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RESEARCH ARTICLE

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Hyperglycemia and gestational diabetes suppress placental glycolysis and mitochondrial function and alter lipid processing

Amy M. Valent[1] | **Haeri Choi**[1,2] | **Kevin S. Kolahi**[3] | **Kent L. Thornburg**[2,3]

[1]Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynecology, Oregon Health and Science University, Portland, OR, USA

[2]Center for Developmental Health, Knight Cardiovascular Institute, Oregon Health and Science University, Portland, OR, USA

[3]School of Medicine, Oregon Health and Science University, Portland, OR, USA

Correspondence
Amy M. Valent, Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynecology, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Mail Location L-458, Portland, OR 97239, USA.
Email: valent@ohsu.edu

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Abstract

The degree that maternal glycemia affects placental metabolism of trophoblast cell types [cytotrophoblast (CTB) and syncytiotrophoblast (SCT)] in pregnant persons with gestational diabetes mellitus (GDM) is unknown. We tested the hypotheses that (a) hyperglycemia suppresses the metabolic rates of CTB and SCT; and (b) low placental metabolic activity from GDM placentas is due to decreased oxygen consumption of CTB. Trophoblast cells isolated from GDM and non-GDM term placentas were cultured for 8-hour (CTB) and following syncytialization at 72-hour (SCT) in 5 mM of glucose or 25 mM of glucose. Oxygen consumption rates, glycolysis, ATP levels, and lipid droplet morphometries were determined in CTB and SCT. In CTB from GDM placentas compared to control CTB: (a) oxidative phosphorylation was decreased by 44% (41.8 vs 74.2 pmol O_2/min/100 ng DNA, $P = .002$); (b) ATP content was 39% lower (1.1×10^{-7} vs 1.8×10^{-7} nM/ng DNA, $P = .046$); and (c) lipid droplets were two times larger (31.0 vs 14.4 μm^2/cell, $P < .001$) and 1.7 times more numerous (13.5 vs 7.9 lipid droplets/cell, $P < .001$). Hyperglycemia suppressed CTB glycolysis by 55%-60% (mean difference 20.4 [GDM, $P = .008$] and 15.4 [non-GDM, $P = .029$] mpH/min/100 ng DNA). GDM SCT was not metabolically different from non-GDM SCT. However, GDM SCT had significantly decreased expression of genes associated with differentiation including hCG, GCM1, and syncytin-1. We conclude that suppressed metabolic activity by the GDM placenta is attributable to metabolic dysfunction of CTB, not SCT. Critical placental hormone expression and secretion are decreased in GDM trophoblasts.

KEYWORDS

BODIPY, cytotrophoblast, gestational diabetes, hyperglycemia, lipids, metabolism, mitochondria, mitochondrial respiration, placenta, syncytiotrophoblast

1 | INTRODUCTION

Babies born to mothers with gestational diabetes mellitus (GDM) often grow disproportionately large or can be growth restricted, both of which predispose them to a lifetime risk of metabolic disturbances including obesity and diabetes.[1-4] This metabolic complication affects up to 1 in 10 US pregnancies.[5] A defining feature of GDM is excessive maternal

Abbreviations: CTB, cytotrophoblast; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; GDM, gestational diabetes mellitus; LD, lipid droplet; NEFA, non-esterified fatty acid; OCR, oxygen consumption rate; SCT, syncytiotrophoblast.

insulin resistance and hyperglycemia, which lead to increased maternal-fetal glucose flux, fetal hyperinsulinemia, and variable fetal growth patterns. The range of hyperglycemia among pregnant persons with GDM varies substantially due to maternal metabolic heterogeneity and variable therapeutic compliance. Nevertheless, pregnant persons with GDM may have extended episodes of plasma glucose levels that exceed the commonly accepted normal range.[6] While GDM is currently defined by alterations in glucose homeostasis, GDM is also independently associated with elevated plasma triglycerides and non-esterified fatty acids (NEFA).[7] Maternal plasma lipid concentrations more strongly correlate with fetal growth than do glucose concentrations.[8-12]

Fetal nutrient acquisition is regulated by placental transport systems, many of which are metabolically expensive and require ample ATP generated primarily through oxidative phosphorylation. During the last trimester, the human placenta extracts approximately 40% of the oxygen delivered to the conceptus. Despite its sixfold greater mass, the fetus extracts a similar amount of oxygen.[13] Placental oxygen consumption supports ATP production required for protein synthesis, steroid hormone production, and nutrient transport systems by trophoblast cells. Placentas from pregnant persons with GDM exhibit compromised metabolism and lower rates of oxygen consumption.[14,15]

Alterations in placental metabolism have ramifications for materno-fetal nutrient transport, gas exchange, and the production of pregnancy-sustaining hormones.[14,16,17] The primary placental tissues that regulate these processes are the two villous trophoblastic layers of the placenta that comprise the primary interface between maternal and fetal blood. The first is the syncytiotrophoblast (SCT), a multinucleated layer in contact with maternal blood derived from the differentiation and fusion of the second layer, the cytotrophoblast (CTB) which is composed of mononucleated, progenitor cells. In normal pregnancies, CTB are the primary oxygen consumers in the placenta.[18] They exhibit greater fuel flexibility than SCT, by virtue of their ability to readily metabolize amino acids, glucose, or fatty acids under varying fuel conditions.[18] However, the comparative roles of these two trophoblast cell types have yet to be determined in pregnancies complicated by GDM.[14,19] Understanding their separate roles is particularly important because they may contribute differently to the pathological compromises made by the placenta among pregnant persons with GDM. At present, hyperglycemia has not been shown to affect fatty acid uptake in pregnancies complicated by pre-existing diabetes but is positively associated with placental triglyceride levels.[20] The degree to which CTB and SCT are independently affected by high maternal blood glucose levels has not been investigated.

Thus, the objective of this study was to determine the degree to which glycemic conditions alter the metabolic activities and mitochondrial lipid processing by the two villous trophoblast cell types (CTB and SCT) from GDM and non-GDM placentas. Based on previous studies,[18,21] we hypothesized that in GDM placentas: (a) metabolism would be more suppressed in CTB compared to SCT; (b) that the metabolic rates of CTB and SCT would be further suppressed under conditions of hyperglycemia; and that (c) hyperglycemia would alter trophoblast lipid processing. To test these hypotheses, trophoblast cells isolated from term, normal, and GDM placentas were studied under euglycemic and hyperglycemic conditions.

2 | MATERIALS AND METHODS

2.1 | Subjects

Pregnant persons undergoing a scheduled cesarean delivery at the Oregon Health & Science University obstetric unit from 2016 to 2018 were screened for enrollment. This study was approved by the Institutional Review Board (#5684). Placentas from individuals with singleton, non-anomalous fetuses without the diagnosis of GDM (non-GDM, referent) or with the diagnosis of GDM and using insulin for glycemic management were included for this study. Pregnant persons with GDM managed with insulin were specifically chosen to minimize the metabolic heterogeneity observed in lifestyle-managed GDM (ie, diet-controlled). The referent group was specifically chosen as non-obese subjects because prior studies have shown that even under nutrition-controlled settings, obese pregnant persons have higher baseline glucose levels than normal weight individuals.[12] Therefore, to avoid the potential influence of relative hyperglycemia on placental function from obese pregnancies, we chose our referent group to be non-obese and without GDM.

Exclusions included pregnancies complicated by multifetal gestation, fetal chromosomal or structural anomalies, preeclampsia or hypertensive spectrum, immunosuppressive conditions, or other chronic maternal morbidities. Maternal and neonatal data were collected from medical records. From prior studies comparing trophoblast metabolism from GDM and non-GDM placentas, we chose to include at least $n = 6$ in each group based on a calculated sample size, assuming a 50% reduction in mitochondrial respiration among CTB from pregnant persons with GDM, a power of 90%, and a two-sided confidence interval of 95%.

2.2 | Human villous CTB isolation and culture

Primary human CTB were isolated and cultured in vitro to investigate the functional and morphological effects of GDM on villous trophoblast.[22] All placentas were processed and

sampled within 30 minutes of delivery. CTB cells were isolated using a trypsin-DNAse I-dispase II digestion followed by Percoll enrichment, modified from previously described protocols.[23,24] In brief, chorion, and maternal decidua were removed, and 40-50 g of villous placental tissue was finely minced and thoroughly washed using 1x phosphate-buffered saline (PBS). Villous fragments underwent three sequential 20-25 minutes (37°C) digestions in 0.25% Trypsin (Gibco, Waltham, MA USA), dispase II 1 mg/mL (Gibco, Waltham, MA USA), and 200 U/mL DNAse I (Roche, Basel, Switzerland). CTB was isolated using a Percoll (GE Healthcare Bio-sciences AB, Uppsala, Sweden) discontinuous density gradient centrifugation at 1200 rcf for 25 minutes (room temperature). Purity of trophoblast isolations was assessed by positive immunohistochemical staining of cyokeratin-7 (MAI-06315, Thermo Fisher Scientific, Waltham, MA USA), a marker of trophoblast cells. All isolations included for study comprised of >90% pure, viable CTB, as we have previously published.

Isolated CTB were plated at an optimal density of 3×10^5 cells/cm^2 and cultured in Minimum Essential Media alpha GlutaMAX without nucleosides (MEMα, Gibco, Waltham, MA USA) supplemented with 10% FBS (Gibco, Waltham, MA USA), 100 U/mL penicillin, 2 mM L-glutamine, 25 mM 4-(2-Hydroxyethyl)-1-Piperazinee thanesulfonic Acid (HEPES, Fisher BioReagents, Fisher Scientific, Pittsburgh, PA USA), and 100 μg/mL streptomycin, replaced every 24 hours, and incubated at 37°C 5% CO_2. Cells were studied in "euglycemic" (5 mM) and "hyperglycemic" (25 mM) media conditions to mimic normal and abnormal states of glycemia that may be encountered among pregnant persons with diabetes. CTB undergo fusion and differentiation into multinucleated, syncytialized giant cells by 72 hours in vitro culture, which mimics the syncytialization process in vivo.[22] Therefore, cells were compared between two time points; 8-hours to study trophoblasts prior to differentiation as cytotrophoblasts (CTB) and 72-hours to study differentiated, multinucleated, syncytiotrophoblast (SCT).[18,21,25,26]

2.3 | Metabolic flux analyses

Primary human CTB cells (40 000/well) were plated onto Seahorse XFe96 culture microplates (©Agilent Technologies, Inc, Santa Clara, CA USA). The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XFe96 flux analyzer (©Agilent Technologies, Inc, Santa Clara, CA USA) to derive the parameters of mitochondrial respiration and glycolysis. At the time of assay, Seahorse XF Assay Medium (©Agilent Technologies, Inc, Santa Clara, CA USA) supplemented with 1 mM of pyruvate and 4 mM of L-glutamine or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM of L-glutamine, 143 mM of NaCl, and 3 mg of phenol red replaced culture medium. After a 1 hour incubation at 37°C, cell cultures underwent a Agilent Seahorse XF Cell Mito Stress or Glycolytic stress test (©Agilent Technologies, Inc, Santa Clara, CA USA). Compounds that target the electron transport chain [1 μM oligomycin (ATP synthase inhibitor; Sigma-Aldrich, St. Louis, MO USA), 5 μM of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (mitochondrial uncoupling agent), and a mixture of 1 μM of Rotenone (mitochondrial complex I inhibitor) and 1 μM of Antimycin A (Mitochondrial complex III inhibitor); Sigma-Aldrich, St. Louis, MO USA] are serially injected to measure the key parameters of metabolic function. To interrogate glycolytic function in the metabolic analyzer, 10 mM of glucose, 1 μM of oligomycin, and 50 mM of 2-Deoxyglucose (hexokinase inhibitor; Sigma-Aldrich, St. Louis, MO USA) were injected in to the cell cultures sequentially. OCR and ECAR were recorded in at least three wells for each condition for three cycles following each timed injection.[27] All experimental measurements were normalized to DNA content using Quant-iT PicoGreen dsDNA kit (Molecular Probes Invitrogen, Eugene, OR USA).

2.4 | Mitochondria and lipid labeling, imaging, and quantification

Isolated CTB were plated on glass coverslips or 96-well microplates and cultured for 8 or 72 hours in complete growth medium. MitoTracker Red CMXRos (500 nM; Molecular Probes Invitrogen, Eugene, OR USA), a dye that stains mitochondria and accumulation is dependent upon membrane potential, and MitoTracker Green FM (200 nM; Molecular Probes Invitrogen, Eugene, OR USA), a dye used to localize mitochondria regardless of membrane potential, or BODIPY-493/503 (Molecular Probes Invitrogen, Eugene, OR USA), a dye used to label neutral lipid droplets (LD), were added and incubated for a total of 1 or 2 hours, respectively, prior to fixation. Wheat Germ Agluttinin-CF640R (4 μg/mL; Biotium, Fremont, CA USA) was added for the final 20 minutes of incubation to demarcate the plasma membrane. Pre-warmed 3.7% of paraformaldehyde (pH 7.4) in complete growth medium was added and incubated for 30 minutes at 37°C, 5% CO_2 for fixation. For cells labeled with MitoTracker CMXRos only, the coverslips were submersed into −20°C acetone for 10 minutes post-fixation and subsequently washed up to three times in PBS. The coverslips were counterstained for 20 minutes with Hoechst 33 258 (2 μg/mL; Molecular Probes Invitrogen, Eugene, OR USA) to label nuclei and mounted with Slowfade Diamond (Molecular Probes Invitrogen, Eugene, OR USA) before confocal microscopy.

High-resolution confocal microscopy was performed to image the trophoblast cells (Zeiss 880 LSM Confocal with Airyscan, 63 × High NA objective; NA = 1.4). The Nyquist sampling interval was used for all spatial dimensions. Each field of view was comprised of serial Z-"stack" images measuring 67.5 μm by 67.5 μm (x-y) and 2 μM total thickness. All raw, 32-channel single-color images were processed using automatically determined Airyscan parameters in Zen software (©Zeiss Microscopy, Jena, Germany). Images acquired by BioTek Cytation 5 20x objective (BioTek Instruments, Inc, Winooski, VT USA) were used to quantify mitochondrial mass and lipid droplet number and size. Over 1000 cells per group were measured with results representing triplicates of non-GDM and GDM from at least three different placentas. Processed images were analyzed using Fiji software (LD) and BioTek Synergy software (mitochondrial mass) using automatic thresholding to segment mitochondria and LD.[28]

The uptake of BODIPY-C_{16}, a very long-chain fatty acid, was quantified in vitro as previously described with modifications.[26] CTB were cultured in 96-well plates (Corning Life Sciences, Tewksbury, MA USA) for 8 and 72 hours as described above. Cells were pre-incubated in 50 μL of serum-free MEM-α for 1 hour. An assay solution (50 μL) with a final concentration of 5 μM of BODIPY, 5 μM of FAF-BSA, and 5 mg/mL of Trypan Blue was added to each well, mixed, and incubated for 15 minutes at 37°C with 5% CO_2. The cells were washed with HBSS with calcium and magnesium (Hanks Balanced Salt Solution; Gibco, Waltham, MA USA) and the fluorescence (485 nm excitation, 525 nm emission) was measured using spectrophotometric analysis (BioTek Synergy H1 Hybrid Multi-Mode Reader; BioTek Instruments, Inc, Winooski, VT USA) and normalized to total protein using a bicinchoninic acid method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA USA).

2.5 | Quantification of mRNA expression

After 8 or 72 hours of culture, TRIzol Reagent was added, total RNA was isolated using the chloroform method,[29] cleaned with EZNA MicroElute RNA Clean Up kit (Omega BioTek, Norcross, GA USA), and assessed using spectroscopy at 260 nm/280 nm. Using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA USA), reverse transcription of 1 μg of RNA to cDNA was performed and stored at −20°C until desired use. Quantitative polymerase chain reaction (qPCR) was performed using the Stratagene Mx3005P Thermocycler (©Agilent Technologies Inc, Santa Clara, CA USA). The qPCR amplicons were detected by fluorescent detection of SYBR Green (Power SYBR Green Master Mix; Applied Biosystems, Carlsbad, CA USA). For each primer pair, standard curve samples, unknowns, and no template controls were run in triplicate. Following cycling, the melt curve of the resulting amplicon was analyzed to ensure that a single product was detected. Using the manufacturer's respective standard curves (MxPro QPCR; ©Agilent Technologies, Inc; Santa Clara, CA USA), mRNA quantification was achieved. Primers for CD36, CGB, CPT1α, GCM1, GLUT1, PDK4, SLC27A2, SLC27A4, SYNCYTIN-1, and GAPDH were studied (Table S1). Relative mRNA expression was expressed as a ratio of the gene of interest to the reference gene (GAPDH) for each sample, which did not differ at 8 vs 72 hours.

2.6 | Protein analysis

2.6.1 | Western blot analysis

Total cell lysates were prepared in radioimmunoprecipitation assay buffer plus protease cocktail inhibitors (1 mM of phenylmethylsulfonyl fluoride and 1 mM of sodium fluoride). The lysates were centrifuged at 13 000 rpm for 30 minutes to remove cellular debris. The bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA USA) determined the supernatant protein concentration. Lysates were mixed with SDS-PAGE sample buffer (2.5% of SDS and 0.002% of bromophenol blue, 10% of glycerol, and 713.5 mM of β-mercaptoethanol) and boiled for 10 minutes. TEMED and 10% of Ammonium Persulfate were added to EZ-Run Protein Gel Solution for gel preparation and samples were run evenly. The gels were transferred on to a nitrocellulose membrane, washed with 1x TBS, and blocked with 5% skim milk in TBST at room temperature for 30 minutes.

Western immunoblot assays quantified the levels of β-hCG, CPT1α, SLC27A2, and GLUT1, which have been successfully used in human trophoblasts.[18,21] Antibodies for β-hCG (1 μg/mL, 1:1000, Dako Product A0231 Agilent; Santa Clara, CA USA), CPT1α (1 μg/mL, 1:1000, Abcam ab128568; Cambridge, United Kingdom), SLC27A2 (1 μg/mL, 1:1000, Biorbyt orb153480; Cambridge, United Kingdom), and GLUT1 (0.2 μg/mL, 1:1000, Invitrogen PA5 16 793; Carlsbad, CA USA) were diluted in skim milk and rocked overnight. The antibodies were detected using anti-mouse and anti-rabbit IgG (1:1000, Cell Signaling 7076 & 7074; Danvers, MA USA) and visualized using a highly sensitive chemiluminescence system (UVP ChemiDoc Imager; Bio-Rad, Hercules, CA USA). Densitometry to quantify protein bands was performed using Fiji software. Protein expression was normalized to the loading control protein (ACTIN) for each sample. ACTIN expression did not differ at 8 vs 72 hours.

2.6.2 | ELISA analysis

Cell culture supernatants were collected after 72 hours of incubation as stated above. Venous blood (10 mL) was collected from participants on the day of their delivery in EDTA tubes, placed immediately on ice, and processed within 90 minutes from collection by centrifugation at 2000 g for 10 minutes. Plasma was collected, divided into aliquots, and frozen at −80°C until future analysis. Cell supernatant and maternal plasma β-hCG were measured using commercially available ELISA kits purchased from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions.

2.7 | Statistical analysis

Two-way ANOVA with Sidak's post hoc testing was used to compare the metabolic fluxes and hormone expression between non-GDM and GDM samples. Simple and multiple linear regression were performed to determine the relationship of maternal demographics and 2-hour oral glucose tolerance test (2h-OGTT) indices and placental function as defined by the metabolic and hormone analyses. Corrected paired Student's *t* test was used to compare CTB and SCT within the same cultured samples. Mitochondria and LD numbers/area were analyzed using the Cytation-5 imager and Gen5 data analysis software. An unpaired Student's *t* test with Bonferroni correction was used to compare mitochondrial volume ratios. Demographic data were analyzed using Stata 14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). All placental test comparisons were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA) and presented as mean ± SD unless noted otherwise. *P* values <.05 were considered statistically significant.

3 | RESULTS

Maternal, neonatal, and placental characteristics are reported in Table 1. The GDM cohort had significantly higher 2h-OGTT indices and prepregnancy or first trimester BMI compared to non-GDM cohort. Maternal age (hormone concentration: $P = .303$; metabolic assays: $P = .756$) and early BMI (hormone: $P = .436$; metabolic: $P = .599$) did not correlate with trophoblast hormone production or metabolic function. The relationship of glycemia measured at the time of 2h-OGTT, prior to GDM interventions, and trophoblast function was measured using regression analysis. An inverse relationship with increasing glycemia and declining trophoblast function was found (Supplement 1). When adjusting for maternal age, race, and prepregnancy or first trimester BMI, *hCG* mRNA expression continued to be significantly correlated with the 1h- and 2h-OGTT time points (1h: $P = .011$, $r^2 = 0.949$; 2h: $P = .022$, $r^2 = 0.902$) and CTB oxygen consumption rate continued to be significantly correlated with the 1h-OGTT time point ($P = .016$, $r^2 = 660$). Isolated CTB from GDM and non-GDM placentas recapitulated the well described cell aggregation and fusion in culture to form differentiated, multinucleated syncytialized giant cells that mimicking mature SCT (Figure 1).

TABLE 1 Maternal and delivery characteristics

Variable	GDM (n = 14)	Non-GDM (n = 24)	*P* value
Age (years; mean ± SD)	33 ± 5	34 ± 4	.505
Race/ethnicity (n%)			.741
Non-Hispanic White	64%	71%	
Non-Hispanic Black	7%	8%	
Hispanic/Latina	14%	4%	
Asian	14%	17%	
Parity (median [IQR])	1 [1, 2]	1 [1, 2]	.913
Glucose tolerance test (mg/dL; mean ± SD)			
Fasting	94 ± 6	81 ± 5	<.001
1-hour	185 ± 27	128 ± 21	<.001
2-hour	141 ± 31	102 ± 23	<.001
Pre-pregnancy BMI (kg/m^2; mean ± SD)	35.8 ± 7.7	22.7 ± 2.7	<.001
GA at delivery (weeks; mean ± SD)	$38^{1/7} \pm 0^{6/7}$	$38^{6/7} \pm 1^{2/7}$	.281
Birth weight (grams; mean ± SD)	3453 ± 632	3506 ± 419	.758
Ponderal index, (g/cm^3; mean ± SD)	2.72 ± 0.28	2.68 ± 0.37	.765
Birth weight: Placenta weight ratio	7.20 ± 2.16	6.69 ± 0.95	.326
Male fetus	39%	61%	.804

Note: Continuous variable are presented as mean ± SD or median [interquartile range]. Dichotomous variables are presented as percent.

3.1 | GDM trophoblasts demonstrate significantly lower expression β-hCG and other markers of syncytialization

Because β-hCG is produced primarily by the syncytial layer of the placenta, we quantified its protein levels at 8 (CTB) and 72-hours (SCT) of trophoblast culture to demonstrate the well reported rise β-hCG with syncytialization in SCT compared to CTB in vitro (Figure 2A,D,E,F).[30] Beta-hCG mRNA and protein expression and peptide secretion was significantly lower in SCT from GDM placentas compared to non-GDM placentas. These findings were not attributable

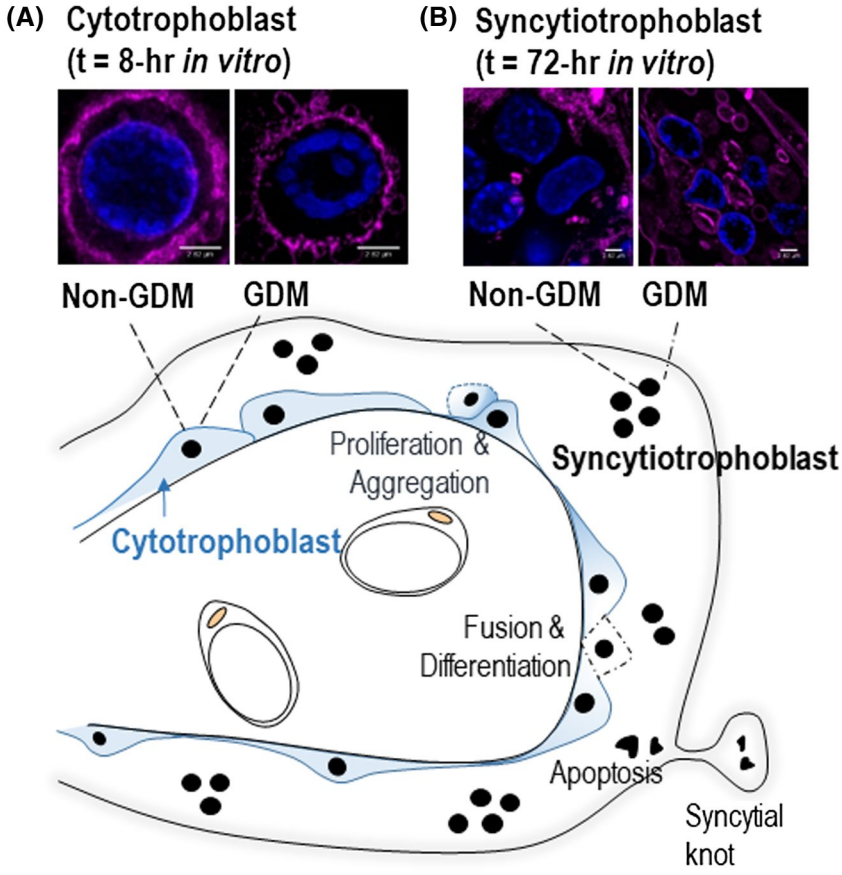

FIGURE 1 In vitro, isolated cytotrophoblast cells fuse and differentiate to multinucleated syncytiotrophoblasts. The CTB process of proliferation, fusion, and differentiation to regenerate the SCT layer can be recapitulated in vitro from placentas complicated by GDM and non-GDM. After plating CTB (shaded in blue) have spheroid shape with filopodia (A) and continue to aggregate, fuse, and differentiate to form sheet-like SCT by 72 h (B). Wheat germ agglutinin (magenta) used for plasma membrane, Hoechst dye (blue) used for nuclear labeling. Images represent trophoblasts from n = 1 GDM and non-GDM placenta; however, observed in all n = 38 placentas used for the study

to hyperglycemic culture conditions, differences in the rates of differentiation nor the timing of peak expression between the two cohorts (Figure 2A,D,E,F). Significantly lower levels of β-hCG were measured in maternal plasma among GDM pregnant persons than non-GDM (Figure 2G). Other markers of syncytialization (GCM1 and Syncytin-1) were also significantly decreased in GDM compared to non-GDM trophoblasts.

3.2 | GDM placentas rely on glycolytic activity in CTB for energy production

CTB from GDM placentas had overall lower oxygen consumption rate (OCR) compared to non-GDM CTB (Figure 3), regardless of the level of glycemia (5 or 25 mM glucose) [GDM 41.8 vs non-GDM 74.2 pmol O_2 min^{-1}/100 ng DNA, $P = .002$]. Interestingly, the basal OCR of SCT was not different between GDM and non-GDM (Figure 3B). When CTB from GDM placentas were maximally challenged with FCCP, they had a significantly lower mitochondrial respiratory capacity than from non-GDM placentas, which was not impacted by differences in glycemic conditions (Figure 3A,C). The mitochondrial respiratory capacity of SCT derived from GDM placentas was unchanged by the FCCP challenge compared to non-GDM trophoblasts (Figure 3C). Our findings suggest that oxygen consumption in CTB from GDM placentas is suppressed regardless of glycemic conditions.

As mentioned in the methods, we used extracellular acidification rate (ECAR) as an index of glycolytic activity. Under euglycemic glucose conditions (5 mM), glucose utilization was similar in CTB from both GDM and non-GDM placentas. However, under conditions of hyperglycemia (25 mM glucose), both non-GDM and GDM CTB had significantly lower levels of glycolytic activity (Figure 3E: non-GDM, 27.3 [25 mM] vs 42.7 mpH min^{-1}/100 ng DNA [5 mM], $P = .029$; GDM, 25.3 vs 45.7 mpH min^{-1}/100 ng DNA, $P = .008$). SCT from non-GDM and GDM placentas had similarly low ECAR activity regardless of glycemic conditions. Although the maximal glycolytic capacities were greater in CTB compared to SCT, they were similar between GDM and non-GDM CTB and SCT in both euglycemic and hyperglycemic conditions (Figure 3F). Thus these studies demonstrated that oxygen consumption was suppressed only in CTB, not SCT, in placentas derived from mothers with GDM. Additionally, glycolytic activity in CTB was significantly suppressed under hyperglycemic conditions in cells from both GDM and non-GDM placentas.

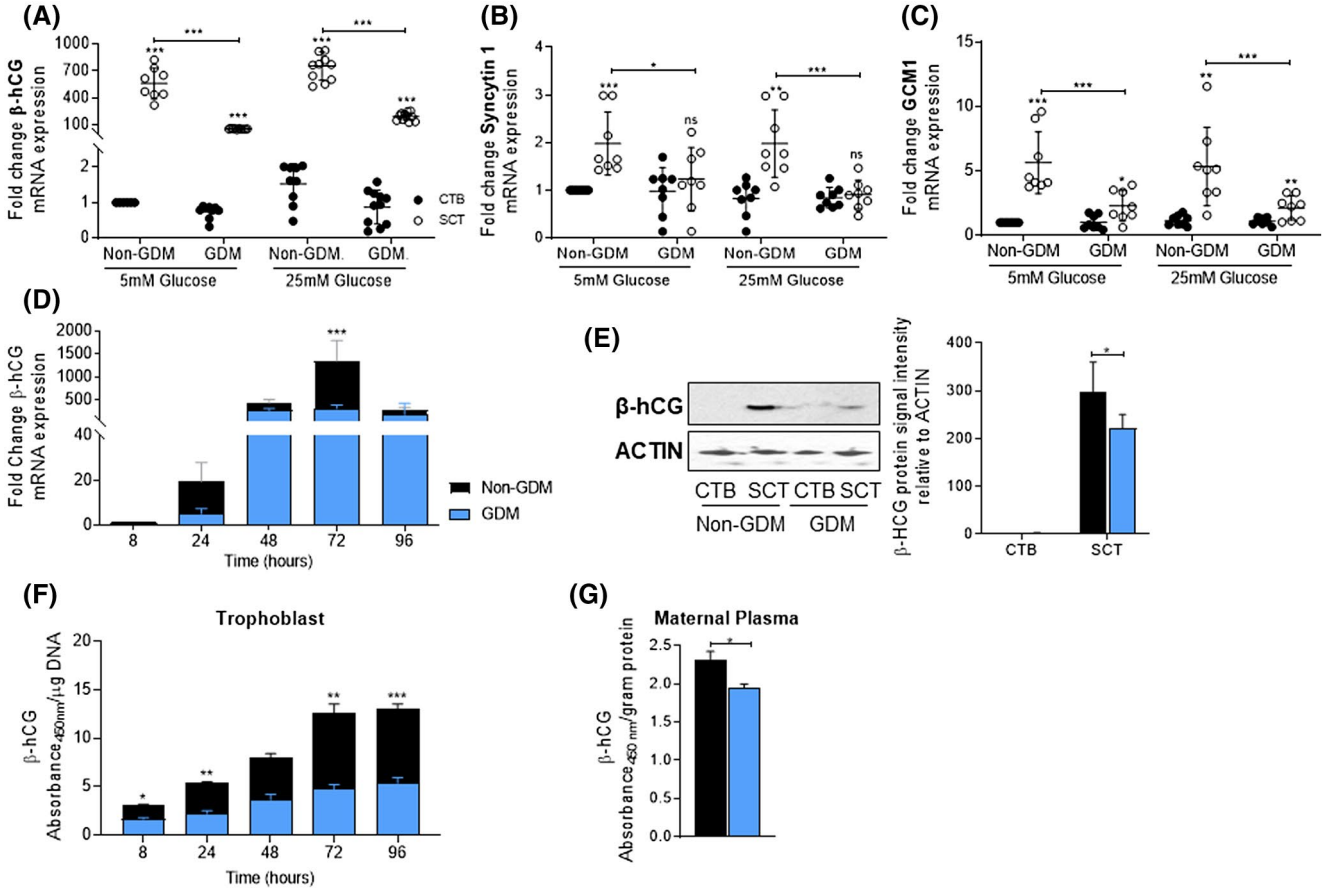

FIGURE 2 Suppression of markers of syncytialization in GDM trophoblasts. β-hCG is a specific marker for trophoblast differentiation and both gene and protein expression significantly increases by 72 hours (SCT) compared to CTB in vitro (A, D). GDM SCT had decreased gene (A) and protein expression (E) compared to non-GDM SCT that was not attributable to the differences in timing of differentiation as measured by mRNA expression (D) or ELISA in the cell supernatant (F). Pregnant persons with GDM had significantly lower levels of β-hCG than non-GDM persons (G). This β-hCG suppression was consistent with other markers of syncytialization including Syncytin-1 and GCM1 (B, C). Duplicates of n = 3 (E); duplicates of n = 4 (B-D, F); duplicates n = 5 (G); duplicates with n = 6 (A). Mean ± SD; CTB [•] vs SCT [○] (asterisk over circles), GDM (blue bars) vs non-GDM (black bars) [asterisk above bars], 5 mM of glucose vs 25 mM of glucose: ***$P < .001$, **$P < .01$, *$P < .05$

3.3 | Lipid metabolism pathways decreased in GDM placentas

To investigate potential pathways that could contribute to the suppression of mitochondrial metabolism, we studied key genes known to be important in lipid and glucose metabolism (Figure 4). *SLC27A2* (FATP2) is highly expressed in the placenta and important for long-chain fatty acid acylation, transport, and trafficking; expression is significantly higher in CTB than in SCT.[21] Compared to non-GDM CTB, CTB from GDM placentas had much lower mRNA and protein expression of *SLC27A2*. Protein expression in 5 mM of glucose and 25 mM of glucose conditions were similar (data not shown). *CD36* (fatty acid translocase) and *SLC27A4* (FATP4) have been well described to be found in the SCT.[31] *SLC27A4* expression was not significantly different between GDM and non-GDM trophoblasts. *CD36* expression was slightly increased in GDM SCT but was no longer significantly different in hyperglycemic conditions. The mRNA and protein expression of *CPT1α*, the enzyme that shuttles fatty-acyls into mitochondria for β-oxidation, was suppressed in GDM CTB as compared to non-GDM CTB.

GLUT1 is the primary isoform involved in the transplacental transport of glucose via facilitated diffusion.[32] No significant difference in mRNA or protein expression for GLUT1 was observed between non-GDM and GDM trophoblasts. However, under hyperglycemic conditions, GDM CTB had significantly lower mRNA expression of GLUT1 than under euglycemic conditions. Because maintaining a balance between energy demand and supply is critical for the placenta, pyruvate dehydrogenase kinase 4 was investigated. It is one of the most widely expressed isozymes that influence the activity of pyruvate dehydrogenase complex which catalyzes the oxidative decarboxylation of pyruvate. GDM SCT and in hyperglycemic conditions GDM CTB had significantly higher expression of PDK4, which may underlie the decreased glucose utilization and increased lipolysis and lipid accumulation.

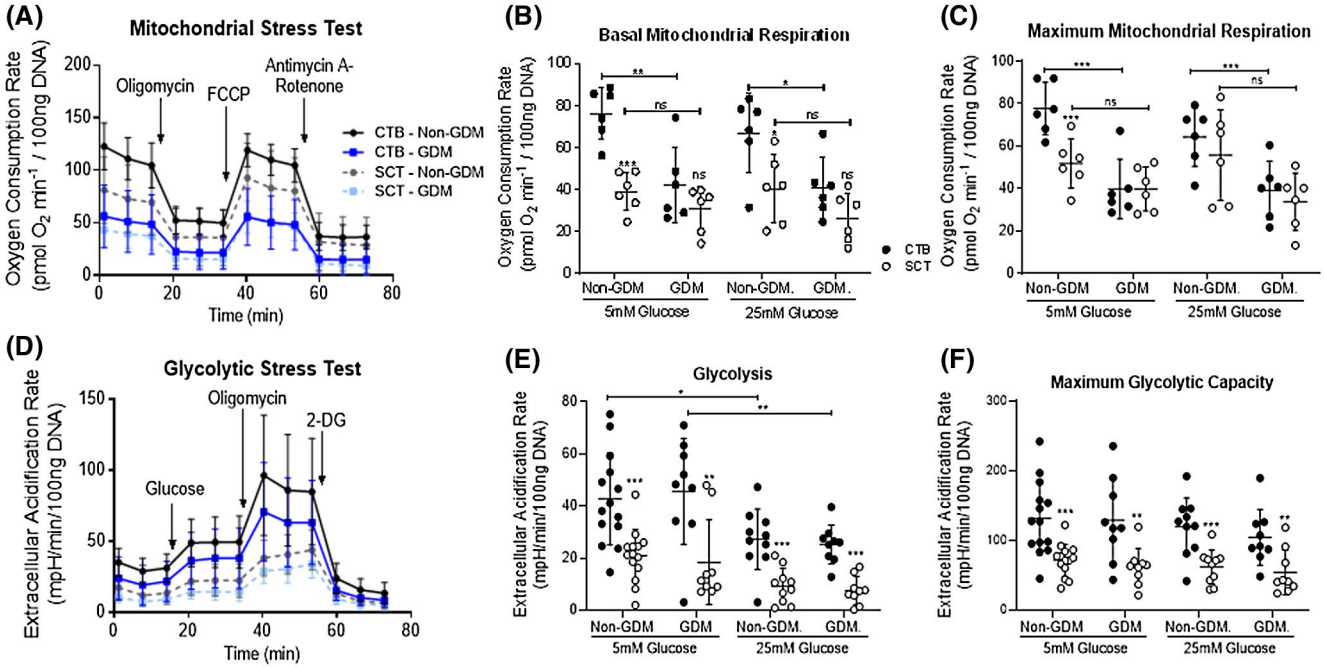

FIGURE 3 Mitochondrial respiration and glycolytic rates from GDM and non-GDM placentas. Human CTB (●) were isolated from term, GDM and non-GDM placentas and studied in vitro before and after differentiation into SCT (○) in 5 mM of glucose and 25 mM of glucose conditions. Oligomycin, FCCP, a mixture of Rotenone and Antimycin A, glucose, or 2-Deoxyglucose were injected to interrogate mitochondrial respiration and glycolytic metabolism using Agilent Seahorse XFe96 Analyzer (A and D). OCR at baseline (B) and maximal mitochondrial respiratory capacity (C) with FCCP was significantly decreased in GDM CTB compared to non-GDM CTB. Hyperglycemic conditions significantly reduced CTB glycolysis as measured by ECAR (E). Already suppressed SCT were not metabolically different between GDM and non-GDM placentas. Triplicates of n = 6-14 placentas for each group. Mean ± SD; CTB [●] vs SCT [○] (asterisk over circles), GDM vs non-GDM (asterisk above bars), 5 mM of glucose vs 25 mM of glucose (asterisk): ***$P < .001$, **$P < .01$, *$P < .05$; ns: non-significant

3.4 | GDM is associated with decreased active mitochondria and ATP production

Active mitochondria are characterized by their electrical potential across the inner mitochondrial membrane, which is the driving force for ATP production. MitoTracker Red, a dye that accumulates in living mitochondria in a membrane potential-*dependent* manner provides a visual indicator of mitochondria activity. Figure 5A,B shows that mitochondria in CTB have greater fluorescent intensity than does SCT, when measured at the same settings. However, mitochondrial activity in CTB from GDM placentas was significantly decreased when compared to non-GDM CTB (53.3 vs 67.7 μm^2/cell; mean difference 14.4 ± 5.8, $P = .021$); no difference was detectable between non-GDM and GDM SCT. The lower mitochondrial activity observed in CTB was not secondary to a difference in the total mitochondrial volume between GDM and non-GDM trophoblasts (Figure 5C) or glycemia (data not shown). As expected from the lower mitochondrial activity, ATP content was lower in GDM compared to non-GDM CTB regardless of glycemia (Figure 5D; 1.1×10^{-7} vs 1.8×10^{-7} nM/ngDNA, $P = .0457$).

3.5 | Fatty acid processing is altered in GDM cytotrophoblasts

Mitochondria import fatty acids to generate the necessary metabolic intermediates for β-oxidation and sustain cellular ATP levels. In most cells, lipid droplets accumulate when mitochondrial beta-oxidation is compromised under conditions where NEFA concentrations are high. LD can mitigate cellular damage by sequestering potentially lipotoxic fatty acids and storing them as non-cytotoxic triacylglycerides.[33,34] CTB from placentas with GDM had greater numbers and larger LD than non-GDM CTB (Figure 6A-C). No differences in LD size were found between GDM or non-GDM SCT but GDM SCT had greater LD number. Using a fluorescent palmitate analog (BODIPY-C_{16}), we found no differences in fatty acid uptake between the non-GDM and GDM CTB and the same was true for SCT regardless of glycemia (data not shown).

4 | DISCUSSION

We tested three hypotheses: in GDM placentas: (a) metabolism would be more suppressed in CTB compared to SCT;

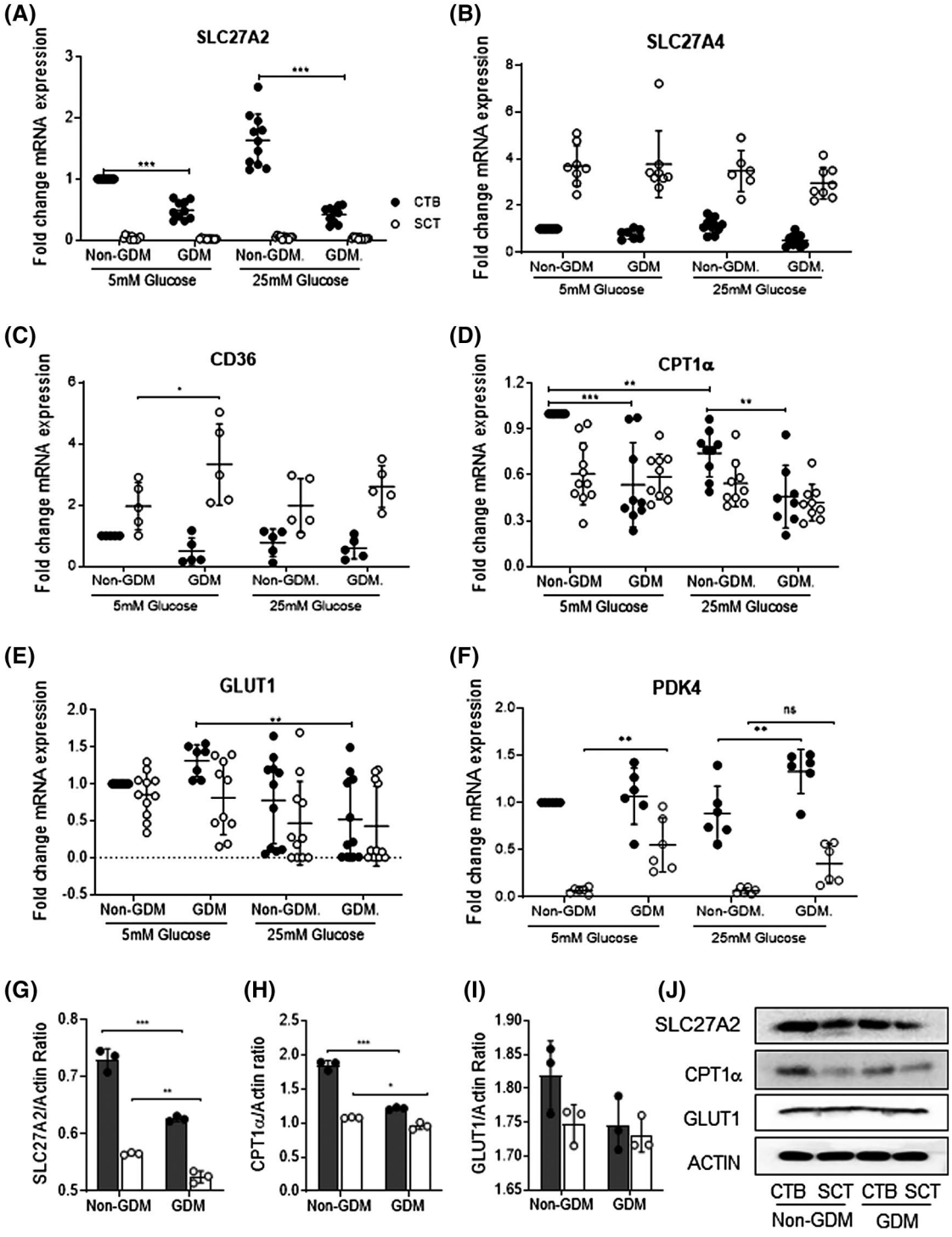

FIGURE 4 GDM affects fatty acid processing. GDM is associated with reduced gene and protein expression for (A, G) *SLC27A2* (primary transporter for long-chain fatty acids) and (D, H) *CPT1α* (essential for fatty acid oxidation) in CTB without significant differences appreciated in *SLC27A4* and *CD36* (B, C). Glucose transport (E; *GLUT1*) expression in CTB from GDM placentas was decreased in conditions of hyperglycemia and PDK4 (F) was increased, suggesting metabolic inflexibility. Duplicates with n = 6 (A, D, E); duplicates with n = 4 (B, C, F); n = 3 (G-J). Mean ± SD; CTB (●) vs SCT (○) (asterisk above ○), GDM vs non-GDM (asterisk over bar), 5 mM of glucose vs 25 mM of glucose (asterisk): ***$P < .001$, **$P < .01$, *$P < .05$; ns: non-significant

(b) that the metabolic rates of CTB and SCT would be further suppressed under conditions of hyperglycemia; and that (c) hyperglycemia would suppress mitochondrial oxidative phosphorylation and lipid processing. We reasoned that decreased oxygen consumption by the placenta would likely affect the cells that were most metabolically active. We had previously shown that CTB are the most metabolically active cell type in the human placenta.[18] In keeping with the first

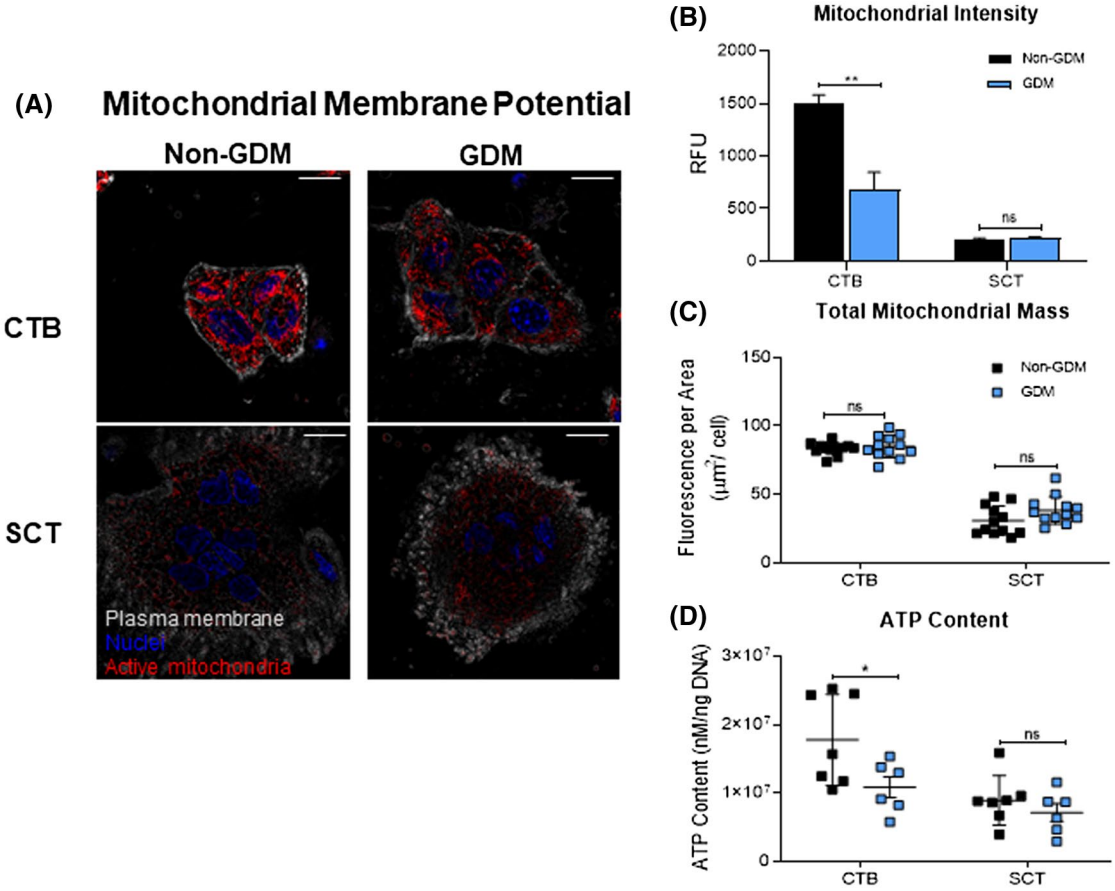

FIGURE 5 Mitochondrial activity is decreased in GDM CTB compared to non-GDM CTB without differences in mitochondrial mass. The active mitochondria in CTB are more intensely fluorescent (increased accumulation with increased membrane potential) than SCT, and GDM CTB demonstrate significantly less active mitochondria than non-GDM CTB (A, B; duplicate with n = 3). Total mitochondrial mass was quantified using MitoTracker Green and no difference was appreciated between GDM and non-GDM trophoblasts (C; duplicate with n = 6). Intracellular ATP content was significantly less in GDM CTB than non-GDM CTB (D; duplicate with n = 3). Mean ± SD; GDM [blue] vs non-GDM [black] (asterisk over bar), CTB vs SCT (asterisk): ***$P < .001$, **$P < .01$, *$P < .05$; ns: non-significant. Scale bar 10 μm

hypothesis, we found that the CTB appears to be responsible for the suppressed oxygen consumption rates in term human trophoblast complicated by GDM. Our hypothesis notwithstanding, we were surprised by the magnitude of oxygen consumption suppression by diabetes in CTB, a cell that is ordinarily highly fuel flexible.[18]

Glucose is the predominant fuel source for the placenta and our previous data showed that CTB from normal placentas use glucose preferentially for energy production and have higher glycolytic capacity than SCT.[18,35,36] We hypothesized that hyperglycemia would further suppress metabolic activity beyond that seen by GDM alone, which was supported by the data. Our current findings show that the glycolytic activity of CTB in both GDM and non-GDM placentas can be maintained under euglycemic conditions but suffer a greater metabolic insult under hyperglycemic conditions. Other cell types such as hepatocytes, pancreatic islet cells, and myocytes have been demonstrated to have decreased glucose uptake, glucose oxidation, and impaired metabolic flexibility under chronic hyperglycemic conditions, which may underlie the progressive cellular dysfunction observed in diabetes and insulin-resistant states.[37,38]

CTB not only continue to have suppressed mitochondrial respiration but also suppressed glycolytic activity under hyperglycemic conditions. A prior study reported increased glucose uptake in GDM SCT with increased *GLUT1* protein in the basal membrane, which was not isolated in our experiments.[39] We found that *GLUT1* expression was downregulated in CTB from GDM placentas under hyperglycemic conditions; others have similar findings in isolated trophoblasts.[40] Moreover, indices of the 2h-OGTT performed mid-pregnancy correlated inversely with oxygen consumption rate of CTB and its hormone production. These findings are profound because CTB from GDM placentas appear to be the primary cell type metabolically affected , which may contribute to the overall suppressed placental function and subsequent adverse outcomes of offspring.

With regard to our third hypothesis, while our data did demonstrate decreased oxidative phosphorylation in CTB

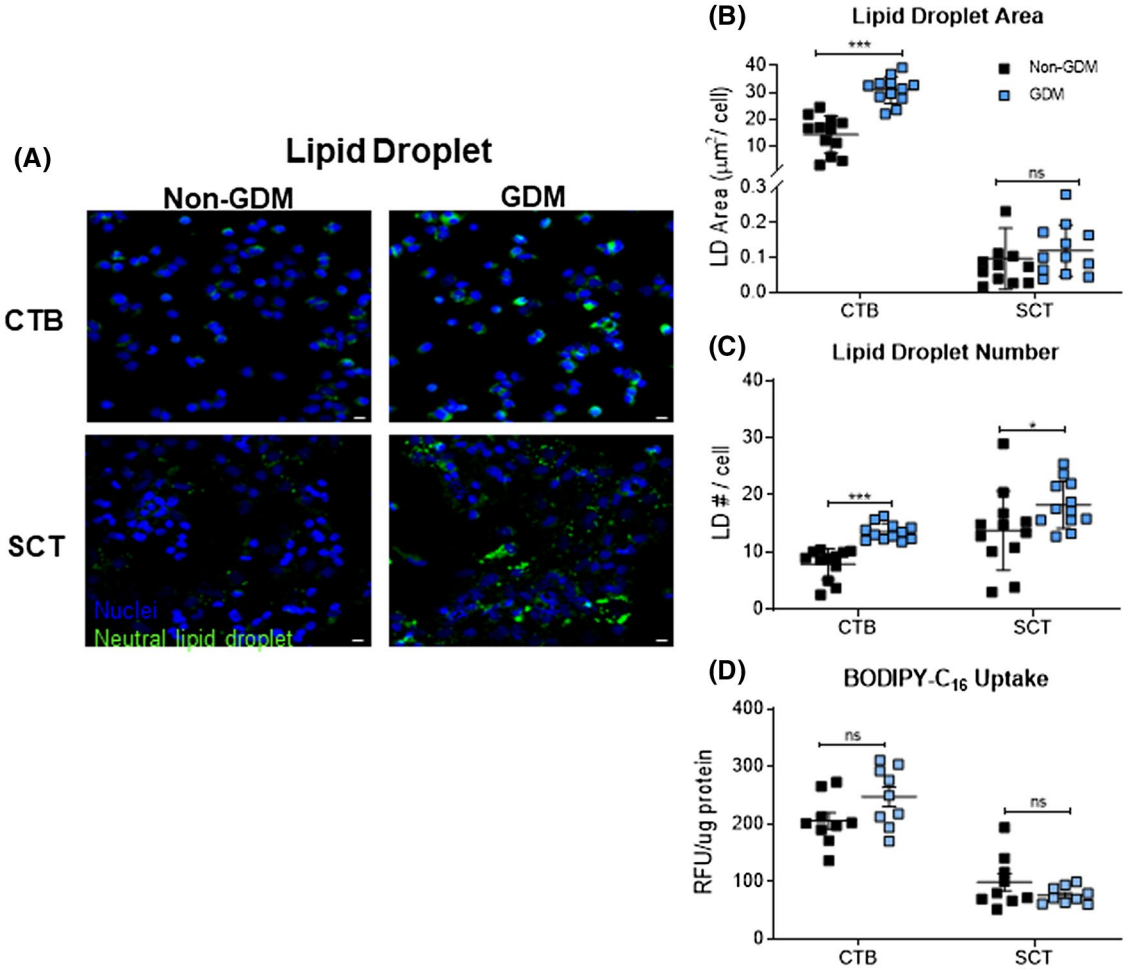

FIGURE 6 Cytotrophoblasts from GDM placentas have increased lipid droplet area and number compared to non-GDM placentas without a change in fatty acid uptake. Lipid droplet area and number were identified using BODIPY-493/503 (A; n = 3) and measured (B andC; duplicate with n = 6) in isolated human, term CTB after 8 and 72 h (SCT). LD size and number were greater in CTB from GDM than non-GDM placentas. BODIPY-C_{16} uptake in to the cells was quantified, normalized to protein and not found to be different between GDM and non-GDM (D; duplicate n = 5). Mean ± SD. GDM [blue box] vs non-GDM [black box] (asterisk over bar), CTB vs SCT (asterisk): ***$P < .001$, **$P < .01$, *$P < .05$; ns: non-significant. Scale bar 10 μm

from GDM mothers, glycemic conditions did not further suppress oxygen consumption rates beyond the effect of GDM alone, in contrast to what we predicted. Our data showed decreased mitochondrial activity and increased LD size and formation in GDM CTB.

It is likely that enlarged lipid droplets signify compromised beta oxidation as seen in other tissues.[41] These findings in CTB have not been previously reported to our knowledge.

The placenta consumes oxygen at a high rate through glycolysis and oxidative phosphorylation as required to generate enough ATP to support the many placental processes required for normal fetal development.[13] Consistent with our previous work, we found that CTB have higher rates of oxygen consumption and glycolysis compared to SCT.[18] CTB are unique in their ability to generate ATP from high rates of glycolysis and oxidative phosphorylation simultaneously. Prior studies have demonstrated decreased mitochondrial function and fatty acid oxidation from isolated trophoblasts and placental explants from GDM placentas.[14,42] However, the present study was designed to differentiate the metabolic contributions of CTB and SCT. Visiedo et al demonstrated that non-GDM trophoblasts have reduced fatty acid oxidation through inhibition of CPT1 under high glucose conditions.[19] In keeping with that finding, we found that CTB from GDM placentas have lower *CPT1α* mRNA and protein expression. This results in a lower capacity for mitochondria to shuttle long-chain free fatty acids into the mitochondrion, which shifts the metabolic dominance to glycolysis.

The placenta plays a role in NEFA esterification, a key step in transporting fatty acids to the fetus.[43] Prior studies have reported increased placental triglyceride content and circulating non-esterified NEFA among persons with GDM.[19,44] Under these conditions, elevated NEFA concentrations drive

increases in lipid accumulation in trophoblast tissues. Lipid accumulation is known to be mildly toxic and has detrimental effects on cellular function in many cell types. CTB have a higher capacity for esterification of NEFA in intracellular lipid pools than do SCT.[21,26] The accumulation of LD in CTB from GDM placentas that we observed is consistent with the poorly functioning mitochondria where beta oxidation is compromised. Our data suggest that LD may provide a lipid buffering system that sequesters NEFA when fatty acid processing or mitochondrial activity is compromised.[45] These findings in placentas from GDM may be important as NEFA and their derivatives play important roles in regulating cellular signaling cascades and contribute to NEFA-driven modulations in metabolism, inflammatory responses, and programed cell death. Potential links between lipid accumulation in the CTB and mitochondrial dysfunction, as suggested in the experiments reported here, have not been investigated.

We acknowledge the limitations of our experimental approach, including the fact that term trophoblast cells were studied in isolation of other cell types in vitro. In our previous studies, we showed that the uptake of long-chain NEFA analog, BODIPY-C_{12} is similar in isolated CTB to those CTB found in placental explants.[21,26] The study of term trophoblast cells does not necessarily apply to trophoblast function at earlier ages. The strengths of the study include the fact that our investigations used only primary, human trophoblast cells from either clinically normal pregnancies or placentas from pregnant persons with GDM requiring insulin therapy. We did not use immortalized cell lines. Studying isolated cell types allows us to investigate metabolic function without the potential influence of other cell type composition differences.

Our findings in this study suggest that the metabolic phenotype of trophoblasts from GDM are significantly different from non-GDM, particularly the CTB. Although the lack of metabolic differences observed in SCT of GDM and non-GDM placentas could be attributable to their longer duration of ex vivo culture, there are increasing levels of hormone expression in both GDM and non-GDM cultured cells that peak at 72 hours, indicating their cellular functional status throughout syncytialization. Moreover, isolated GDM and non-GDM trophoblasts experiments were the same and performed in parallel to reduce the variability of culture conditions and continued to demonstrate differences between metabolic activities and expression of hormone production. Our findings suggest an abnormal CTB differentiation to SCT in GDM pregnancies but our studies were not designed to characterize differentiation.

Although maternal obesity is known to have associated clinical complications similar to GDM, more recent studies have demonstrated that GDM independent of BMI is associated with increased risks for inflammation, lipid abnormalities, and fatty acid oxidation.[19,44,46] Our control group consisted of placentas from non-obese individuals without GDM, which decreases the potential confounding effect of the relative hyperglycemia observed in obese compared to normal weight pregnant persons.[12,47] Prior studies have demonstrated trophoblast metabolism is affected by GDM independent of obesity.[15] We appreciate that sex is a biologic variable that may affect CTB function. Our findings had relatively robust significance with inclusion of both sexes that were similar in number between the two cohorts but the study was inadequately powered to differentiate the roles of sex.

Hyperglycemia is a hallmark for pregnant persons with GDM. We have shown that metabolic function of placental CTB is compromised in individuals with GDM but less evident in SCT. The GDM CTB cells have a lower capacity for mitochondrial oxygen consumption and overall decreased active mitochondria even under euglycemic conditions. Our data suggest that during periods of hyperglycemia the placenta's capacity to produce energy is further compromised with a decreased glycolytic capacity and lower ATP production. Dysregulation of NEFA in GDM CTB suggest detrimental consequences for placental metabolism, inflammatory responses, and cell death programs all known to be associated with lipotoxicity.[48] Metabolic inflexibility, as defined by insufficient glucose utilization and increased fatty acids, is a common feature of diabetes, heart disease, and insulin resistance.[49] An increased PDK4 expression suggests a dysregulation of glucose metabolism, but whether it is the result of abnormal transcriptional upregulation vs a causative factor underlying the metabolic shift cannot be determined by our study design.

GDM is associated with significant perinatal outcomes, including fetal demise, large- and small-for-gestational age infants, cesarean deliveries, preeclampsia, birth injuries, and neonatal hypoglycemia, which are associated with maternal hyperglycemia in addition to other factors such as lipid abnormalities.[6,50,51] Although we understand that placental metabolism is compromised in GDM pregnancies, we can speculate that CTB plays a central role in GDM-related adverse perinatal outcomes. Moreover, our results demonstrate that glycolytic metabolism of trophoblast is compromised under chronic hyperglycemic conditions, supporting the importance of glycemic control during pregnancy for the mother, fetus, and placenta.

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co-authors have a conflict of interest as described above that is relevant to the subject matter or materials included in this work.

CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS

A.M. Valent, K.S. Kolahi, and K.L. Thornburg designed the research; A.M. Valent, K.S. Kolahi, and H. Choi performed the research; A.M. Valent, K.S. Kolahi, and H. Choi analyzed the data; K.L. Thornburg contributed analytic tools; A.M. Valent wrote the paper.

ORCID

Amy M. Valent https://orcid.org/0000-0003-3832-2024

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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