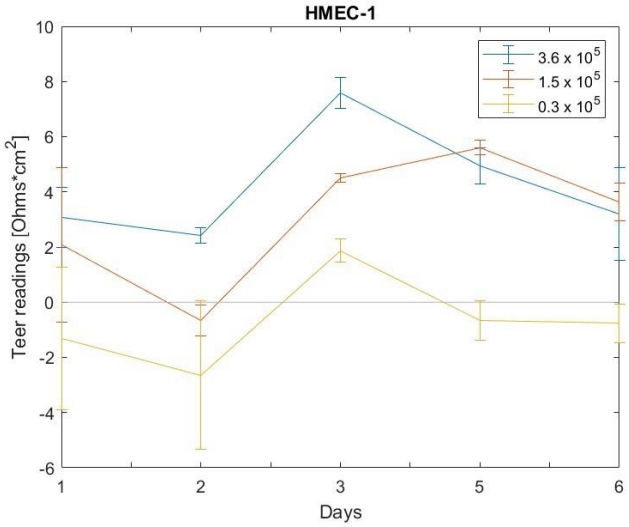
Due to the faulty probe, experimentation on the optimization of seeding density and time to confluence was repeated. In this experiment both TEER and FITC-dextran methods were used for each day that data was collected. The decision to attempt both measurement techniques at once was made with the hopes that the data from the first experiment with FITC-dextran could be corroborated while optimization was also completed.

BeWo b30 results

The BeWo b30 cells seemed to react poorly to the repeated exposure to FITC-dextran. On day one post seeding (before the cells were exposed to FITC-dextran) seeding densities 2.4 x 10^5 cells/cm^2 and 1.0 x 10^5 cells/cm^2 had resistance values greater than the control, indicating that cells were present and beginning to grow. By day three, the resistance values for all seeding densities seem to indicate cell death. On days four through seven resistance measurements stay either below zero or close to zero indicating that the cells did not recover from exposure to FITC-dextran (Figure 4). This result, when considered with the first FITC-dextran experiment on the BeWo b30 cells seemed to indicate that the BeWo b30 cells respond, as

Figure 4: Resistance measurements from FITC-dextran and TEER experiment. (Left) BeWo b30 resistance measurements over seven days after seeding. (Right) HMEC-1 resistance measurements over 6 days after seeding. Control values were subtracted from resistance measurements making the control zero for all days.





opposed to passively block, FITC-dextran (Figure 2). Results needed to move forward from optimization were again not achieved.

HMEC-1 results

The HMEC-1 cells were not as severely affected by the repeated exposure to FITC-dextran. The resistance measurements increased up to the third day, however they were lower than expected for confluent values and resistance started to decrease on the fourth- and fifth-days post seeding (Figure 4). It is possible that residual FITC-dextran remained in the wells even after removing the FITC-dextran solution and replacing it with media after incubation. This exposure could have limited the growth of the HMEC-1 cells, preventing them from reaching full confluence.

Discussion

Through this research we have developed a scaffold for creating a co-culture model and instituted the first steps of its creation. We have confirmed and quantified FcRn expression in BeWo b30 cells and HMEC-1 cells. We have also begun the optimization process for both cell types individually and narrowed down potential metrics.

Future Directions

In continuing towards the creation of a co-culture model, proper seeding densities and time to confluence for both BeWo b30 and HMEC-1 cells must be decided on. By using the results of this paper, potential options may be narrowed down. This decision may then be made by further literature review, which helped us decide our initial metrics, or further experimentation. Once these optimal conditions are defined, antibody transport assays may be carried out with those metrics. After optimization and IgG transcytosis characterization is complete for both cell types individually, optimization of the cells grown in co-culture may occur, followed by antibody transport assays in the co-culture as well.

Additionally, HMEC-1 cells are currently being genetically engineered to express another receptor found in the mECs that potentially contributes to placental IgG transfer, FcγRIIb. Antibody transport assays could be run on these cells individually, then the HMEC-1 FcγRIIb cells could replace the original, or wild type (WT), HMEC-1 cells in the co-culture model. The data collected in these experiments could be compared to HMEC-1 WT cells in both the single-culture and co-culture models. These experiments would offer insights into the specific role of FcγRIIb within the placenta as it relates to IgG transport.

Limitations of Co-Culture Model

Because the placental composition develops over pregnancy, different amounts of antibody transfer occur during each trimester. The co-culture model is unable to account for these changes and, therefore, cannot give insights into the difference in IgG transfer between trimesters. The amount of STBs and mECs present in the placenta varies over the different trimesters as well which cannot be modeled with the co-culture method because of the necessity for a single confluent layer on the membrane[10]. To address this problem, the same lab is working on a computational model that will be representative of the placenta as it changes over time. Additionally, parts of this model could be validated with the co-culture models mentioned above.

End Matter

Author Contributions and Notes

R.E.W carried out immunofluorescence staining and imaging and provided code for immunofluorescence analysis. M.E.T. aided in acquiring immunofluorescence images and carrying out immunofluorescence analysis.
M.E.T. carried out all other experimentation and data analyses, while consulting and being aided by R.E.W.

The authors declare no conflict of interest.

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Supplemental Figure 1: P-values for each day of BeWo b30 and HMEC-1 TEER values.

	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
BeWo b30	0.001	0.002	0.004	0.331	0.107	0.160
HMEC-1	0.375	0.038	0.127	0.708		

Supplemental Figure 2: BeWo b30 and HMEC-1 TEER Standard deviations for each seeding for each day of measurements.

	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
SD: 3.2	1.40	2.67	2.63	4.59	4.67	4.39
SD: 1.0	2.93	3.68	2.62	4.18	4.07	6.84
SD: 0.5	2.08	5.47	2.10	4.59	2.35	3.93

Supplemental Figure 3: An image of the broken connector piece of the resistance probe for the EVOM2.