Mitochondria respond to a range of stimuli and function in energy production and redox homeostasis. However, little is known about the developmental and environmental control of mitochondria in the placenta, an organ vital for fetal growth and pregnancy maintenance in eutherian mammals. Using respirometry and molecular analyses, the present study examined mitochondrial function in the distinct transport and endocrine zones of the mouse placenta during normal pregnancy and maternal inhalation hypoxia. The data show that mitochondria of the two zones adopt different strategies in modulating their respiration, substrate use, biogenesis, density, and efficiency to best support the growth and energy demands of fetoplacental tissues during late gestation in both normal and hypoxic conditions. The findings have important implications for environmentally induced adaptations in mitochondrial function in other tissues and for compromised human pregnancy in which hypoxia and alterations in placental mitochondrial function are associated with poor outcomes like fetal growth restriction.

Mitochondria are multifunctional organelles. Their primary role is in ATP generation by oxidative phosphorylation (OXPHOS) using substrates derived from β-oxidation and the tricarboxylic acid cycle. They are also involved in cell signaling via production of reactive oxygen species (ROS) and other molecules, which affect cell homeostasis and survival. ROS are a normal byproduct of OXPHOS but, when produced in excess, e.g., during disrupted oxygen (O2) or substrate supply (1), they can cause oxidative stress and damage DNA, lipids, and proteins (2). In endocrine tissues, like the placenta, mitochondria also synthesize steroids, which consumes O2 independently of OXPHOS (3). Consequently, mitochondria vary in number and function between different cell types in relation to metabolic needs (4).

The placenta has a high energy requirement. It synthesizes hormones and other molecules for pregnancy maintenance and actively transports a range of substrates to the fetus for growth and development (4, 5). It also requires energy for its own metabolism, growth, and morphological remodeling (6). In all mammals, placental energy demand is met primarily by OXPHOS (7), thus the placenta has a significant O2 requirement, using 50 to 70% of O2 taken up from the uterine circulation at a mass-specific rate of consumption higher than in adult liver (7). As the fetus grows, the demands on placental energetics increase, yet total mass-specific uteroplacental O2 consumption changes little, if at all, from mid to late gestation in sheep and humans (7, 8). However, there are gestational changes in placental oxidative stress and expression of the mitochondrial-related proteins in several species (9–12), which suggest that function of placental mitochondria changes developmentally to meet the increasing fetal demands for growth toward term.

The placenta is known to adapt its morphology and transport characteristics to optimize fetal growth during suboptimal conditions in several species (4). In humans, hypoxia is the main cause of fetal growth restriction at high altitude and is a common feature of pregnancy complications at sea level (13). In pregnant rodents and guinea pigs, inhalation hypoxia adapts placental morphology and nutrient transport to the fetus dependent upon the degree, timing, and length of O2 restriction (14–19). Changes in placental mitochondrial function are also seen in compromised human pregnancies (3) and in nutritionally induced fetal growth restriction in rodents, in association with changes in mitochondrial function and biogenesis (20, 21). However, the extent to which placental mitochondrial function adapts to environmental cues like hypoxia remains unclear.

Here we comprehensively examine the functional phenotype of placental mitochondria during the last third of mouse pregnancy and in response to maternal inhalation hypoxia in relation to the temporal changes in fetoplacental growth. In rodents, unlike humans, the endocrine and transport functions of the placenta are carried out by structurally distinct regions, the junctional zone (Jz) and labyrinthine zone (Lz), respectively, which differ in morphology, cellular composition, and blood flow (4). Consequently, we investigated mitochondrial function of the two zones separately.

**Results**

*Mitochondrial Respiratory Capacity in the Placenta with Gestation.* C57BL/6J mice were time-mated and the ontogeny of mitochondrial function determined in the placental Jz and Lz on day (D) 14, 16, and 19 of pregnancy (term = ∼D20). This covers the period when mouse fetuses grow most rapidly in absolute terms. We used three respirometry assays to assess the capacity for substrate use and electron transfer system (ETS) function in saponin-permeabilized placental samples, initially in the absence of ADP (O2 consumption without ATP generation; LEAK) and then following the addition of ADP (OXPHOS state). First, Py-supported respiration was measured in the LEAK (Py2) and OXPHOS (Py2) states in the presence of malate. Second, palmitoyl carnitine (Pal)-supported respiration was measured in the LEAK (Pal) and OXPHOS (Pal) states also in the presence of malate. Third, a substrate titration was used to elucidate ETS capacity. Initially, LEAK state respiration in pla...
the presence of complex I-linked substrates, glutamate and malate was recorded (GMPs) before OXPHOS was stimulated (GMPs), and, finally, succinate was added (GMSs) to measure OXPHOS capacity when electron entry via complexes I and II of the ETS was saturated. The respiratory medium comprised 0.5 mM EGTA, 3 mM MgCl₂, 0.6 mM O₂, 20 mM taurine, 10 mM KH₂PO₄, 20 mM Hepes, 1 mg/mL of BSA, 60 mM K-lactobionate, 110 mM sucrose, pH 7.1.

Both Lz and Jz were able to use Py and Pal as respiratory substrates, although respiratory rate varied between D14 and D19. In the Jz, PyL declined between D14 and D19 (Fig. 1A). In contrast, Jz PalL remained stable gestationally (Fig. 1B). The Jz displayed no gestational changes in either PyP or PalP; however, respiratory control ratios (RCRs; OXPHOS/LEAK) for Py and Pal increased from D16 to D19 (Fig. 1A–C). GMp declined between D16 and D19 in the Jz (SI Appendix, Fig. S2). However, GMp and GMSp did not vary between D14 and D19, in line with Jz size (Fig. 1D and SI Appendix, Figs. S1 and S2).

In the Lz, PyP and PalL declined between D14 and D19 (Fig. 1A and B). PyP in the Lz was also greatest at D14 and decreased toward term (Fig. 1A). PalL in the Lz instead remained stable between D14 and D19. The RCR for Py was lower in the Lz on D19, relative to D14 (Fig. 1C), due to the greater decline in OXPHOS than LEAK respiration. In contrast, Pal RCRs in the Lz were greatest between ages (Fig. 1C). GMp and GMSp in the Lz were greatest on D14 and/or D16, with values decreasing by D19 (Fig. 1D and SI Appendix, Fig. S2). These data suggest more active mitochondrial function at the earlier ages when the Lz is growing most rapidly (SI Appendix, Fig. S1). The lower rates of Lz O₂ consumption with age, particularly with Py, also suggest O₂ and glucose may be spared by the placenta for fetal transfer during the rapid phase of fetal growth (SI Appendix, Fig. S1).

**Mitochondrial Proteins in the Placenta with Gestation.** Next, we investigated whether there were ontogenic changes in the levels of proteins regulating mitochondrial content and function in the two zones. In the Jz, abundance of both citrate synthase (CS), a marker of mitochondrial density, and UCP2 declined with increasing gestational age (Fig. 1E). Similarly, Jz abundance of peroxisome proliferator-activated receptor gamma coactivator-1-alpha (PGC1α), a regulator of mitochondrial biogenesis, showed a trend to decrease gestationally (Fig. 1E, \( P = 0.054 \)). There was an overall effect of gestational age on protein carbonylation, a marker of oxidative stress, in the Jz; values appeared highest on D16 versus D14 and D19 (relative abundance mean ± SEM: D14, 100 ± 7%; D16, 171 ± 31%; D19: 98 ± 22%; \( P < 0.05 \)). However, there was no significant change in the Jz abundance of any ETS complex between D14 and D19 (Fig. 1E).

Unlike the Jz, Lz expression of CS and PGC1α were unaffected by gestational age (Fig. 1F). Abundance of Lz UCP2 was similar on D14 and D16, but decreased by D19 (Fig. 1F). Lz abundance of ETS complexes II and IV (Fig. 1F) and protein carbonylation were greater on D16, relative to D14 or D19 (D14, 100 ± 8%; D16, 182 ± 32%; D19: 98 ± 15%; \( P < 0.05 \) for D16 versus D14 and D19). Thus, the functionally distinct zones show ontogenic differences in abundance of mitochondrial-regulatory proteins that relates to their respiratory capacity and the pattern of fetoplacental growth during the last third of pregnancy.

**Hypoxia and Placental Mitochondrial Respiratory Capacity.** Adaptation in placental mitochondrial function may optimize fetal growth and survival when maternal availability of resources, like inspired O₂, are limited. Thus, we sought to address whether placental mitochondrial function is altered by hypoxia in a severity-dependent fashion that relates to the known placental support of fetal growth in these conditions (15). We assessed the effects of both moderate hypoxia (13% inspired O₂) between D11 and D16 or D14 and D19 and severe hypoxia (10% inspired O₂) from D14 to D19 on placental mitochondrial function on D16 and D19 of pregnancy, relative to normoxic dams (21% O₂ N). These levels of hypoxia would be equivalent to altitudes of ~3,700 m and ~5,800 m, over which
range human and rodent populations decrease from significant to sparse levels (15). As 13% O₂ from D11 to D16 and 10% O₂ from D14 to D19 were associated with reductions in maternal food intake (15), additional groups of normoxic dams were pair-fed (PF) to match the intakes of the 13% O₂ and 10% O₂ dams for the same periods of pregnancy, to discriminate between the effects of hypoxia and hypoxia-induced hypophagia. Previously, we have shown that fetal growth is unchanged at D16 and only marginally decreased (by 5%) at D19 in 13% O₂ whereas pup weight is reduced by 20% in 10% O₂ dams at D19, with intermediate values in the PF dams (15). There was no effect of hypoxia or pair feeding on placental weight at either D16 or D19, although Lz volume was increased by 13% O₂ on D16, and Jz volume increased by 10% O₂ on D19 (15).

Early Exposure.

Respiratory capacity. Py-supported Jz respiration was unaffected by 13% O₂ on D16. However, Jz Palₚ in 13% O₂ dams was greater than in N dams but similar to PF (Fig. 2B). Palₚ in the Jz of 13% O₂ dams did not differ from N or PF mice; however, values were higher in PF mice, relative to N dams (Fig. 2B). There was no effect of 13% O₂ on Py- or Pal-supported RCRs or GMₜ, GMₚ, and GMSₚ in the Jz (Fig. 2C and D and SI Appendix, Fig. S3A).

In the Lz, the majority of differences in placental mitochondrial respiratory function were seen between the 13% O₂ and PF dams, with intermediate values in the N group (Fig. 2). Although not different from N dams, Py-supported LEAK and Pal-supported LEAK and OXPHOS respiration rates and RCRs were reduced in the Lz on D16 in 13% O₂ relative to PF dams (Fig. 2A–C). GM₀, GMₚ, and GMSₚ were diminished by 13% O₂ compared with either N or PF dams (Fig. 2A–C and SI Appendix, Fig. S3A).

Mitochondrial proteins. In the Jz, CS and PGC1α abundance was reduced by 13% O₂ relative to N mice on D16 (Fig. 2E). However, Jz CS was greater in 13% O₂ mice than in PF, while PGC1α did not differ between 13% O₂ and PF. Abundance of UCP2 and ETS complexes in the Jz was not affected by 13% O₂ compared with N dams. Jz UCP2 abundance was also not different from that of PF mice, but values for the PF group were increased relative to N mice (Fig. 2E). Moreover, complexes I and II were greater and ATP synthase was lower in the Jz of 13% O₂ mice, relative to PF (Fig. 2E). There was no effect of 13% O₂ Jz protein carbonylation (SI Appendix, Fig. S4A).

In the Lz at D16, abundance of PGC1α was greater in both 13% O₂ and PF mice, compared with N dams (Fig. 2F). The Lz abundance of ETS complex III and ATP synthase was lower in 13% O₂ dams compared with both N and PF dams (Fig. 2F). In the Lz, other ETS complexes, CS, and UCP2 abundance were unaffected by 13% O₂. However, Lz complex I was more abundant in PF relative to N dams. There was also no effect of 13% O₂ on Lz protein carbonylation (SI Appendix, Fig. S4A). Thus, 13% O₂ affected mitochondrial phenotype differentially in the Jz and Lz at D16.

Late Exposure.

Respiratory capacity. On D19, Pyₜ and Pyₚ supported respiration and Py-supported RCRs in the Jz or Lz were unaffected by 13% O₂ or 10% O₂ (Fig. 3A–C). However, Jz Palₚ was lower in 13% O₂, relative to N. It also tended to be lower than N values in 10% O₂ and PF mice, which were similar to each other. However, Jz Pal-supported RCRs, as well as GM₀, GMₚ, and GMSₚ, were unchanged by either 13% O₂ or 10% O₂ (Fig. 3C and SI Appendix, Fig. S3B).

In the D19 Lz, both Palₚ and Palₚ were lower in both hypoxic groups, relative to N dams (Fig. 3B). Dams PF to the 10% O₂ group also exhibited lower Lz Palₚ, versus N dams (Fig. 3B). In the Lz, Pal-supported RCRs in 13% O₂ and 10% O₂ dams were similar to N and the respective PF dams (Fig. 3C). GM₀, GMₚ, and GMSₚ was lower in Lz of 13% O₂ and 10% O₂ dams, relative to N dams (SI Appendix, Fig. S3B and Fig. 3D, by t test for 13% O₂ dams). GM₀, GMₚ, and GMSₚ values in the Lz for PF were intermediate between N and 10% O₂ dams (Fig. 3D and SI Appendix, Fig. S3B).

Mitochondrial proteins. On D19, Jz CS, PGC-1α, and UCP2 abundances were increased in 13% and 10% O₂ mice, relative to N, but
Ferruzzi-Perri et al. and tended to increase with hypoxia, with a significant difference between 13% O₂ and N dams (Fig. 3F). Lz expression of PGC-1α tended to increase with hypoxia, with a significant difference between 13% O₂ and N dams (Fig. 3F).

Discussion

This study in mice shows that placental mitochondria use both fatty acids and carbohydrates as respiratory substrates and adapt their function ontogenically during normal pregnancy and in response to environmental hypoxia. It is comprehensive in demonstrating that there are Lz- and Jz-specific changes in mitochondrial respiration, efficiency, substrate use, biogenesis, density, and ETS complex abundances that depend on gestational age, nutritional intake, and the degree of maternal hypoxia. There were also zonal and age-related differences in placental oxidative stress during normal and hypoxic pregnancy, which probably relate to changes in mitochondrial ROS production with potential consequences for cell damage more widely. These data also emphasize the dynamic nature of mitochondrial phenotype and complexity of the physiological and molecular mechanisms regulating placental mitochondrial function in response to environmental cues.

In both the Lz and Jz, ADP-coupled O₂ consumption rates were similar when respiration was supported by either Py or Pal. Previous studies have shown that the placenta expresses fatty acid oxidation enzymes and that trophoblasts oxidize fatty acids in vitro (22, 23). Indeed, the activity of certain fatty acid oxidation enzymes in the human placenta is as high as those in adult liver (23). The present study demonstrates that the placenta can use fatty acid oxidation to support mitochondrial ATP production, in part fulfilling its requirements for growth, transport, and hormone synthesis. Defects in placental fatty acid oxidation in complicated pregnancies may therefore contribute to the poor fetoplacental growth common in these diseases (22, 24).

At the earlier gestational ages studied, the Lz had greater Py and total OXPHOS respiration rates than the Jz. Thus, the energy demands of the transport zone appear greater than those of the endocline zone at this stage of gestation, consistent with the rapid growth, morphological remodeling, and synthesis of proteins required for Lz nutrient transport between D14 and D16 (25–27). Therefore, both pyruvate (Py)-supported and maximal Lz respiration rates and RCRs declined toward term, but there were no apparent alterations in Lz mitochondrial biogenesis and density, as indicated by the CS and PGC-1α abundances, that could explain this ontogenic change. The lower rates of Lz mitochondrial O₂ consumption, particularly with Py toward term, suggest O₂ and glucose may be spared by the placenta for transfer to the fetus during its rapid growth phase. Indeed, OXPHOS rates are lower for the transporting syncytiotrophoblast than the proliferative cytotrophoblast in the term human placenta (28). Since Lz Pal₄ was unaffected by gestational age, while Pal₃ declined, fatty acids may become more important substrates for meeting the energy demands placed on the Lz for transport by the rapidly growing fetus (29). Unlike the Lz, in the Jz, the coupled respiratory rates with Pal and Py and the maximum OXPHOS capacity were stable across the last third of pregnancy, in line with Jz weight and the steady energetic requirements for hormone production (5). The maintained Jz respiratory capacity between D14 and D19 occurred despite decreasing CS abundance. However, the RCRs increased,
suggesting that Jz mitochondria become more efficient near term. Indeed, abundance of UCP2 was low in both the Jz and Lz, at D19, which might reduce proton leak and increase coupling of O$_2$ consumption to ATP production.

Since UCP2 attenuates ROS production, the high level of Lz UCP2 abundance at D14 and D16 may help protect against oxidative damage at the time when the Lz is growing most rapidly and placental O$_2$ delivery is still low (27). Indeed, Lz oxidative stress was highest at D16 in the current study. By D19, Lz oxidative stress and UCP2 abundance had decreased, in parallel with the known increase in uteroplacental blood flow toward term in the mouse (29). The ontogenic change in UCP2 abundance in the Lz, therefore, appears to reflect placental O$_2$ availability and the fetoplacental demands for growth. At the earlier ages, the high level of Lz UCP2 appears to set the limit to rapid ROS accumulation and the risk of oxidative damage, whereas, at D19, the low UCP2 abundance will increase the efficiency of mitochondrial ATP production by minimizing proton leak when Lz energy requirements are at their highest to support fetal growth.

In this study, placental mitochondria adapted not only developmentally but also in response to environmental hypoxia during late gestation. Exposure to 5 d of 13% O$_2$ led to an overall reduction in Lz mitochondrial oxidative capacity at both D16 and D19. These changes appear to have been a result of hypoxia alone, because maternal inhalation hypoxia, with the Lz normally less well oxygenated than the Lz (39), it may be more resilient to further reductions in O$_2$ levels than the Lz, although, at D19, abundance of UCP2 in the Jz increased in both hypoxic and the PF groups, which may have protected against excessive ROS production associated with hypoxia and hypophagia. Whatever the mechanisms involved, overall maintenance of Jz respiration rates during hypoxia may help prevent maternal inflammation and other endocrine activities that are essential for pregnancy (5).

While our study has clear strengths, it also has some limitations. Both the Lz and Jz are heterogeneous and vary in cellular composition and mitochondrial ultrastructure during gestation (27, 40). Furthermore, the human placenta (41) and other tissues [e.g., adipose tissue (42)] contain subpopulations of mitochondria with specific functions. Studies using single-cell isolation will be helpful in establishing the contribution of placental cell types and mitochondrial subpopulations to the respiratory profile and function of the Lz and Jz in normoxia and hypoxia. The contribution of O$_2$ consumption by non-OXPHOS–related processes (such as steroid and nitric oxide production) could also be explored in future work using ETS inhibitors (e.g., rotenone and antimycin). Indeed, studies using the mitochondrial-targeted antioxidant MitoQ suggest non-OXPHOS-related processes may have a significant effect on substrate exchange, placental secretions, and fetal growth in hypoxic rat dams (17, 43).

In summary, our data show that the mouse placental Lz and Jz adopt different strategies at the mitochondrial level to support the growth and other energy-demanding functions of both the placenta and fetus during normal and hypoxic pregnancy (SI Appendix, Table S1). More broadly, our data emphasize that mitochondrial function in the placenta is highly adaptable over the course of normal gestation and in response to environmental cues, which appears to help support normal fetal growth. Our
findings are also important clinically, as hypoxia and altered mitochondrial function are reported in the placenta of human pregnancies with poor outcomes such as fetal growth restriction (3). The heterogeneity of pregnancy outcomes for women with gestational hypoxia may be explained, in part, by differences in the adaptive responses of placental mitochondria. Our work, therefore, highlights placental mitochondria as possible mediators and targets for intervention, in hypoxia-induced fetal growth restriction in sea-level and high-altitude human pregnancies.

Materials and Methods
All procedures described were approved by the Ethical Review Committee of the University of Cambridge (Cambridge, United Kingdom) and were carried out in accordance with UK Animals (Scientific Procedures) Act 1986 as previously reported (15). Either on D14, D16, and D19 (ontogeny study) or on D16 or D19 (hypoxia study), dams were killed by cervical dislocation. For the hypoxia study, all dams were anesthetized before death with an i.p. injection of fentanyl-Huanison and midazolam in sterile water (1:1:2, 10 μg/ml; Janssen Animal Health). The uterus was removed, and each fetus and corresponding placenta were weighed. Two placenta from each litter were separated into Lz and Jz. Zones from one placenta were snap-frozen for quantification of protein abundance. Zones from the other placenta were immediately taken for analysis of mitochondrial respiratory capacity. Data are presented as means ± SEM and were analyzed by one-way or two-way ANOVA with Bonferroni post hoc tests using IBM SPSS statistics or by t test using Excel with statistical significance determined by P < 0.05. For fetal and placental weights, statistics were performed using litter means.

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