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Exercise prevents the adverse effects of maternal obesity on placental vascularization and fetal growth.

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Abstract

More than one third of pregnant women in the USA are obese and maternal obesity (MO) negatively affects fetal development, which predisposes offspring to metabolic diseases. The placenta mediates nutrient delivery to fetuses and its function is impaired due to MO. Exercise ameliorates metabolic dysfunction due to obesity, but its effect on placental function of obese mothers has not been explored. Here C57BL/6J female mice were randomly assigned into two groups fed either a control or a high-fat diet (HFD), and then the mice of each diet were further divided into two sub-groups with/without exercise. In HFD-induced obese mice, daily treadmill exercise during pregnancy reduced body weight gain, lowered serum glucose and lipid concentration, and improved insulin sensitivity of maternal mice. Importantly, maternal exercise prevented fetal overgrowth (macrosomia) induced by MO. To further examine preventive effects of exercise on fetal overgrowth, placental vascularization and nutrient transporters were analyzed. Vascular density and the expression of vasculogenic factors were reduced due to MO but recovered by maternal exercise. On the other hand, the contents of nutrient transporters were not substantially altered by MO or exercise, suggesting that the protective effects of exercise in MO-induced fetal overgrowth were primarily due to alteration of placental vascularization and improved maternal metabolism. Furthermore, exercise enhanced down-stream insulin signaling and activated AMP-activated protein kinase in HFD placenta. Taken together, maternal exercise prevented fetal overgrowth induced by MO, which were associated with the improved maternal metabolism and placental vascularization in obese mothers with exercise.

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Author contributions

M.D. planned and designed the study with contribution from J.S.S. The authors participated in the animal work (J.S.S., X.L., Q.T., L.Z., Y.C., Y.H., and J.D.), data analyses (J.S.S., and M.D.), and data interpretation (J.S.S., S.A.C., M-J.Z., and M.D.). J.S.S was involved in the writing, and M.D. reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

Maternal exercise; maternal-fetal exchange; nutrient transport; insulin resistance

Introduction

Obesity and sedentary life style are major risk factors for metabolic syndrome and complications, including cardiovascular disease, type 2 diabetes mellitus, and certain types of cancers (Hruby *et al.*, 2016; Kerr *et al.*, 2017; Leiva *et al.*, 2017). Currently, over 30% of pregnant women in the United States are obese and with additional one third are overweight (Ogden *et al.*, 2012), which predisposes their children to obesity and metabolic diseases (McCurdy *et al.*, 2009; Vogt *et al.*, 2014). Therefore, interventions which can prevent or reduce the development of metabolic dysfunction in offspring of obese mothers are imperative.

The placenta performs nutrient/oxygen exchanges between the mother and her fetuses, and syncytiotrophoblasts which mediate nutrient/gas exchange have high oxygen consumption rates (Carter, 2000) and energy expenditure (Jones & Rolph, 1985). The placental function is impaired due to maternal obesity (MO), which impedes vasculogenic/angiogenic development of the placenta (Stuart *et al.*, 2018). Obesity and HFD intake further upregulate placental nutrient transporters such as GLUT1 and GLUT3, and stimulate placental mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, inducing placental and fetal overgrowth (Aye *et al.*, 2015). Moreover, offspring, overgrown during fetal development (macrosomia) due to maternal obesity, had impaired glucose tolerance, elevated fasting insulin level, and increased adiposity and metabolic diseases (Stanford *et al.*, 2017; Barbour & Hernandez, 2018). Thus, maintaining proper vasculogenesis and placental function in obese mothers is the critical step for preventing metabolic dysfunction in the offspring (Bairagi *et al.*, 2016).

Exercise during pregnancy is known to be beneficial for both maternal health and fetal development (Gregg & Ferguson, 2017; Stanford *et al.*, 2017). However, the beneficial effects of exercise on placental vascularization and nutrient transport have not been examined. Exercise may facilitate placental development through activating AMP-activated protein kinase (AMPK), a key kinase increasingly implicated in cell differentiation and tissue development (Kaufman & Brown, 2016). In addition, exercise stimulates muscle to secrete myokines including apelin, and apelin mediates placental vasculogenesis and its deficiency is linked to preeclampsia (Gilbert, 2017; Ho *et al.*, 2017). Moreover, exercise affects the down-stream signaling pathways of insulin and insulin-like growth factors, including mTORC1 signaling (Ogasawara *et al.*, 2014), as well as suppresses low-grade inflammation associated with obesity (Ringseis *et al.*, 2015). In the current study, we found that maternal exercise improved maternal metabolic health and prevented fetal overgrowth induced by maternal HFD; we further found that the enhanced placental vasculogenesis and improved maternal metabolic health might explain the beneficial effects of maternal exercise to fetal development.

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Materials and Methods

Ethical Approval.

All animal procedures have been conducted in accordance with the guidelines of the National Institutes of Health (NIH) and according to the protocol approved by the Institute of Animal Care and Use Committees (IACUC) at Washington State University. The investigators understand the ethical principles under which the *Journal of Physiology* operates and that their work complies with the animal ethics checklists (Grundy, 2015).

Animals and diets.

Eight-week-old C57BL/6J female mice (The Jackson Laboratory, Bar Harbor, ME, USA) were randomly assigned into two groups (n = 12 /group) fed *ad libitum* either a control diet (10% energy from fat, D12450J, Research Diets, New Brunswick, NJ, USA) or an high-fat diet (60% energy from fat, D12492, Research Diets) for 8 weeks to induce obesity. When the dams in the HFD group had gained over 25% of their initial body weight (Fig 2A), showing HFD-induced obesity (Aye *et al.*, 2015; Yang *et al.*, 2016). At one week preconception, the diets were changed to a control diet (CON, 10% energy from fat, D12450J, Research Diets) or an obesogenic diet (HFD, 45% energy from fat, D12451, Research Diets). Female mice were mated with eight to ten-week-old male mice (The Jackson Laboratory) fed with regular chow. The mating was determined by examining vaginal smears. Additionally, both maternal groups were divided into two subgroups with/without exercise during gestation (n = 6 for each treatment). During treatments, food and water were provided *ad libitum*, and body weight and food intake were monitored daily. At embryonic day 18.5 (E18.5), after 5 h of fasting, pregnant mice were anesthetized by carbon dioxide inhalation and euthanized by cervical dislocation, consistent with previous reports (Zou *et al.*, 2017). Placenta was collected after removing uterine tissue, umbilical cord, and other extraembryonic membranes. All female and male fetuses were collected and studied, following the fundamental principles for animal models (Dickinson *et al.*, 2016) and the animals in research: reporting *in vivo* experiments (ARRIVE) guidelines (Kilkenny *et al.*, 2012). Mice were housed at 22°C on 12 h-light/12 h-dark cycles (Figure 1).

Endurance treadmill exercise.

To customize with exercise, all mice were subjected to flat treadmill exercise at 10 m/min for 10 min, 3 times per week before mating. Then, the maximal oxygen consumption rates (VO_2max) for control and obese mice were measured using treadmill respiratory measurement system (Oxymax Fast 4 lane modular treadmill system, Columbus Instruments, Columbus, OH, USA). The exercise intensity for CON and HFD mice was set based on VO_2max rates, which are commonly used in previous studies (Petrosino *et al.*, 2016). Briefly, based on the gestation stages, maternal mice were separated into three periods, E1.5 to E7.5, E8.5 to E14.5, and E15.5 to E16.5. The VO_2max rates were measured on E1.5, E8.5, and E15.5. The exercise intensity was set as follows, 40% (E1.5 to E7.5), 65% (E8.5 to E14.5), and 50% (E15.5 to E16.5) of VO_2max, based on the exercise guidelines in pregnancy (Zavorsky & Longo, 2011). Each exercise regimen was composed of three steps, warming up (5 m/min for 10 min), exercise (10 to 14 m/min for 40 min), and cooling down (5 m/min for 10 min), which were performed at the same time every morning.

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The treadmill speeds during the exercise step were set to 11/14/12.5 m/min for E1.5-E7.5/E8.5-E14.5/E15.5-E16.5 respectively in CON+EX mice and 10/13/12 m/min for HFD+EX mice. Sedentary CON and HFD mice were placed on the treadmill for an hour daily with the speed set at 0 m/min. Mice were not subjected to exercise between E16.5 to E18.5 (two days before euthanization) in order to avoid the acute effects of exercise on samples collected.

Metabolic studies.

Two days before tissue collection (E16.5), the metabolic rates of oxygen consumption (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER), and heat production were analyzed during 24 h (light: quiescent phase / dark: active phase) using an indirect open circuit calorimetry system (Comprehensive Lab Animal Monitoring System [CLAMS], Columbus Instruments, Columbus, OH, USA). During measurement, mice were fed *ad libitum* with the respective diets and water provided (Wang *et al.*, 2017). The fat and carbohydrate oxidation was calculated as previously described (Peronnet & Massicotte, 1991).

Thermal imaging.

Surface temperatures were measured using an E6 Thermal Imaging Infrared Camera (FLIR Systems, Wilsonville, OR, USA) before euthanization at E18.5.

Hand grip strength.

A grip strength meter (Columbus Instruments, Columbus, OH, USA) was used to measure the maximal grip strength (average of 5 times measurement). In addition, the endurance grip strength was assessed by sequentially performing 10 times of measurements.

Serum analysis.

Before mating, blood samples were collected from tail tip following 5 h fasting. At E18.5, following 5 h fasting, maternal blood was further collected through cardiopuncture under deep anesthesia *via* carbon dioxide inhalation, and used for measuring glucose and insulin levels. Moreover, fetal blood was collected from fetuses through the retroorbital sinus into the conical tube for centrifuge. Blood glucose was measured using blood glucose monitoring system, and insulin was measured using the mouse high range insulin enzyme-linked immunosorbent assay (ELISA; ALPCO, Salem, NH, USA). The homeostatic model assessment of insulin resistance (HOMA-IR) and pancreatic β-cell function (HOMA-%B) were calculated according to the following formula: HOMA-IR [Fasting insulin in μU/mL × (fasting blood glucose in mg/dL × 0.055)/22.5] and HOMA-%B [(360 × fasting insulin in μU/mL)/(fasting blood glucose – 63)] (Matthews *et al.*, 1985; Ghasemi *et al.*, 2015; Lemaitre *et al.*, 2018).

Gene expression.

The experimental approach was consistent with the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin *et al.*, 2009). Briefly, at E18.5, the placenta was collected and frozen in liquid N_2 and stored at −80°C until analysis. Total RNA was isolated by TRIzol reagent (Invitrogen, Grand Island, NY, USA), and cDNA

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was synthesized using extracted mRNA with the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Relative mRNA expression was determined by quantitative real-time PCR (IQ5, Bio-Rad) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), and *18S* rRNA was used as a reference gene for normalization (Yang *et al.*, 2016). Primer sequences are listed in Table 1.

Immunoblotting analysis.

Proteins were extracted from collected frozen placental tissues at −80°C using 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), and the protein concentration of lysates was determined by the Bradford Assay (Bio-Rad). The following antibodies were used for the detection of phosphorylation and total protein levels: phosphorylated insulin receptor substrate 1 [p-IRS-1 (Ser789); 2389S], insulin receptor substrate 1 (IRS-1; 3407S), p-Akt (Ser473; 9271), phosphorylated AMPKα [p-AMPKα (Thr172); 2535], AMPKα (2532), phosphorylated mammalian target of rapamycin [p-mTOR (Ser2448); 2971], p-4E-BP1 (Thr37/46; 9459), 4E-BP1 (9452), p-Erk½ (Thr202/Tyr204; 4370), Erk½ (4695), GLUT1 (12939), tumor necrosis factor α (TNF-α; 3707), interleukin-1β (IL-1β; 12242), and interleukin-6 (IL-6; 12153), all of which were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Akt (sc-5298), mTOR (sc-517464), vascular endothelial growth factor (VEGF; sc-7269), endogenous ligand for the G-protein coupled APJ receptor (APLN; sc-293441), and hypoxia-inducible factor 1α (HIF1α; sc-13515) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). β-Actin and β-tubulin were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). IRDye 800CW goat anti-rabbit secondary antibody (1:10,000) and IRDye 680 goat anti-mouse secondary antibody (1:10,000) were purchased from LI-COR Biosciences (Lincoln, NE, USA). Immunoblotting analysis was performed using an Odyssey infrared imaging system (Li-COR Biosciences) for detecting target proteins.

Histological analyses.

Fresh placenta tissues were fixed for 24 h at room temperature in PBS containing 4% paraformaldehyde, and then embedded in paraffin. The tissue sections at 5 μm-thickness were prepared for hematoxylin and eosin (H&E) staining or immunocytochemical (ICC) staining following deparaffinization as previously described (Wang *et al.*, 2017). For measuring the ratio of cross-sectional area (CSA) of the junctional/labyrinth zones and CSA of the placenta, each zone was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). For ICC staining, sections of labyrinth zone were heated in Tris-EDTA buffer for 20 min, blocked within 5% goat serum in Tris-buffer saline (TBS) containing 0.3% Triton X-100 for 2 h. Then, samples were incubated with purified anti-mouse CD34 primary antibody (#119301; 1:100; BioLegend, San Diego, CA, USA) or a mouse monoclonal endothelial nitric oxide synthase (eNOS) primary antibody (sc-136977; 1:50; Santa Cruz Biotechnology, Dallas, TX, USA) overnight and Alexa Fluor 488 anti-rat IgG secondary antibody (#405418:1,000; BioLegend) for 1 h. Sections were mounted with a mounting medium (Vector Laboratories, Burlingame, CA, USA), and fluorescence was examined using an EVOS® FL color Imaging System (Mill Creek, WA, USA). Oil Red O staining was performed on placenta cryosections, as previously described (Louwagie *et al.*,

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2018). Placenta samples including labyrinth zone were excised, fixed with paraformaldehyde and embedded in OCT (Thermo 6502, Thermo Fisher Scientific, Waltham, MA, USA). Tissue sections at 10 μm-thickness were prepared and stained with Oil Red O. Sections were mounted with a mounting medium (Vector Laboratories, Burlingame, CA, USA), and images were obtained using an EVOS® XL Core Imaging System (Mill Creek, WA, USA).

Data presentation and statistical analyses.

Data were analyzed and visualized using GraphPad Prism 7 for Windows (GraphPad Software, San Diego, CA, USA), and expressed as the mean ± s.e.m. Statistical analyses were performed using one- or two-way ANOVA (diet×exercise), with Bonferroni post hoc analysis (SAS Institute Inc., Cary, NC, USA). *P* values less than 0.05 (* or #), 0.01 (** or ##) or 0.001 (*** or ###) were statistically different.

Results

Effects of maternal obesity and exercise intervention on maternal characteristics.

An overview of the experimental schematic diagram for the assignment of diets, preconception treatments, exercise, and experimental analyses is shown in Fig. 1. Before mating, HFD group had higher body weight, in accordance with higher caloric intake (Fig. 2A,B). Consistently, the fasting glucose, insulin, and HOMA-IR were significantly elevated in HFD mice (Fig. 2C–F), consistent with previous reports (Fu *et al.*, 2016; Zou *et al.*, 2017). After mating, HFD mice with exercise training gained less weight compared to HFD without exercise, despite of no difference in food intake (Fig. 2G–I). At E18.5, maternal mice were sacrificed following 5 h fasting. The weights of inguinal white adipose tissue (ingWAT) and gonadal WAT (gonWAT) were higher in HFD mice, which was mitigated by exercise (Fig. 2J). On the other hand, exercise intervention during gestation increased the weight of intrascapular brown adipose tissue (iBAT), but HFD did not affect iBAT weight (Fig. 2J). Blood glucose, insulin, and HOMA-IR in HFD-induced obese mothers were higher than those of control mothers; on the other hand, exercise training alleviated these changes (Fig. 2K–M). For HOMA-%B index, which indicates the insulin secretion by pancreatic β cells (Matthews *et al.*, 1985), was decreased only in maternal mice fed with a control diet and with exercise (Fig. 2N), suggesting exercise stimulated insulin-independent utilization of glucose.

Exercise training improves muscle strength and energy expenditure in maternal mice challenged with HFD.

Maternal obesity and HFD lead to a loss of aerobic exercise capacity including reduced total exercise time and distance, but the maximal oxygen consumption during a single bout of exhaustive exercise was not altered (Fig. 3A–C). In addition, relative maximal grip strength and endurance grip strength were significantly reduced due to HFD but recovered following exercise (Fig. 3D,E). At E15.5, we further measured oxygen and CO_2 consumption, and the respiratory ratio. HFD reduced oxygen and CO_2 consumption, which was recovered in HFD mice with exercise. On the other hand, no significant changes in VO_2 and VCO_2 between CON and CON with exercise mice were detected (Fig. 3G,H). The HFD profoundly

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decreased the respiratory exchange ratio, showing increased utilization of fatty acids, which was not altered by exercise (Fig. 3I). Quantitatively, HFD increased the fat oxidation and decreased carbohydrate oxidation, but exercise training increased the carbohydrate oxidation of HFDEX mice in the dark cycle (Fig. 3K,L). These data were consistent with earlier reports (Romijn *et al.*, 1993; Schrauwen *et al.*, 2000). Moreover, the surface temperature was increased in HFD mice with exercise (Fig. 3F).

Exercise intervention of obese mothers prevents fetal overgrowth.

Placental weight of obese mice did not differ from control mice, but it was higher in exercised mice (Fig. 4A). HFD increased the fetal body weight at E18.5, which was alleviated by exercise intervention (Fig. 4B). There was no difference in the litter sizes among groups (Fig. 4C). Placental efficiency, as determined by fetal weight divided by the weight of placenta, increased by HFD, consistent with a previous report (Jones *et al.*, 2009), but decreased by exercise training (Fig. 4D). The mRNA expression of nutrient transporters was also analyzed. The mRNA expression of lipoprotein lipase (*Lpl*) and *Cd36* (fatty acid transporter) was higher, and that of glutamine transporter *Snat1* and amino acid transporter *Snat2* was lower in HFD compared to CON mice at E18.5, which were reversed by exercise training (Fig. 4H). Consistently, blood glucose, insulin, and insulin resistance were elevated in fetuses from HFD mothers without exercise, which were alleviated in both female and male fetuses of HFD mothers with exercise (Fig 4E–G). However, no differences between female and male fetuses in glucose and insulin levels were observed (Fig. 4E,F). Additionally, the positive correlations in the blood glucose and insulin levels between mothers and fetuses were observed (Fig. 4J–M). To explore whether increased expression of fatty acid transporters led to lipid accumulation in the placenta, we performed Oil Red O staining. The amount of lipid droplets was higher in HFD placenta, which was prevented through maternal exercise training (Fig. 4I). Placental inflammation during pregnancy affects placental development and function, which induces fetal abnormal growth (Howell & Powell, 2017). The contents of inflammatory cytokines, TNF-α and IL-1β in the placental tissue were increased in HFD mice, while exercise down-regulated their levels. On the other hand, the protein level of IL-6 was reduced in obese mice while exercise increased its content (Fig. 4N). Unlike TNF-α and IL-1β, IL-6 has both anti-inflammatory and inflammatory effects dependent on the context, and exercise is known to elevate IL-6 levels (Petersen & Pedersen, 2005).

Exercise intervention reverses impaired placental vascularization in obese mice.

Placental vascularization is critical for its proper function (Ho *et al.*, 2017). The area of junctional zone, which contains spongiotrophoblast cells, was decreased by HFD and the area of labyrinth zone was increased due to HFD, which were reversed by exercise training (Fig. 5A–D,I,J). The CD31, CD34, and eNOS are markers of endothelial cells (Hu *et al.*, 2016; Lacko *et al.*, 2017). Based on immunohistochemical CD34 and eNOS staining, and immunoblotting of CD31 of E18.5 placenta, the vascular density was reduced in HFD but recovered by exercise training (Fig. 5K–M). Recently, the APLN, also known as apelin, was identified as a potent mediator of placental vascularization (Ho *et al.*, 2017), and the apelin content was decreased due to HFD but highly upregulated following exercise training (Fig. 5O). In agreement, factors regulating angiogenesis, including VEGF, VEGFR1, and hypoxia

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induced factor 1α (HIF1α) were dramatically increased by exercise training except CCN family member 1 (CCN1) which is an extracellular matrix-associated angiogenic inducer (Mo *et al.*, 2002), and glial cells missing gene (GCM1) which is a chorion-specific transcription factor. (Fig. 5N,O).

Maternal exercise training enhances AMPK, mTORC1, and insulin signaling in the placenta.

AMPK is a master regulator of cell metabolism activated by exercise (Niederberger *et al.*, 2015), which is a critical mediator of remodeling in many tissues (Fu *et al.*, 2016; Yang *et al.*, 2016; Yao *et al.*, 2017; Zhu *et al.*, 2018). In addition, a negative correlation of placental AMPK phosphorylation in relation to body mass index (BMI) and birth weight was reported (Jansson *et al.*, 2013). In this study, AMPK phosphorylation was downregulated in the placenta of obese mice, while exercise dramatically upregulated the phosphorylation of AMPK (Fig. 6C). On the other hand, growth factors, including insulin-like growth factor 2 (IGF-2) and fibroblast growth factor 2 (FGF2), stimulate mTORC1 signaling to enhance placental development; in addition, mTORC1 and AMPK function as nutrient and energy sensors, controlling protein synthesis (Kim *et al.*, 2002; Hardie, 2014; Carroll & Dunlop, 2017). In the present study, the mRNA expression of growth factors, *Fgf2* and *Frfr2,* was profoundly decreased in HFD group, but recovered by exercise (Fig. 6A). No differences in *Igf2* and *Igfbp1* mRNA expression were found. (Fig. 6A). As the downstream mediators of growth factor-initiated signaling, Akt phosphorylation was suppressed in the HFD placenta, but recovered by exercise (Fig. 6D). Consistently, phosphorylation of mTOR and 4E-BP1 was elevated in EX and HFD+EX mice, but no differences between CON and HFD mice were observed. (Fig. 6B). The impairment of IRS-1 and Erk phosphorylation in HFD mice was also reversed by exercise (Fig. 6D). Collectively, these data demonstrated that maternal exercise intervention activated AMPK and enhanced mTORC1 mediated growth signaling which were attenuated in response to maternal obesity.

Discussion

Through mediating nutrients, oxygen and waste exchange between mothers and their fetuses, adaptive remodeling in placenta has profound effects on fetal development. Maternal obesity and metabolic dysfunction is known to negatively affect fetal development, which has long-term metabolic impacts on their offspring (Zou *et al.*, 2017). Placental function is altered by maternal obesity (Brett *et al.*, 2014), triggering low efficient nutrient flux to the fetus and hampering fetal development independent with nutrient levels in the maternal side (Gaccioli *et al.*, 2013). Exercise improves metabolic health of obese populations. Though the beneficial efforts of exercise in preventing the adverse effects of maternal obesity on fetal and placental development have been reported previously (Nathanielsz *et al.*, 2013; Mangwiro *et al.*, 2018a; Mangwiro *et al.*, 2019), detailed mechanisms remain poorly defined. In this study, we found that maternal obesity impaired placental vasculogenesis/angiogenesis, which was reversed by maternal exercise training, preventing fetal overgrowth resulted from maternal obesity.

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Placental nutrient transporters mediate cross-placental nutrient delivery. In the present study, we found that the expression of glutamine and amino acid transporters was downregulated, but fatty acid transporters were increased in HFD mice, consistent with a previous report (Nam *et al.*, 2017). Nonetheless, the overall changes in the contents of nutrient transporters were quite small, suggesting that changes in nutrient transporter abundance might not explain observed changes in placental function. Consistently, in humans, maternal obesity elevates the umbilical vein glucose level and increases placental size, but not the abundance of nutrient transporters, suggesting placental expansion associated with obesity may explain neonatal macrosomia (Acosta *et al.*, 2015).

In addition to the density of nutrient transporters, the placental vasculature system mediates blood circulation and thus is also critically important for nutrient transport. Poor placental vasculature due to HFD reduces oxygenation of the fetal tissues, resulting in poor neonatal survival (Hayes *et al.*, 2012). Furthermore, chronic inflammation due to MO induces dysregulation of placental angiogenesis (Kim *et al.*, 2018). On the other hand, exercise can suppress inflammation due to obesity (Antunes *et al.*, 2018), which may promote placental angiogenesis (Reyes & Davenport, 2018). In addition, enlargement of junctional zone in placenta increases the spongiotrophoblast cells which is important for maintaining the endocrine function within the junctional zone (Coan *et al.*, 2006; Burton & Fowden, 2012). Labyrinth zone, known as the fetal side in placenta and containing dense capillaries and syncytiotrophoblasts, is the site for nutrition and oxygen interaction between the mothers and her fetuses (Burton & Fowden, 2012). In the present study, the area of the labyrinth zone was increased but vessel density was reduced in HFD mice; on the other hand, exercise training reversed these adverse changes induced by MO. Consistently, exercise training increased while MO reduced eNOS content in labyrinth zone of HFD mice, which might partially explain placental dysfunction in HFD mice because eNOS deficiency blocks uteroplacental blood flow and nutrient transport (Cureton *et al.*, 2017).

Recent studies increasingly implicate AMPK in regulating tissue development, including vascularization (Park *et al.*, 2008). Interestingly, we observed that AMPK phosphorylation was suppressed due to maternal obesity, but there was a profound increase in AMPK phosphorylation in exercised mice, which suggests an AMPK-mediated mechanism regulating vasculogenesis and nutrient/oxygenic exchanges in placenta. Furthermore, the angiogenic markers including *Vegfa* and *Vegfr*, and a hypoxic marker, *Hif1a*, were elevated in exercised mice, which correlated with AMPK phosphorylation (Skeffington *et al.*, 2016), suggesting a possible causal link between exercise-induced hypoxic/vasculogenic condition and AMPK activation (Skeffington *et al.*, 2016). In alignment, AMPK knockdown inhibited placental nutrient transport function (Carey *et al.*, 2014), and AMPK signaling is essential for hypoxia-induced angiogenesis in endothelial cells (Nagata *et al.*, 2003). The effect of exercise on angiogenesis could also be mediated by apelin (Ho *et al.*, 2017). Apelin has been considered as an exercise-induced hormone that can mediate angiogenesis in the skeletal muscle (Son *et al.*, 2017). In addition, apelin promotes angiogenesis through activating AMPK activation in myocardial microvascular endothelial cells (Yang *et al.*, 2014), and apelin is known to stimulate vasculogenesis in placenta (Kurowska *et al.*, 2018). Consistently, our data showed increased AMPK phosphorylation and apelin protein level in

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the placenta following exercise training, demonstrating the possible role of apelin in mediating AMPK activation and placental angiogenesis stimulated by exercise.

The mTORC1-mediated signaling regulates protein synthesis, which interacts with AMPK to regulate Akt phosphorylation and protein synthesis (Inoki *et al.*, 2012). In our study, we found that both AMPK and mTORC1 signaling was attenuated in HFD placenta, which was reversed by exercise, suggesting that maternal exercise training might prevent fetal overgrowth in obese mice via normalizing placental AMPK and mTORC1 signaling. The down-regulation of mTORC1 in HFD placenta was consistent with the previous observations that obesity and associated insulin resistance inhibit mTORC1 signaling (Ost *et al.*, 2010; Kleinert *et al.*, 2014). However, in a previous study, activation of placental mTORC1 signaling was observed in obese women (Jansson *et al.*, 2013). The discrepancy in mTORC1 signaling due to obesity might be explained by the presence of insulin resistance or chronic inflammation (Jiang *et al.*, 2014; Kleinert *et al.*, 2014). Indeed, in insulin resistant mice, Akt and mTORC1 phosphorylation is suppressed (Li *et al.*, 2018) and exercise can stimulate phosphorylation of Akt (Kido *et al.*, 2017). In agreement, maternal obesity in our mouse model might be associated with insulin resistance, which was alleviated by exercise. To be conclusive, however, insulin stimulation could be applied, which might directly assess the changes in insulin sensitivity.

Finally, in order to mimic the situation in obese mothers, in this study, we induced female obese first and then mated. Therefore, maternal obesity should be the major factor inducing changes in placental function, but also includes the effect of HFD, mimicking the situation of obese mothers in humans. In this study, we only measured the vascular density based on the expression of markers and immunohistochemical staining, which showed up-regulation of vasculogenesis in placenta of obese mothers with exercise. It will be better if the placental transport function could be directly measured.

In summary, our data show that, for the first time to our knowledge, maternal exercise enhanced placental vasculogenesis and prevents fetal overgrowth, which might be mediated by activation of AMPK, mTORC1, and insulin signaling in the placenta, all of which were suppressed by maternal obesity and HFD intake. Collectively, these data support the compelling possibility that AMPK might be a key factor mediating placental function altered by obesity and exercise training.

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Abbreviations

MO	maternal obesity
HFD	high-fat diet
mTORC1	mammalian target of rapamycin complex 1
AMPK	AMP-activated protein kinase

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VO_2max	maximal oxygen consumption rate
VO_2	oxygen consumption rate
VCO_2	carbon dioxide production
RER	respiratory exchange ratio
ELISA	enzyme-linked immunosorbent assay
HOMA-IR	homeostatic model assessment of insulin resistance
HOMA-%B	homeostatic model assessment of pancreatic β-cell function
H&E	hematoxylin and eosin
ICC	immunocytochemical
ingWAT	inguinal white adipose tissue
gonWAT	gonadal white adipose tissue
iBAT	intrascapular brown adipose tissue
Lpl	lipoprotein lipase; IRS-1, insulin receptor substrate 1
TNF-α	tumor necrosis factor α; IL-1β, interleukin-1β
IL-6	interleukin-6
VEGF	vascular endothelial growth factor
APLN	endogenous ligand for the G-protein coupled APJ receptor
VEGFR1	VEGF receptor 1
HIF1	hypoxia induced factor 1
eNOS	endothelial nitric oxide synthase
CCN1	CCN family member 1
GCM1	glial cells missing gene
IGF-2	insulin-like growth factor 2
FGF2	fibroblast growth factor 2

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Key points summary

- Maternal exercise improves metabolic health of maternal mice challenged with high-fat diet.
- Exercise intervention of obese mothers prevents fetal overgrowth.
- Exercise intervention reverses impaired placental vascularization in obese mice.
- Maternal exercise activates placental AMP-activated protein kinase, which was inhibited due to maternal obesity.

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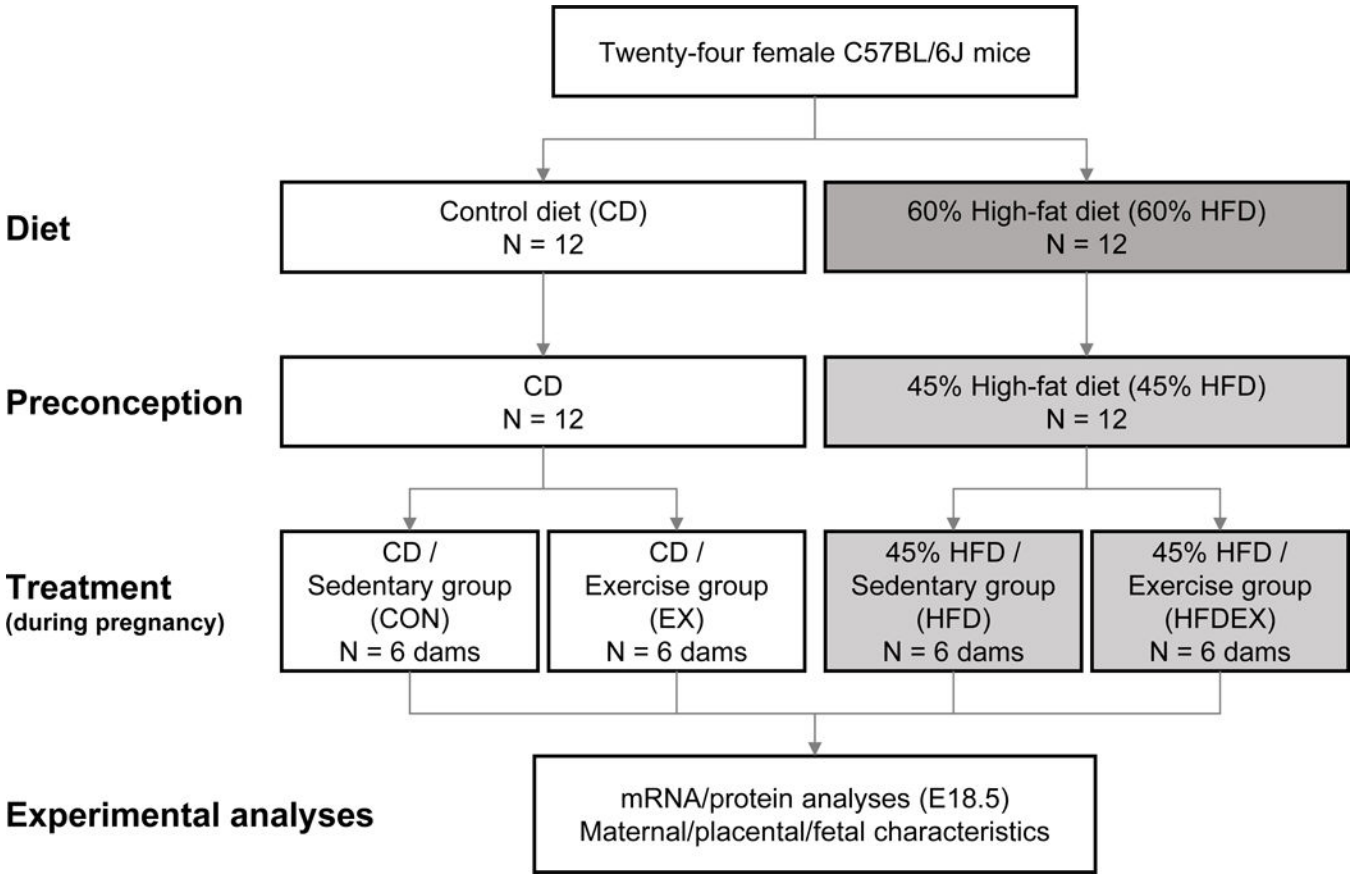

Figure 1.
Flow chart illustrating the allocation of mice and treatments.

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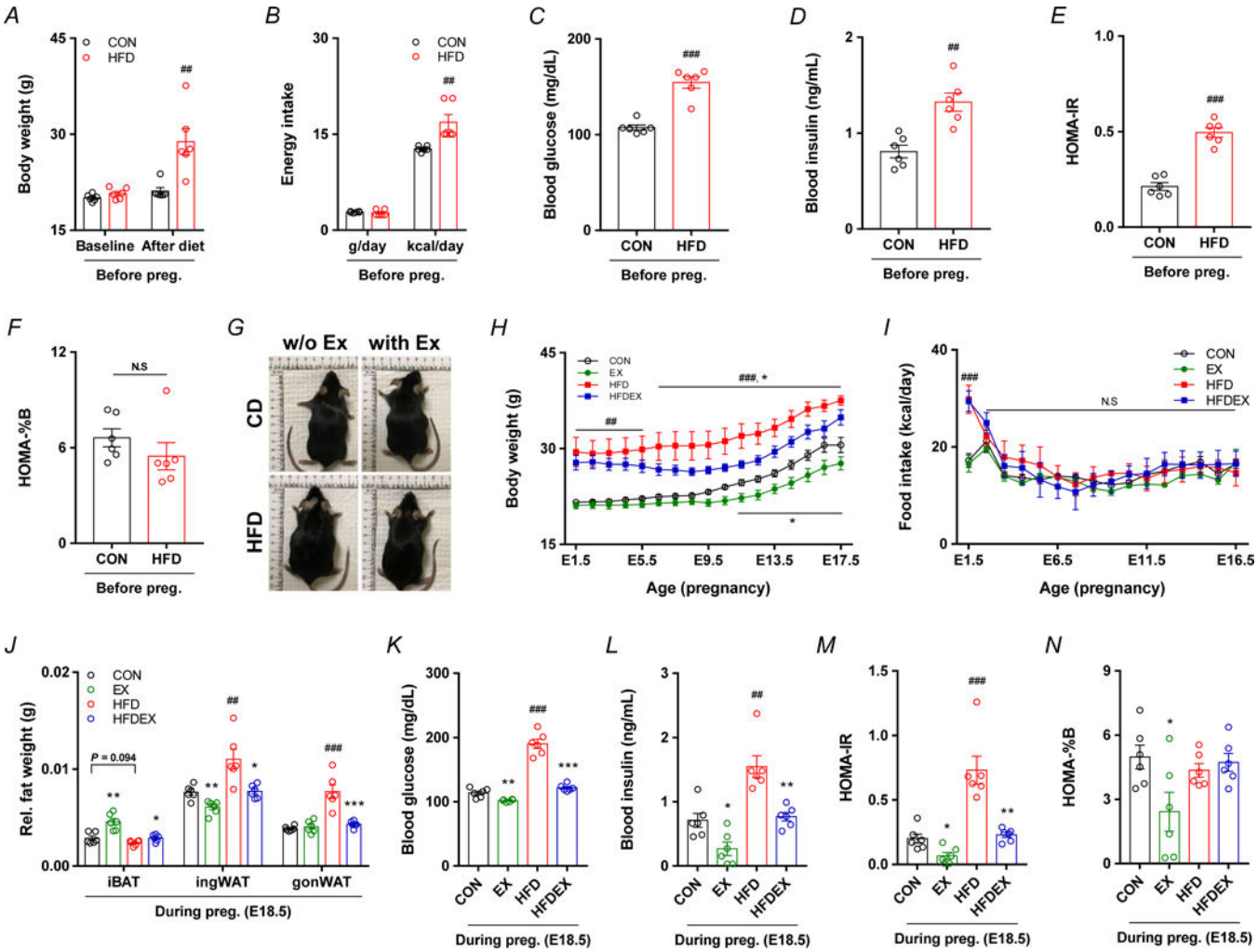

Figure 2.

Effects of maternal obesity and exercise intervention on maternal characteristics. ***A-D***, Data were collected following 8 weeks of diet intervention before mating. Body weight (*A*) and energy intake (*B*) of CON and HFD maternal mice before pregnancy. Blood glucose (*C*) and insulin (*D*) following 5 h of fasting in CON and HFD maternal mice before pregnancy. ***E-F***, Calculated insulin resistance (*E*) and pancreatic β cell function (*F*) following 5 h of fasting in CON and HFD maternal mice before pregnancy. ***G-N***, Data were collected from maternal mice at E18.5, including representative whole body images (*G*), body weight (*H*), food intake (*I*), and relative fat wet weight (*J*) in maternal mice at E18.5, as well as blood glucose (*K*), insulin (*L*), and calculated insulin resistance (*M*) and pancreatic β cell function (*N*). Data are expressed as the mean ± s.e.m. n = 6. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ in CON vs. EX or HFD vs. HFDEX; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ in CON vs. HFD by two-tailed Student's *t*-test (***A-F, J-N***) or ANOVA with post hoc Bonferroni multiple comparison analysis (***H,I***).

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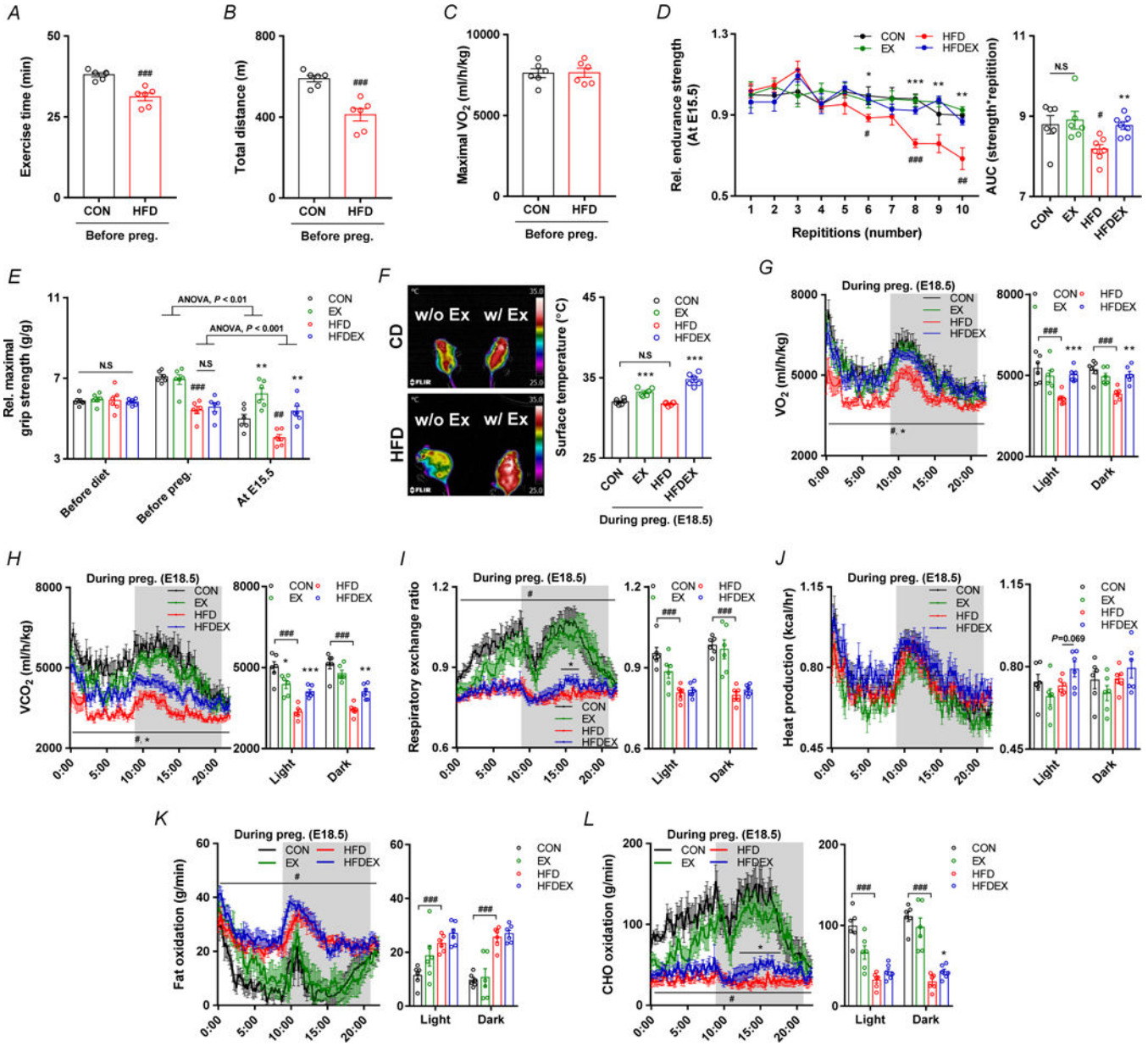

Figure 3.

Maternal exercise training improves exercise capacity and energy expenditure in HFD-induced obese and pregnant mice. ***A-C***, Maximal aerobic capacity test of female mice before mating, including exercise time (*A*), total distance (*B*), and maximal oxygen consumption levels (*C*). ***D-F***, Muscle strength of maternal mice at E15.5: relative endurance grip strength (*D*) and forelimb grip strength (*E*), and representative thermogenic images (*F*). ***G-L***, Metabolic parameters of maternal mice at E16.5: Time-resolved oxygen consumption (*G*), time-resolved carbon dioxide production (*H*), respiratory exchange ratio (RER) (*I*), Time-resolved heat production (*J*), time-resolved fat oxidation (*K*) and time-resolved carbohydrate oxidation (*L*); dark phase is marked as dark background; right inset depicts calculated means as indicated. Mean ± s.e.m. n = 6. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 in CON vs. EX

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or HFD vs. HFDEX; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ in CON vs. HFD by two-tailed Student's *t*-test (***A-E, G-L***), ANOVA with post hoc Bonferroni multiple comparison analysis (***D***, ***E***).

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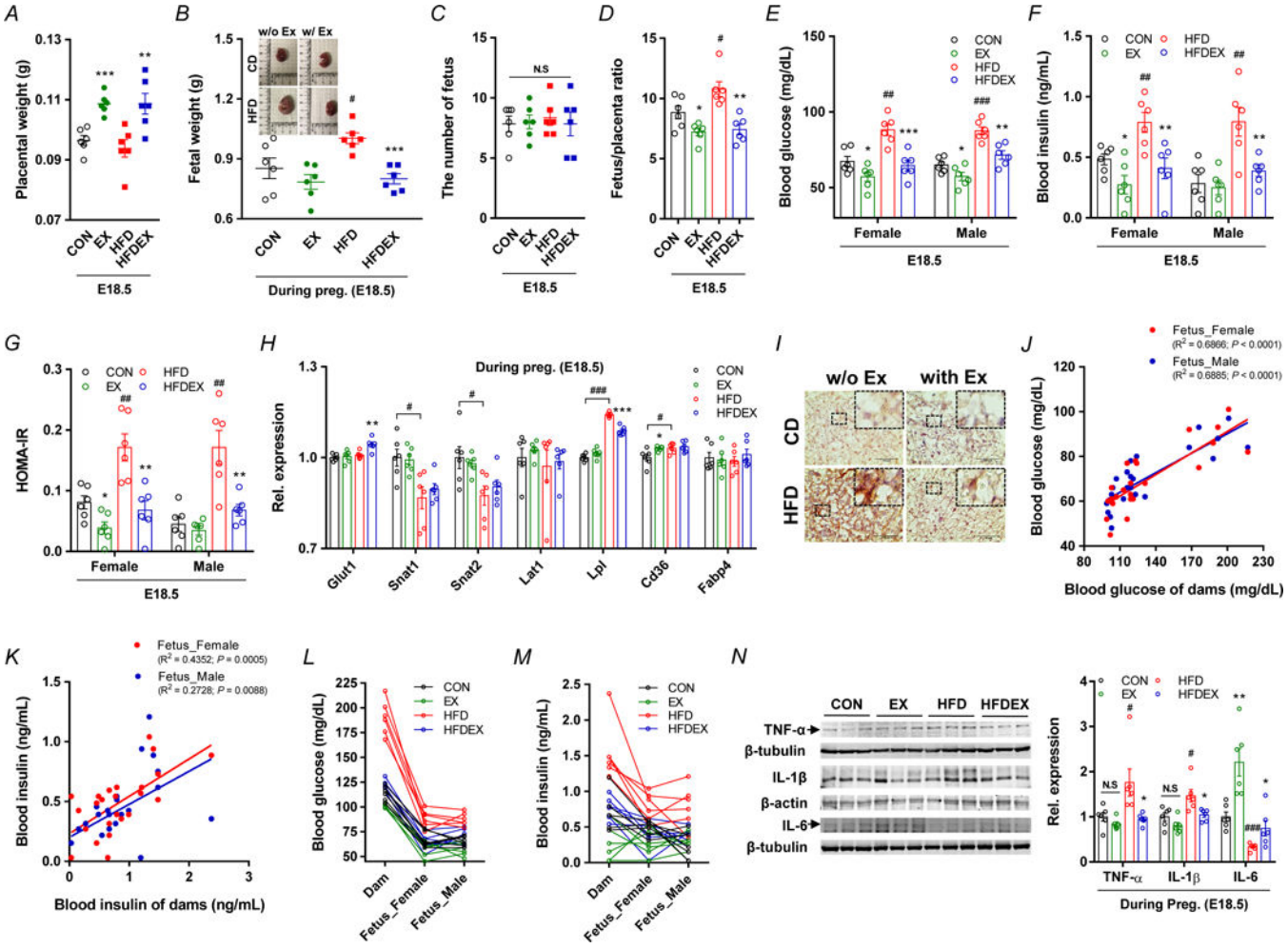

Figure 4.

Maternal exercise training regulates placental efficiency and nutrient transport, and fetal metabolic parameters. ***A-D,*** Placental parameters of CON or HFD with/without exercise mice: placental weight (*A*), representative fetal images and weight (*B*), the number of fetuses per dam (*C*), and placental efficiency (*D*). ***E-G,*** Blood glucose (*E*), insulin (*F*), and insulin resistance (*G*) in E18.5 fetuses. ***H,*** mRNA levels of different nutrient transport markers in CON or HFD mice with/without exercise training. ***I,*** Representative images of Oil Red O staining in the placenta, Scale bar, 100 μm. ***J-M***, *Pearson* correlations (*J,K*) and distributions (*L,M*) in the blood glucose and insulin levels between dams and fetuses. ***N***, Cropped western blots of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in the placenta (β-tubulin or β-actin were used as the loading control, n = 6 per group). Data are expressed as the mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in CON vs. EX or HFD vs. HFDEX; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ in CON vs. HFD by two-tailed Student's *t*-test (***A-H, L***) followed by multiple comparison and *Pearson* correlations analysis (***J,K***).

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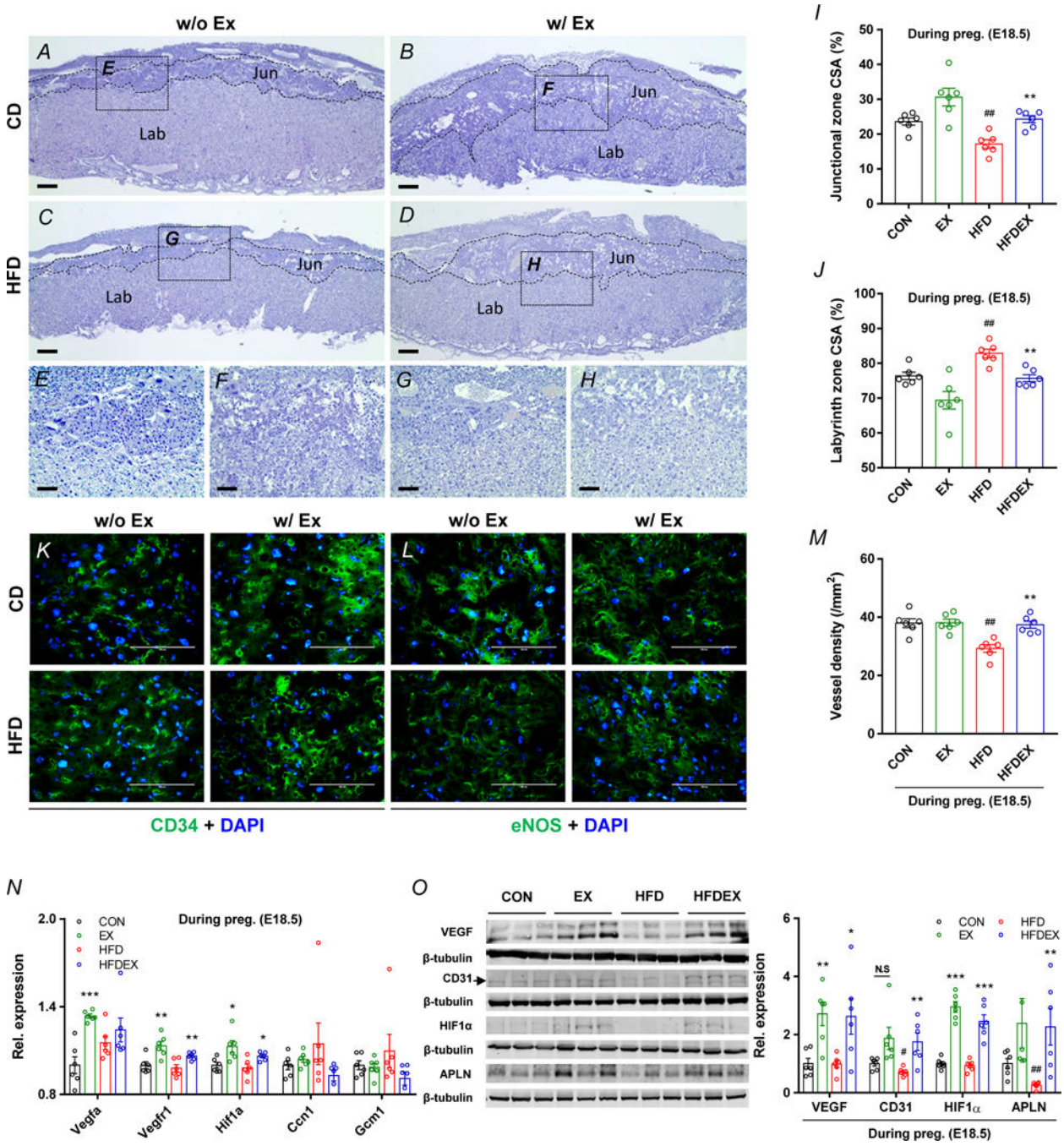

Figure 5.

Maternal exercise training reverses impaired placental vascularization in HFD-mice. ***A-H,K,L***, Representative images of hematoxylin and eosin (H&E), CD34 and eNOS immunocytochemistry (ICC) staining of placenta from CON or HFD mice with/without exercise, Scale bar, 500 μm (*A-D*), 100 μm (*E-H,K,L*), junctional zone (Jun) and labyrinth zone (Lab). ***I-J,*** Cross-sectional area (CSA) of junctional zone (*I*) and labyrinth zone (*J*) at E18.5 in placenta. ***M,*** Vessel density per mm^2 in labyrinth zone of placenta. ***N***, mRNA levels of different vasculogenic markers in CON or HFD mice with/without exercise. Expression

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was normalized by ΔC_t values. ***O***, cropped western blots of VEGF, CD31, and APLN in the placenta (β-tubulin was used as the loading control) from CON or HFD with/without exercise. Data are expressed as the mean ± s.e.m. n = 6. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ in CON vs. EX or HFD vs. HFDEX; $^{\#\#}P < 0.01$ in CON vs. HFD by two-tailed Student's *t*-test followed by one-way ANOVA with post hoc Bonferroni multiple comparison analysis (***I-O***).

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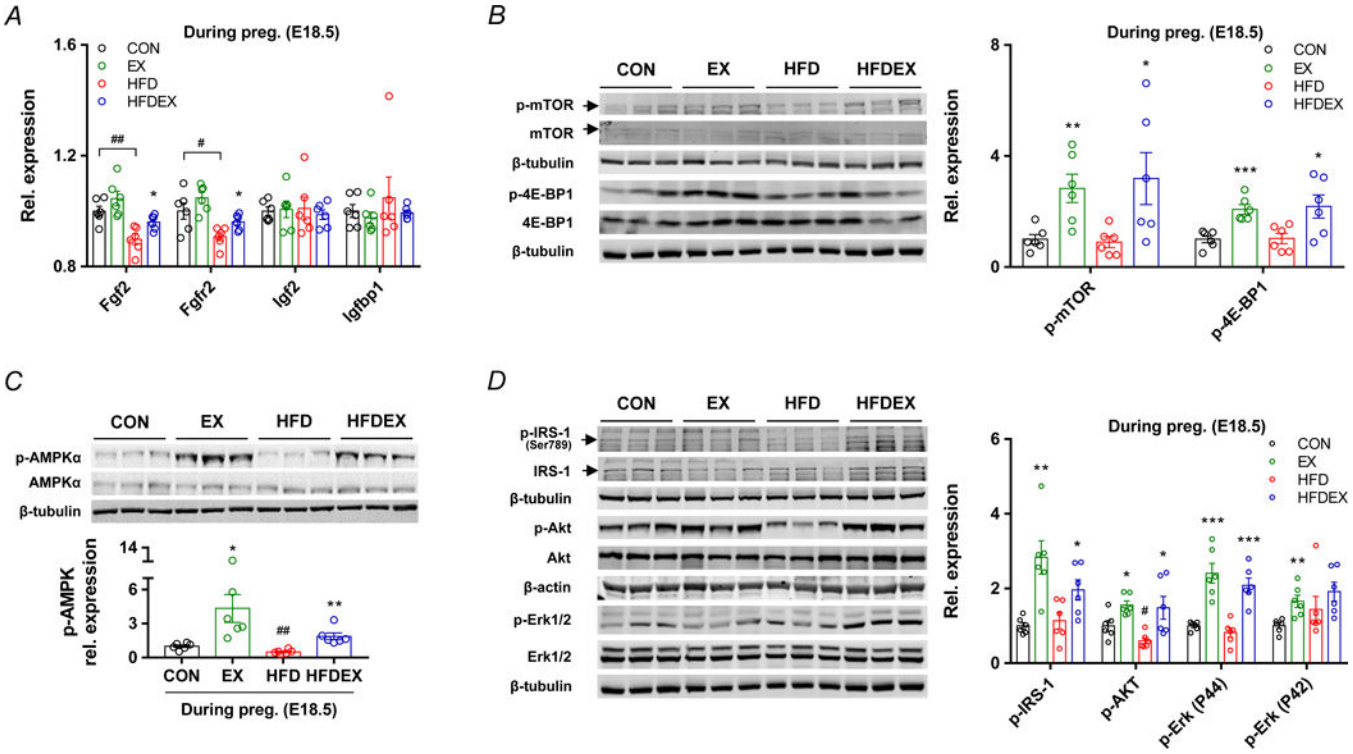

Figure 6.

Maternal exercise training activates AMPK/mTORC1/insulin signaling in the placenta of HFD mice with exercise. ***A***, mRNA levels of growth factors in CON or HFD mice with/without exercise mice (n = 6 per group). Expression was normalized by ΔC_t values. ***B***, cropped western blots of mTOR phosphorylation and 4E-BP1 phosphorylation in the placenta (the total mTOR or total 4E-BP1 were used as the reference). ***C***, cropped western blots of AMPK phosphorylation in the placenta (the total AMPK was used as the reference). ***D***, cropped western blots of IRS-1 phosphorylation, and Akt phosphorylation, and Erk½ phosphorylation in the placenta (the total IRS-1, total Akt, and total Erk½ were used as the references). Data are expressed as the mean ± s.e.m. n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in CON vs. EX or HFD vs. HFDEX; $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ in CON vs. HFD by two-tailed Student's *t*-test (***A***-***D***).

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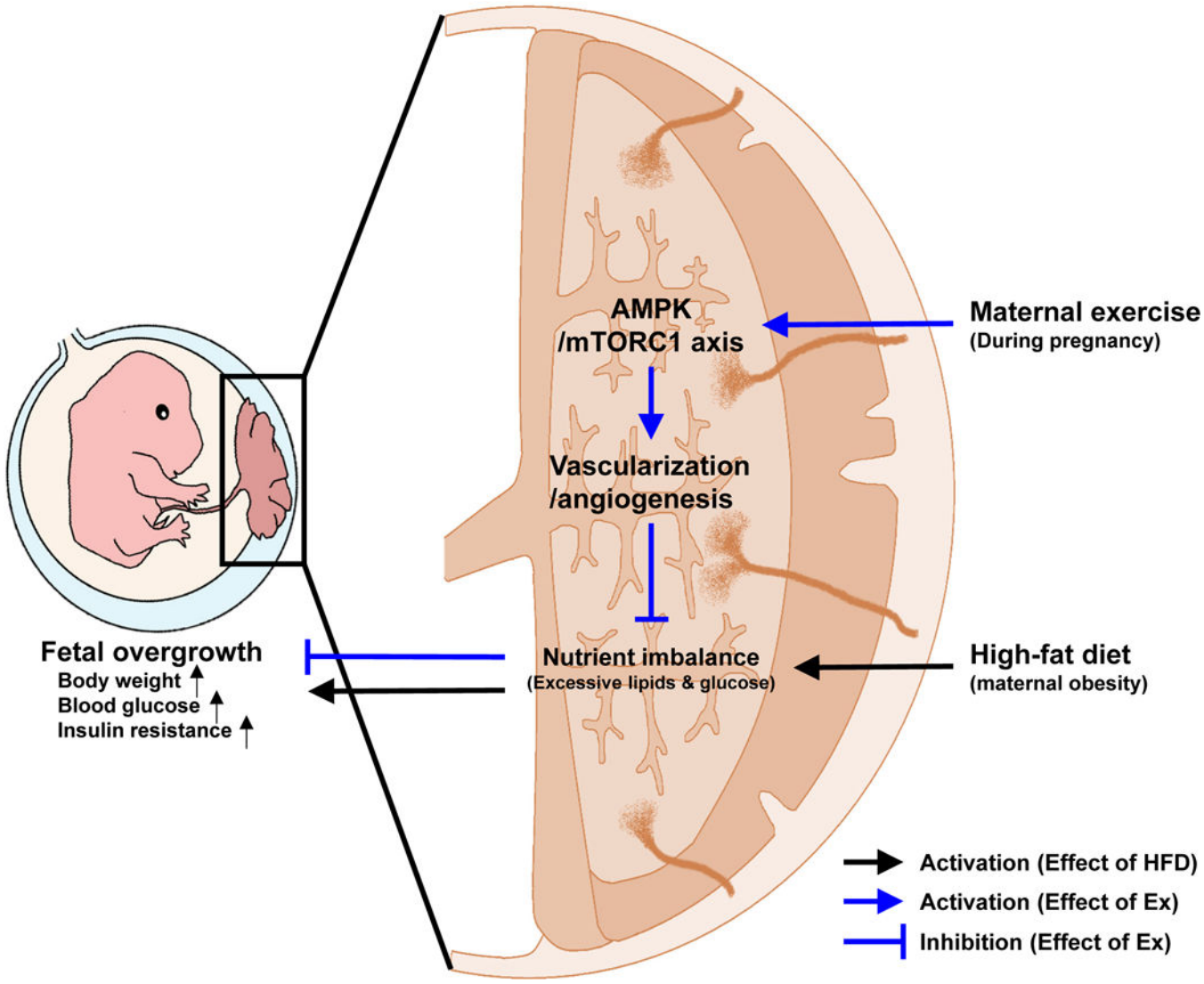

Figure 7.

Potential schematic mechanisms of maternal exercise to prevent fetal overgrowth due to high-fat diet-induced obesity in mothers.

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Table 1.

qPCR primer sequences used for gene expression analyses

Gene Name	Forward primer sequence	Reverse primer sequence
Vegfa	5’-TGGACCCTGGCTTTACTGCT-3’	5’-GCAGTAGCTTCGCTGGTAGA-3’
Vegfr1	5’-TGTGCACATGACGGAAGGAA-3’	5’-GTATTGGTCTGCCGATGGGT-3’
Fgf2	5’-GGCTGCTGGCTTCTAAGTGT-3’	5’-GTCCCGTTTTGGATCCGAGT-3’
Fgfr2	5’-CACGACCAAGAAGCCAGACT-3’	5’-CTCGGCCGAAACTGTTACCT-3’
Hif1a	5’-AGGATGAGTTCTGAACGTCGAAA-3’	5’-CTGTCTAGACCACCGGCATC-3’
Ccn1	5’-AGAGGCTTCCTGTCTTTGGC-3’	5’-CCAAGACGTGGTCTGAACGA-3’
Gcm1	5’-GAAGGCGGACAGGCTTTGA-3’	5’-GTTTCACGTAGGAGTCCGGC-3’
Glut1	5’-GCGGGAGACGCATAGTTACA-3’	5’-CAGCCCCGTTACTCACCTTG-3’
Snat1	5’-GGGCATAAGGTACACCGAGG-3’	5’-CAACGTGCACCTGTTTACCG-3’
Snat2	5’-ACGTGGATCCCGAAAACCAG-3’	5’-CCAAGGATTCCACTGCCCAC-3’
Lat1	5’-GGGAAGGACATGGGACAAGG-3’	5’-GCCAACACAATGTTCCCCAC-3’
Lpl	5’-GAAAGGGCTCTGCCTGAGTT-3’	5’-TAGGGCATCTGAGAGCGAGT-3’
Cd36	5’-TGAATGGTTGAGACCCCGTG-3’	5’-TAGAACAGCTTGCTTGCCCA-3’
Fabp4	5’-CGACAGGAAGGTGAAGAGCATCATA-3’	5’-CATAAACTCTTGTGGAAGTCACGCCT-3’
Igf2	5’-CGCCCCAGCCCTAAGATAC-3’	5’-GGGTATGCAAACCGAACAGC-3’
Igfbp1	5’-TGTTTCTTGGCCGTTCCTGA-3’	5’-GAGAAATCTCGGGGCACGAA-3’
18S	5’-GTAACCCGTTGAACCCCATT-3’	5’-CCATCCAATCGGTAGTAGCG-3’