Placental mitochondria adapt developmentally and in response to hypoxia to support fetal growth

Amanda N Sferruzzi-Perri[1], Josephine Higgins[1], Owen Vaughan[1], Andrew Murray[1], Abigail Fowden[1]

[1]University of Cambridge

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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 feto-placental 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 | metabolism | fetus | placenta | hypoxia

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 (TCA) cycle. They are also involved in cell signalling via production of reactive oxygen species (ROS) and other molecules, which affect cell homeostasis and survival. ROS are a normal by-product of OXPHOS but, when produced in excess e.g. during disrupted oxygen (O_2) or substrate supply (1), they can cause oxidative stress and damage DNA, lipids and proteins (2). In endocrine tissues, like the placenta, mitochondria also synthesise steroids, which consumes O_2 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 synthesises 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 remodelling (6). In all mammals, placental energy demand is met primarily by OXPHOS (7), thus the placenta has a significant O_2 requirement, using 50-70% of O_2 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 utero-placental O_2 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 towards 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 O_2 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 are the first to 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 feto-placental 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 ETS (electron transfer system) function in saponin-permeabilised placental samples, initially in the absence of ADP (LEAK state) and then following the addition of ADP (OXPHOS state). First, pyruvate (Py)-supported respiration was measured in the LEAK (Py_L) and OXPHOS (Py_P) states in the presence of malate. Second, palmitoyl carnitine (Pal)-supported

Significance

Mitochondria are the primary source of ATP for placental growth, transport and hormone synthesis. However, to date, little is known about the developmental regulation or functional significance of placental mitochondria during normal or suboptimal intrauterine conditions, such as oxygen deprivation (hypoxia). Here we show that in the placenta, mitochondria adapt their use of oxygen and nutrients (carbohydrate and fat) to best support both placental growth and function, as well as fetal development, during normal and hypoxic conditions. These data are significant because they improve our mechanistic understanding of human pregnancies compromised by fetal growth restriction at sea level and high altitude.

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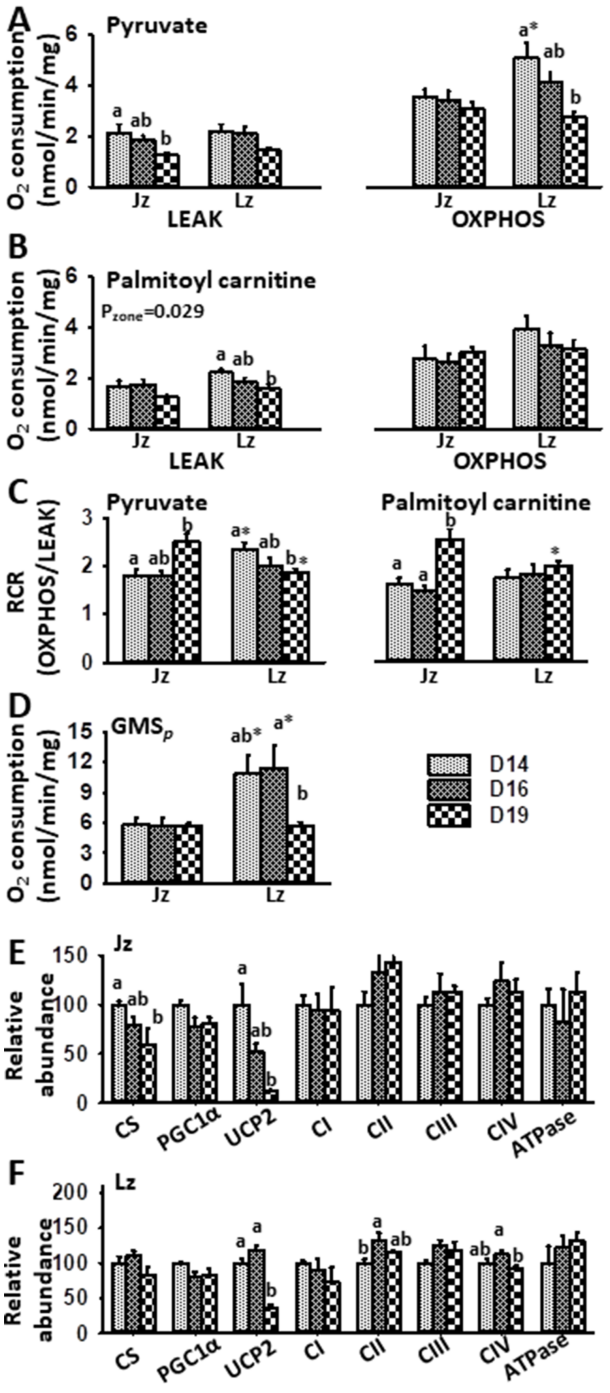

Fig. 1. Mitochondrial respiration and associated protein abundance in the last third of pregnancy. Oxygen consumption in LEAK and OXPHOS states using pyruvate (Py) and palmitoyl carnitine (Pal) (B), RCRs for Py and Pal (C) and OXPHOS respiration in the presence of malate, glutamate and succinate (GMS_P) (D) in the placental Jz and Lz, as well as, protein abundance in the Jz (E) and Lz (F) at D14, D16 and D19 of pregnancy. Analysed by two way ANOVA (age and zone) with Bonferroni post-hoc tests. Different letters represent a significant difference between gestational ages, within a zone ($p<0.05$); * denotes a significant difference of Lz to Jz, for a given age ($p<0.05$). Outcome of ANOVA is shown if pair-wise comparisons were not significant. n=6-10 per age for A-D and n=5-6 for E-F per treatment. ATPase, ATP synthase; CI-IV, ETS complexes I to IV.

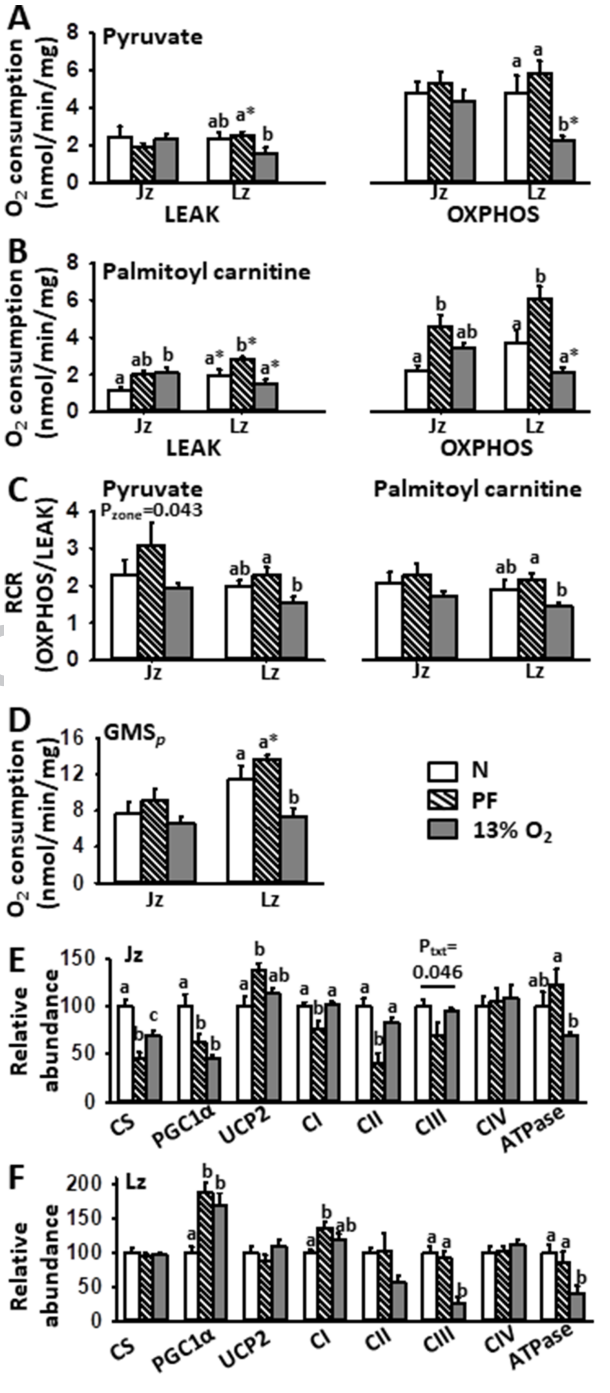

Fig. 2. Mitochondrial respiration and associated protein abundance at D16 in response to hypoxia. Oxygen consumption in LEAK and OXPHOS states using pyruvate (Py) and palmitoyl carnitine (Pal) (B), RCRs for Py and Pal (C) and OXPHOS respiration in the presence of malate, glutamate and succinate (GMS_P) (D) in the placental Jz and Lz, as well as, protein abundance in the Jz (E) and Lz (F) following exposure to 13% O_2 from D11-D16 or pair feeding normoxic animals to the food intake of mice in 13% O_2 (PF). Analysed by two way ANOVA (treatment and zone) with Bonferroni post-hoc tests. Different letters represent a significant difference between treatments, within a zone ($p<0.05$); * denotes a significant difference of Lz to Jz ($p<0.05$). Outcome of ANOVA is shown if pair-wise comparisons were not significant. n=6-10 for A-D and n=5 for E-F per treatment. ATPase, ATP synthase; CI-IV, ETS complexes I to IV; txt; treatment.

respiration was measured in the LEAK (Pal_L) and OXPHOS (Pal_P) states also in the presence of malate. Third, a substrate titration was used to elucidate ETS capacity. Initially, LEAK state respiration in the presence of complex I-linked substrates,

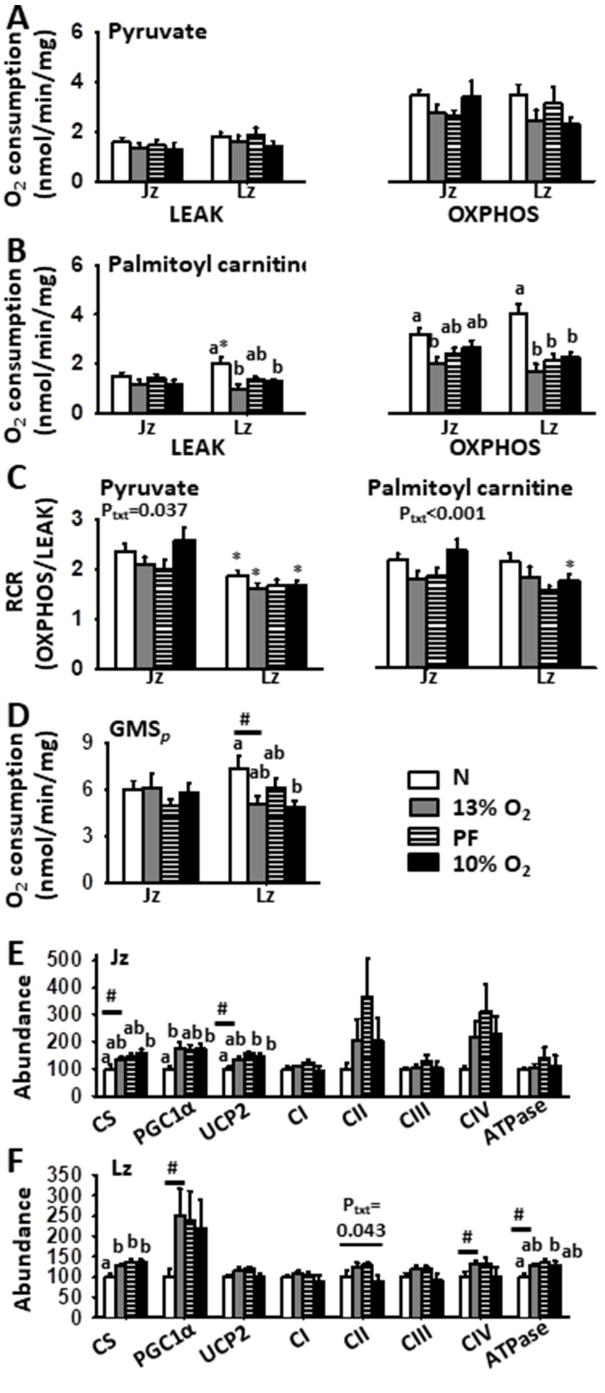

Fig. 3. Mitochondrial respiration and associated protein abundance at D19 in response to hypoxia. Oxygen consumption in LEAK and OXPHOS states using pyruvate (Py) and palmitoyl carnitine (Pal) (B), RCRs for Py and Pal (C) and OXPHOS respiration in the presence of malate, glutamate and succinate (GMS_P) (D) in the placental Jz and Lz, as well as, protein abundance in the Jz (E) and Lz (F) following exposure to 13% O_2 or 10% O_2 from D14-D19 or pair feeding normoxic animals to the food intake of mice in 10% O_2 (PF). Analysed by two way ANOVA (treatment and zone) with Bonferroni post-hoc tests. * denotes a significant difference of Lz to Jz ($p<0.05$); different letters represent a significant difference between treatments, within a zone ($p<0.05$). Outcome of ANOVA is shown if pair-wise comparisons were not significant. n=9-16 for A-D and n=6-9 for E-F per treatment. ATPase, ATP synthase; CI-IV, ETS complexes I to IV; txt; treatment.

glutamate and malate was recorded (GM_L) before OXPHOS was stimulated (GM_P) and finally, succinate was added (GMS_P) 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 $MgCl_2.6H_2O$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 1 mg/ml 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, Py_L declined between D14 and D19 (Fig. 1A). In contrast, Jz Pal_L remained stable gestationally (Fig. 1B). The Jz displayed no gestational changes in either Py_P or Pal_P, however respiratory control ratios (RCRs; OXPHOS/LEAK) for Py and Pal increased from D16 to D19 (Fig. 1A-C). GM_L declined between D16 and D19 in the Jz (SI Appendix, Fig. S2). However, GM_P and GMS_P did not vary between D14 and D19 in line with Jz size (Fig. 1D and SI Appendix, Fig. S1 and S2).

In the Lz, Py_P and Pal_L declined between D14 and D19 (Fig. 1A-B). Py_P in the Lz was also greatest at D14 and decreased towards term (Fig. 1A). Pal_P 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 stable with age (Fig. 1C). GM_L, GM_P and GMS_P 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 O_2 consumption with age, particularly with Py, also suggest O_2 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 if 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 feto-placental growth during the last third of pregnancy.

Hypoxia and placental mitochondrial respiratory capacity

Adaptation in placental mitochondrial function may optimise fetal growth and survival when maternal availability of resources, like inspired O_2 are limited. Thus, we sought to address if 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 O_2) between D11 and D16 or D14 and D19 and severe hypoxia (10% inspired O_2) from D14 to D19 on placental mitochondrial function on D16 and D19 of pregnancy, relative to normoxic dams (21% O_2; N). These levels of hypoxia would be equivalent to altitudes of ~3700 m and ~5800 m, over which range human and rodent populations

decrease from significant to sparse levels (15). As 13% O_2 from D11 to D16 and 10% O_2 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% O_2 and 10% O_2 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% O_2, whereas pup weight is reduced by 20% in 10% O_2 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% O_2 on D16 and Jz volume increased by 10% O_2 on D19 (15).

Early exposure

Respiratory capacity: Py-supported Jz respiration was unaffected by 13% O_2 on D16. However, Jz Pal_L in 13% O_2 dams was greater than in N dams but similar to PF (Fig. 2B). Pal_P in the Jz of 13% O_2 dams did not differ from N or PF mice, however values were higher in PF mice, relative to N dams (Fig. 2A). There was no effect of 13% O_2 on Py or Pal-supported RCRs or GM_L, GM_P and GMS_P in the Jz (Fig. 2C-D and SI Appendix, Fig. S3A).

In the Lz, the majority of differences in placental mitochondrial respiratory function were seen between the 13% O_2 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% O_2 relative to PF dams (Fig. 2A-C). GM_L, GM_P and GMS_P were diminished by 13% O_2 compared to 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% O_2, relative to N mice on D16 (Fig. 2E). However, Jz CS was greater in 13% O_2 mice than in PF while PGC1α did not differ between 13% O_2 and PF. Abundance of UCP2 and ETS complexes in the Jz was not affected by 13% O_2 compared with N dams. Jz UCP2 abundance with 13% O_2 was also not different to 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 lower in the Jz of 13% O_2 mice, relative to PF (Fig. 2E). There was no effect of 13% O_2 Jz protein carbonylation (SI Appendix, Fig. S4A).

In the Lz at D16, abundance of PGC1α was greater in both 13% O_2 and PF mice, compared with N dams (Fig. 2F). The Lz abundance of ETS complex III and ATP-synthase was lower in 13% O_2 dams compared with both N and PF dams (Fig. 2F). In the Lz, other ETS complexes, CS and UCP2 abundance were unaffected by 13% O_2. However, Lz complex I was more abundant in PF relative to N dams. There was also no effect of 13% O_2 on Lz protein carbonylation (SI Appendix, Fig. S4A). Thus, 13% O_2 affected mitochondrial phenotype differentially in the Jz and Lz at D16.

Late exposure

Respiratory capacity: On D19, Py_L and Py_P respiration and Py-supported RCRs in the Jz or Lz were unaffected by 13% O_2 or 10% O_2 (Fig. 3A-C). However, Jz Pal_P was lower in 13% O_2, relative to N. It also tended to be lower than N values in 10% O_2 and PF mice, which were similar to each other. However, Jz Pal-supported RCRs, as well as GM_L, GM_P and GMS_P were unchanged by either 13% O_2 or 10% O_2 (Fig. 3C and SI Appendix, Fig. S3B).

In the D19 Lz, both Pal_L and Pal_P were lower in both hypoxic groups, relative to N dams (Fig. 3B). Dams pair-fed to the 10% O_2 group also exhibited lower Lz Pal_P, versus N dams (Fig. 3B). In the Lz, Pal-supported RCRs in 13% O_2 and 10% O_2 dams were similar to N or the respective PF dams (Fig. 3C). GM_L, GM_P and GMS_P was lower in Lz of 13% O_2 and 10% O_2 dams, relative to N dams (Fig. S3B and 3D, by t-test for 13% O_2 dams). GM_L, GM_P and GMS_P values in the Lz for PF were intermediate between N and 10% O_2 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% O_2 mice, relative to N, but were not different to their respective PF group (Fig. 4E, t-test for 13% O_2 dam). There was no effect of hypoxia on Jz abundance of ETS complexes (Fig. 3E) or protein carbonylation (SI Appendix, Fig. S4B). In the Lz, CS was more abundant in 13% O_2 and 10% O_2 than N dams or the respective PF group (Fig. 3F). Lz expression of PGC-1α tended to increase with hypoxia, with a significant difference between 13% O_2 and N dams (Fig, 3F, t-test). Overall, there was a significant effect of treatment on Lz abundance of complex II (Fig. 3F). Lz expression of complex IV and ATP-synthase was greater in 13% O_2, but not 10% O_2 relative to N dams (Fig. 3E, by t-test). ATP synthase abundance in the Lz was increased by PF compared to N (Fig. 3F). Protein carbonylation was ~2-fold greater in the Lz of 10% O_2 mice, than in all other experimental groups (SI Appendix, Fig. S4B). Thus, hypoxia, independent of the severity, affects mitochondrial profile differentially in the Jz and Lz at D19.

Discussion

To our knowledge, this study in mice is the first to show 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 novel 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 O_2 consumption rates were similar when respiration was supported by either pyruvate or palmitoyl carnitine. Previous studies have shown that the placenta expresses fatty acid oxidation enzymes and that trophoblasts oxidise fatty acids *in vitro* (22, 23). Indeed, the activity of certain fatty acid oxidation enzymes in the human placenta are 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 feto-placental 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 endocrine zone at this stage of gestation, consistent with the rapid growth, morphological remodelling and synthesis of proteins required for Lz nutrient transport between D14 and D16 (25-27). Thereafter, both pyruvate-supported and maximal Lz respiration rates and RCRs declined towards 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 O_2 consumption particularly with pyruvate towards term, suggest O_2 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 Pal_P was unaffected by gestational age, whilst Pal_L 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 utero-placental blood flow towards term in the mouse (29). The ontogenic change in UCP2 abundance in the Lz, therefore, appears to reflect placental O_2 availability and the feto-placental demands for growth. At the earlier ages, the high level of Lz UCP2 appears to act to limit ROS accumulation and the risk of oxidative damage whereas, at D19, the low UCP2 abundance will increase the efficiency of mitochondrial ATP production by minimising 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 five days 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 dams in normoxia but pair-fed to the 13% O_2 intake typically showed increased, rather than decreased rates of Lz respiration. As fatty acids require around 8-11% more O_2 to generate a given amount of ATP (6), the specific reduction in Lz fatty acid supported O_2 consumption in hypoxic mice at D19 may be a more efficient way of producing ATP in hypoxic conditions near term. There may also be a greater reliance on glycolytic ATP production, as placental accumulation and transplacental transfer of glucose and amino acids are increased, while active transport of amino acids is maintained in 13% O_2 dams (15). These reductions in mitochondrial respiration and the change in relative substrate use with 13% O_2 would be beneficial in sparing O_2 for transfer to the rapidly growing fetus and are consistent with reports of normal fetal oxygen consumption rates in high altitude human pregnancies (30). In line with this, fetal growth was maintained or only marginally (5%) reduced at D16 and D19, respectively, despite 5 days of hypoxia (15).

In 13% O_2 dams at D16, the reduced Lz respiration was associated with decreased abundance of ETS complex III and ATP-synthase whereas complex I abundance was increased. As the major mitochondrial source of ROS in hypoxia is complex III (31), its lower abundance in the Lz of 13% O_2 dams may serve to limit the excessive production of hypoxia-induced ROS. Consistent with this, we found no change in the levels of Lz oxidative stress in 13% O_2 dams at D16. PGC1α was enhanced in the D16 Lz in 13% O_2 dams, suggesting more mitochondrial biogenesis at this stage. However, this was likely to be due to hypoxia-induced hypophagia as values in PF dams were similar to those in 13% O_2 at D16, consistent with the enhanced mitochondrial biogenesis seen previously in the placenta of nutrient-restricted rats (20). At D19, lower respiration rates in hypoxic dams were paradoxically associated increased abundance of complex II, IV, ATP-synthase, PGC1α and CS. Although reductions in oxidative respiration in hypoxic tissues, including the placenta, are generally associated with decreased mitochondrial biogenesis and ETS complexes (16, 32, 33) hypoxia has also been shown to stimulate mitochondrial biogenesis and lead to greater mitochondrial content in different cell types (34). Moreover, placentas of women evolutionarily adapted to high altitude contain more syncytial mitochondria than those of native sea level populations (35). Enhanced mitochondrial formation in response to 13% O_2 may thus reflect an adaptive attempt to increase Lz bioenergetic capacity. As mitochondria are susceptible to ROS-mediated damage, their enhanced biogenesis in the Lz of 13% O_2 dams may be important for replacing damaged mitochondria and preventing excessive ROS production at D19, when there was no evidence of placental oxidative stress.

The effects of more severe hypoxia (10% O_2) on Lz mitochondrial respiration and protein abundance in late pregnancy were generally similar to those observed with 13% O_2. However, in contrast to 13% O_2 dams, placental accumulation and transport of glucose was not upregulated whilst amino acid transport and fetal growth were reduced in 10% O_2 dams at D19 (15). These differences were not entirely explained by the hypophagia in the 10% O_2 dams; fetuses of PF dams were growth restricted compared to normoxic animals but significantly larger than their hypoxic counterparts. Placental amino acid transport capacity of PF dams has also been shown to be intermediate between that of the normoxic and 10% O_2 groups at D19 (15). Therefore, the more pronounced defects in placental amino acid transport and fetal growth restriction induced by 10% O_2 at D19 may be accounted for by the more pronounced Lz oxidative stress in these dams. Certainly, placental oxidative stress is a common feature of fetal growth restriction in human pregnancy and reduces amino acid uptake in human trophoblasts *in vitro* (36, 37). Collectively, our findings suggest that the changes in Lz mitochondrial and transport phenotype with 10% O_2 at D19 are due to complex regulatory interactions between maternal undernutrition, O_2 availability and the degree of ROS production and oxidative stress. This probably involves at least two molecular pathways (eg, HIF and mTORC1) and energy sensing molecules such as adenosine monophosphate-activated protein kinase (AMPK) which is known in increase in abundance specifically in the mouse Lz during late gestation (38).

Compared with the Lz, maternal inhalation hypoxia had little effect on Jz mitochondrial respiration or oxidative stress levels at either gestational age. Since the Jz is devoid of fetal capillaries and does not function in fetal O_2 transfer (4), there is little need to downregulate its respiratory pathways as a mechanism to spare O_2 for fetal use during hypoxia at either age. Jz mitochondrial density, biogenesis and abundance of specific ETS complexes were altered by hypoxia in an age-related manner, with decreased PGC1α, CS and ATP-synthase abundance in 13% O_2 dams on D16 but increased protein abundances at D19 irrespective of the degree of hypoxia. However, there were similar alterations in Jz mitochondrial respiration, biogenesis and density in animals pair fed to the intakes of the hypoxic groups at both D16 and D19, indicating that many of the changes seen during hypoxia may have been driven largely by maternal undernutrition. Since the Jz is 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 maintain steroidogenesis 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 heterogenous and vary in cellular composition and mitochondrial ultrastructure during gestation (27, 40). Furthermore, the human placenta (41) and other tissues (eg, adipose tissue (42)) contain subpopulations of mitochon-

dria 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 (eg rotenone and antimycin). Indeed, studies using the mitochondrial-targeted antioxidant, MitoQ suggest non-OXPHOS 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 (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 appear 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 on mice were carried out under the UK Animal (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 anaesthetised prior to death with an intraperitoneal injection of fentanyl-fluanisone and midazolam in sterile water (1:1:2, 10 µg/ml, Janssen Animal Health, High Wycombe, UK). The uterus was removed and each fetus and corresponding placenta were weighed. Two placentas 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 analysed 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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