EXAMINING THE RELATIONSHIP BETWEEN PLACENTAL EXTRACELLULAR MATRIX AND TROPHOBLAST DIFFERENTIATION

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by

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Examining the Relationship Between Placental Extracellular Matrix and Trophoblast Differentiation

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DEDICATION

I whole-heartedly dedicate this to my daughter Penelope Jean. You were named after the woman who taught me to be strong, and I hope to one day do the same for you. You are my reason.

Besides, they'll see how beautiful I am and be ashamed

--Langston Hughes

ABSTRACT OF THE THESIS

Examining the Relationship Between Placental Extracellular Matrix and Trophoblast Differentiation
by
Trishana M. Norquist
Master of Science in Biology with a Concentration in Molecular Biology
San Diego State University, 2015

Extracellular matrix is the biological scaffold which provides both structural support as well as factors which support cellular differentiation and tissue/organ function. Cytotrophoblasts (CTB) are the epithelial stem cells in the placenta which can give rise to both villous syncytiotrophoblast (STB) and invasive extravillous trophoblast (EVT). Modified differentiation of CTBs resulting in too few EVTs underlies the abnormalities and symptoms observed in preeclamptic placentas. Studies investigating the plating of progenitor cells on their tissue-specific ECM demonstrated the effect of ECM on cellular processes such as differentiation, proliferation, survival, and adhesion. The results of the study presented here reveal for the first time that primary CTBs can be maintained in an undifferentiated state when cultured on human placental ECM. ECM was prepared from minced third trimester placental tissues following decellularization, detergent washes and nucleic acid treatment. The resulting matrix was dehydrated by a lyophilizer and reconstituted in 0.1M acetic acid before plating. Through the novel approach of decellularizing a human placenta, it was found that clinically normal placentas had 3.64 times higher ECM yield than preeclamptic placentas. Primary CTBs were isolated from term placental tissues per published protocols and cultured either on tissue culture plastic as a control, or 1 mg/mL placental ECM. Differentiation was monitored based on morphology, hCG production, and qPCR for lineage-specific markers. Primary CTBs were also monitored for ECM effects on cell proliferation, apoptosis, and adhesion. With isolated term CTBs, cells plated on placental ECM, both normal (nlECM) and preeclamptic (peECM) mostly remained mononuclear, produced significantly less hCG, and showed significantly lower expression of markers of STB differentiation, including hCGα, hCGβ, Syncytin, and CSH-1 by qPCR. This study demonstrates that placental extracellular matrix can maintain the undifferentiated state of primary CTBs, while having little to no effect on proliferation, survival, and adhesion. This study also suggests that composition of the extracellular matrix does not play a substantial role in driving modified trophoblast differentiation observed in preeclamptic placenta.

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LIST OF ABBREVIATIONS

CSH-1	Chorionic somatomammotropin hormone 1 (placental lactogen)
CTB	Cytotrophoblast
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EVT	Extravillous trophoblast
hCGα	Human chorionic gonadotropin alpha
hCGβ	Human chorionic gonadadotropin beta
hESC	Human embryonic stem cell
iPSC	Induced pluripotent stem cell
nlECM	Normal placental extracellular matrix
p63	Tumor protein p63
peECM	Preeclamptic placental extracellular matrix
STB	Syncytiotrophoblast

INTRODUCTION

Preeclampsia, the most common form of hypertensive disease of pregnancy (HDP), occurs in 3-5% of all pregnancies and is the cause of 15-25% maternal mortality (Burleigh et al., 2007; Fritz, Jain, & Armant, 2014). The fetal syndrome of preeclampsia includes perinatal death, birth before 34 weeks gestation, and intrauterine growth restriction (Burleigh et al., 2007). Both maternal and fetal cardiovascular risks are increased by HDP, leading to post-labor cardiovascular diseases (Fritz, jain, & Armant, 2014). Currently, preeclampsia remains a disorder of unknown cause (Burleigh, 2007). What is known of preeclamptic CTBs is that an overproduction of STBs leads to a depletion in the amount of CTBs able to differentiate into EVTs, thus inhibiting the amount of oxygen able to reach the fetus and increasing the amount of hCG released into the maternal bloodstream.

INTRICACIES OF THE PLACENTA

The placenta is a tumor-like, transient organ vital to fetal life and development. Its unique branching structure allows the maternal body to provide all of the oxygen and nutrients the fetus needs for a successful gestation in utero (Pavlov et al., 2014; Sanchis, Cristofolini, Taglialegna, & Merkis, 2011). There are different types of trophoblast epithelial cells in the placenta, each of which has a specific role to play during placental growth. CTBs are known as the placental “stem cells” because they can differentiate into both trophoblast lineage subtypes: EVTs and STBs. EVTs are invasive trophoblasts that play a role in maternal spiral arteries remodeling into specialized uteroplacental arteries (Flynn, Semple, & Woodhouse, 2006). Due to this role, the presence of EVTs ensures optimal exchanges between the maternal and fetal circulation (Flynn, Prestwich, Semple, & Woodhouse, 2008). STBs arise from CTB fusion forming a multinucleated cell layer, and provide an interface for gas and nutrient exchange. They are also the primary cell

type responsible for secretion of the pregnancy hormone, human chorionic gonadotropin (hCGβ).

The yield of CTBs from a third trimester human placenta averages 80 million cells, and lose proliferation within 7 days. JEG3s, a human choriocarcinoma cell line, can be treated as CTBs that will differentiate into the STB lineage (Singhai & Martin, 2001). The ability to proliferate for weeks and the secretion of hCGβ by JEG3s make them ideal candidates for large scale CTB-based experiments.

PLACENTAL ECM

Placental ECM has only been studied as a possible vascular scaffold for plastic surgeons to perform reconstructive, corrective, and cosmetic procedures in combination with adipose tissue (Abousleiman, Reyes, McFetridge, & Sikavitsas, 2008; DeQuanch, Yuan, Goldstein, & Christman, 2010). Like placental ECM, umbilical veins have been decellularized and repopulated with vocal fold primary fibroblasts or used as a musculoskeletal scaffold, for restoration purposes (Chaiworapongsa, Chaemsalthong, Yeo, & Romero, 2014; Hopper, Woodhouse, & Semple, 2003). The extracellular microenvironment provides molecular factors that stimulate cell behavior, such as differentiation (Chan, Rodriguez, & McFetridge, 2009; Sibai, Dekker, & Kupferminc, 2005).

ECM AND THE STEM FIELD

The extracellular microenvironment provides molecular factors that stimulate cell behavior, such as differentiation (Chan et al., 2009; Sibai et al., 2005). Tissue-specific ECM as a coating for cell culture is a novel approach for inducing tissue-specific effects on stem cell behavior (French et al., 2012; DeQuanch, Mezzano, et al, 2010). ECMs that have been tested as a coating for cell cultures include those derived from skin, fat, heart, skeletal muscle, brain, pericardium, and liver (Sibai et al., 2005).

It was originally hypothesized that nlECM would contain enough environmental factors to direct undifferentiated hESCs into the trophoblast lineage, without any additional factors. Unfortunately, as shown in Figure 2B-D, undifferentiated hESCs did not adhere to nlECM. More recently, it has been shown that adherence to tissue-specific

ECM is improved when human pluripotent stem cells (hPSCs), including hESCs and induced pluripotent stem cells (iPSCs), have been differentiated into the progenitor cell type specific to that tissue (Li et al., 2013; Sibai et al., 2005). The use of various tissue-specific ECMs mimic the native microenvironment of those progenitor cells more closely than individual purified proteins and commercially available mixed ECM, such as Matrigel and Geltrex (Li et al., 2013). Tissue-specific ECMs have also proven to be more physiologically relevant, for subtle differences in the composition of the ECM play a large role on cell phenotype.

RATIONALE

Normal human primary cytotrophoblasts provide the best cellular model of the trophoblast prior to disease. For this reason, these cells have been chosen for this study. Various studies have shown that progenitor cells can be maintained longer, and differentiate better, when cultured on their tissue-specific ECM (Sibai et al., 2005). However, to date, there have not been any studies that have utilized placental ECM as a potential coating for culturing trophoblasts *in vitro*. This study strives to understand the role that purified placental extracellular matrix plays in trophoblast differentiation, comparing the effects of culturing trophoblasts on ECM derived from normal and diseased placentas. The **hypothesis** of this thesis is that **compared to Geltrex, Tissue-Culture Plastic, and other commercially designed cell culture coatings, placental ECM will provide a more suitable cell culturing environment to maintain CTBs *in vitro*.** The goal of this study is to determine if normal placental ECM can serve as a culturing method to study cytotrophoblasts without influencing proliferation, adhesion, and cell death. Additionally, this study tests the hypothesis that preeclamptic placental ECM enhances trophoblast differentiation *in vitro,* possibly contributing to the depletion of these cells observed in preeclamptic placenta *in vivo.*

MATERIALS AND METHODS

DONATION OF HUMAN PLACENTA

All placental tissue collections were done under protocols approved by the Human Research Protections Program Committee of the University of California, San Diego Institutional Review Board, and following patients' consent. Clinically-diagnosed normal placentas came from non-high risk patients. Clinically-diagnosed preeclamptic placentas were collected under the parameters that patients had hypertension (mild: 140/90, severe: 160/110) while pregnant, chronic proteinuria (without diagnosed urinary tract infection), as well as signs and symptoms of preeclampsia (headaches, epigastric pain, intrauterine growth restriction, etc.).

ISOLATION OF HUMAN PRIMARY CTBS

Third trimester placentas were processed within an hour after cesarean section delivery. After being washed with 1x PBS and antibiotics, villous placental tissue was minced, transferred to a cell dissociation sieve, and once again rinsed with 1x PBS. Three enzyme digestion steps followed using 0.25% trypsin and 300 U/mL DNase I each step, until CTBs were separated on a Percoll gradient. Third trimester primary CTBs were then resuspended and plated in Iscove's Dulbecco's modified Eagle medium with 10% fetal bovine serum and antibiotics.

DECELLULARIZING HUMAN PLACENTAS

Placental extracellular matrix was prepared from minced term placental tissues separated into maternal and fetal sides (see Figure 1A). For the purpose of this study, only the maternal side of the placenta was used after decellularization, in experiments. Whole human placentas were decellularized by stirring in 800mL of 0.1% wt/vol of sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) with 1% penicillin/streptomycin, with cellular remnants decanted every 24 hours and refilled to

800mL for 6-7 days, until tissue was decellularized (Herrera-Garcia & Contag, 2014) (Figure 1B-F). A DNase/RNase step was done to ensure nucleic digestion, prior to final 24 hours detergent rinse. A sample of decellularized placental ECM was frozen in Tissue Tek O.C.T. for future histological analysis. Resulting matrix was dehydrated, lyophilized, and milled. Prior to cell culture, placental matrix was solubilized by enzymatic digestion with porcine pepsin (sigma) and pH balanced with NaOH and 10x PBS, using previously modified protocols; then lyophilized once more and stored at -80 °C (Sibai et al., 2005).

TROPHOBLAST CELL-CULTURE TECHNIQUES

Frozen placental extracellular matrix was brought to room temperature before being reconstituted in sterilized, deionized water to bring the ECM to 8.18mg/mL, its determined physiological concentration calculated through its dry weight and amount of added solvents. The liquid ECM is then resuspended with enough 0.1M acetic acid to bring the placental ECM to the desired concentration. The liquid ECM was then used to completely cover the tissue culture well, and incubated at 37 °C for at least 1.5 hours. Following two washes with sterile 1x PBS, cells were plated. Cells are kept in normoxia overnight, and then immediately placed in hypoxic conditions for two days (5% in XVIVO System) to allow proper differentiation (Flynn, Prestwich, Semple, & Woodhouse, 2007). This is then followed with four days back in normoxia, with cell and media collection on Day 3 and Day 7.

H9-DERIVED CTBS

H9 human Embryonic Stem Cells grown Geltrex + Stempro are cultured for two days in EMIM, a minimal media (KO-DMEM/F12 [89%], NEAA [0.01%], BSA [10%], Heparin [1%]), followed by four days of EMIM + BMP4. After six day protocol, cells exhibit first trimester CTB characteristics and can be used as such for one re-plating.

HCG-BETA ELISA ASSAY

Supernatants from the cell culture were collected on Day 3 and Day 7, allowing the total level of hCGβ secreted to be quantified with the use of an hCG ELISA Kit (Calbiotech). Steps were followed according to the manufacturer's protocol. Results were

then normalized to the cellular DNA content measured by DNeasy Kit (Qiagen) and collected the same day as the supernatant.

REAL TIME RT-PCR

mirVana RNA Isolation Kit (Ambion) was prepared from cultured cells according to the manufacturer's protocol to isolate total RNA. Nanodrop ND-1000 Spectrphotometer (Thermo Science) was then used to determine the purity and concentration of the RNA sample isolated. mRNA was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad) following manufacturer's protocol. Real-Time PCR was done in triplicate using iQ SYBER Green (Bio-Rad). Relative mRNA expression levels were normalized to 18S rRNA and determined using the ΔΔCt method. Human primer sequences used are as follows 5'—3':

- CSH-1 (Fwd): ACAGAAACAGGTGGGGTCAAG
- CSH-1 (Rev): TTATTAGGACAAGGCTGGTGGG
- p63 (Fwd): CTGGAAAACAATGCCCAGA
- p63 (Rev): AGAGAGCATCGAAGGTGGAG
- Syncytin (Fwd): TACTCCTGCCCGATAGATGA
- Syncytin (Rev): CCGTTTTTCTTAACAGTGGGT
- CGA (Fwd): CAACCGCCCTGAACACATCC
- CGA (Rev): CAGCAAGTGGACTCTGAGGTG
- CGB (Fwd): TGAGATCACTTCACCGTGGTCTCC
- CGB (Rev): TTTATACCTCGGGGTTGTGGGG

IMMUNOFLUORESCENCE DOUBLE STAINING

Third trimester primary CTB cells were plated on glass coverslips to analyze cell morphology during trophoblast differentiation. CTBs were fixed for 15 minutes in 2% paraformaldehyde and then permeabilized in 0.2% Triton X-100 in PBS for 5 minutes. Rabbit EGFR-α antibody (Abcam) and Alexa dye-conjugated phalloidin and secondary antibodies (Molecular Probes) were used per manufacturer's protocol. Vectashield mounting media containing DAPI was used to mount coverslips. STBs were identified as cells having 3 or more nuclei and little to no expression of EGFR-α.

Cell Death and Proliferation Assays

Cell survival was assessed through Annexin-7AAD flow cytometry. JEG3s were plated in 10cm culture dishes at 50% confluence in DMEM 1x + 2% Fetal Bovine Serum + penicillin/streptomycin. After one day, JEG3s were subjected to 2ng/mL staurosporine (STS), a stress stimulus, in serum-free DMEM 1x+p/s overnight. All cells, along with those that no longer adhered to the dish, were collected, centrifuged and re-suspended in the binding buffer. JEG3s were then stained with Annexin and 7AAD and analyzed by flow cytometry to allow quantification of live, necrotic, early and late stage apoptosis. Cell proliferation was assessed through Brdu-7AAD flow cytometry. JEG3s were plated in 10cm culture dishes at 100% confluence in culture media. After one day, JEG3s were subjected to Brdu treatment for 45 minutes. Only the cells that adhered to the dish were collected, centrifuged and re-suspended in the binding buffer. JEG3s were then stained with Brdu and 7AAD and analyzed by flow cytometry to analyze the effects of placental ECM on the JEG3s' cell cycle. Both nlECM and peECM results were compared to tissue culture plastic that had not been coated.

CyQuant Direct Assay

JEG3s were plated in triplicate at 10,000 cells per well in a 96-well black flat bottom plate with a total volume of 100µL/well. Media used was DMEM 1x + 2% Fetal Bovine Serum + penicillin/streptomycin. The cellular DNA content was measured by the binding of a fluorescent dye to indirectly determine cell number using a CyQUANT Direct Cell Proliferation Assay (Invitrogen) and fluorescence intensity was measured on a Tecan SpectraFluor plate reader at excitation and emission wavelengths of 485nm. Cells were cultured on nlECM, peECM, or control in normoxia.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5 software. One-way ANOVA with Tukey's post-tests were used to compare multiple groups. P-values less than 0.05 were considered statistically significant, and error bars represent standard error of the mean (SEM).

RESULTS

NORMALIZE PLACENTAL ECM COATING METHODS

Following decellularization protocol (Figure 1), brief histological views using hematoxylin and eosin (H&E) confirmed the absence of cells. Placental ECM had a physiological concentration of 8.18mg/mL and was able to be reconstituted at chosen concentrations through the use of deionized water bringing matrix to starting concentration, and 0.1M acetic acid bringing matrix to desired concentration. Adherence of undifferentiated hESC onto nlECM was done with 6-well plate, coated 4-wells with nlECM reconstituted with 0.1M acetic acid and deionized water, at concentrations of 4mg/mL, 2mg/mL, 1mg/mL, and 0.5mg/mL, followed by two hours in incubator and two PBS rinses to determine most conducive ECM environment. Fifth well was coated using Geltrex as an adherence and morphology control. H9 hESCs were plated at 100,000 cells/well in DMEM/F12+L+Glut+HEPES (80%), Knockout Serum (20%), Glutamax (0.01%), MEM Non-essential amino acids (0.01%), and β-ME (0.001%). When adherence did not occur with the placental ECM (see Figure 2), H9 derived CTBs were used to determine parameters of ECM concentration for future experiments. With 12-well plate, 8-wells were coated with nlECM reconstituted with 0.1M acetic acid and deionized water, at the same duplicate concentrations. Ninth and tenth wells were coated using Geltrex as an adherence and morphology control; eleventh and twelfth wells were coated with 0.1M acetic acid as a carrier control. H9-derived CTBs were plated at 200,000 cells/well in Feeder Conditioned Media +BMP4. Supernatants from cell culture Day 3 were collected, allowing level of total hCGβ secreted to be quantified. It was shown (see Figure 3D) that CTBs had normalized hCG levels most close to the control when plated on 1mg/mL nlECM. CTB morphology was also most similar to control's (see Figure 3A-C).

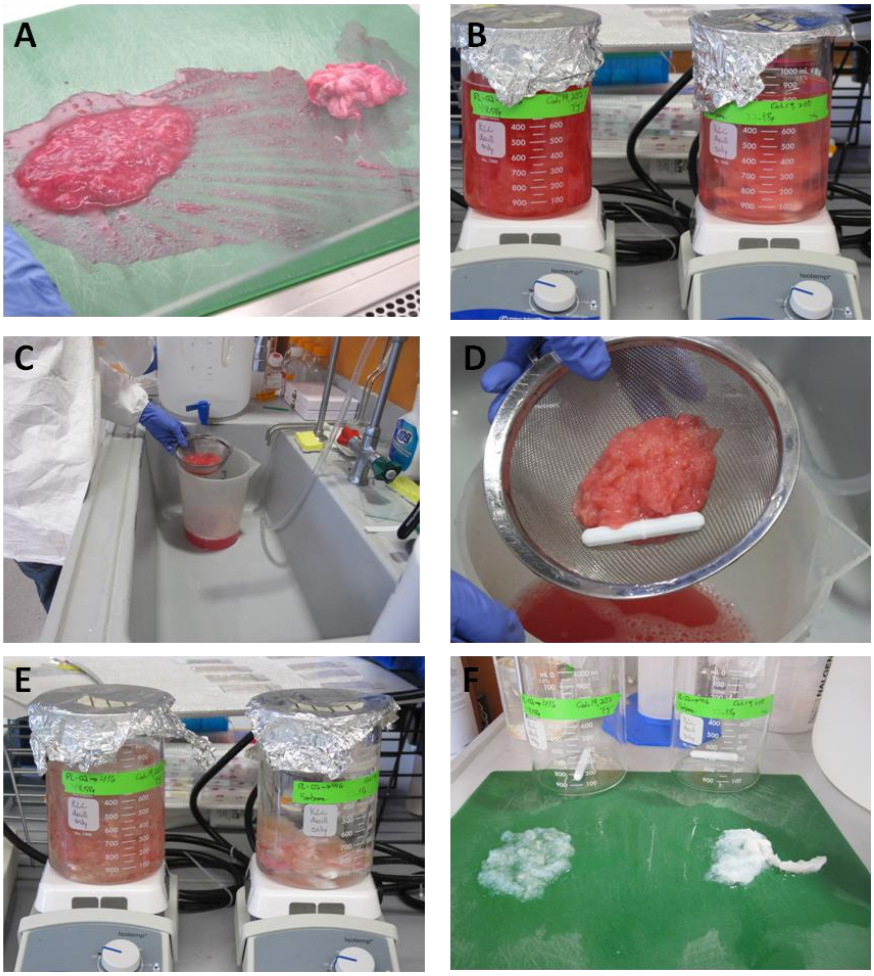

Figure 1. Decellularization of third trimester normal human placenta. (A) Separation of maternal and fetal sides followed by (B-E) multiple SDS washes and rinses. (F) Final result after 7 days of washes.

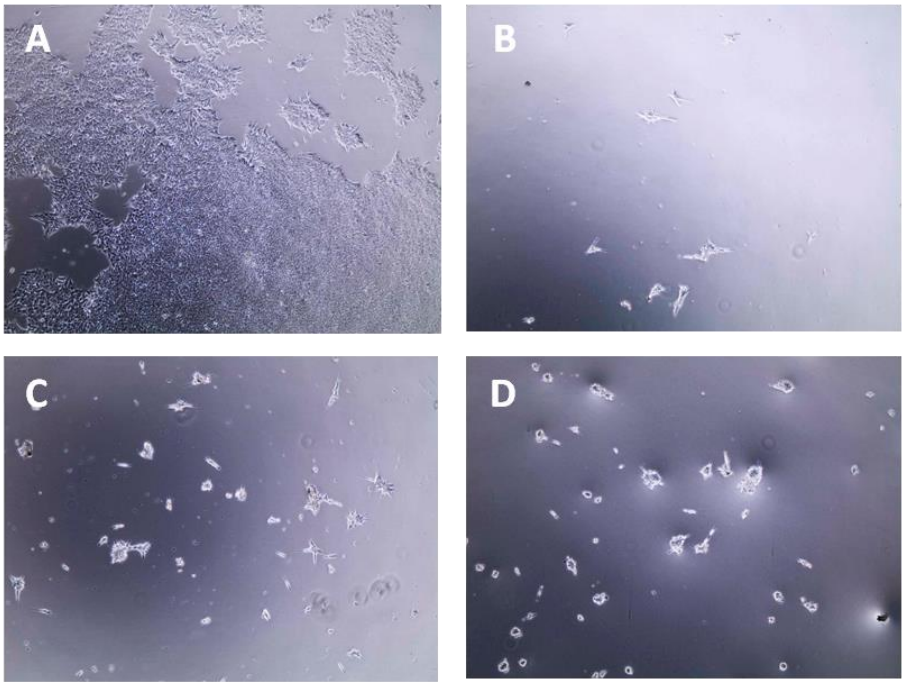

Figure 2. Testing undifferentiated hESC on placental ECM. (A) Geltrex (B) 4mg/mL nlECM (C) 2mg/mL nlECM (D) 1mg/mL nlECM.

nlECM Maintains Primary CTBs in Undiferentiated State

Cell morphology during differentiation was observed in third trimester primary CTBs using immunofluorescence EGFR-α and Phalloidin double staining. Primary CTBs were plated in two 6-well plates at 3 million cells per well in Third Trimester Media (Iscoves DMEM (90%) +FBS (10%) + penicillin/streptomycin (0.001%)), with collection times Day 1, 3, and 6. One plate was pre-coated with nlECM at 1mg/mL. The control plate remained as tissue-culture plastic. Using EGFR as a CTB marker, primary CTBs plated on nlECM displayed less EGFR expression, indicated with block arrows, than those plated on tissue-culture plastic by Day 6 (see Figure 4). A new group of third trimester primary CTBs were plated at 3 million cells per well using serum-free Third Trimester Media on three wells coated with nlECM, with one well as tissue-culture plastic for control. Supernatants and cells were collected on Day 3 and Day 7. hCGβ secretion was inhibited in CTBs plated on nlECM when compared to control (see Figure 5). When compared to the control, nlECM also showed little change in p63, a marker for undifferentiated CTBs (see Figure 5).

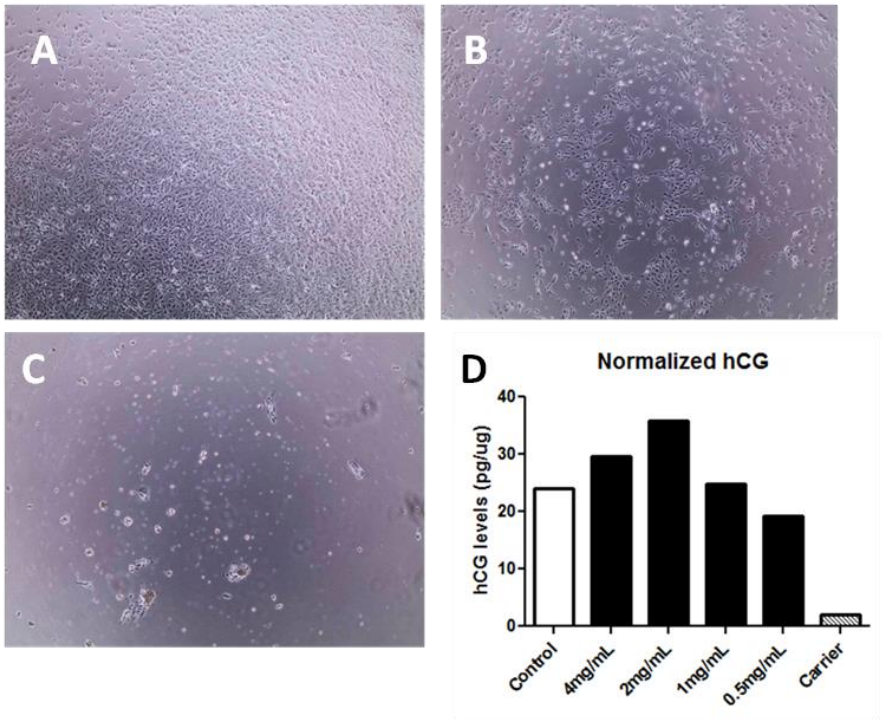

Figure 3. Normalization of placental ECM coating methods. (A) Geltrex: control (B) 1mg/mL nlECM (C) 0.1M acetic acid: carrier control (D) hCGβ ELISA.

mRNA expression levels of Syncytin, a fusion marker, were also lower for primary cells cultured on nlECM at both Day 3 and Day 7 when compared to primary CTBs cultured on tissue-culture plastic. Little change in the amount of morphology differentiation was observed. Hormonal differentiation was inhibited at an mRNA level on nlECM, as shown through markers hCGα and hCGβ.

peECM Inhibits Differentiation of CTBs in vitro

Third trimester primary CTBs were cultured on 6-well plates coated with peECM, nlECM, and tissue-culture plastic at 3 million cells per well using serum-free Third Trimester Media. Triplicates of supernatants and cells were collected on Day 3 and Day 7. hCGβ secretion was inhibited in third trimester primary CTBs plated on both nlECM and peECM when compared to control (see Figure 6). mRNA expression levels of hCGβ (indicating hormonal differentiation) and CSH-1 (a mature STB marker) both increased by Day 7 for all samples. These results were confirmed three more times with n (2) for

both nlECM and peECM, revealing significant difference between nlECM and control, as well as peECM and control (see Figure 7).

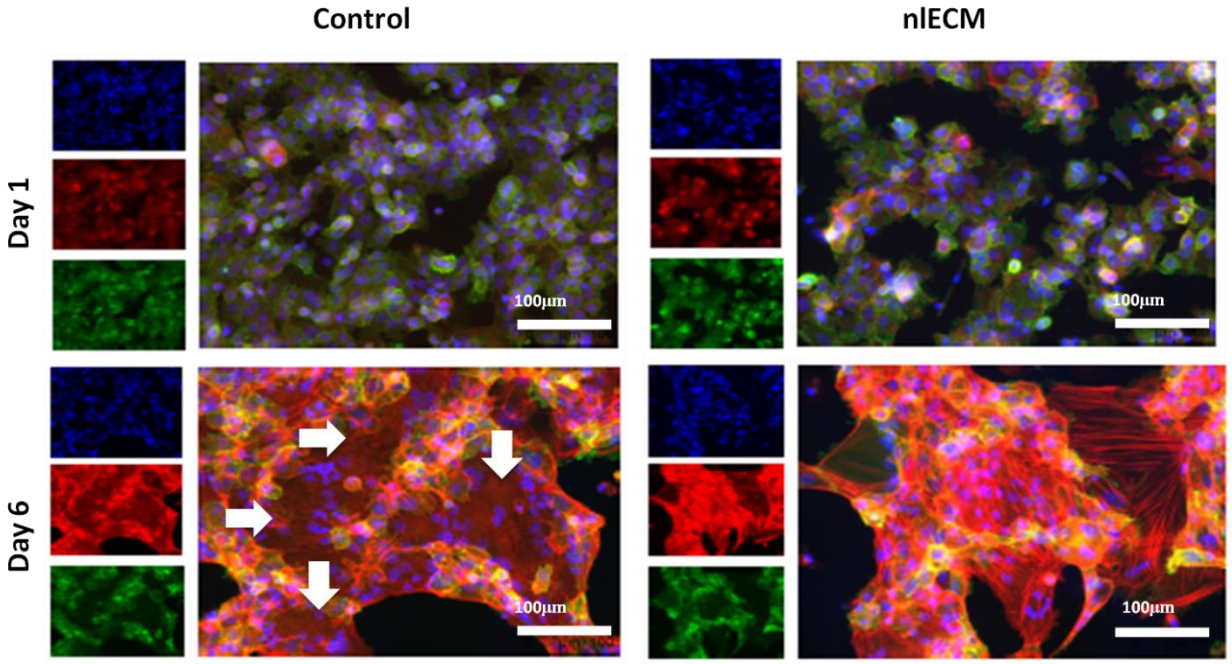

Figure 4. Immunofluorescence double staining on nlECM, with EGFR-α (green), Phalloidin (red), and Dapi (blue). Block arrows indicate CTB fusion into STB cell layers.

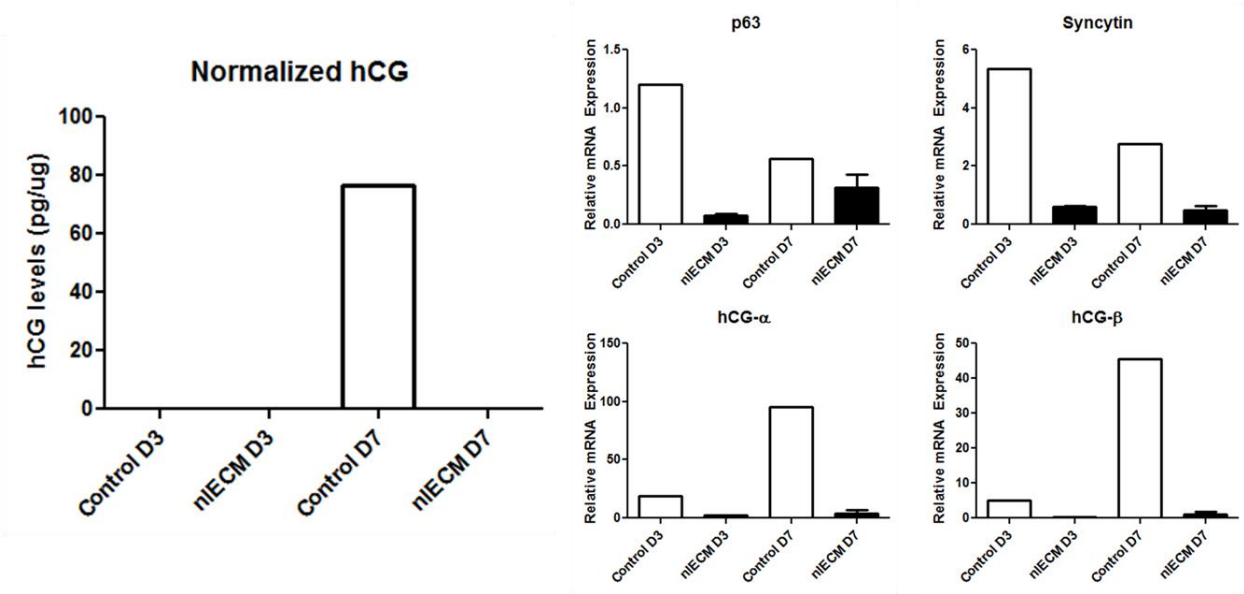

Figure 5. nlECM maintains undifferentiated CTBs.

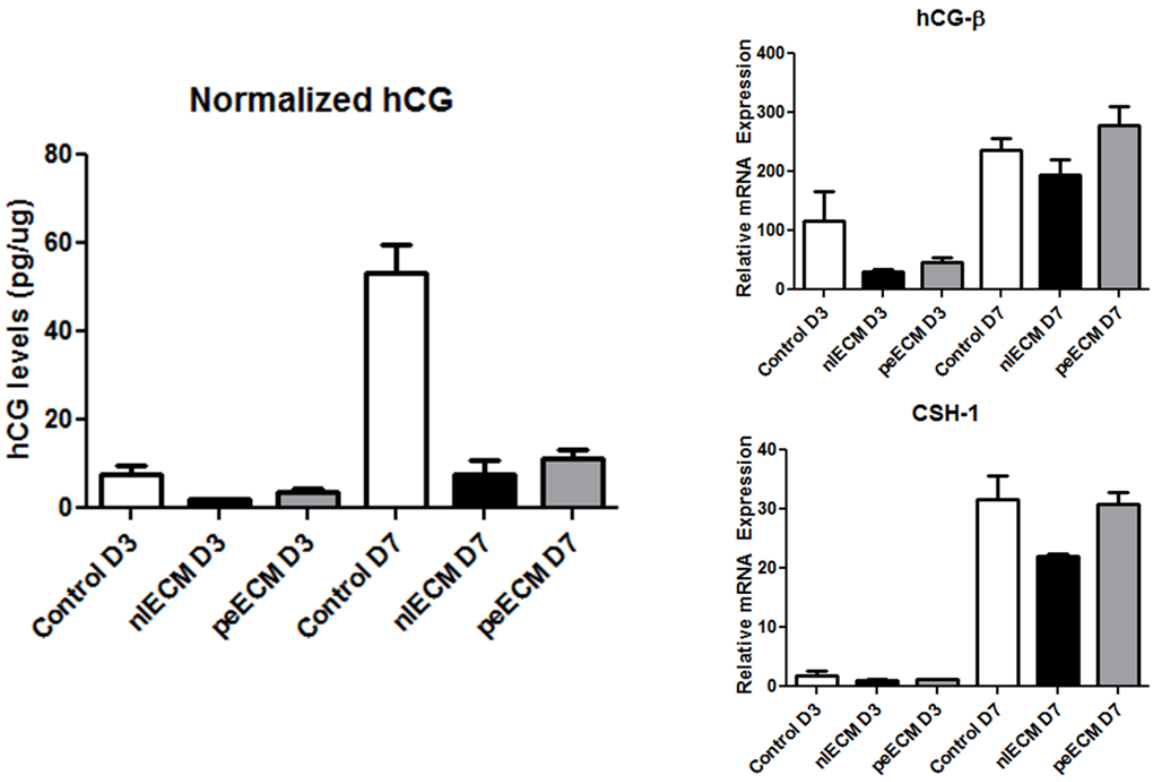

Figure 6. peECM inhibits differentiation of CTBs *in vitro*.

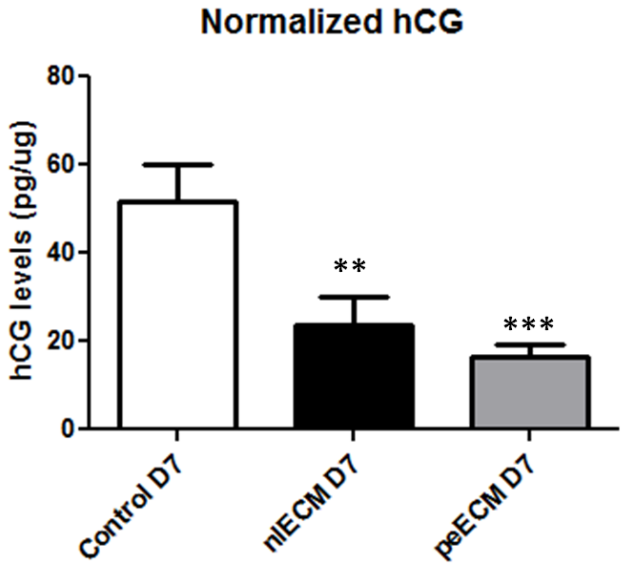

Figure 7. peECM and nlECM significantly inhibit hCGβ secretion.

Proliferation, Adhesion, and Cell Death Unaffected by Placental ECM

Cell proliferation on nlECM and peECM was assessed with JEG3s using Brdu-7AAD flow cytometry and CyQUANT proliferation assay. JEG3s were analyzed for cell number changes from Day 2 to Day 4 in the CyQUANT assay; G_1 checkpoint, S-phase, and G_2 checkpoints were analyzed in the Brdu-7AAD experiment. No significant difference was observed at any point in the cell cycle between nlECM, peECM, and control (see Figure 8). Cell adhesion was also analyzed by plating triplicates of 50,000, 100,000, and 150,000 cells per well in a 96-well flat bottom plate. Media used was DMEM 1x +2% Fetal Bovine Serum +penicillin/streptomycin. Cell concentration was measured by CyQuant Direct Cell Proliferation Assay (Invitrogen) after one night in normoxia. No significant difference in cell adherence was observed between ECM and tissue-culture plastic (see Table 1). Cell survival was evaluated through Annexin-7AAD flow cytometry. Both nlECM and peECM revealed no significant change in cell death when compared to control (see Figure 9).

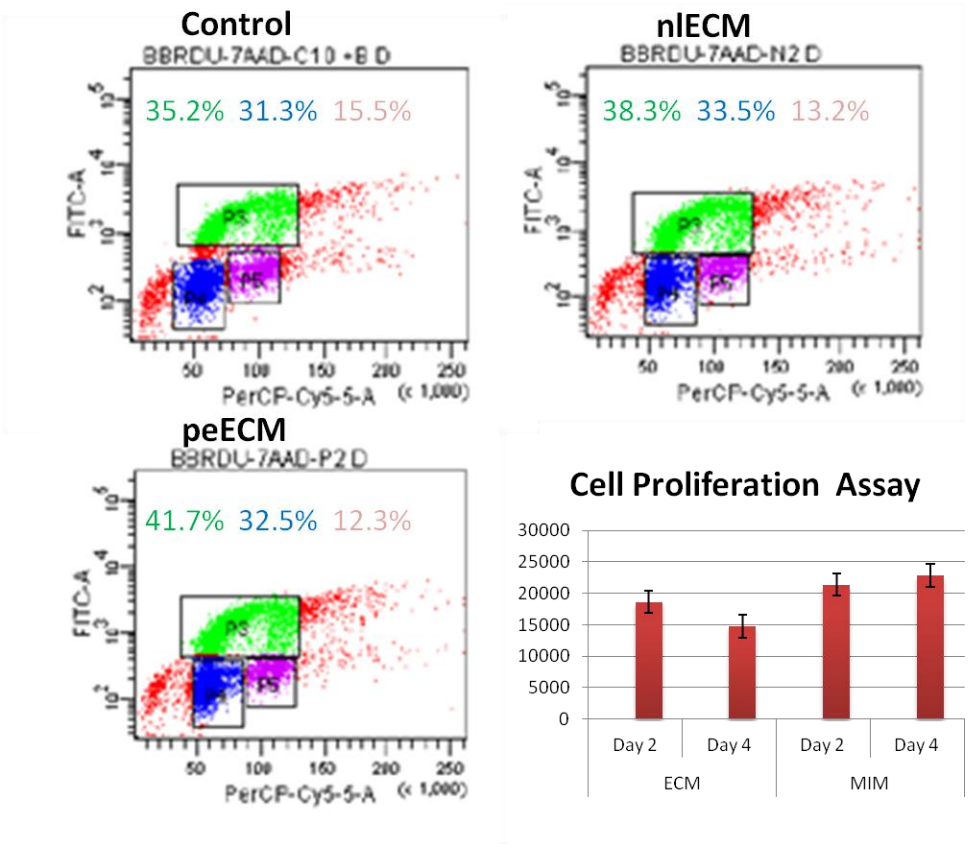

Figure 8. Proliferation unaffected by placental ECM. G_1 Checkpoint (blue), S-phase (green), and G_2 Checkpoint (purple).

Table 1. Cell Adhesion Unaffected by Placental ECM. Average of Triplicates.

	50,000 cells	100,000 cells	150,000 cells	Overall Average
control	30143.33	36709.67	39041.67	35298.22
nlECM	31749.33	35042	37058.67	34616.67
peECM	33868.67	31700	39272.67	34947.11

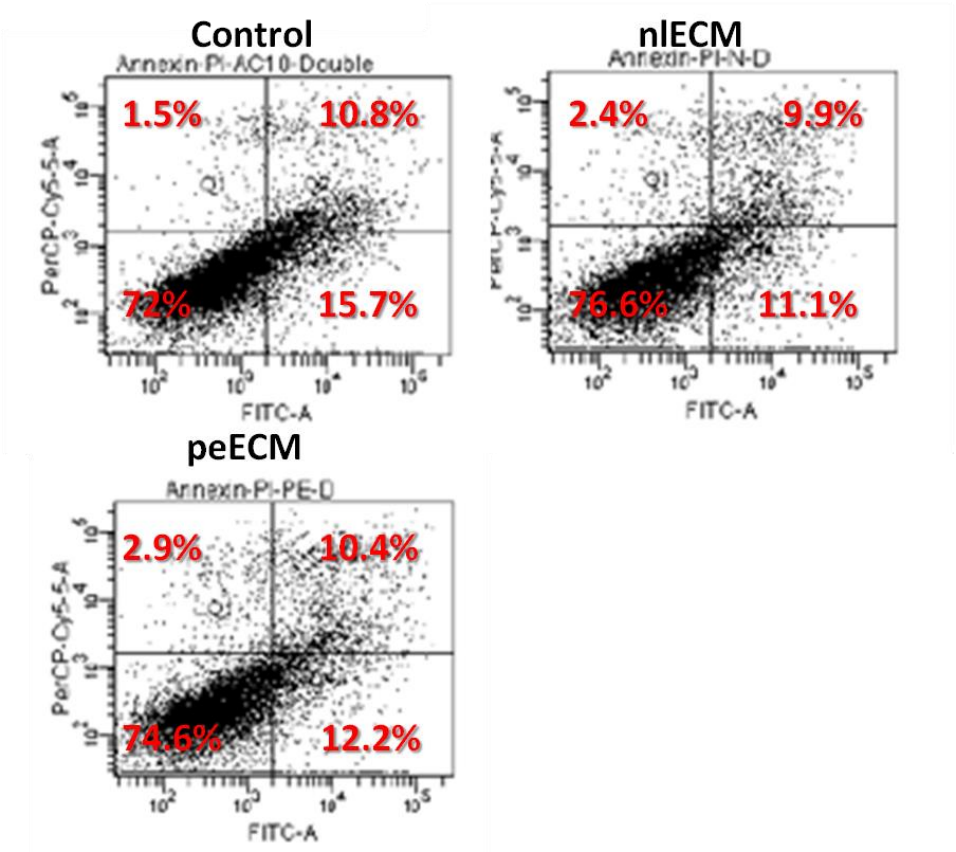

Figure 9. Cell death unaffected by placental ECM. Necrotic (upper left), living (lower left), late stage apoptotic (upper right), and apoptotic (lower right).

DISCUSSION

One quarter of all maternal mortality in the United States is caused by preeclampsia. This placental disease negatively impacts the fetus, and without intervention ultimately leads to intrauterine growth restriction with an estimated fetal weight of <10% (Tache et al., 2013). Current treatment for patients diagnosed with preeclampsia is primarily preventative. Undiagnosed high risk patients (those with pre-existing health conditions) are usually instructed to exercise daily, begin an aspirin regiment, and are encouraged to limit weight gain, stress, and sodium intake. Stress and sodium both aggravate the high blood pressure characteristic of preeclampsia, and can greatly increase the risk of long-term heart disease post-labor and post-diagnosis (Tache et al., 2013; Uzan, Carbonnel, Piconne, Asmar, & Ayoubi, 2011). Once diagnosed, a patient is put on blood thinners, bed rest, and monitored closely for what typically leads to pre-term labor. Great care is taken to avoid pre-mature birth, which carries extremely elevated mortality rates for both the mother and fetus.

Preeclampsia is caused by a fundamental breakdown in the CTBs' ability to properly differentiate into functioning vascular pathways (Burleigh et al., 2007; Fritz et al., 2014). A large amount of CTBs will differentiate into STBs leading to a depletion of CTBs able to differentiate into the necessary amount of EVTs for proper oxygen flow to the fetus. This lack of oxygen and blood flow to the fetus creates a hypertensive effect on both mother and fetus (Fritz et al., 2014). Pathologically, the preeclamptic placenta is less developed than the normal placenta, and yet secretes a larger quantity of hCGβ due to the overpopulation of STBs. Determining exactly how differentiation breaks down is key to understanding this disease – and ultimately, a cure.

Numerous *in vitro* studies have analyzed the molecular pathways and environmental factors that cause preeclampsia through mice models and human trophoblast cell lines (Burleigh et al., 2007; Tache et al., 2013). Extracellular matrix is the biological scaffold that provides the factors and structure needed for organs/tissues to

function, and yet there have been no studies to date utilizing human placental extracellular matrix to monitor primary trophoblast differentiation *in vitro* (Chan et al., 2009). The findings of this study demonstrate for the first time a connection between placental ECM (normal and preeclamptic) and trophoblast differentiation *in vitro*.

This study has created an ideal standard protocol to use with all types of placental extracellular matrix *in vitro*. Third trimester minced human placentas were completely decellularized through a protocol specific to human placentas (see Figure 1). The placental ECM was then turned into a tissue-culture coating for use in *in vitro* trophoblast studies. Placental ECM was compared to Geltrex, a leading commercial product, to determine standard concentration. From the beginning, the goal was to use solely undifferentiated hESCs for the experiments to reveal that the placental ECM factors alone were enough to push hESCs into the trophoblast lineage. Unfortunately, hESCs were immediately found unsuitable in their undifferentiated state because they would not adhere to the placental ECM (Figure 2). A possible explanation may be that the placentas were collected during the third trimester, making the gestational age tissue-unspecific. First trimester placental ECM may provide a more tissue-specific environment for the hESCs in the future.

The course of the study was then redirected to determine how primary CTBs (tissue-specific to third trimester human placentas) would differentiate *in vitro*. Through the use of H9-derived CTBs (a cell type similar to primary CTBs), nlECM was shown to have similar effects as Geltrex when coated at 1mg/mL, both morphologically and functionally (see Figure 3). By determining 1mg/mL as the concentration most similar to standard tissue-culture coatings, a standard protocol and conditions for the use of human placental ECM were established.

An important step in the differentiation pathway is for the CTBs to fuse together and become STBs. This fusion is what creates multinuclear STBs that have an immense amount of cytoplasm (Sanchis et al., 2011). With primary CTBs collected from a third trimester normal human placenta, fusion was shown to occur more with CTBs plated on tissue-culture plastic than with nlECM, indicating that nlECM maintains CTB morphology *in vitro* better than the leading control (see Figure 4). nlECM was also demonstrated to functionally maintain the CTBs in their undifferentiated state through an

hCGβ secretion analysis and mRNA expression of trophoblast markers (see Figure 5). When introducing peECM, both nlECM and peECM demonstrated a way to functionally maintain CTBs in their undifferentiated state (see Figure 6) while having little to no effect on cell proliferation, adhesion, or death (see Figures 8-9). By ruling out proliferation, adhesion, and death, it can be concluded that the effects observed, both morphologically and functionally, are being produced by the ECM.

Given the physiological differences between normal and preeclamptic human placentas when CTBs are differentiating *in vivo*, CTBs cultured on peECM *in vitro* were predicted to exhibit higher levels of hCGβ secretion than both nlECM and the control. But even with a fresh primary CTB sample and an n(2) performed three times for both nlECM and peECM in triplicate, the hCGβ ELISA assay's results were significant in showing that peECM inhibited CTB differentiation into STBs similarly to nlECM (see Figure 7).

However, the lack of significant difference between trophoblasts cultured on nlECM and peECM does not completely rule out ECM as a contributor to preeclampsia. There was a large difference in percent yield noted during the decellularization of normal placentas and preeclamptic placentas during the early stages of the study. The average ECM yield for normal placentas was 11.6mg/g, compared to 3.2mg/g for preeclamptic placentas – an almost four-fold difference. The amount of ECM present, rather than the molecular differences of the ECM, may have a greater influence in this placental disease. Since both ECMs were brought to the same concentration for *in vitro* studies, the difference that is seen *in vivo* cannot be observed.

This study has created a protocol to decellularize human placentas for use in *in vitro* cell culture at 1mg/mL, and has demonstrated that both nlECM and peECM maintain CTBs in an undifferentiated state *in vitro* without negative effects on cell proliferation, death, or adhesion. Future studies would aim to profile placental ECM through mass spectrometry, tensile strength tests, and hydroxyproline content. By characterizing the nlECM and peECM, more insight into the similarities seen *in vitro* and *in vivo* can be revealed. The use of normal primary CTBs can be expanded upon as well; this study could be repeated with the use of isolated preeclamptic primary CTBs. Unfortunately, the use of preeclamptic primary CTBs is beholden to the disease; CTBs

differentiate into STBs in such vast numbers that the amount of CTBs able to get isolated would only be enough for one small project per preeclamptic placenta. A superior isolation protocol of primary CTBs from preeclamptic placentas would have to be created first. In conclusion, this study allows other researchers to now utilize nlECM and peECM to study CTBs, CTB-based diseases, and eventually to determine the mechanism that allows CTBs to differentiate into the EVT lineage.

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