Research Article
doi.org/10.1002/cbic.202300410
Chemistry Europe
European Chemical Societies Publishing

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Impact of Metabolic Stress on BeWo Syncytiotrophoblast Function**

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During placental formation, cytotrophoblasts (CTBs) fuse into multinucleate, microvilli-coated syncytiotrophoblasts (STBs), which contact maternal blood, mediating nutrient, metabolite, and gas exchange between mother and fetus, and providing a barrier against fetal infection. Trophoblasts remodel the surrounding extracellular matrix through the secretion of matrix metalloproteinases (MMPs). Maternal obesity and diabetes mellitus can negatively impact fetal development and may impair trophoblast function. We sought to model the impact of metabolic stress on STB function by examining MMP and hormone secretion. The BeWo CTB cell line was syncytialized to STB-like cells with forskolin. Cell morphology was examined by electron microscopy and immunofluorescence; phenotype was further assessed by ELISA and RT-qPCR. STBs were exposed to a metabolic stress cocktail (MetaC: 30 mM glucose, 10 nM insulin, and 0.1 mM palmitic acid). BeWo syncytialization was demonstrated by increased secretion of HCGβ and progesterone, elevated syncytin gene expression (*ERVW-1* and *ERVFRD-1*), loss of tight junctions, and increased surface microvilli. MetaC strongly suppressed syncytin gene expression (*ERVW-1* and *ERVFRD-1*), suppressed HCGβ and progesterone secretion, and altered both MMP-9 and MMP-2 production. Metabolic stress modeling diabetes and obesity altered BeWo STB hormone and MMP production *in vitro*.

Introduction

The placenta is critical for the progression of pregnancy. It serves as the maternal-fetal interface mediating the transport of gas, nutrients, bioactive molecules, and waste products between the mother and fetus.[1] Trophoblasts are the most abundant cell type of the placenta, and their function is critical to fetal development. During the initial phase of gestation, trophoblasts form a thick cellular layer on the maternal endometrium which is responsible for supplying the early-stage embryo with nutrients.[1a,2] As the embryo develops, trophoblasts differentiate and reorganize to form the placenta and serve as a barrier between the maternal and fetal vasculature.[3] Trophoblasts differentiate along the villous or the extravillous pathway.[4] Villous trophoblasts cover the chorionic villi and are involved in gas/nutrient exchange between mother and fetus, while extravillous trophoblasts (EVT) invade into the uterine wall myometrium. Both villous and extravillous trophoblasts have been implicated in releasing hormones and proteinases into their surrounding micro-environments to promote normal functions of the placenta.[4b]

Metabolic conditions such as obesity and diabetes mellitus increasingly affect women during pregnancy and have detrimental effects on maternal-child health outcomes. Worldwide, more than 20 million births each year are impacted by some form of hyper-glycemia in pregnancy, including gestational diabetes mellitus (GDM) or preexisting diabetes.[5] When coexisting, diabetes and obesity are sometimes referred to collectively as diabesity.[6] These metabolic diseases can be accompanied by elevated levels of circulating saturated fat, glucose, and insulin; what we refer to as "metabolic stressors".

Cytotrophoblasts (CTBs) serve as the placenta's primary metabolic cells, forming organized, branching, villous structures that invade the wall of the uterus into the maternal decidua[7] Syncytiotrophoblasts (STBs) are CTB-derived multi-nucleated cells that originate from the villous pathway[4b] to form a single cellular layer covering the CTB layer.[3] They are in direct contact with maternal blood and secrete the hormone human chorionic gonadotropin beta (HCGβ).[8] Microvilli coat the surface of STBs, increasing surface area and aiding in nutrient and gas exchange.[7] The STBs are among the first placental cells to

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[**] *A previous version of this manuscript has been deposited on a preprint server (https://doi.org/10.20944/preprints202210.0453.v1)*

encounter maternally-derived biochemicals and play a significant role in protecting the fetus from infection and other stressors.[7,9] The role of STBs in innate immune defense against infection is well-demonstrated.[10] Harm to trophoblast structure and/or function could have downstream negative effects on fetal development and the health of offspring.

BeWo cells are a human trophoblast-derived choriocarcinoma cell line similar to primary villous CTBs that can be induced to syncytialize (fuse into multi-nucleated, giant cells) into STBs and are therefore used to model syncytialization of villous trophoblasts.[11] Forskolin (FSK) is used to induce the syncytialization of BeWo cells and acts by stimulating adenylyl cyclase and generating the second messenger molecule cAMP, which is required for BeWo syncytialization.[12] Trophoblast syncytialization is associated with an increase in secreted HCGβ[13] as well as progesterone, a key hormone required to maintain pregnancy. BeWo cells secrete both of these hormones in response to cAMP agonists such as FSK.[13b,14] Considering the body of previous research supporting the use of BeWo cells as an in vitro tool to study trophoblasts,[11c,13a,c,15] we chose to subject BeWo cells to an in vitro model of metabolic stress, composed of high levels of glucose, insulin, and saturated fat (in the form of the ω-6 free fatty acid palmitic acid; C16:0).

Interestingly, although previous research using the BeWo trophoblast cell line revealed that high concentrations of glucose did not affect the amount of HCGβ protein expressed after syncytialization,[16] and the saturated fat, palmitate, did not affect cell viability or caspases-3 activity,[17] there are relatively few studies of this type, particularly ones combining multiple metabolic stressors together. Given the rising prevalence of both obesity and diabetes complicating the same pregnancies, we chose a combined stress-or-approach, one that has been evaluated by other investigators.[18]

Syncytin-1 (Syn-1) and −2 (Syn-2) are proteins involved in mediating fusion of CTBs into STBs during placental development, encoded by the genes *ERVW-1* and *ERVFRD-1*, respectively.[19] Cell-to-cell fusion is essential for the formation of the STB layer, which protects the fetus from microbial threat and allows for nutrient transfer between mother and fetus. Previous work in BeWo cells has shown an FSK-induced upregulation of HCGβ and *ERVFRD-1* transcripts,[20] while another study showed that *ERVW-1* expression was an early event and *ERVFRD-1* expression was a later event in the syncytialization process.[21]

Matrix metalloproteinases (MMPs) are a family of extracellular matrix degrading proteins that are involved in the breakdown of extracellular matrix in normal physiological processes, including embryonic development and tissue remodeling.[22] Extravillous trophoblasts have an invasive property that is possible through the secretion of MMPs, specific to the type IV collagens, referred to as gelatinase A and B (MMP-2 and −9, respectively) .[22] Previous work has shown that MMPs are important mediators of trophoblast invasion[23] and MMP-2 and MMP-9 are critical to this process.[24] MMP-2 is also secreted by BeWo cells, and in much larger quantities than MMP-9.[22] MMP secretion by BeWo cells could be an artifact of their origin from a choriocarcinoma. However, previously published literature shows that primary villous trophoblasts can also secrete MMPs 2 and 9,[24–25] but relatively little is known as to what role this plays. One recent study suggested that MMP-2 and −9 release by villous trophoblasts promote reformation of the placental basal lamina as part of tissue remodeling to accommodate the placenta expansion.[25b] Other processes that MMPs are involved with are regulation of ion metabolism,[26] platelet aggregation,[27] and vasoconstriction.[28]

The impact of metabolic stress has been examined on placental immune cell functions. For example, our lab previously demonstrated that palmitate induced the activation of the nucleotide-binding oligomerization domain-like receptor (NLR) Family Pyrin Do-main Containing 3 (NLRP3) inflammasome in human primary placental macrophages (Hofbauer cells), which was associated with increased interleukin (IL)-1β secretion and apoptotic cellular death.[18b] Neither high glucose nor high insulin concentrations alone provoked these effects and neither augmented the impact of palmitate itself.[18b] We therefore sought to utilize all three components of this model of metabolic stress (hyper-insulinemia, hyperglycemia, and high levels of the saturated fat palmitate) to determine the extent to which villous STB function might be affected, reporting here that production of the hormones progesterone and HCGβ, and the matrix remodeling enzymes MMP2 and MMP9, were significantly altered by this metabolic stress *in vitro*.

Results and Discussion

BeWo cells treated with forskolin syncytialize into syncytiotrophoblast (STB) cells: BeWo cells were treated with FSK to model syncytialization of trophoblasts and supernatants were harvested at 24, 48, and 72 hr post-FSK treatment. Analysis of supernatants by ELISA revealed that HCGβ secretion occurred very gradually over 72 hr from non-FSK-exposed BeWo cells, which are referred to as CTBs. At both 24 and 48 hr following FSK administration, a statistically significant increase in secreted HCGβ above vehicle control (0.2% DMSO) was seen (24 hr: $p \leq 0.01$; 48 hr $p \leq 0.05$) but this significance was lost by 72 hr, although HCGβ secretion was still elevated over vehicle control (Figure 1A). FSK also induced progesterone production, and by 72 hr there was a statistically significant increase above vehicle control ($p \leq 0.05$) (Figure 1B).

We next isolated RNA from BeWo cells to quantify expression of *ERVW-1* (Syncytin-1) and *ERVFRD-1* (Syncytin-2). Cells treated with 20 μM FSK demonstrated an increase in *ERVW-1* and *ERVFRD-1* gene expression at all time points. Specifically, at 24 hr there was a trend for increased expression of both *ERVW-1* (Figure 1C) and *ERVFRD-1* (Figure 1D), although non-significant. By 48 hr there was a significant increase above control for both *ERVW-1* ($p \leq 0.01$) and *ERVFRD-1* ($p \leq 0.05$). By 72 hr there were variable results: *ERVW-1* was non-significantly induced while *ERVFRD-1* was significantly different from control ($p \leq 0.05$). Note that a much larger fold change increase in *ERVFRD-1*

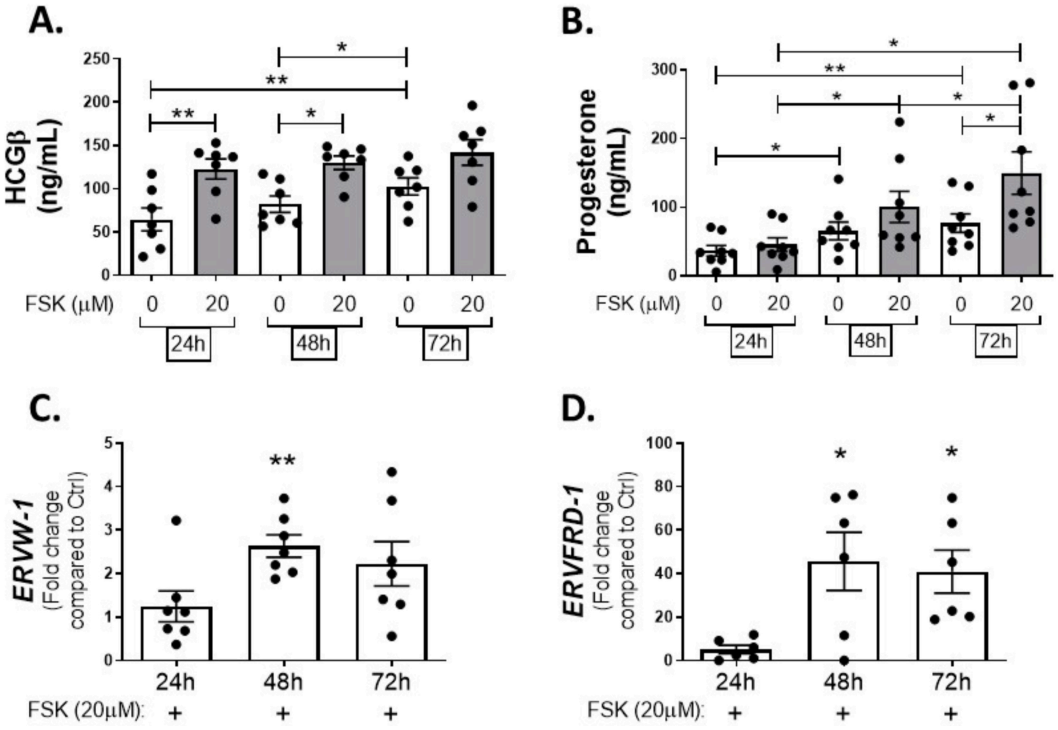

Figure 1. Forskolin treatment increases BeWo secretion of HCGβ and progesterone, as well as *ERVW-1* and *ERVFRD-1* gene expression. (A, B) 500,000 BeWo cells were treated with 0.2% DMSO or 20 μM FSK (grey bars) for 24–72 hr; HCGβ (A) and progesterone (B) secretion were quantified by ELISA (n = 7–8). (C–D) 500,000 cells per well at 24 hr, 48 hr, and 72 hr respectively (n = 6–7); RNA analyzed for gene expression changes in *ERVW-1* and *ERVFRD-1*. 1-way repeated measures ANOVA with Tukey post-test; mean ± SEM; $*p < 0.05$, $**p < 0.01$.

expression was observed compared with *ERVW-1* expression upon treatment with FSK.

BeWo cells were stained with antibodies against the tight-junction protein ZO-1, HCGβ, and the nuclear stain DAPI. Across all time points following exposure to FSK, more HCGβ and less ZO-1 staining were observed, consistent with the formation of giant multinucleated cells lacking individual cellular borders or tight junctions (Figure 2A–F).

Our SEM images revealed the cellular morphology of BeWo CTB cells (vehicle control) and BeWo STB cells (FSK-treated) (Figure 3). Specifically, at 24 hr when BeWo cells were treated with 100 μM FSK (Figure 3B), microvilli structures similar to those seen in other epithelial cells[29] were observed that were not present in vehicle control cells (CTBs) (Figure 3A).

Previously published data showed trophoblast syncytialization was associated with an increase in secreted HCGβ and an increase in *ERVW-1* gene expression.[13] Our data presented here showed a significant increase in secreted HCGβ by 24 hr and a trend towards increased expression of both *ERVW-1* and *ERVFRD-1*. Thus, we selected 24 hr FSK treatment to generate STBs for all experiments moving forward, and BeWo cells treated with FSK are herein referred to as STB cells.

Metabolic stress (MetaC) alters BeWo STB hormone secretions: As previously conducted,[18a,b] we modeled metabolic stress in BeWo STB cells with a cocktail referred to as MetaC (30 mM glucose, 10 nM insulin, and 0.1 mM palmitic acid). This cocktail did not demonstrate toxicity to BeWo STB cell viability as measured by flow cytometry (data not shown). We treated BeWo cells with 20 μM FSK for 24 hr prior to exposure to FSK and MetaC concurrently for an additional 24 hr. MetaC treatment significantly reduced the secretion of HCGβ by BeWo STB cells (FIGURE 4 A) ($p \leq 0.05$), and progesterone secretion followed a similar decreasing trend although not significant (FIGURE 4B). Metabolic stress had a negative impact on the ability of BeWo STBs to secrete the pregnancy hormones HCGβ and progesterone. Interestingly, we saw a significant down-regulation in both *ERVW-1* and *ERVFRD-1* gene expression after MetaC treatment (Figure 4C, 4D) ($p \leq 0.001$, $p \leq 0.0001$, respectively).

MetaC significantly alters BeWo MMP-9 and MMP-2 secretion and total MMP activity: Extravillous trophoblasts have an invasive property that is possible through the secretion of MMPs, (specifically, MMP-9 and MMP-2),[22–23] so we wanted to determine if MetaC affected the secretion of these MMPs in villous STB cells since previous literature shows these cells also secrete MMPs, despite the unknown role this might play.[25] BeWo cells were treated with 20 μM FSK to generate STB cells for 24 hr prior to exposure to FSK and MetaC concurrently for an additional 24 hr. Supernatants were assayed for secreted MMP-9 and MMP-2, and we observed MMP-9 decreasing and MMP-2 increasing with MetaC treatment. More specifically, at 24 hr we saw a decreasing trend in BeWo STB MMP-9 secretion (Figure 4E) when compared to vehicle control, and a statistically significant increase in secreted MMP-2 (FIGURE 4F) ($p \leq 0.05$). In general, we note that BeWo STB cells secreted quantitatively more MMP-2 than MMP-9. We also examined total MMP activity

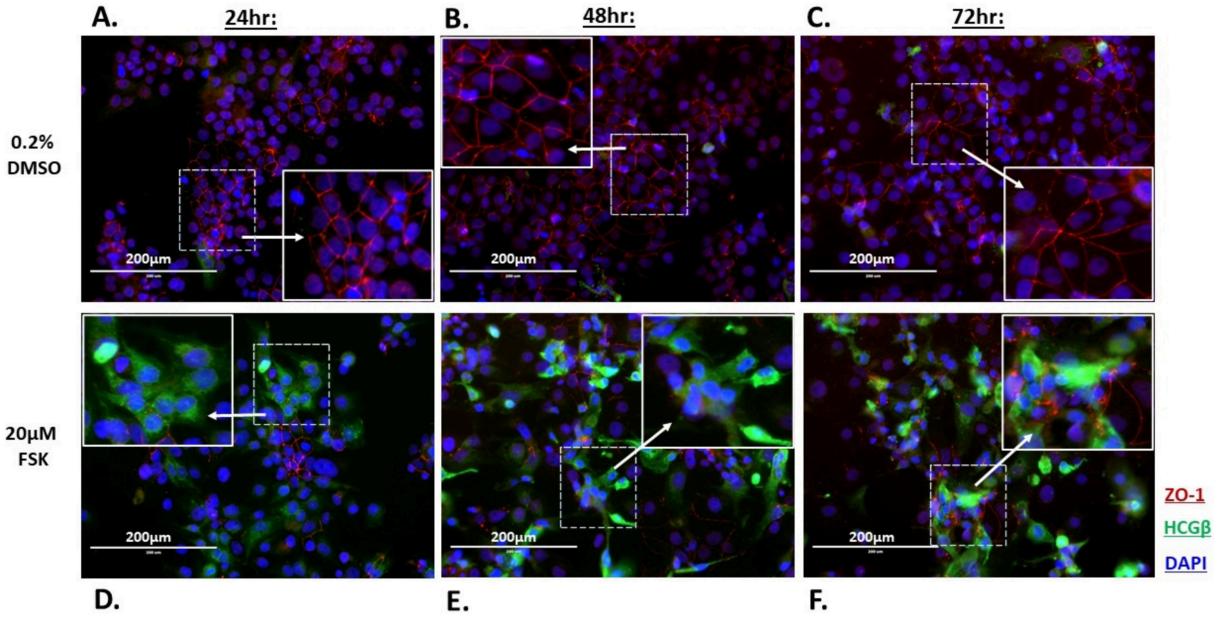

Figure 2. BeWo CTBs transform into STBs with forskolin treatment, as shown by loss of tight junctions and enhanced HCGβ production. 500,000 BeWo cells were treated with 0. 2% DMSO or 20 μM FSK for 24 hr (A, D), 48 hr (B, E), or 72 hr (C, F) and cells were stained with antibodies against ZO-1 (red), HCGβ (green), and DAPI (blue). Representative images are shown (20X images were obtained), and images are shown as merged channels. Scale bar shown on the image. Dotted-line boxes depict an area that we zoomed in on (shown in solid-line box with arrow).

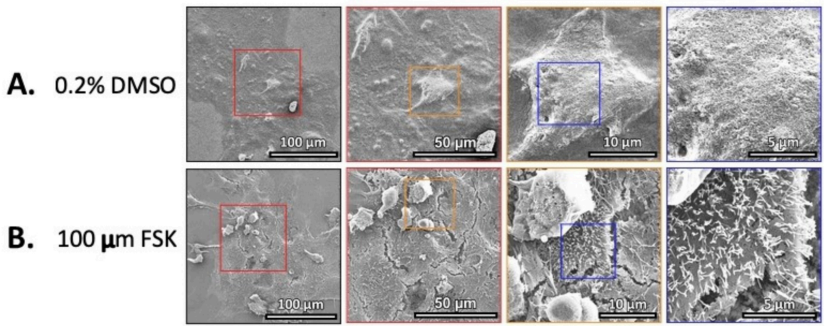

Figure 3. Forskolin treatment results in the formation of microvilli structures on the surface of BeWo STB cells at 24 hr. 500,000 BeWo cells per well were treated with (A) 0.2% DMSO or (B) 100 μM FSK for 24 hr and SEM images were taken. Scale bars are shown on images. Boxes within images depict area zoomed in for subsequent image.

using a commercially available assay that does not distinguish among individual MMPs. We detected a statistically significant decrease in global MMP activity in BeWo STB cell supernatants at 24 hr (FIGURE 4G) ($p \leq 0.01$). Taken together, MetaC treatment affected BeWo STB MMP secretion and activity, possibly resulting in a negative effect on trophoblast cells' role in embryonic development and tissue remodeling related to growth of the placenta throughout pregnancy.

Discussion

In this study we model villous trophoblast syncytialization using a standard model of BeWo CTB cells treated with FSK.[1b,11c] We interrogated this system to assess the impact of one model of metabolic stress (high glucose, insulin, and saturated free fatty acid exposure) on the process of syncytialization and the generation of hormones, soluble inflammatory mediators, and matrix-remodeling enzymes. Syncytialization was successfully induced for BeWo cells using FSK treatment, as demonstrated by significant increases in HCGβ and progesterone secretion, supporting previous research by others.[12] We were also able to successfully detect significant increases in two common gene transcripts associated with syncytialization, *ERVW-1* and *ERVFRD-1*, which supports previous work done by Saha *et al.*[20] and Vargas *et al.*[21] We confirmed our hormone ELISA results by completing immunofluorescent staining and saw increased HCGβ and decreased ZO-1 immunostaining, indicating the merging of nuclei to form giant multinucleated cells, a hallmark of the STB. We did not observe 100% fusion of these cells at the time points tested (Figure 2), which suggests there is a combination of CTBs and STBs in the experimental cultures, which is supported by previous work done by Wang *et al.*[30] showing 30–40% fusion after 48 hours of FSK treatment. This

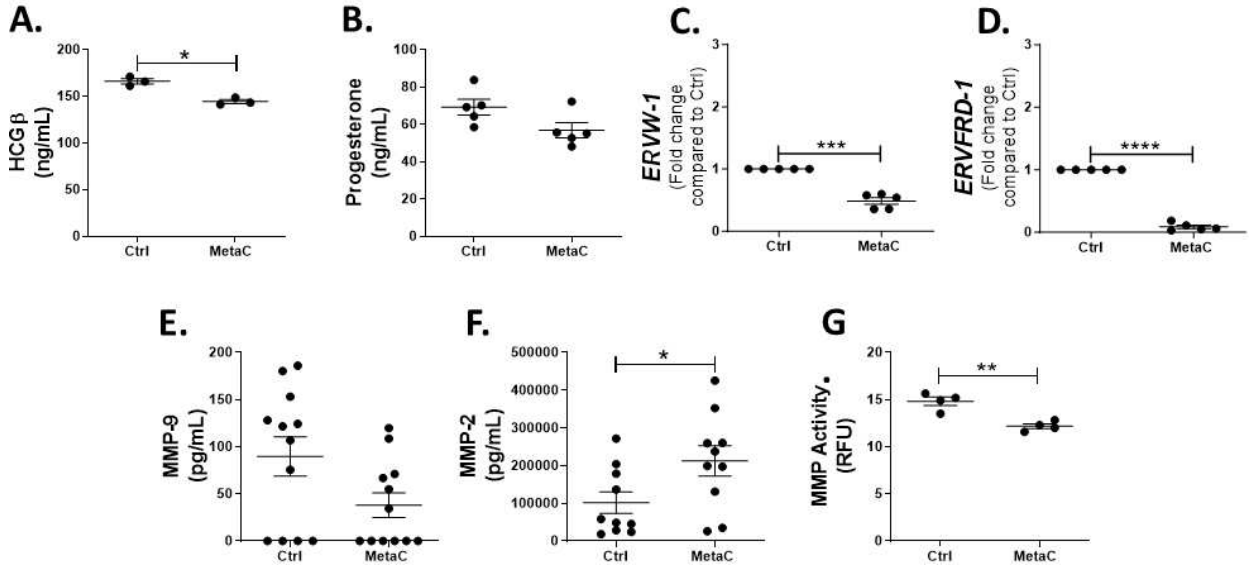

Figure 4. MetaC treatment decreases BeWo STB hormone secretion, syncytialization genes, and matrix metalloproteinase (MMP) secretion & activity. 500,000 BeWo cells per well treated for 24 hr with 20 μM FSK followed by 24 hr treatment with MetaC cocktail (30 mM glucose + 0.1 mM palmitate + 10 nM insulin). Supernatants harvested for (A) HCGβ analysis by ELISA (n = 3), (B) progesterone analysis by ELISA (n = 5), (E) MMP-9 analysis by ELISA (n = 12), (F) MMP-2 analysis by ELISA (n = 10), and (G) global MMP activity (n = 4). RNA was harvested from cells for (C) *ERVW-1* and (D) *ERVFRD-1* gene expression analysis (n = 5 each). Paired t-test analysis; mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

may explain the variability we see in *ERVW-1* and *ERVFRD-1* gene expression after syncytialization with FSK (FIGURES 1 C, 1D). Lastly, we identified by SEM microvillar structures on the surface of FSK-treated BeWo STB cells at 24 hr that were lacking in vehicle control (CTB) cells. Taken together, our data support previous evidence that FSK effectively provokes BeWo CTB cell transformation into multinucleated STB cells, a suitable *in vitro* tool for studying STB formation.[12,20–21]

Metabolic conditions increasingly affect women during pregnancy and can have detrimental effects on maternal-child health outcomes,[5] which can be accompanied by elevated levels of circulating saturated fat, glucose, and insulin; what we refer to as "metabolic stressors". We previously modeled this state by using a formerly studied cocktail of stressors containing high insulin, glucose, and the saturated fat palmitate, referred to as "MetaC",[18b] on placental macrophages and reported cellular consequences including NLRP3 inflammasome activation, increased secretion of IL-1β, and increased apoptotic cell death. In this current body of work, metabolic stress had a significant impact on the ability of BeWo STBs to secrete the pregnancy hormones HCGβ and progesterone, and how this might affect the ability of the STB cells to maintain their role in protecting the fetus from insult or injury is unknown. We did observe a significant suppression of both *ERVW-1* and *ERVFRD-1* when STBs were treated with MetaC for 24 hours, suggesting a detriment in the ability of trophoblasts to properly syncytialize in the presence of high levels of circulating saturated fat, insulin, and glucose.

Extravillous trophoblasts (EVT) secrete extracellular matrix-remodeling enzymes such as the MMPs 9 and 2[22–23,24a] in order to invade uterine wall. Villous trophoblasts have also been shown to secrete MMPs 9 and 2,[25c] although the role for secreting these is currently unclear. We report here that BeWo STBs secrete MMP-9 and MMP-2 and noted that there is more MMP-2 being secreted than MMP-9, supporting previous work by Morgan *et al.*[22] We observed a strong but nonsignificant decrease in secreted MMP-9 after MetaC treatment of BeWo STBs, and a significant increase in STB-secreted MMP-2. Total MMP activity was quantified, and we found a significant decrease in BeWo STB MMP activity, though it is unclear which MMPs are contributing to that phenomenon, a point requiring further study in the future. Since the invasiveness of EVTs is dependent on MMP secretion,[23] it is interesting to speculate that MetaC might alter the trophoblasts' ability to properly invade the decidua to start building the architecture of the placenta, though our model focuses more on villous trophoblasts than EVTs. Such speculation will need to be evaluated in future studies.

These results in combination suggest a possible negative effect of metabolic stress on STB function, potentially negatively impacting the STB's ability to protect the fetus from infection and microbial threats. Metabolic stress might also have a severe impact on the structural integrity of the STB cell layer, which might allow room for pathogens and microbes to pass through and reach the fetus. Furthermore, another potential negative impact on STB structural integrity could allow for poor mediation of nutrients between the mother and fetus, possibly leading to poor fetal health or pre-term birth. This work is summarized in Figure 5.

Several limitations accompanied this research. First, the BeWo cell line used in these studies was not a primary cell line from human placenta. Rather, BeWo cells are a human trophoblast-derived choriocarcinoma cell that can be induced to syncytialize just as human CTB cells syncytialize *in vivo*. The

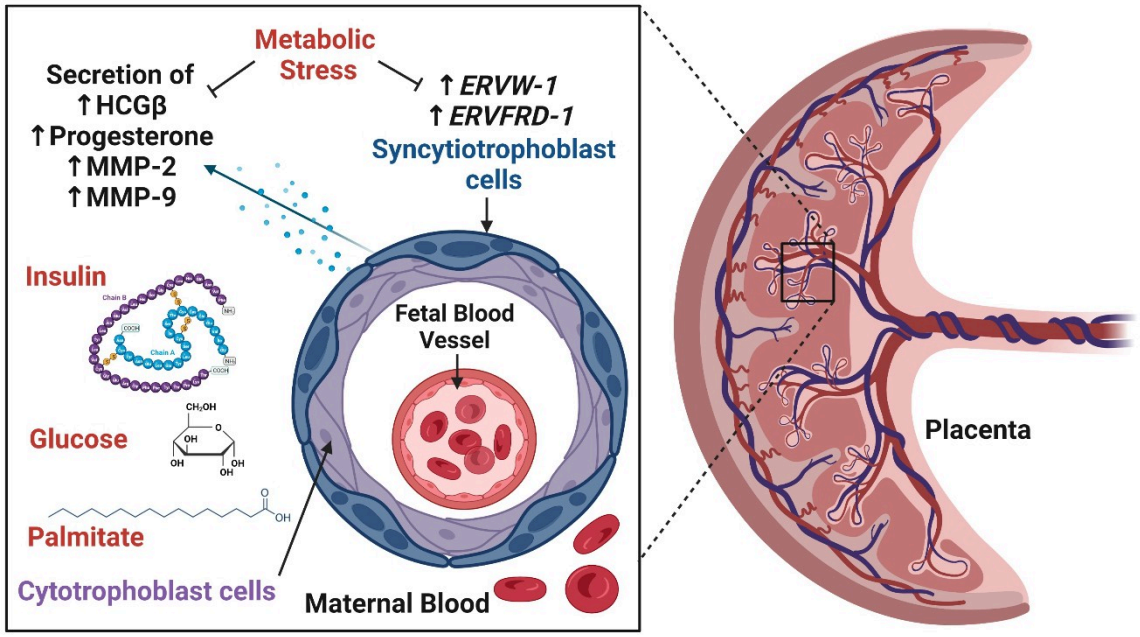

Figure 5. Summary of research findings. Multi-nucleated syncytiotrophoblast cells exhibit enhanced expression of syncytin 1 (*ERVW-1*) and syncytin 2 (*ERVFRD-1*) as well as secretion of progesterone, human chorionic gonadotropin beta (HCGβ), matrix metalloproteinase 2 (MMP-2), and matrix metalloproteinase 9 (MMP-9). Metabolic stress such as that imposed by the presence of insulin, glucose, and palmitate inhibits secretion of progesterone, HCGβ, MMP-2, and MMP-9 by syncytiotrophoblast cells. Created with BioRender software.

choriocarcinoma source of this cell line could confound the results as many cancer cells increase production of MMP's to promote invasion and dissemination.[31] Second, the process of syncytialization in this model is artificially induced by FSK, which is not a physiological substance in humans. Also, FSK treatment does not result in 100% STB fusion, thus resulting in a likely population of CTBs and STBs in experimental cultures. Third, we focus our work here on villous trophoblasts, but the vast majority of MMPs 2 and 9 are released by EVTs to invade the uterine tissue early in pregnancy. The role of villous trophoblasts secreting MMPs is less well defined. One recent study by Sawicki *et al.*[25b] demonstrated that villous trophoblasts do secrete MMP-2 and MMP-9, particularly in differentiated syncytiotrophoblasts. The authors found that secretions preferentially occurred at the basal surface of cells which may function to allow placental expansion, particularly in the third trimester of pregnancy.[25b] We also note that future studies will be necessary to assess the extent to which effects of metabolic stress on the expression of syncytialization-associated genes syncytin 1 (*ERVW-1*) and syncytin 2 (*ERVFRD-1*) correlate with morphological changes in exposed trophoblasts. Lastly, the metabolic stress cocktail is a simplified model of true diabesity. We focused on a limited number of endpoints and a broader study including a systems-biology approach may reveal more insights.

Conclusions

In summary, we report successful and reproducible STB transition from BeWo CTB cells, where HCGβ and progesterone secretions are enhanced, *ERVW-1* and *ERVFRD-1* gene expressions are elevated, and MMP-2 is secreted in much larger amounts than MMP-9. MetaC, as a surrogate of metabolic stress, demonstrated a potential negative impact on BeWo trophoblast function as evidenced by decreased pregnancy hormone production (HCGβ and progesterone), significant downregulation of Syncytin-related genes, as well as alterations in STB ability to secrete MMPs. To our knowledge, the effects of exposure to saturated fats (such as palmitate) on trophoblast function have not been studied in the context of a diabesity model system *in vitro*. Further research needs to be conducted to comprehensively characterize the cellular morphology and the impact of metabolic stress on trophoblast syncytialization. Additional research could specify whether metabolic stress negatively impacts the structural formation and integrity of STBs to the extent that pathogens and microbes are able to pass through the placental barrier and assault the fetus. Such findings could have implications regarding the relationship between fetal health and diabesity, as well as pre-term birth and pregnancy infections.

Experimental Section

Reagents: F–K12 culture medium (containing L-glutamine and sodium bicarbonate) and the human BeWo CCL-98 cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA). This cell line represents a villous CTB cell that can syncytialize into STBs using FSK.[1b,11c] Charcoal-stripped and dextran-treated fetal bovine serum (FBS) was purchased from HyClone Laboratories (South Logan, UT). Antibiotic-antimycotic solution (penicillin, streptomycin, and amphotericin), phosphate

buffered saline solution (PBS) Hank's balanced salt solution (HBSS), 0.25% Trypsin EDTA, trypsin, and DNA-free DNase Treatment kit were purchased from Life Technologies (Carlsbad, CA). Taqman Fast Advanced Master Mix for QPCR was purchased from Applied Biosystems (Thermo-Fisher). TRIzol reagent, normal calf serum, ZO-1 monoclonal antibody (clone ZO1-1 A12, Alexa Fluor 594), GAPDH, *ERVW-1, & ERVFRD-1* Taqman Gene Expression Assays were purchased from Invitrogen (Carlsbad, CA). Paraformaldehyde (PFA) was purchased from Al-fa Aesar (Ward Hill, MA). Trypan Blue was purchased from Thermo Scientific (Waltham, MA). iQ Supermix and iScript cDNA Synthesis kits for RT-qPCR were purchased from Bio-Rad Laboratories (Hercules, CA). Prime Time Gene Expression Assays (forward & reverse primers + FAM probe) and Master Mix for Syncytin-1 (*ERVW-1*) and Syncytin-2 (*ERVFRD-1*) RT-qPCR were purchased from Integrated DNA Technologies (IDT, Coralville, IA). RNeasy Mini Kit was purchased from Qiagen (Germantown, MD). NucBlue Live Cell Stain (DAPI), goat anti-mouse IgG secondary antibody (Alexa Fluor 488), and Aqua Live/Dead viability dye were purchased from Life Technologies (Eugene, OR). An antibody against HCGβ (Alexa Fluor 594) was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Free HCGβ and progesterone ELISA kits were purchased from DRG International (Springfield, NJ). Forskolin (*Coleus forskohlii*, FSK), Triton X-100, glycine, $CaCl_2$, gelatin, Tween 20, glucose, $MgSO_4$, bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from Millipore-Sigma (Burlington, MA). Normal goat serum (NGS) and the MMP Total Activity Assay were purchased from Abcam (Cambridge, MA). Novolin human insulin was purchased from Novo Nordisk (Plainsboro, NJ). Palmitic acid (palmitate, C16:O) was obtained from Nu-Chek Prep (Elysian, MN). MMP-9 and MMP-2 ELISA kits were purchased from R&D Systems (Minneapolis, MN).

***In vitro* syncytialization**: BeWo cells were cultured in F-12 K medium containing 10% FBS and 1% antibiotic-antimycotic (complete media) and maintained every two to three days, at which point cells were passaged by using Trypsin-EDTA for lifting and fresh complete media for counting using trypan blue. For syncytialization, BeWo cells were cultured in 6-well cell culture plates with 500,000 cells per well in 1 mL complete media and rested overnight. In the morning, cells were treated with FSK under three concentration conditions: 0 μM FSK with 0.2% DMSO as the vehicle control, 20 μM FSK, or 100 μM FSK (in the case of scanning electron microscopy (SEM) imaging only). Supernatants were collected at the 24 hr, 48 hr, and 72 hr time points (before daily media changes with fresh FSK, to keep FSK exposure constant) and stored at −80 °C for ELISA analysis. Cells were then washed with PBS, fixed with 4% PFA, and stored at 4 °C for future batched immunofluorescent staining and imaging. In some instances, 1 mL of TRIzol reagent was added to the cells following super-natant collection, and cells were subsequently scraped, collected, and stored at −80 °C for RNA analysis. In other instances, cells were imaged on the SEM for microvilli identification.

Metabolic stress treatment: BeWo cells were cultured in 6-well cell culture plates with 500,000 cells per well in 1 mL complete media and rested overnight. In the morning, BeWo cells were exposed to 20 μM FSK for 24 hr, followed by treatment with 30 mM glucose + 0.1 mM palmitate + 10 nM human insulin (together known as a metabolic cocktail, or MetaC)[18a] for another 24 hr. Supernatants were saved for MMP and hormone ELISA analysis, and RNA was banked for *ERVW-1, & ERVFRD-1* gene expression analysis by QPCR.

Flow cytometry: BeWo STB cells were harvested after MetaC treatment using nonenzymatic cell dissociation buffer and cells were stained with Aqua Live/Dead viability dye for cytotoxicity/cellular death assessment by flow cytometry as acquired on a LSRII Cytometer.

Real-Time quantitative PCR (RT-qPCR): Total RNA was extracted from BeWo cells that were stored in TRIzol reagent using the RNeasy-Mini kit. DNAse treatment was then performed before RNA concentrations were subsequently quantified using a Thermo Scientific Nanodrop Spectrophotometer. 400 ng of RNA was reverse transcribed into cDNA using the Bio-Rad iScript cDNA Syn-thesis kit with the following parameters: (25 °C for 5 min (priming), 46 °C for 20 min (reverse transcription (RT)), and 95 °C for 1 min (RT inactivation)) on a ProFlex PCR System (Applied Biosystems). cDNA preparation concentrations were quantified again using the Nano-drop and 1000 ng cDNA was used in RT-PCR as described (in triplicate): Taqman Fast Advanced Master Mix was used with the *GAPDH*, *ERVW-1 (Hs02341206_g1), & ERVFRD-1 (Hs01942443_s1)* Taqman Gene Expression Assays for MetaC treatment analysis, while PrimeTime Gene Expression Master Mix was used with the *ERVW-1* (NM_001130925.2) primers (forward: AGGCAAAGACAG-GAGGTAAAG (Sense); probe: ATCATT-GTCCCTCCTGCTGTGCTC (Anti-Sense); reverse: GGCTCGAAGACTTGGGTTTAT (Anti-Sense)), and the *ERVFRD-1* (GI# 405754) primers (forward: CTGGGAGAG-CACAACAT-CAA (Sense); probe: ATCTTGGCCGCATTGATGAACTGC (Antisense); re-verse: CCAGAGTGTCCCTGTTGTATTT (Antisense)) for *ERVW-1 & ERVFRD-1* gene analysis after FSK-treatment. cDNA template was omitted for internal PCR controls (no template control). The following parameters were used for amplification on the Bio-Rad Step One Plus thermocycler (fast setting): 95 °C for 3 min (polymer-ase activation and DNA denaturation), followed by 40 cycles of 95 °C for 12 sec (denaturation) and 60 °C for 45 sec (annealing/extension). Data were analyzed using Step One Plus software.

Field-emission gun scanning electron microscopy (SEM): Follow-ing FSK treatment as indicated above in *In Vitro* Syncytialization, BeWo cells were incubated in 2.0% PFA and 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer for at least 12 hr prior to sequential dehydration with increasing concentrations of ethanol. Samples were dried at the critical point using a CO_2 Drier (Tousimis, Rockville MD) mounted onto an aluminum stub and sputter coated with 80/20 gold-palladium. A thin strip of colloidal silver was painted at the sample edge to dissipate sample charging. Samples were imaged with an FEI Quanta 250 field-emission gun scanning electron microscope.

Immunofluorescent staining: Following FSK treatment as indicated above in *In Vitro* Syncytialization, BeWo cells were washed with PBS and fixed with 4% PFA for 15 min while gently rocking. Cells were then washed 3 times with PBS and stored at 4 °C in PBS until batched antibody staining. Briefly, fixed BeWo cells were incubated on a rocker at room temperature with 1 mL of permeabilization buffer (0.1% Triton X-100) for 10 min and subsequently washed with PBS 3 times (5 min each) before incubating on a rocker with 1 mL of blocking buffer (PBS containing glycine and NGS) for 30 min at room temperature. Cells were then stained with primary antibodies HCGβ (1:100), and ZO-1-AF594 (1:100) rocking for 1 hr at room temperature protected from the light. BeWo cells were then washed 3 times with wash buffer (PBS containing BSA, Tween 20, and gelatin) for 5 min each time. Cells were then incubated with the corresponding secondary antibody goat anti-mouse (Alexa Fluor 488) at 1:2000 for 30 min rocking at room temperature in the dark. Cells were then washed 3 more times with PBS before being DAPI stained and imaged on the EVOS microscope. Images were taken using an EVOS microscope with 20X magnification.

Measurement of hormones, cytokines, and MMPs: Free HCGβ, progesterone, MMP-9, and MMP-2 were each quantified in our experimental supernatants using commercially available ELISA kits following the manufacturer's instructions, and each sample was analyzed in duplicate.

Measurement of total MMP activity: MMP activity was quantified in our experimental supernatants using an MMP Activity Assay kit (abcam, ab112146). Each sample was measured in duplicate. The MMP assay protocol uses a fluorescence resonance energy transfer (FRET) peptide as a generic MMP activity indicator. In the intact FRET peptide, the fluorescence of one part is quenched by another. After cleavage into two separate fragments by MMPs, the fluorescence is recovered. This kit is designed to check the general activity of MMP enzymes and doesn't give an individual read-out for each MMP.

Preprint disclosure

An earlier version of this work was submitted as a preprint to *Preprints.org* and can be found here.

Funding: This research received no external funding.

Acknowledgements

Author Contributions: D.M.A conceived and designed the study with assistance from L.M.R.; M.H. conducted the experiments and collected the data with supervision from L.M.R. and D.M.A.; L.M.R. conducted the statistical analyses and generated the figures; A.J.E. consulted on experimental design and data interpretation; R.S.D. and J.I.O. conducted the MMP activity assay, as well as the SEM imaging. All authors contributed to manuscript writing and editing. All authors have read and agreed to the published version of this manuscript.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: developmental origins of health and disease · diabetes · obesity · placenta · syncytialization

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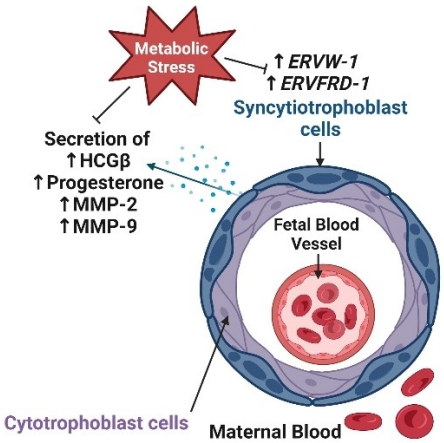
Manuscript received: May 31, 2023
Revised manuscript received: October 6, 2023
Accepted manuscript online: October 6, 2023
Version of record online: ■■, ■■



Multi-nucleated syncytiotrophoblast cells exhibit enhanced expression of syncytan 1 (*ERVW-1*) and syncytin 2 (*ERVFRD-1*) as well as secretion of progesterone, human chorionic gonadotropin beta (HCGβ), matrix metalloproteinase 2 (MMP-2), and matrix metalloproteinase 9 (MMP-9). Metabolic stress (such as insulin, glucose, and palmitate) inhibits secretion of progesterone, HCGβ, MMP-2, and MMP-9 by syncytiotrophoblast cells. Created with BioRender.

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Impact of Metabolic Stress on BeWo Syncytiotrophoblast Function