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Research Article

Exercise enhances placental labyrinth trophoblast development by activation of PGC-1α and FNDC5/irisin†

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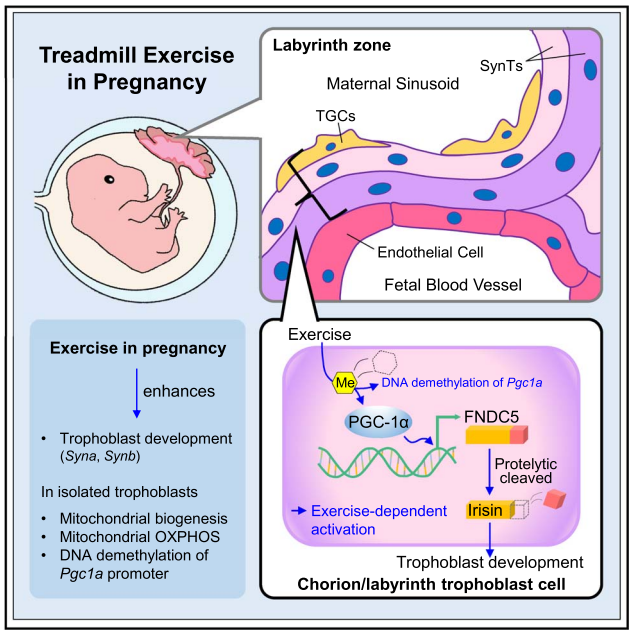
Abstract

Placental chorion/labyrinth trophoblasts are energy demanding which is met by the mitochondrial oxidative phosphorylation. Exercise enhances placental development and mitochondrial biogenesis, but the underlying mechanisms remain poorly understood. To address, female C57BL/6 J mice were randomly assigned into two groups: a control group and an exercise (EX) group. All animals were acclimated to treadmill exercise for 1 week before mating, but only the EX group was subjected to daily exercise during pregnancy from embryonic day (E) 1.5 to E16.5. Placenta were collected at E18.5 for biochemical and histochemical analyses, and primary trophoblast cells were isolated from the E18.5 placenta for further analyses. The data showed that exercise during pregnancy promoted the expression of syncytiotrophoblast cell markers, indicating trophoblast cell differentiation, which was closely associated with elevated mitochondrial biogenesis and oxidative metabolism in the E18.5 placenta. In addition, exercise during pregnancy activated peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α), which was associated with upregulated placental α-ketoglutarate and the expression of isocitrate dehydrogenases and ten-eleven translocations, facilitating DNA demethylation of the *Pgc1a* promoter. Furthermore, exercise upregulated fibronectin type III domain containing 5 expression and the secretion of its cleaved form, irisin, which is known to activate PGC-1α. These data suggest that exercise-induced activation of PGC-1α, via epigenetic modifications, is responsible for promoting mitochondrial energy metabolism and chorion/labyrinth trophoblast development.

Summary Sentence

Exercise during pregnancy induced DNA hypomethylation of *Pgc1a* promoter, which enhances FNDC5/irisin activation, mediating chorion/labyrinth trophoblast development.

Graphical Abstract



Key words: exercise, pregnancy, placenta, trophoblast cell, mitochondria, DNA methylation.

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Key points

- Exercise during pregnancy upregulates the expression of chorion/labyrinth trophoblast cell markers.
- Exercise enhances placental mitochondrial biogenesis and oxidative metabolism in primary trophoblast cells, which is mediated by DNA demethylation of the *Pgc1a* promoter.
- PGC-1α synergizes with FNDC5/irisin to enhance differentiation of primary trophoblast cells.

Introduction

Gestational complications including gestational diabetes mellitus (GDM) and preeclampsia are associated with suppressed placental vascularization [1, 2]. Placental insufficiency results in intrauterine growth retardation, leading to fetal growth restriction and offspring metabolic dysfunction [3–6]. Placental dysfunction and insufficiency occur due to abnormal trophoblast invasion and impeded labyrinth chorion development in the early stage of pregnancy [7, 8]. Thus, proper labyrinth trophoblast development is required for the fetoplacental development [9].

Placental trophoblast development requires energy which is provided via the mitochondrial Krebs cycle and glycolysis [10–12]. GDM predisposes mitochondrial dysfunction in placental trophoblast cells [13]. Peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α in protein or *Pgc1a* in gene) is a coactivator of transcription factors related to mitochondrial biogenesis and activity, and it regulates the expression and activation of irisin, a cleaved form of fibronectin type III domain containing 5 (FNDC5), known as a key hormone stimulating placental trophoblast differentiation [14], whereas the mechanisms underlying the role of PGC-1α and irisin axis in regulating exercise-mediated placental trophoblast development remain unclear.

Epigenetic modifications dynamically occur during the placental development [12, 15, 16], which include DNA (de)methylation, histone modifications, and non-coding RNAs, playing important roles in proper fetoplacental development [12, 15–17]. For example, ablation of DNA methyltransferases (DNMTs), including DNMT1 and DNMT3L, in the placenta results in chorioallantoic fusion defects and labyrinth malformation [18, 19]. Exercise during pregnancy induces DNA demethylation of the *Pgc1a* promoter in the fetus [20–22], and the PGC-1α-irisin axis is dynamically upregulated by exercise training [23]. However, exercise-responsive epigenetic modifications in the *Pgc1a* promoter in the placenta have not been investigated. The activation of PGC-1α and irisin in the labyrinth trophoblast cells in response to exercise remains to be examined.

In this study, we hypothesized that exercise during pregnancy improves placental trophoblast development, which is associated with enhanced placental mitochondrial biogenesis and oxidative metabolism. In particular, we examined DNA demethylation of the *Pgc1a* promoter in the placenta due to exercise, together with increased FNDC5/irisin expression, which enhances mitochondrial biogenesis and oxidative metabolism, facilitating placental chorion/labyrinth development.

Materials and methods

Ethics

All animal work was conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities followed by the Animals in Research: Reporting In Vitro Experiments guidelines [24] was approved by the Institutional Animal Care and Use Committee at the Washington State University (Protocol #ASAF 6704) and University of Maryland School of Medicine (Protocol #AUP-00000151).

Mice

Eight-week-old female C57BL/6 J mice (Jackson Laboratory, Bar Harbor, ME, USA) were randomly assigned into two groups: (1) a control (CON) group and (2) an exercise (EX) group. All animals were acclimated to treadmill exercise training for 1 week before mating. Then, female mice were mated with age-matched male C57BL/6 J mice fed on a chow diet, and mating was determined by the presence of vaginal smear. Animals were housed on a reverse 12-h dark/light cycle and fed ad libitum with a rodent control diet (D12450J, Research Diets, New Brunswick, NJ, USA).

Treadmill exercise training protocol

The treadmill exercise protocol was followed by the principles of progressive loading in exercise intensity and duration based on our previous studies [21, 25]. Briefly, 1 week before mating, acclimation to treadmill exercise was performed, followed by the intensity (exercise duration and speed) for 10 min (10 m/min), three times per week. After mating, EX group performed treadmill exercise training during pregnancy from embryonic day 1.5 (E1.5) to E16.5 every morning. Based on our exercise protocol for pregnant mice [25], training was separated into three stages: E1.5–E7.5 (intensity: 40% of VO_2max), E8.5–E14.5 (65% of VO_2max), and E15.5–E16.5 (50% of VO_2max). Each bout of exercise included warming up (5 m/min for 10 min), main exercise (10 to 14 m/min for 40 min), and cool down (5 m/min for 10 min). As a CON group, pregnant mice of CON were placed on a flat treadmill that did not move for an hour every morning the same as EX group.

Tissue collection

Two days after the last bout of treadmill exercise, E18.5 placental samples were collected and stored at −80°C for analyzing gene and protein expression or 4% paraformaldehyde (PFA) solution for histological analysis based on our previous study [26].

Primary trophoblast cell culture

After removing the decidua, placentas with decidua removed were utilized for isolating trophoblast cells from the E18.5 placenta as previously described [27]. Briefly, fresh placentas were placed in ice-cold dissociation buffer containing collagenase type IA after sample collection immediately and minced coarsely with scissors. After a 45-min incubation in a 37°C bath, undigested material was removed via a 100 μm cell strainer, and cells were centrifuged (500 × *g*; 5 min), washed, and then placed in a Percoll solution by mixing Percoll:10×

medium 199:wash solution (9:1:13) to isolate trophoblast cells. High speed centrifugation (30 000 × *g*; 30 min; 4°C) was then employed to yield trophoblast giant and syncytiotrophoblast (SynT) layer I and II cells [27, 28], which were transferred to new tubes, washed, and centrifuged (500 × *g*; 5 min). Then, the cells were resuspended and cultured in NCTC-135 culture medium (#N3262, MilliporeSigma, Burlington, MA) at 37°C. After at least 72 h of culture, differentiated cells were stored for further biochemical analyses. Primary trophoblast cells were high purity and viability, which were determined by the expression of cytokeratin-7 and β-hCG and by counting over 80% of cytokeratin-7 positive cells, as described in the previous study [27, 29].

Gene expression

The TRIzol reagent (Invitrogen, Grand Island, NY, USA) was utilized for extracting total RNA from homogenized frozen tissues. cDNA was synthesized using reaction and reverse transcriptase of iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). mRNA expression was quantified using PowerUp SYBR Green Master Mix (Applied Biosystems, Waltham, MA) and a real-time quantitative PCR machine (QuantStudio 3, Applied Biosystems). *18S* rRNA and/or *36B4* were utilized for normalization and mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method, as described in our previous studies [21, 26, 30, 31]. Primer information is listed in Table 1.

Western blotting

Lysis buffer (100 mM Tris–HCl pH 6.8, 2.0% SDS, 20% glycerol, 0.02% bromophenol blue, 5% 2-mercaptoethanol, 100 mM NaF, and 1 mM Na_3VO_4) was used for protein isolation. The concentration of protein lysates was measured by the Bradford methodology (Bio-Rad) based on our previous study [21, 26, 30]. In the primary antibodies, OxPhos cocktail (#45–8099, RRID:AB_2533835) antibody was purchased from Thermo Fisher Scientific (Rockford, IL, USA). E-cadherin (#610181, RRID:AB_397580) was purchased from BD Biosciences (San Jose, CA, USA). Monocarboxylate transporter 4 (MCT4) (#22787–1-AP, RRID:AB_11182479), PGC-1α (#66369–1-IG, RRID:AB_2828002), FNDC5 (#23995–1-AP, RRID:AB_2879394), β-tubulin (#66240–1-IG, RRID:AB_2881629), and β-actin (#66009–1-IG, RRID:AB_2687938) were also purchased (Proteintech, Rosemont, IL, USA). StarBright Blue520 goat anti-rabbit IgG (#12005869, RRID:AB_2884949) and StarBright Blue700 goat anti-mouse IgG (#12004158, RRID:AB_2884948) secondary antibodies (dilution 1:5000) were purchased from Bio-Rad. ChemiDoc MP Imaging System (Bio-Rad) was used for detecting the target proteins as previously described [30, 32]. Uncropped western blot images are provided in Supplementary Figure 1.

Methylated DNA immunoprecipitation

Twenty μg total DNA was extracted from isolated trophoblast cells using lysis buffer (20 mM Tris–HCl, 4.0 mM EDTA, 20 mM NaCl, 1% SDS) and proteinase K solution (20 mg/ml; in the base solution of 50 mM Tris–HCl, 10 mM $CaCl_2$). The DNA was diluted using tris-EDTA (TE) buffer, then sonicated. Antibodies (5hmC, #51660, RRID:AB_2799398; 5mC, #28692, RRID:AB_2798962; and IgG, #7054, RRID:AB_2099235) were purchased from Cell Signaling Technology (Danvers, MA, USA) and added into denatured DNA. The DNA-antibody complexes were pulled

Table 1. Primer sequences used for qPCR analysis.

Gene Name	Forward/ Reverse	Primer sequence
Cdx2	F	5′-AGTGAGCTGGCTGCCACACT-3′
	R	5′-GCTGCTGCTGCTTCTTCTTGA-3′
Esrrb	F	5′-AGTACAAGCGACGGCTGG-3′
	R	5′-CCTAGTAGATTCGAGACGATCTTAGTCA-3′
Elf5	F	5′-ATTCGCTCGCAAGGTTACTCC-3′
	R	5′-GGATGCCACAGTTCTCTTCAGG-3′
Prl2c2	F	5′-AACGCAGTCCGGAACGGGG-3′
	R	5′-TGTCTAGGCAGCTGATCATGCCA-3′
Gjb3	F	5′-GGGATGGTACACAGGGCTTCT-3′
	R	5′-CATCTCCTTTCTTTCACTGTGCTTT-3′
Ctsq	F	5′-AATTGGCTATGGTTATGTGGGA-3′
	R	5′-TCACACAGTAGGGTATTGGG-3′
Synb	F	5′-TCCGGAAAGGGACCTGCCCA-3′
	R	5′-CAGCAGTAGTGCGGGGTGCC-3′
Syna	F	5′-CCTCACCTCCCAGGCCCCTC-3′
	R	5′-GGCAGGGAGTTTGCCCACGA-3′
Plgf	F	5′-TGAAGGCATGTAGAGGGGAC-3′
	R	5′-CACTCTGCCTGTGTTCCAGA-3′
Igf2	F	5′-GGAGACATACTGTGCCACCC-3′
	R	5′-CTCACCCCACATTGCAGAATTAC-3′
Igfbp1	F	5′-TGTTTCTTGGCCGTTCCTGA-3′
	R	5′-GAGAAATCTCGGGGCACGAA-3′
Igfbp7	F	5′-GCGAGCAAGGTCCTTCCATA-3′
	R	5′-GTTCTGTCCGCTGAACTCCA-3′
Fgf2	F	5′-GGCTGCTGGCTTCTAAGTGT-3′
	R	5′-GTCCCGTTTTGGATCCGAGT-3′
Fgfr2	F	5′-CACGACCAAGAAGCCAGACT-3′
	R	5′-CTCGGCCGAAACTGTTACCT-3′
Cd36	F	5′-TGAATGGTTGAGACCCCGTG-3′
	R	5′-TAGAACAGCTTGCTTGCCCA-3′
Cpt1a	F	5′-TCGCTCATTCCGCCGC-3′
	R	5′-GAGATCGATGCCATCAGGGG-3′
Cpt2	F	5′-TTCTGCAGTGCGGTTTCTGA-3′
	R	5′-GTGTCACTTCTGGCAGGGTT-3′
Acadm	F	5′-AGGGTTTAGTTTTGAGTTGACGG-3′
	R	5′-CCCCGCTTTTGTCATATTCCG-3′
Acaa2	F	5′-GAACGAAGCTTTTGCCCCTC-3′
	R	5′-CTTTCCACCTCGACGCCTTA-3′
Hadh	F	5′-CCATCTTTGCCAGCAACACG-3′
	R	5′-GCACGGGGTTGAAAAAGTGG-3′
mt*Cox1*	F	5′-ATGTTCTATCAATGGGAGC-3′
	R	5′-TCTGAGTAGCGTCGTGGT-3′
mt*Cox2*	F	5′-AGACGAAATCAACAACCC-3′
	R	5′-GGAAGTTCTATTGGCAGA-3′
mt*Atp6*	F	5′-AACCTGGTGAACTACGAC-3′
	R	5′-GATGTTACTGTTGCTTGAT-3′
mt*Atp8*	F	5′-ATGCCACAACTAGATACAT-3′
	R	5′-TAGTGATTTTGGTGAAGG-3′
mt*Nd1*	F	5′-CACTATTCGGAGCTTTACG-3′
	R	5′-TGTTTCTGCTAGGGTTGA-3′
mt*Nd2*	F	5′-ACAACCCATCCCTCACTC-3′
	R	5′-ATTTTGGTAAGAATCCTGTT-3′
mt*Nd3*	F	5′-CCTAACGCTAATTCTAGTTG-3′
	R	5′-GACGTGCAGAGCTTGTAG-3′
mt*Nd4*	F	5′-TCCTCAGTTAGCCACATAGCA-3′
	R	5′-AGGCAGAATAGGAGTGATGATG-3′
mt*Nd5*	F	5′-ATAACCGCATCGGAGACA-3′
	R	5′-TGGTAGTCATGGGTGGAG-3′
mt*Nd6*	F	5′-ACAAAGATCACCCAGCTA-3′
	R	5′-GGAGTTATGTTGGAAGGA-3′
Pgc1a	F	5′-CCATACACAACCGCAGTCGC-3′
	R	5′-GTGGGAGGAGTTAGGCCTGC-3′
Pparg	F	5′-GCATGGTGCCTTCGCTGA-3′
	R	5′-TGGCATCTCTGTGTCAACCATG-3′

(*Continued*)

Table 1. Primer sequences used for qPCR analysis.

Gene Name	Forward/ Reverse	Primer sequence
Tfam	F	5′-CCAAAAAGACCTCGTTCAGC-3′
	R	5′-CTTCAGCCATCTGCTCTTCC-3′
Nrf1	F	5′-GCACCTTTGGAGAATGTGGT-3′
	R	5′-CTGAGCCTGGGTCATTTTGT-3′
Cox7a1	F	5′-CAGCGTCATGGTCAGTCTGT-3′
	R	5′-AGAAAACCGTGTGGCAGAGA-3′
Idh1	F	5′-ACATGCATATGGGGACCAATACAGA-3′
	R	5′-TTCAAAGTCATGTACCATGTATGTCACC-3′
Idh2	F	5′-GCAGTTCATCAAGGAGAAGCTCATC-3′
	R	5′-CACACTTGACAGCCACACTGTACTTCT-3′
Idh3	F	5′-TCAAGGAAGTGTTCAAGGCTGCTG-3′
	R	5′-GATGGCAACTTTGTTCTCCTTCATG-3′
Tet1	F	5′-CACCCTGTGACTGTGATGGAGGTA-3′
	R	5′-ACTATCTTCTCAATCCGGATTGCCTT-3′
Tet2	F	5′-AAGGATGCAATCCAGACAAAGATGAA-3′
	R	5′-TTTAGCAATAGGACATCCCTGAGAGCT-3′
Tet3	F	5′-GAACTCATGGAGGATCGGTATGGA-3′
	R	5′-CAGCTTCTCCTCCAGTGTGTGTCTT-3′
Fndc5	F	5′-ATGAAGGAGATGGGGAGGAA-3′
	R	5′-GCGGCAGAAGAGAGCTATAACA-3′
Apln	F	5′-GTTTGTGGAGTGCCACTG-3′
	R	5′-CGAAGTTCTGGGCTTCAC-3′
Bdnf	F	5′-CTCAGGCAGAATGAGCAATG-3′
	R	5′-AGCCGTCTGTGCTCTTCACT-3′
Il6	F	5′-CTGATGCTGGTGACAACCAC-3′
	R	5′-TTTTCTGCAAGTGCATCATCGT-3′
Il15	F	5′-GAGGCCAAGAAGAGTTCTGGAT-3′
	R	5′-TGCCCAGGTAAGAGCTTCAA-3′
Sparc	F	5′-GGCCCGAGACTTTGAGAAGA-3′
	R	5′-AATGTTCCATGGGGATGAGG-3′
Adipoq	F	5′-GCACTGGCAAGTTCTACTGCAA-3′
	R	5′-GTAGGTGAAGAGAACGGCCTTGT-3′
Pgc1a-MeDIP A	F	5′-CAGGAGATTTGAGTTATTATGTGAGCA-3′
	R	5′-TGAAGTAACGCTTAGAGAGAGAGGAA-3′
Pgc1a-MeDIP B	F	5′-TTCCTCTCTCTCTAAGCGTTACTTCA-3′
	R	5′-CTTACTACAGTCCCCAGTCACATGA-3′
Pgc1a-MeDIP C	F	5′-TCATGTGACTGGGGACTGTAGTAAG-3′
	R	5′-CCAGCTCCCGAATGACGCCA-3′
36B4	F	5′-TCCAGGCTTTGGGCATCA-3′
	R	5′-CTTTATCAGCTGCACATCACTCAGA-3′
18S	F	5′-GTAACCCGTTGAACCCCATT-3′
	R	5′-CCATCCAATCGGTAGTAGCG-3′

down with EcoMag beads (#MJA-102, Bioclone Inc., San Diego, CA, USA). The immunoprecipitated DNA was used for qPCR quantification using PowerUp SYBR Green Master Mix and the QuantStudio 3 machine (Applied Biosystems). Relative enrichment folds were normalized to that of IgG, and primer information is listed in Table 1.

Gas chromatography–mass spectrometry

Homogenized frozen tissues were utilized for extracting proteins using lysis buffer (20 mM Tris–HCl, 4.0 mM EDTA, 20 mM NaCl, and 1% SDS). Proteins were used for gas chromatography–mass spectrometry analysis, as described in our previous study [21].

Histological analysis

Placental samples fixed in the 4% PFA solution for 24 h were embedded in paraffin. About 5-μm-thick sections of placentas were used for immunocytochemical (ICC) staining, as described in our previous studies [30, 31]. Primary antibodies for PGC-1α (#66369–1-IG, RRID:AB_2828002, dilution 1:50, Proteintech) and CDH1 (#610181, RRID:AB_397580, dilution 1:50, BD Biosciences) were used. For the secondary antibodies, anti-mouse IgG1 Alexa 488 (#406626, RRID:AB_2715989, dilution 1:250, BioLegend, San Diego, CA, USA) and anti-mouse IgG2a, κ Alexa 594 (#400280, dilution 1:250, BioLegend) were used. Images were captured using a Nikon Eclipse Ni microscope (Nikon Instrument Inc., NY, USA).

Statistical analysis

Data were presented as mean ± s.e.m. and visualized by Prism 9 (GraphPad Software, San Diego, CA, USA). Student *t*-tests between the CON and experimental groups were performed using SPSS Statistics Version 21 (IBM Corp., Armonk, NY, USA). The significance was statistically defined as $P < 0.05$, and the number of samples and *P* values for each measurement is indicated in each figure legend.

Results

Exercise during pregnancy enhances placental chorionic development

We previously demonstrated that maternal obesity impedes vascularization in placental labyrinth zone, and this adverse outcomes due to maternal obesity were prevented in response to exercise during pregnancy, leading to facilitating fetal growth [26]. We also showed that placental angiogenesis and nutrient activity are associated with mitochondrial biogenesis and development [30]. To explore whether exercise develops placental labyrinth zone-specific cell-type, we analyzed gene expression related to placental cell types, based on the developmental process of placental cells (Figure 1A). Exercise during pregnancy may increase the stem-cell pool, exhibited by higher expression of *Cdx2* (Figure 1B). In addition, the expression levels of *Syna* and *Synb*, as SynT layer cell markers showing trophoblast differentiation into SynT cells, were higher in response to exercise during pregnancy (Figure 1B). Consistent with gene expression of SynT cell markers, the protein levels of MCT4, as a SynT marker, and E-cadherin, as an epithelial cell marker, and gene expression of placental growth factor (*Plgf*) were upregulated by exercise training (Figure 1C and D). By contrast, exercise in pregnancy did not affect the expression of trophoblast giant cell (TGC)/glycogen cell (GlyT) lineage markers, including *Prl2c2*, *Gjb3*, and *Ctsq* (Figure 1B). Together, exercise promotes the expression of SynT gene and protein markers, which might well contribute to the labyrinth formation of the placenta.

Maternal exercise enhances placental mitochondrial biogenesis and oxidative metabolism

Exercise rescues the impairment of placental mitochondrial biogenesis and oxidative metabolism due to maternal obesity [30]. To test whether exercise directly enhances mitochondrial biogenesis and oxidative metabolism, gene expression and protein levels related to mitochondriogenesis and

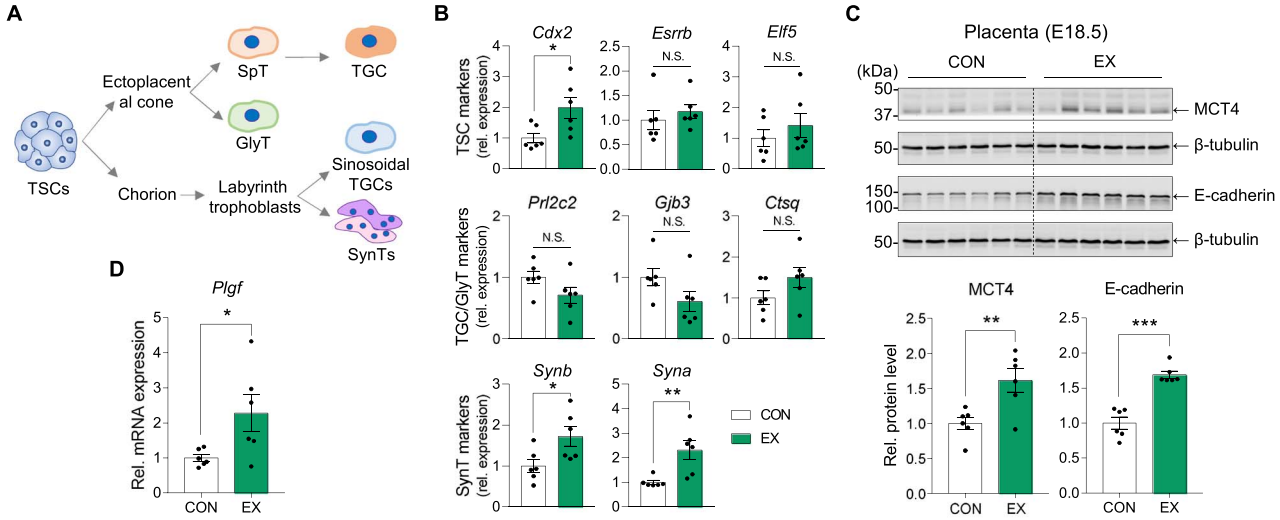

Figure 1. Exercise in pregnancy enhances placental chorionic development. (A) A schematic diagram of placental cell development. (B) Gene expression of TSC, TGC/GylT, and SynT layer I and II markers ($n = 6$). (C) Cropped western blot images (up) and relative levels (down) of MCT4 and E-cadherin (β-tubulin were used for normalization; $n = 6$/group). (D) mRNA expression of *Plgf* ($n = 6$). Data are presented as the mean ± SEM, and each dot represents one litter. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by unpaired Student *t*-test (B–D).

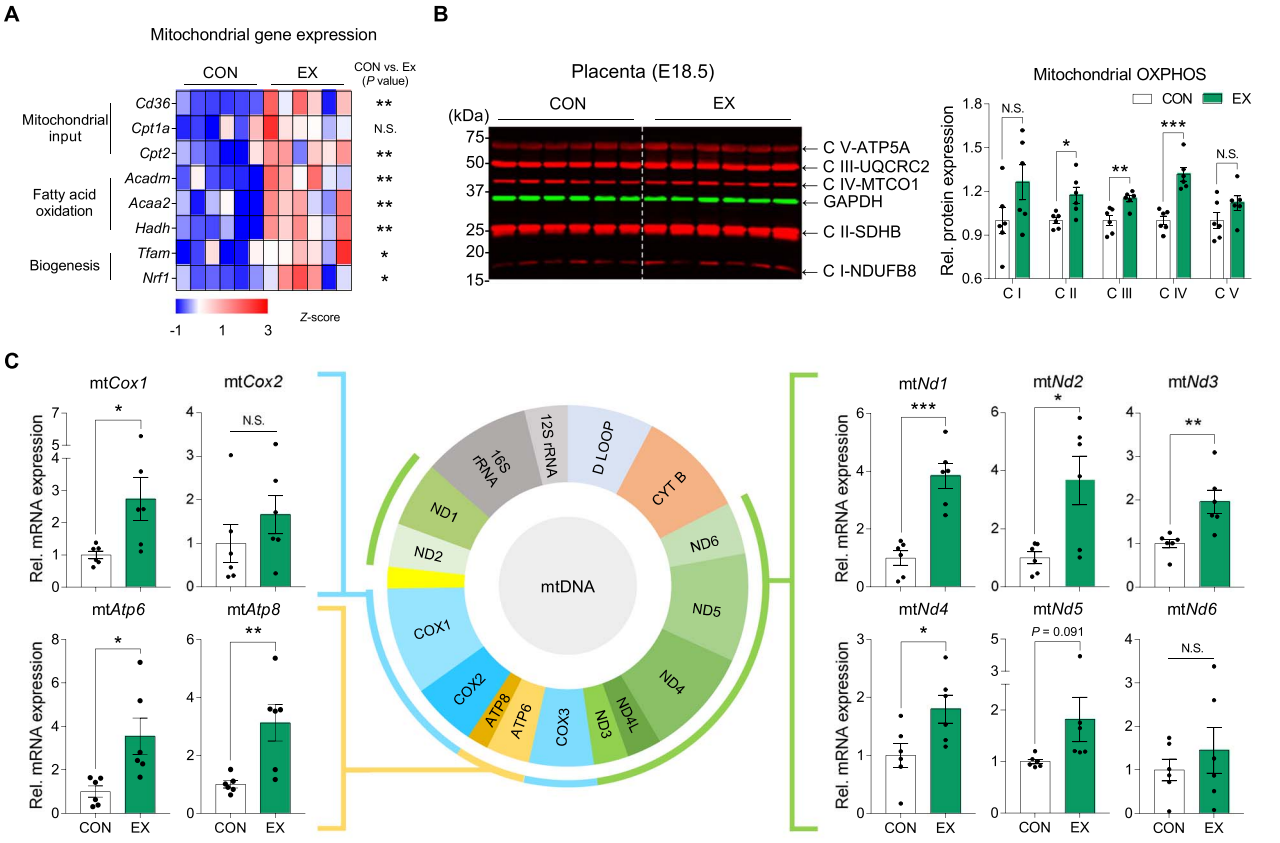

Figure 2. Exercise expresses placental mitochondrial biogenic and encoding genes. (A) Gene expression of placental mitochondrial input (*Cd36*, *Cpt1a*, *Cpt2*), fatty acid oxidation (*Acadm*, *Acaa2*, *Hadh*), and biogenesis (*Tfam*, *Nrf1*) in response to exercise ($n = 6$). (B) Cropped western blot images (left) and relative means (right) of OXPHOS at post intervention (GAPDH was used for normalization; $n = 6$). (C) Mitochondrial DNA (mtDNA) gene expression in the placenta following exercise during pregnancy ($n = 6$). Data are presented as the mean ± SEM, and each dot represents one litter. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by unpaired Student *t*-test (A–C).

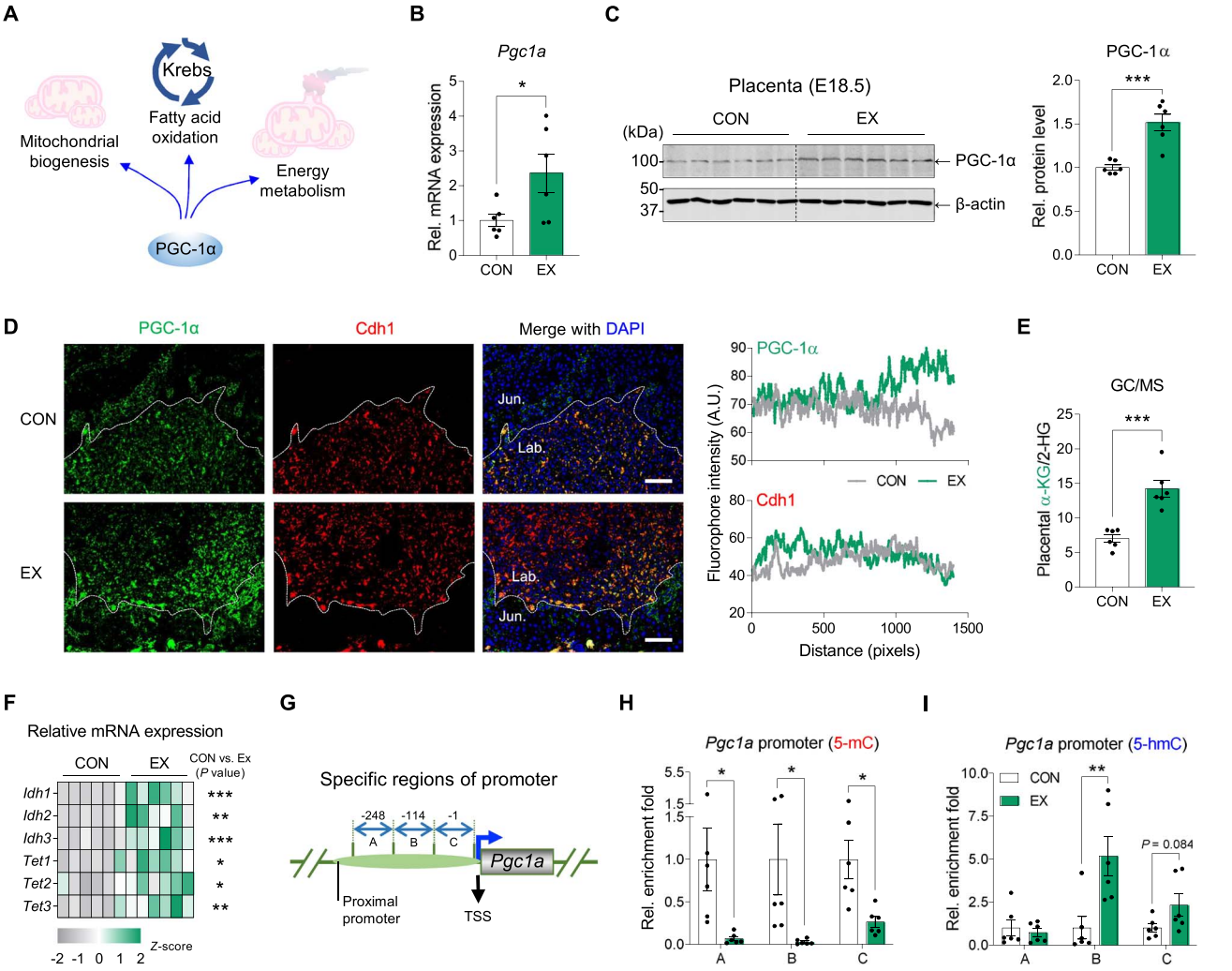

Figure 3. Exercise in pregnancy enhances PGC-1α expression by DNA hypomethylation. (A) A diagram showing the role of PGC-1α in mitochondrial biogenesis, fatty acid oxidation, and energy metabolism. mRNA expression of *Pgc1a* (B) and western blots of PGC-1α (C) in response to exercise (β-actin was used for normalization; $n = 6$). (D) Representative images of ICC staining (left) and fluorophore intensity (right) of PGC-1α and CDH1 in response to exercise. (E) The ratio of α-KG to 2-hydroxyglutarate (2-HG) in E18.5 placenta ($n = 6$). (F) mRNA expression of IDHs and TETs in E18.5 placenta ($n = 6$). (G) Diagram of three regions of the *Pgc1a* proximal promoter. (H, I) 5-methylcytosine (5-mC; H) and 5-hydroxymethylcytosine (5-hmC; I) enrichment fold of E18.5 placenta ($n = 6$). Data are presented as the mean ± SEM, and each dot represents one litter. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by unpaired Student *t*-test (B–I).

oxidative phosphorylation (OXPHOS)/metabolism were analyzed. Exercise in pregnancy increased the expression of mitochondrial input genes (*Cd36* and *Cpt2*), fatty acid oxidative marker genes (*Acadm*, *Acaa2*, and *Hadh*), and OXPHOS protein markers (Figure 2A and B), indicating enhanced placental mitochondrial biogenesis and respiration. Consistently, mitochondrial encoding genes related to the mitochondrial respiration chain and OXPHOS were highly expressed in exercised placentas (Figure 2C). These data showed that exercise in pregnancy enhanced mitochondrial biogenic genes and oxidative metabolism in the placenta.

Placental labyrinth development is associated with epigenetically activated DNA demethylation in the *Pgc1a* promoter by activation of α-ketoglutarate and ten-eleven translocations

Consistent with placental mitochondrial biogenesis in response to exercise during pregnancy, PGC-1α, as a metabolic co-activator responsible for mitochondrial biogenesis, fatty acid oxidation, and energy metabolism, was upregulated at both transcriptional and translational levels following exercise in pregnancy (Figure 3A–C). In addition, PGC-1α positive cells were histochemically increased in exercised placentas (Figure 3D). To test relevant mechanisms underlying epigenetic modifications, we analyzed α-ketoglutarate (α-KG) and ten-eleven translocations (TETs), and respective DNA (de)methylation of the *Pgc1a* promoter. α-KG, as an intermediate of the Krebs cycle, is a co-factor required for TET-mediated DNA demethylation [33–36]. We previously demonstrated that maternal exercise increased α-KG levels in fetal brown fat and muscle, which were consistent with the enhanced mitochondrial biogenesis in the respective tissues [21, 22]. Consistent with our previous discoveries, we found that exercise training increased placental α-KG levels (Figure 3E) and the gene expression of isocitrate dehydrogenases (IDHs) (Figure 3F), which catalyze the reaction from isocitrate to α-KG. Furthermore, we showed that exercise in

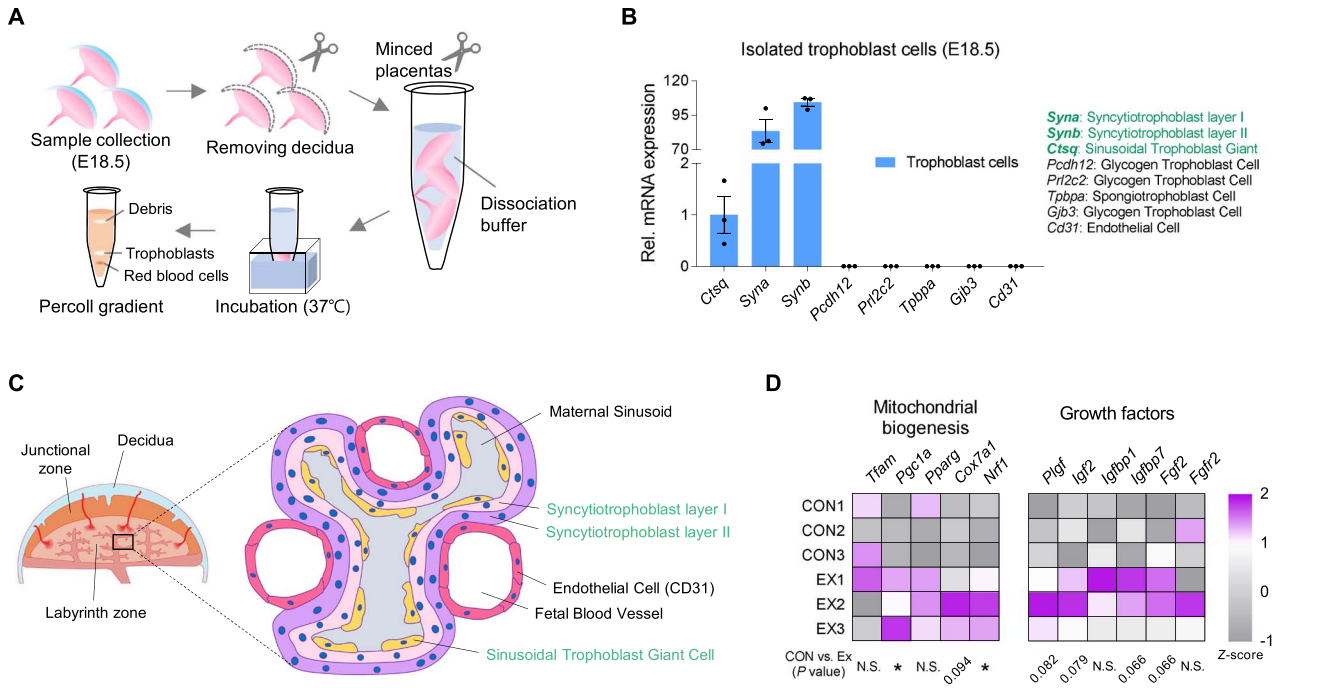

Figure 4. Placental labyrinth trophoblast cells mediate mitochondrial biogenesis and development in response to exercise in pregnancy. (A) The procedure isolating placental trophoblast cells. mRNA expression of placental cell type-specific markers (B) and structure of placental labyrinth zone mainly consisted to sinusoidal trophoblast giant and SynT cells (C). (D) Gene expression of mitochondrial biogenesis (*Tfam*, *Pgc1a*, *Pparg*, *Cox7a1*, *Nrf1*) and growth factors (*Plgf*, *Igf2*, *Igfbp1*, *Igfbp7*, *Fgf2*, *Fgfr2*) in E18.5 placenta ($n = 6$). Data are presented as the mean ± SEM, and each dot represents one litter. $^*P < 0.05$ by unpaired Student *t*-test (D).

pregnancy elevated the expression of TET genes (Figure 3F) and enhanced DNA demethylation (5-hmC) but reduced DNA methylation (5-mC) in the three promoter regions (A, B, and C) of *Pgc1a* gene of the placenta (Figure 3G–I). Together, these data strongly suggest that exercise during pregnancy induces epigenetic modifications of the *Pgc1a* promoter, which activate PGC-1α and mitochondrial biogenesis in the placenta.

Mitochondrial biogenesis and placentokine secretion in the isolated placental labyrinth trophoblasts of exercised mice

Given that maternal exercise expressed labyrinth trophoblast cell markers (Figure 1B) and PGC-1α histochemical presence at the labyrinth zone (Figure 3D), we isolated trophoblast cells from E18.5 placentas and measured developmental markers during trophoblast cell differentiation (Figure 4A). Isolated labyrinth trophoblast cells are composed of sinusoidal trophoblast giant and SynT cells (Figure 4B and C). To test whether placental mitochondrial biogenesis is regulated in the placental trophoblast cells of exercised mice, we measured mitochondriogenic gene expression, including *Tfam*, *Pgc1a*, *Pparg*, *Cox7a1*, and *Nrf1*. We found that trophoblast cells isolated from E18.5 placentas of exercised mice had elevated expression of *Pgc1a* and *Nrf1* genes (Figure 4D). Because molecular mediators generated from placental labyrinth trophoblast cells can be delivered into the fetal circulation to regulate fetal development [21, 37], we further analyzed exercise-induced biomarkers in the isolated primary trophoblast cells. Exercise during pregnancy dynamically activated gene expression of *Fndc5*, brain-derived neurotrophic factor (*Bdnf*), and secreted protein acidic and rich in cysteine (*Sparc*) (Figure 5B). Together, mitochondria in the placental labyrinth trophoblasts of exercised mice are activated due to exercise, which is associated with elevated expression of FNDC5.

Exercise during pregnancy induces DNA demethylation of PGC-1α promoter and activates FNDC5/irisin

A previous study showed that placental trophoblast differentiation is promoted by irisin, known as a cleaved form of FNDC5 [23], via AMP-activated protein kinase (AMPK) activation [14]. Consistent with previous studies, FNDC5/irisin was elevated in the E18.5 placenta and isolated trophoblast cells of exercised mice (Figure 5A and B) Also, our previous study showed that exercise during pregnancy activated AMPK and respective downstream pathways [26]. As a mediator to activate FNDC5 gene expression, in the *Pgc1a* promoter, DNA methylation (5-mC) was reduced, and DNA demethylation (5-hmC) was elevated in the isolated trophoblast cells from the E18.5 placenta of exercised mice (Figure 5C). Taken together, these data suggest that exercise during pregnancy enhances PGC-1α expression via DNA demethylation and induces FNDC5/irisin activation, potentially leading to chorion/labyrinth trophoblast development (Figure 5D).

Discussion

Maternal physiological conditions, including obesity, GDM, and undernutrition, have profound impact on placental and fetal development [26, 38, 39]. As a therapeutic tool, exercise in pregnancy improves placental angiogenesis and nutrient delivery [26, 40]. In the current study, we observed that exercise during pregnancy plays an essential role in placental trophoblast formation and differentiation through increasing

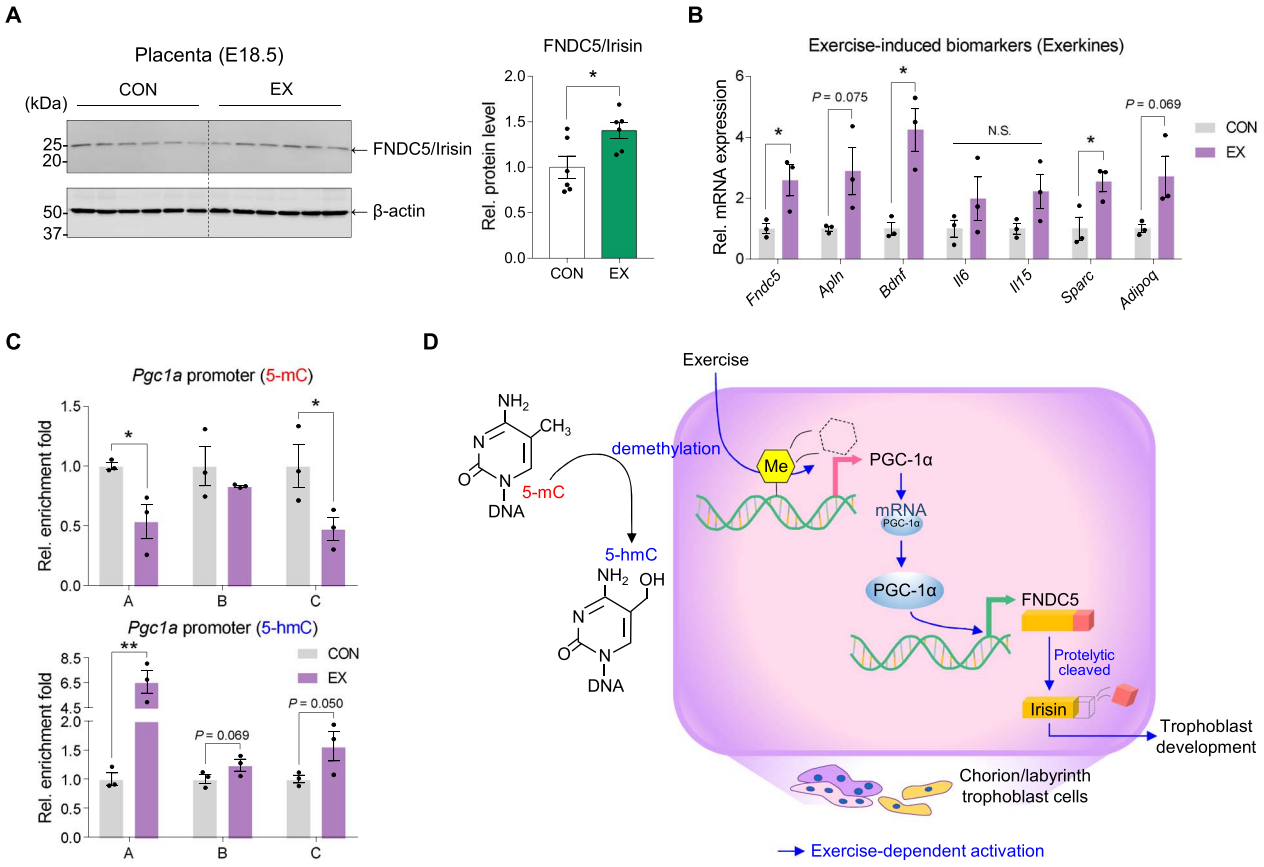

Figure 5. Exercise-induced placental PGC-1α expression via DNA demethylation coactivates FNDC5 expression. (A) Cropped western blot images (left) and relative levels (right) of FNDC5/irisin in the E18.5 placenta ($n = 6$) (β-actin was used for normalization). (B) Gene expression of exercise-induced biomarkers in the isolated placental trophoblast cells ($n = 3$). (C) 5-mC (up) and 5-hmC (down) relative enrichment fold of trophoblast cells isolated from E18.5 placenta ($n = 3$). (D) A suggested schematic diagram. Data are presented as the mean ± SEM, and each dot represents one litter. $^*P < 0.05$ by unpaired Student *t*-test (A–C).

mitochondrial biogenesis and oxidative metabolism. Of note, mitochondrial biogenesis in placental trophoblast cells was highly associated with PGC-1α activation, which was associated with a decrease of DNA methylation in the *Pgc1a* promoter. Furthermore, exercise-induced PGC-1α activation was concomitant with the activation of FNDC5/irisin, potentially promoting placental chorion/labyrinth trophoblast formation and differentiation.

The placenta comprises several cell types, including sinusoidal trophoblast giants, SynTs, glycogen trophoblasts, spongiotrophoblasts (SpTs), and endothelial cells [11, 41]. Trophoblast stem cells (TSCs) are precursors of the differentiated placental cells, which are derived from the polar trophectoderm or extraembryonic ectoderm by fibroblast growth factor 4 (FGF4) [42]. The ectoplacental cone, generated by the proliferation of the polar trophectoderm in the blastocyst, forms the junctional zone of the placenta, leading to differentiation into TGCs, SpTs, and glycogen trophoblast cells [43]. In addition, TSCs also differentiate to SynTs during early embryogenesis, which potentially facilitates labyrinth layer development [44].

Mitochondrial biogenesis is essential for healthy fetoplacental growth [45]. Of note, recent studies suggested that the activation of placental mitochondrial biogenesis and OXPHOS predisposes normal placentation, emphasizing the importance of placental mitochondrial function [46, 47]. For example, fatty acids (FAs) can be utilized by placenta [48] for fatty acid oxidation [49]. Absorbed FAs are transported into mitochondria through the acyl-carnitine shuttle system, including carnitine palmitoyltransferase 1 (CPT1) and CPT2, which are oxidized into acetyl-CoA through β-oxidation and utilized for the Krebs cycle [50]. However, mitochondrial dysfunction occurs in placental trophoblast cells under pathophysiological conditions, including GDM [13]. On the other hand, exercise has been recognized to enhance mitochondrial activity and energy metabolism [51]. Consistently, we previously showed that exercise in pregnancy protected the adverse effects of obesity in placental mitochondrial biogenesis and oxidative metabolism [30].

Epigenetic modifications include DNA methylations, histone modifications, miRNAs, and non-coding RNAs (ncRNAs) [16, 52]. Based on the previous studies, α-KG is required for TET-mediated DNA demethylation and is a rate limiting factor [33, 53]. Furthermore, 2-hydroxyglutarate (2-HG), known as an α-KG antagonist, may accumulate due to mutations of IDHs which commonly occurs in cancer cells, leading to an increase in DNA methylation [54]. Consistent with the importance of exercise in epigenetic modifications [55], a recent study showed that recreational physical activity pre-pregnancy and during pregnancy alters placental DNA methylation [56].

Exercise-induced PGC-1α-irisin axis has been recognized as a key signaling pathway in enhancing brown adipogenesis and

browning white adipose tissue via upregulation of uncoupling protein 1 [23]. Several studies have demonstrated that irisin, a cleaved form of FNDC5, has a key role in fetoplacental development and healthy pregnancy [57, 58]. Fundamentally, the *Fndc5* gene is highly expressed in the human placenta, and circulating irisin levels are elevated during the entire pregnancy [57]. Moreover, irisin enhances placental trophoblast cell differentiation mediated by the AMPK activation [14].

In conclusion, we discovered that exercise during pregnancy facilitates placental trophoblast development, which is closely related to mitochondrial biogenesis and oxidative metabolism in the placenta. Furthermore, DNA demethylation of the *Pgc1a* promoter in the placenta activates *Pgc1a* gene expression, which was concomitant to the of FDNC5/irisin activation. Thus, maternal exercise during pregnancy enhances placental labyrinth chorionic development, improving placental function and healthy fetoplacentation and pregnancy.

Authors' contributions

S.A.C. and J.S.S. designed, performed experiments, analyzed, interpreted the data, wrote the manuscript, and edited the manuscript. M.D. and M.J.Z. designed, interpreted the data, and edited the manuscript.

Conflict of interest

The authors have declared that no conflict of interest exists..

Data availability

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

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