Adaptive structural and functional evolution of the placenta protects fetal growth in high elevation deer mice

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Hypoxia at high elevations challenges female reproductive physiology in placental mammals, increasing rates of gestational complications. Adaptation to high elevation has limited many of these effects in humans and other mammals, offering potential insight into the developmental processes that lead to and protect against hypoxia-related gestational complications. However, our understanding of these adaptations has been hampered by a lack of experimental work linking the functional, regulatory, and genetic underpinnings of gestational development in locally-adapted populations. Here, we dissect high-elevation adaptation in the reproductive physiology of deer mice, (Peromyscus maniculatus), a rodent species with an exceptionally broad elevational distribution that has emerged as a model for hypoxia adaptation. Using experimental acclimations, we show that lowland mice experience pronounced fetal growth restriction when challenged with gestational hypoxia, while highland mice maintain normal growth by expanding the functional region of the placenta that facilitates nutrient and gas exchange between dam and fetus. We then use region-specific transcriptome analyses to show that adaptive structural remodeling of the placenta is coincident with widespread changes in gene expression within this same compartment. Genes associated with fetal growth in deer mice overlap with genes involved in human placental development, pointing to conserved or convergent pathways underlying these processes. Finally, we overlay our results with genetic data from natural populations to identify a subset of genes that may form the genetic basis of these placental adaptations. Collectively, these experiments advance our understanding of placental adaptation to hypoxic environments and reveal physiological and genetic mechanisms underlying fetal growth trajectories.

altitude | Peromyscus | labyrinth zone | plasticity | gene expression

High elevation (> 2500 m) environments fundamentally challenge terrestrial life through an inescapable and pervasive reduction in oxygen availability. Low oxygen directly limits individual performance, and common physiological responses to low oxygen that accumulate over hours to days spent at high elevation can exacerbate these performance detriments and contribute to altitude-related illnesses (1–3). Reproduction is also compromised at high elevation (4–6), which we should expect to have significant consequences for populations because reproductive outcomes are the ultimate arbiters of Darwinian fitness. The effects of high elevation on reproduction are best documented in humans, for whom residence at high elevations is consistently associated with reduced birth weight and increased risks for birth complications, including intra-uterine growth restriction (4). However, humans with altitude-adapted ancestry (e.g., Tibetan, Quechua) experience reduced risk for these complications (7), presumably reflecting local adaptation in reproductive physiology. In contrast to well-studied cardiopulmonary and metabolic adaptations to high elevation, the reproductive physiology and adaptations that influence fetal growth outcomes remain poorly resolved in humans. To date, a large number of maternal traits, ranging from maternal ventilatory characteristics and uterine artery diameter to microvascular structure in the placenta, have all been hypothesized to contribute to fetal growth outcomes at elevation (5). However, mechanistic links between these traits and fetal growth outcomes remain limited.

The challenges of gestational hypoxia are necessarily shared by all placental mammals, suggesting that evolutionary contrasts within and between species adapted to high elevations can provide novel functional insight into the evolution of reproductive physiology. Indeed, population genomic contrasts in several species have revealed extensive signatures of local adaptation to high elevation (2, 8–11), including numerous examples of convergence in genes with clear *a priori* connections to metabolic or cardiopulmonary functions. However, most genomic scans for elevational selection identify candidate genes without clear phenotypic connections, and elevational adaptation of prenatal reproductive physiology has rarely been

Significance Statement

Residence at high elevation is associated with increased risk for gestational complications, including fetal growth restriction. The underlying drivers of these complications remain poorly understood. Using a high elevation-adapted rodent model, we asked which physiological factors correlated with fetal growth outcomes under hypoxia, and how evolutionary adaptation has modified these traits. We show that deer mice derived from high- and low-elevation populations differ in their susceptibility to fetal growth restriction during gestational hypoxia and that these population-level differences are associated with structural changes in the placenta and broad-scale shifts in gene expression. Our findings point towards structural phenotypes and gene expression profiles that are likely relevant to offsetting the effects of hypoxia on fetal and placental development across mammals.

KW and ZAC conceived of the experiments with input from JMG. KW carried out all animal work and sample collection. KC conducted maternal trait data analysis. KW, ECM, and RMS carried out sequence data analysis and summary. KW completed all other data generation and statistical analysis. KW wrote the manuscript with input and approval from all authors.

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DRAFT

considered outside of humans (5, 6).

Here we use the deer mouse, Peromyscus maniculatus, to understand the physiological and genetic drivers of fetal growth restriction under maternal hypoxia and to identify the evolutionary adaptations that prevent it. Deer mice are an established model for studying adaptive evolution (12), including adaptation to high elevation environments (13–15), and we understand detailed aspects about the physiology and genetic variation that has contributed to persistence of high-elevation deer mouse populations. However, reproductive adaptations have not yet been examined in this system.

Several aspects of deer mouse biology make them a useful comparative model of high elevation adaptation and reproductive physiology. First, high elevation deer mouse populations are derived from lowland populations (16), which mirrors the biogeographic history of many highland human populations (i.e., lowland individuals moving into and adapting to highland environments). Second, wild deer mice exhibit genetic signatures of local adaptation in the face of high rates of gene flow (17, 18), which allows fine-scale resolution of genomic regions that have experienced a history of natural selection at high elevation. Finally, although rodents and humans differ in the degree to which fetal cells invade maternal tissues and developmental of the fetus at term (19), key similarities make deer mice a useful comparative model for studying the effects of hypoxia on gestational outcomes (20). Specifically, deer mice have structurally similar placentas to humans (discoid, hemochorial placentas) (21, 22), and many of the maternal traits that are adjusted to support pregnancy in humans are also remodeled in deer mice (21).

Results and Discussion

Evolutionary adaptation in highland deer mice protects fetal growth under hypoxia. If fetal growth restriction is a fundamental challenge that impacts survival and fitness of mammals at high elevation, (a) lowland-derived deer mice should give birth to smaller pups when gestating under hypobaric hypoxia (hereafter, hypoxia), and (b) highland-adapted deer mice should protect fetal growth. To test this hypothesis, we compared birthweights of deer mouse pups born to dams gestating under normoxia to those born to dams held in hypoxia, using deer mice derived from Ann Arbor, Michigan (250 m above sea level, lowlanders) and Mt. Evans, CO (4300 m ASL, highlanders; Fig. 1A, Table 1). As predicted, pups from lowland dams gestating under hypoxia were nearly 20% smaller (in mass) than their normoxia-gestated counterparts (Fig. 1B; $F_{1,63.1} = 8.39$, P = 0.005). Strikingly, highland dams gestating under hypoxia gave birth to pups that did not differ in mass from their normoxia-gestated counterparts (Fig. 1B; $F_{1,61.9} = 0.03$, P = 0.86), suggesting that natural selection against low birth weights at high elevation has led to adaptations in reproductive physiology that protect fetal growth.

Maternal physiology does not independently explain fetal growth outcomes. To identify the physiological factors shaping fetal growth outcomes in lowland and highland deer mice, we first asked whether maternal physiology explained fetal growth outcomes at mid-gestation (gestational day 18.5-19.5) in dams gestating under normoxia or hypoxia, at which point the fetal growth phenotype is already apparent (Table 1, Fig. 1C; lowland deer mice: P = 0.007; highland deer mice: P = 0.94). One way in which maternal physiology may mediate fetal growth is via nutrient acquisition. Most rodents restrict food intake when held under hypoxia, which can impact fetal growth (22). However, food consumption was not suppressed by hypoxia in lowland or highland deer mice (Figure S1; Table S1). Similarly, gestational hypoxia did not reduce mass gained in lowland or highland dams across pregnancy (Figure S1; Table S1).

Alternatively, maternal cardiopulmonary function could shape fetal growth trajectories under hypoxia, in which case

Table 1. Sample sizes across populations and treatments

Pop.	Treatment	Birth (Dams)	Birth (Pups)	Mid-preg. (Dams)	Mid-preg. (Pups)
Lowland	N	33	104	8	19
Lowland	H	3	8	10	20
Highland	N	26	119	10	120
Highland	H	3	21	9	20

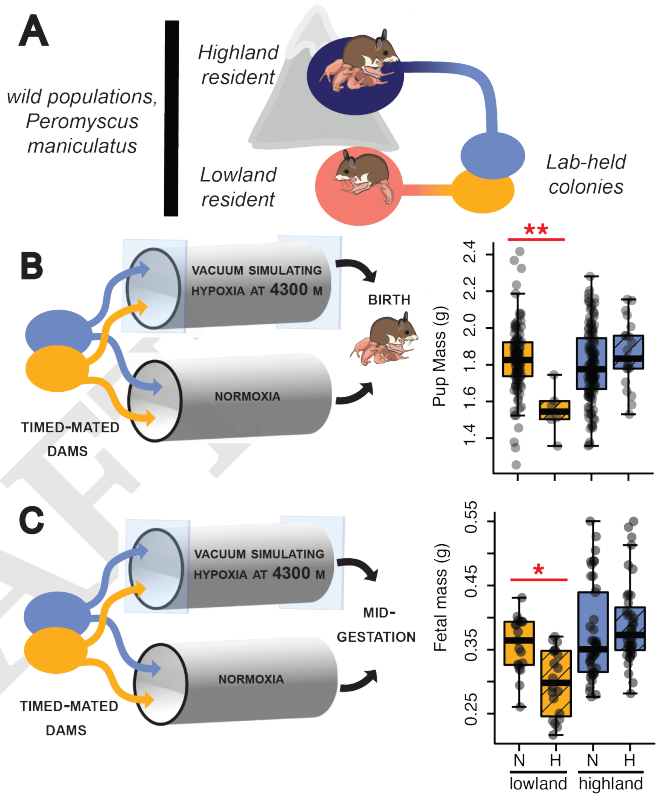

Fig. 1. (A) Deer mouse populations used in experiments were derived from wild-caught populations from low (pink/orange) and high (blue) elevations that have been maintained in lab colonies for at least two generations. (B) To test for population-specific effects of hypoxia on birth weight, time-mated dams from either population were held under hypoxia or normoxia until birth in ventilated chambers made from large PVC pipes. While lowland dams gave birth to smaller pups under hypoxia (right, bar chart; **P=0.005), pups born to highland dams were not affected. Each point represents a single pup from a litter; statistical tests control for litter and dam. (C) To examine mid-gestation phenotypes, timed-mated dams from either population were held under hypoxia or normoxia until mid-gestation (GD 18.5-19.5). Already by this timepoint, pups from lowland dams show fetal growth restriction (right, bar chart; *P = 0.007), whereas pups from highland dams do not. Each point represents a single pup from a litter; statistical tests control for litter. Sample sizes are provided in Table 1.

DRAFT

maternal traits should be associated with fetal mass. We therefore asked whether there were direct or condition-dependent relationships between maternal traits and population or hypoxia treatment (Table S2). We compared variance explained by these models with a model using only the experimental design (i.e., $Population \times Hypoxia$) to ask whether maternal traits explained significant variance in fetal growth, which would suggest that the maternal trait provided better predictive value.

We found that several maternal cardiopulmonary traits were indeed modified by hypoxia (Figure S1, Table S1), however variation in most of these traits was unrelated to fetal mass or failed to improve upon variance explained by the experimental design model (Table S2). That is, while some traits did explain variance among dams, the power to explain fetal growth largely overlapped with the predictive power of the experimental design model which ignored trait variation. We therefore next reasoned that small or interactive contributions of multiple maternal cardiopulmonary traits might influence fetal growth. To test this, we asked whether a combination of maternal physiological parameters explained fetal growth using a principal components analysis (PCA), the output of which is a set of principal components that represent multi-variate metrics (23, 24). Within our PCA of maternal trait measures, the first two principal components summarized 62.7% of the variation in maternal trait data, and the first five principal components explained over 95% of the variation. However, these multi-variate metrics still failed to explain significant variation in fetal growth among populations and treatments (Table S2).

These results suggest that the maternal physiological traits that we measured are not sufficient to explain fetal growth restriction under gestational hypoxia in lowlanders or the protection thereof in highlanders. This result contrasts with work in humans, where maternal physiological traits like hematocrit or pulmonary function do provide predictive value with regards to fetal growth outcomes (4, 5). Furthermore, because maternal physiology interacts broadly with fetal growth and development (25, 26), it seems unlikely that maternal physiology is entirely inconsequential for fetal growth outcomes in deer mice. The absence of evidence for associations between maternal physiology and fetal growth in our experiments may be a function of the limited set of cardiopulmonary traits we measured in a relatively small number of dams; other traits that we did not measure (e.g., pulmonary function, blood pressure) may still directly contribute to fetal growth outcomes. Alternatively or in addition, the effect of maternal traits on fetal growth is dependent on other factors, such as placental development and/or function.

Zone-specific expansion in the highlander placenta contributes to fetal growth preservation. The placenta performs several functions that are essential for successful pregnancies, including gas and nutrient exchange between the maternal and fetal circulatory systems. Altered placental development has been repeatedly linked to fetal growth restriction associated with chronic gestational hypoxia (5, 27), and thus we were interested in whether placental structure or function explained fetal growth outcomes in deer mice. Indeed, we found that placenta mass covaried with fetal mass at mid-gestation in highlanders ($P = 0.03$; Fig. 2A,B, Table S3), suggesting that growth or structural remodeling in the placenta might contribute to protecting fetal growth in highlanders.

Rodent placentas are organized into three functional layers: the decidua, the junctional zone and the labyrinth zone (reviewed in (28, 29)). The decidua is comprised predominantly of maternal tissues, and it is the region where fetal and maternal cell types interact to establish blood flow from maternal circulation to the placenta. The fetal compartments of the placenta include the junctional zone, which plays a key role in the production of hormones producing cell types responsible for vascular remodeling, and the labyrinth zone, where maternal and fetal circulatory systems are brought into close apposition to facilitate gas and nutrient exchange. Because each layer of the placenta performs distinct functions, the relative contribution of each to placental expansion (and, by extension, fetal growth protection) present distinct functional hypotheses about the mechanisms or processes that are contributing to fetal growth outcomes under maternal hypoxia. We therefore asked whether underlying variation in placental structure could explain fetal growth outcomes.

Using immunohistochemistry to differentiate layers along the midline of placentas collected from dams at mid-gestation (Theiler Stage 23-24; Fig. 2C), we found that placentas from highland dams gestating under hypoxia had larger labyrinth zones as a proportion of the fetal placenta ($P < 0.04$ for all contrasts), and there was a trend towards this being true in absolute terms as well ($P < 0.14$ for all contrasts; Table S4). The increase in total mass seen in highland placentas under hypoxia can therefore be attributed to increased volume of the labyrinth zone. We also found that the labyrinth zones of highlanders had a greater proportion of their volume allocated to blood space (Fig. 2E,F), and that the maternal canal (a prominent vascular structure in the placenta though which maternal blood returns to maternal circulation) was largest in placentas from highland dams gestating under hypoxia (Effect of population: $P = 0.009$; Effect of hypoxia: $P = 0.035$; Table S4). Although the hypoxia-induced expansion of the labyrinth zone occurred only in highland mice, the blood space and maternal canal diameter also increased in lowland deer mice under hypoxia, though not to the extent observed in highlanders (e.g., Fig. 2F, Table S4).

Taken together, these results point to vascular organization within the labyrinth zone as a key factor mediating fetal growth under chronic gestational hypoxia. Our data suggest that lowlander placentas display beneficial, but potentially insufficient, structural responses to hypoxia that increase blood delivery and opportunity for nutrient and gas exchange within the placenta. Laboratory strains of mice and rats similarly display greater blood space in the labyrinth zone and an overall increase in placental mass near term, which is thought to contribute to protections in fetal growth and reduced oxidative stress under hypoxia (20, 30). Fetal growth restriction that persists in lowland deer mice and laboratory rodents may therefore reflect insufficiency of these protective responses (i.e., the expansion is not sufficient or early enough in development to overcome limitations imposed by maternal hypoxia) or a requirement for additional adaptations in interacting systems to achieve full protection.

In contrast, the villous portion of the human placenta, which is functionally analogous to the labyrinth zone, tends to be smaller at term in high elevation pregnancies (5). However, humans with highland ancestry display other structural

DRAFT

changes to the vasculature of this compartment that may counteract the overall decrease in volume (5). Thus, structural differences that modify nutrient and gas exchange appear universally relevant to fetal growth outcomes under chronic gestational hypoxia.

Genes involved in gestational hypoxia and high elevation adaptation in humans are associated with fetal growth and hypoxia responses in deer mice. Our findings that structural changes in the placenta explain fetal growth outcomes in deer mice do not preclude the possibility that changes to endocrine signaling or gene expression are also important. In particular, the junctional zone has been a major focus for understanding fetal growth during gestational hypoxia because of its role in coordinating maternal vasculature development at the implantation site (31–35). For these reasons, we were interested in assessing gene expression changes within each compartment, and how these changes may impact fetal growth trajectories.

We performed layer-enriched RNAseq on labyrinth zone and junctional zone/decidua tissues from lowland and highland dams sampled at mid-gestation (Theiler Stage 23-24; N = 19-20 implantation sites per treatment per population for a

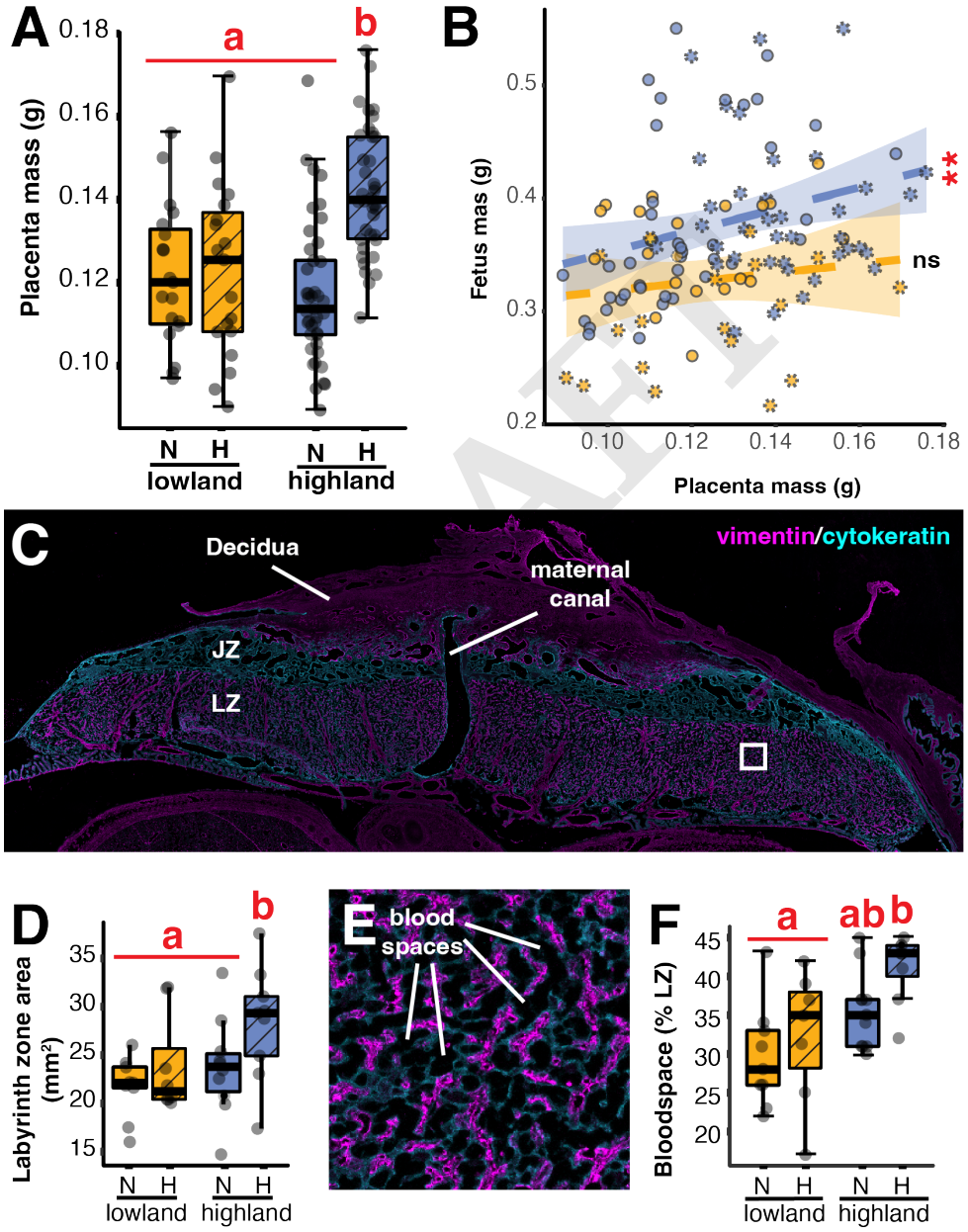

Fig. 2. Structural variation in the placenta explains fetal growth outcomes. (A) Placenta mass at mid-gestation is larger specifically in highlanders gestating under hypoxia. (B) Placental mass positively correlates with fetal mass, but only in highland deer mice. **P=0.03 (C) Compartment sizes and vascular structure within the placenta were quantified from mid-line cryosections that were fluorescently labeled with vimentin and cytokeratin. Maternal canal and decidua are indicated in the figure. The labyrinth zone [LZ] and junctional zone [JZ] are indicated with abbreviations. White box outlines area expanded in panel E. (D) The LZ was relatively and absolutely larger in highlanders under hypoxia than lowlander placentas. (E) Expanded box from panel C showing blood space in LZ in detail. (F) Blood space in the LZ is expanded with hypoxia and particularly in highlanders. Different letters indicate significant differences in mean values at $P < 0.05$.

total N of 79 LZ and 78 JZ; layer enrichment following (36)). After filtering, we detected expression of 14,345 genes in the labyrinth zone, and 14,000 genes in the junctional zone/decidua tissues. To identify genes that were relevant to fetal growth outcomes and adaptive evolution in response to environmental hypoxia, we focused on genes whose expression patterns (i) were correlated with fetal mass, (ii) differed with gestational treatment (hypoxia or normoxia), and/or (iii) differed among populations. We identified genes fitting these categories using a a linear mixed modelling approach (dream, (37)) that accounts for non-independence among samples collected from the same dam. Few genes were differentially expressed between the fetal sexes (18 in the labyrinth zone, and two in the junctional zone/decidua; Table S5) and including this variable did not substantially impact the number or set of genes included in other categories of interest (Table S5). Accordingly, fetal sex was not considered further.

We first examined expression within an *a priori* set of genes hypothesized to be relevant to high elevation adaptation, placental physiology, and fetal growth outcomes in humans (38–46). Placental evolution within and outside of mammals has involved co-opting genetic toolboxes and neofunctionalization of gene duplications such that, even then functional processes involved in placentation are shared across species, the specific underlying genes can vary (47–49). Because of this, we might not expect there to be substantial overlap between human and deer mouse gene sets that are associated with fetal growth outcomes and hypoxia responses. However, overlap between humans and deer mice could point towards universal or convergent mechanisms involved in placental responses to hypoxia that have shared importance across mammals.

Our *a priori* gene list contained 212 genes and 7 receptor/ligand or gene families in humans, for which we were able to identify 253 related genes in deer mice (Table S6). Of these, 204 were expressed in the deer mouse junctional zone/decidua and 208 were expressed within the deer mouse labyrinth zone (Table S6). The expression level of five genes (2.4%) in the junctional zone/decidua and 30 expressed genes (14.4%) in the labyrinth zone were correlated with fetal growth outcomes (Fig. 3A). These proportions represent significant enrichment relative to counts of genes correlated with fetal growth in the full dataset (Fisher's Exact Test; junctional zone/decidua: $P = 3.5$ E-8; labyrinth zone: $P = 0.04$), illustrating that candidate genes in humans are also associated with altitude-dependent variation in fetal growth trajectories in deer mice.

Of the genes in our *a priori* list, nine were significant for at least two of our focal comparisons (fetal mass, population, oxygen treatment, or their interaction) in the junctional zone/decidua, and 21 genes were in the labyrinth zone (Fig. 3B; Table S6). In either tissue, several genes associated with variables of interest have clear connections to hypoxia and/or placental development. For example, in the junctional zone/decidua, we found associations between fetal growth and the expression of fetal hemoglobins *Hba-x* and *Hbb-y* (oxygen transport), *Plin2* (trophoblast cell survival under hypoxia (50)), and *F3* (clotting, pre-eclampsia risk (51, 52)). In the labyrinth zone, we found *a priori* genes associated with outcomes of interest that are involved in diverse processes including nutrient transport (i.e., *Slc32a2*, *Slc38a1*, *Fabp4*; (53, 54)), vascular growth and function (i.e., *Edn1* and *Plagl1*; (55–57)), and trophoblast cell differentiation or survival (i.e., *Pappa2*, *Prkcz*, *Furin*, and *Mmp28*; (58–63)). The two most prominent candidates from human literature, *PRKAA1* and *EDNRA* (38), were not significant for multiple factors of interest in deer mice. However, *Prkaa1* expression in the labyrinth zone was significantly correlated with fetal growth outcomes, and *Ednra* expression in both the labyrinth and junctional zones is constitutively downregulated in highlanders relative to lowlanders. These genes may thus still be of relevance to placental or fetal development under hypoxia in the deer mouse, as they are in humans. Although further work is needed to verify shared functional roles among species, concordance among genes that underlie fetal growth outcomes in humans and deer mice are suggestive of fundamental similarities in how chronic hypoxia shapes placental development and potential evolutionary convergence in how humans and deer mice have mediated these challenges.

Broad-scale gene expression in the labyrinth zone is associated with fetal growth. A major limitation of *a priori* gene sets is that they are less likely to discover novel mechanisms. We therefore surveyed transcriptome-wide patterns of gene expression to identify additional genes associated with fetal growth outcomes. To do so, we combined the results of our differential expression analysis (dream) with an unsupervised network-based approach (WGCNA (64)) to identify sets of genes with correlated patterns of expression (hereafter, gene modules). Module expression can be associated with phenotypic outcomes using the first principle component of gene expression (also known as the module eigengene E; (64)) as a summary of gene expression in the module. We generated modules using a signed approach so that genes within a module were directionally correlated (64). Combining differential expression and co-expression network approaches allowed us to identify both individual genes that were strongly correlated with growth outcomes (i.e., those that were significant in differential expression analyses) as well as putative networks (i.e., gene modules) that co-vary with fetal growth.

In the complete gene set, we found 1,354 genes in the labyrinth zone whose expression correlated with fetal growth and three modules associated with fetal growth containing an additional 582 genes (Fig 4A,C, Tables S7). In striking contrast, there were only three genes in the junctional zone/decidua whose expression correlated with fetal growth, and no gene modules (Fig. 4B; Table S8). The absence of meaningful association between broadscale gene expression in the junctional zone/decidua and fetal growth suggests that junctional zone and decidual cell types are not strongly tied to early fetal growth phenotypes in deer mice. The labyrinth zone, on the other hand, showed patterns of extensive gene expression associations consistent with our histological data.

These associations could be explained by changes in the relative abundance of different cell types within the labyrinth zone. If bulk gene expression predominantly reflected the cell-types that make-up labyrinth zone expansion (and thus fetal growth outcomes), we would also expect to find extensive associations between gene expression in the labyrinth zone and mass of the placenta. However, expression of only 33 genes in the labyrinth zone were associated with placental mass. Differential gene expression in the labyrinth zone associated with fetal growth is therefore unlikely to be a simple result of proliferation of specific cell types within the labyrinth zone. Instead, gene expression associated with fetal growth outcomes

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in the labyrinth zone likely reflects true differential expression that underlies functional changes.

Interestingly, two of the three genes correlated with fetal growth in the junctional zone/decidua were also among those genes correlated with fetal growth in the labyrinth zone with the largest effects sizes (fetal hemoglobin subunits alpha and beta (*Hba-x* and *Hbb-y*). Fetal hemoglobin genes have greater oxygen affinity than their adult counterparts and are critical for moving oxygen from the maternal circulation into fetal circulation (65). Greater expression of fetal hemoglobins could support greater oxygen uptake by fetal tissues, thereby protecting fetal growth under hypoxia. However, we found the opposite pattern: in both the junctional zone/decidua and labyrinth zone, expression of *Hba-x* and *Hbb-y* was negatively correlated with fetal mass (i.e., greater expression of hemoglobins was associated with smaller fetuses). Overexpression of hemoglobins is associated with microvascular damage and endothelial toxicity (66, 67), which may lead to fetal growth restriction. However, for a given expression level of *Hba-x* and *Hbb-y*, highlander fetuses still tended to be larger than lowlander fetuses. Therefore, if hemoglobin expression does contribute to fetal growth via endothelial damage, there must either be additional factors that interact with hemoglobin-related endothelial damage in lowlanders and/or alternate mechanisms by which highlanders are protected from hemoglobin-related endothelial toxicity.

We next focused on the genes correlated with fetal mass in the labyrinth zone to identify a broader set of functions that were likely to influence fetal growth trajectories. Using gene ontology (GO) analysis to evaluate functional enrichment among the genes that were associated with fetal growth within the labyrinth zone, we found enrichment for broad biological processes related to cell replication, division, and differentiation (Table S9). Because these terms were so broad, we also asked whether more specific enrichment categories could be associated with genes that were positively versus negatively correlated with fetal growth. Indeed, among genes with expression positively correlated with fetal growth, we found enrichment for terms specifically linked to blood vessel growth and development (Table S10). In contrast, genes with expression that was negatively correlated with fetal growth remained enriched for broad cell division and replication terms (Table S11).

We also asked whether gene modules that associated with fetal growth had similar functional profiles. Modules were numbered consecutively when ranked by gene content such that the number of genes within the module increased as module number increased (i.e., M2 contains more genes than M1, which was the smallest module). The expression of two of the labyrinth zone gene modules associated with fetal growth (M9 and M12, containing 124 and 271 genes respectively) did not differ by populations or hypoxia treatment, suggesting that these gene sets influenced fetal growth but were not directly responsive to hypoxia. Instead, we focused on gene module M16 (434 genes), which was both correlated with fetal growth and hypoxia-responsive (Fig. 4A,C; Table S7). Consistent with the positive association between M16 and fetal growth (Fig. 4C), genes in this module were enriched for functions tied to angiogenesis and blood vessel formation (Table S12). The positive correlation between this module and fetal growth along with the suppression of these genes in response to hypoxia suggests that the downregulation of these core vascular growth and angiogenic genes contributes to fetal growth restriction in lowland deer mice. However, because highland expression follows similar patterns in the absence of fetal growth restriction, highlanders must possess some adaptations that mediate these effects on fetal growth through alternative or interacting mechanisms.

In search of gene sets that might explain highlander protection of fetal growth, we asked whether any gene modules were significantly differentially expressed by both population and hypoxia, suggestive of a hypoxia-sensitive gene network that has been modified in highlanders relative to lowlanders. In the labyrinth zone, we found five gene modules with evidence of differences in hypoxia-sensitive expression in highlanders and lowlanders, either through interactive (M1 and M13), or additive (M18, M19, M20) effects (Figure S2). Here, we focus on those modules where we found evidence for interactive effects. M1 contained only 30 genes, which were enriched for the broad term "organelle membrane" and included genes associated with endoplasmic reticulum and Golgi apparatus function (Table S12). The larger module M13 contained 350 genes that tend to be up-regulated by lowlanders under hypoxia, but down-regulated by highlanders (Figure S2). The module was functionally enriched for terms tied to RNA metabolism and transcription (Table S12). It also included genes Fosb and Igf2, which are closely connected to placental growth and growth factor regulation (68, 69) and appear in our *a priori* gene set (Table S6). The patterns of gene expression in this module suggest that that regulation of RNA synthesis and metabolism contributes to placental adaptations to altitude and may influence fetal growth outcomes by interacting with vascular growth pathways that were otherwise inhibited by hypoxia. Indeed, general transcriptional repression and decreases in protein production have been previously associated with hypoxic stress in the placenta (70, 71), and thus one way in which highlanders may protect fetal growth is by preventing or avoiding this suppression.

Genes relevant to placental development and fetal growth show signatures of positive selection in highland deer mice. Previous population genomic scans focused on elevational adaptation in other mammals have lacked a detailed understanding of associated placenta-specific developmental responses to gestational hypoxia, including intermediate gene expression changes, limiting progress on potential genotype-to-phenotype connections. To ask whether adaptive evolution in high elevation deer mice has targeted genes or sets of genes relevant to fetal outcomes, we cross-referenced our placental gene sets (including *a priori* candidate genes, those correlated with fetal growth, those differentially expressed among hypoxia treatments, and those with population-specific responses to hypoxia) with genes known to have experienced a history of spatially-varying selection associated with elevation (17). Genes under selection in highland deer mice were previously identified using two metrics: (i) the Population Branch Statistic (PBS; (72)), which is a measure of population differentiation similar to the population fixation index (FST), but uses an outgroup to polarize the direction of selection and here is polarized to high-elevation mice, and (ii) redundancy analysis, which tests for genotypic associations with elevation. Our demographically-informed selection scan identified 1037 genes under selection in the same Colorado population used

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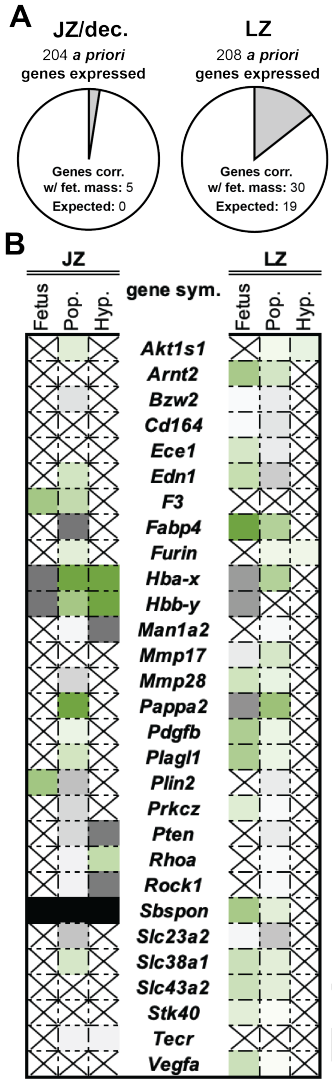

Fig. 3. Figure 3 (A) Pie charts showing enrichment for genes significantly correlated with fetal growth. (B) Summary table showing all *a priori* genes for which at least two categories in one tissue were significant. For all columns, cells are filled in significant correlations such that green cells are positively correlated or up-regulated, and grey cells are negatively correlated or down-regulated. Intensity of the color (i.e., light vs. dark grey) indicates log-fold-change of the effect. Numeric values are provided in Table S6. Black (Sbspon in JZ) indicates absence of expression in that tissue. For the population ("Pop.") column, up-regulation (i.e., green fill) indicates greater expression in lowland deer mice, and down-regulation (i.e., dark grey fill) indicates greater expression in highland deer mice. Crosses indicate no significant relationship.

to source our experimental highland mice (17).

In our labyrinth zone dataset, we found 654 expressed genes that were under spatially varying selection in highland deer mice. Using this full set of expressed genes under selection as a background expectation, we found significant enrichment for genes under selection in our *a priori* gene list ($P = 0.004$). Enrichment selection targets in this gene list reinforces our previous findings that genes of importance to fetal outcomes in humans are also likely to be important in deer mice. Many of these genes coordinate hypoxia responses across many tissue types, such as *Angpt1* and *Epas1*. The *a priori* gene set also contained genes under selection that have other well-established and specific functions connected to placental development, including several matrix metalloproteinases (63, 73) and the vascular endothelial growth factor receptor *Flt1* (74). However, we did not find that targets of selection were overrepresented among genes correlated with fetal growth or among genes that exhibited population specific responses to hypoxia. Collectively, these results suggest that the complex structural and regulatory placental adaptations that buffer fetal growth outcomes in high elevation deer mice might reflect evolutionary changes across relatively few genes. That is, many of the changes in gene expression that track variation in fetal growth may represent correlated outcomes of selection on a small number of variants rather than being the direct targets of selection themselves. However, this inference should be considered preliminary since the genic targets of selection were identified from whole-exome data, which exclude regulatory sequences not closely linked to sequenced exonic regions. Whole genome sequence data will be necessary to clarify relevant sequence variation in regulatory regions of genes that are functionally associated with fetal growth outcomes.

A weighted-ranking approach to stratify genes in the labyrinth zone underlying fetal growth outcomes. The broad-scale changes in gene expression that we have described within the deer mouse placenta poses some difficulties for experimental physiology; the 'best' genes to target for disentangling pathway-specific mechanisms are not immediately obvious among the large sets we have outlined above. Thus, we were interested in a quantitative approach that could be used to contrast or rank genes of interest for further study.

To accomplish this, we calculated a simple aggregate rank for each gene that considered effect size and p-value for associations with fetal growth or differential expression between populations and hypoxia treatment, as well as whether genes were under selection or *a priori* candidates (see SI Methods). This weighted ranking approach has the benefit of identifying genes that would not have been recognized through simple set overlap approaches (i.e., asking which genes were statistically significant for many outcomes of interest) (Fig. 5A). Nonetheless, we did find three genes that overlap with a majority of the gene sets of interest within the top 1% of ranked genes (Fig. 5). The fetal hemoglobin gene *Hba-x* was the second most highly ranked gene; its potential role in fetal growth outcomes is linked to oxygen transport and potential endothelial toxicity has previously been described. The other two genes, *Snx14* and *Thyn1*, are more enigmatic (Fig. 5, inset). Knocking out *Snx14* in house mice is lethal for development and associated with differentiation pathologies in the placental syncytiotrophoblast that result in extreme underdevelopment in the labyrinth zone (75). A more nuanced role for dose-dependent effects of expression on placental development is unknown; however, *Snx14*'s role in labyrinth zone development could contribute to hypoxia-dependent expansion of this zone in highland deer mice. Even less is understood about the role of *Thyn1* in placental development and function, though *Thyn1* abundance in cell-free RNA samples taken in early pregnancy is predictive for later pre-eclampsia development (76). *Thyn1* in the placenta may be implicated in inflammation and immune cell regulation (77–79), though further work is necessary to ascertain a mechanistic association with placental development.

When examined in aggregate, the top 1% of genes in our

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ranking can provide broad insight into functional roles of genes that were associated with many outcomes or factors of interest. As a whole, these genes were enriched for functions that are involved in extracellular matrix organization and collagen metabolism (Table S13). Collagen dysregulation and its metabolism in the extracellular matrix have been linked to vascular defects in the labyrinth zone (80, 81), and thus our weighted ranking again points to angiogenesis-related processes and their differential regulation among populations and treatments as being relevant to hypoxia-dependent fetal growth outcomes. More specifically, this enrichment suggests that processes involved in building vasculature (as opposed to regulatory signals that promote vasculogenesis) are likely closely tied to fetal growth trajectories, and that highland-adapted populations preserve fetal growth under hypoxia through modifying aspects of these processes.

In line with these broader patterns, our top ranked gene (regardless of weighting) is *Muc2*, a gene belonging to the mucin family. Mucin proteins are secreted onto epithelial surfaces where they provide lubrication, chemical barriers, and cell signaling roles (82–84). *Muc2* expression in humans is generally limited to the gastrointestinal epithelium and neoplasms (e.g., (85, 86)), and thus it has not been associated with gestational hypoxia and placental development. However, the production of other mucins are common in the human placenta and they have been implicated in placental development and pathologies. Specifically, overexpression of mucins is associated with inhibition of trophoblast cell motility (87, 88) and dysregulation of placental inflammation (89, 90). Down-regulation of *Muc2* by lowlanders experiencing gestational hypoxia and constitutively lower expression in highlands (Fig. 5B) may therefore support cell movements or migration relevant to vascular growth and placental development.

The top 12 genes in our top 1% of the ranking are provided in Fig. 5B. Many of these genes have putative roles in fetal growth or placental hypoxia responses, which affirms that mechanisms well-studied in other model systems and humans are important in deer mouse placentation and fetal growth. For example, placental expression of *Edn1* and *Abi3bp* in the placenta are associated with hypoxia-related pregnancy complications in humans (20, 57, 91).

Perhaps most compelling, two of our top genes are inti-

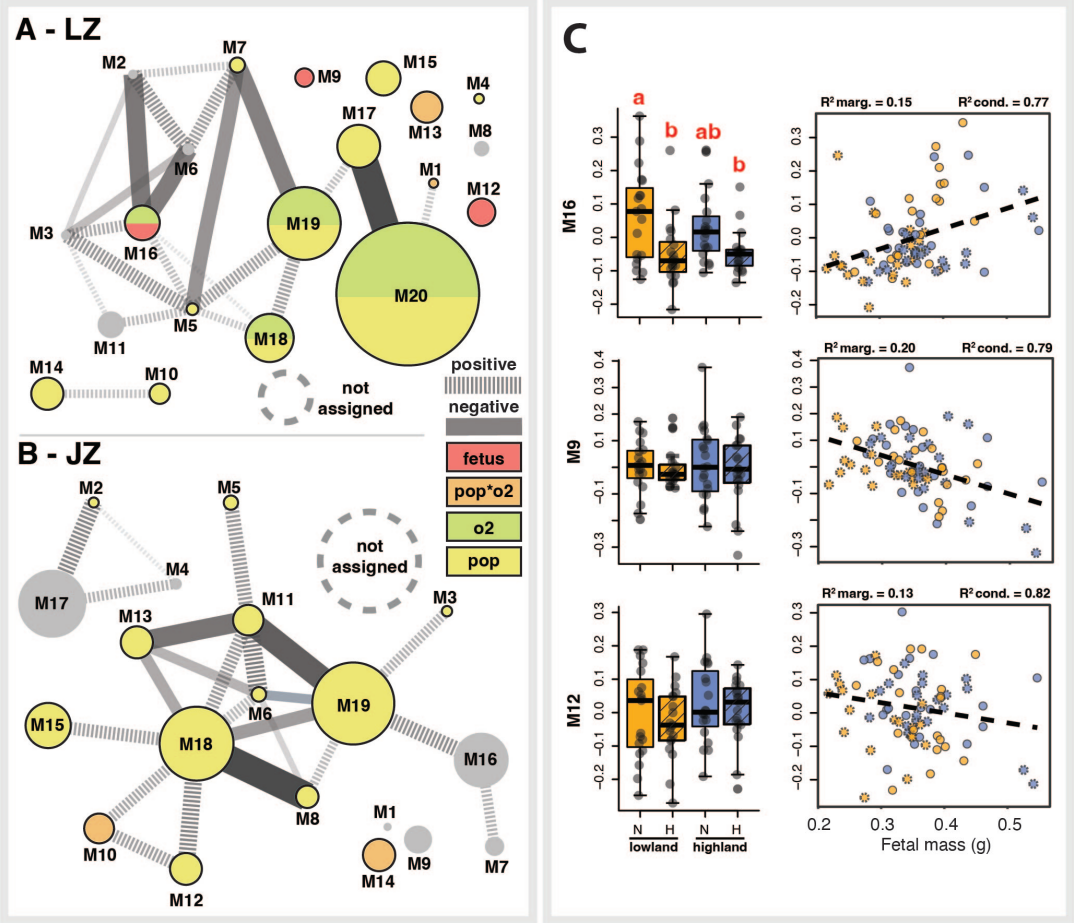

Fig. 4. (A,B) WGCNA module networks illustrating gene expression and module connectivity within the (A) labyrinth zone (LZ) and (B) junctional zone (JZ). Modules (ellipses) are scaled by the number of genes in each module. Correlations between modules of >0.6 R are shown by edges connecting modules. Width and opacity of the edge corresponds to strength of the correlation such that wider and darker edges indicate stronger correlations. Solid edges indicate positive associations, and dashed lines indicated negative correlations. Modules are also colored by their association with outcomes or experimental treatments of interest (fetal growth, red; population-by-hypoxia interactions, orange; hypoxia, green; population, yellow). (C) Plots of eigengenes from the LZ modules correlated with fetal growth (see panel A) showing differences among treatment groups (boxplots, left) and correlation with fetal growth (scatter plots, right). Different letters indicate significant differences in means values at $P < 0.05$. Dashed lines in the scatter plot show the linear relationship between fetal mass and the module eigengene. Pseudo-R-squared values for the linear mixed model are shown above each scatter plot.

mately associated with placental development and fetal growth: *Daam2* and *Pappa2*. Hypoxia-dependent overexpression of *Daam2* has recently been implicated in fetal growth restriction in humans (92). Although *Daam2* expression was not directly correlated with fetal growth in our deer mice, down-regulation of expression is consistent with protective effects for fetal growth. Thus, although the response to hypoxia differs between humans and deer mice, the role in shaping fetal growth outcomes may be similar.

The second candidate, *Pappa2*, has been a gene of interest in fetal growth and hypoxia research for over a decade (20, 60, 61, 76, 93). Overexpression of *Pappa2* has been associated with inhibition of trophoblast migration and pre-eclampsia development (93–95), and indeed we find a strong negative correlation between *Pappa2* expression in the labyrinth zone and fetal growth outcomes in deer mice. However, this gene remains overexpressed in the highlander labyrinth zones (Fig. 5B). Interestingly, Andean women resident at high elevations also display elevated serum concentrations of *Pappa2*, despite its association with pre-eclampsia risk in this population (95). This could suggest either that elevated expression of *Pappa2* is adaptive in other contexts that preclude placental adaptations to expression. Alternatively, post-transcriptional gene regulatory mechanisms, such as those identified our gene module analyses, may mediate the effects high *Pappa2* transcript production in highland deer mouse placentas.

Beyond the data from Andeans, which nevertheless did not assess adaptative function, neither of these genes have previously been suggested as relevant to adaptation to high-elevation in humans or other mammals. Further molecular work on these top candidate genes in a comparative framework is likely to be useful for understanding their functional importance for hypoxia-dependent fetal growth restriction and

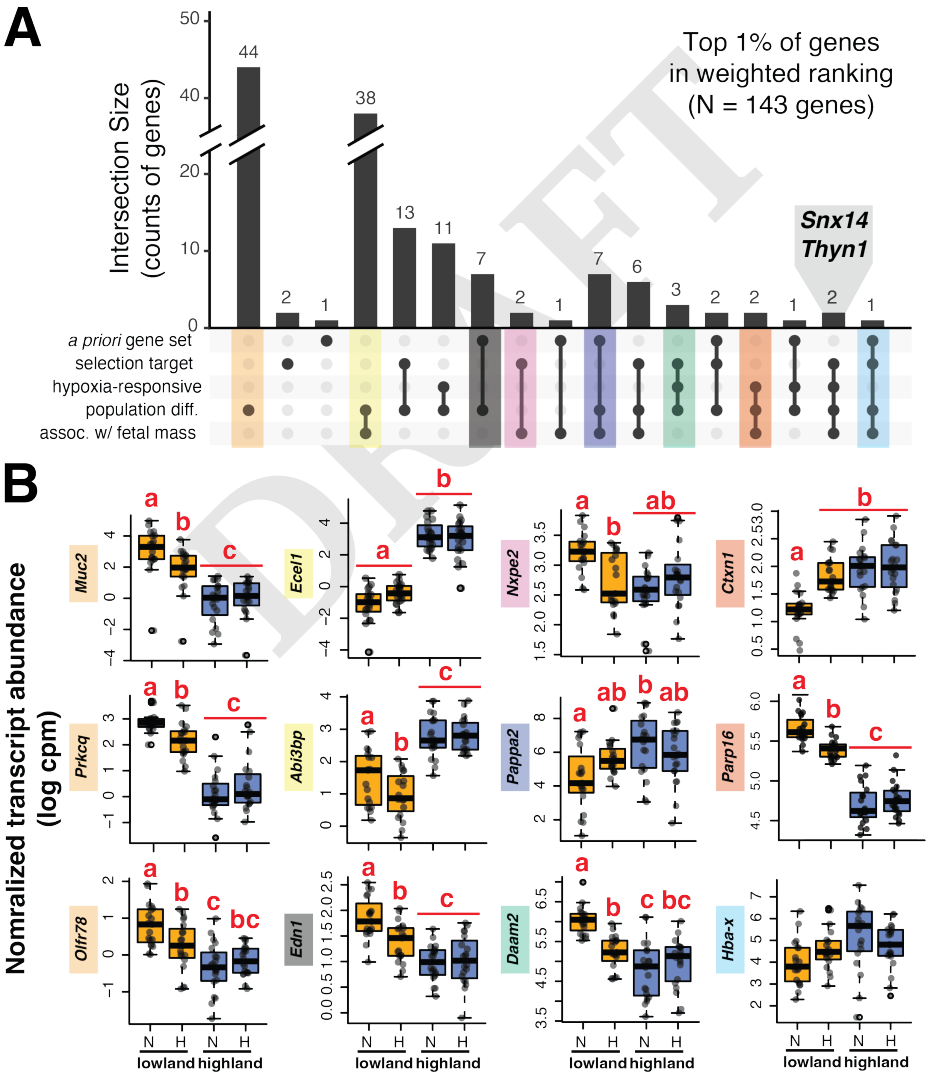

Fig. 5. (A) Upset plot illustrating intersection size based only on FDR-corrected statistical significance in a differential expression framework for all genes within the top 1% of weighted rankings (N = 143). Grey inset shows genes that were significant for 4 categories but that do not appear in panel [B]. (B) Expression differences among populations and gestational treatments for the top twelve (12) candidate genes associated with fetal growth outcomes and population-specific gene regulation based on a weighted ranking system approach (see text and methods for details). Each boxplot shows the normalized transcript abundance as log-transformed counts per million (log cpm) by population and treatment. The specific gene is indicated on the immediate Y axis label. Different letters indicate significant differences in means values at P < 0.05. Colors behind each gene name in indicate which intersection set contains that gene.

adaptative processes that may have modified those associations.

Conclusions. Understanding the basis of reproductive adaptations to altitude has the potential to yield important insights for fields of medicine, physiology, and evolutionary biology. However, progress in these areas has been hampered by the absence of an accessible study system. Here, we show that the deer mouse, Peromyscus maniculatus, an established model for adaptation to high elevation, can be used understand the basis of fetal growth outcomes in lowland and highland populations under hypoxia.

We found both structural and functional evidence that fetal growth under hypoxia is tied to development, and specifically to development and organization of the zone responsible for nutrient and gas exchange. The suppressed expression of genes related to angiogenesis and vascular growth in the placenta appears to be an ancestral response to hypoxia that persists in highland-adapted deer mice. As part of their adaptation to high elevation environments, highland deer mice have overcome these effects through (a) modification of pathways that ultimately promote expansion of the placental compartment responsible for gas and nutrient exchange, (b) alterations to hypoxia-sensitive expression of genes tied to the regulation of RNA transcription and processing, and (c) sequence evolution in genes tied to vascular growth and function.

We also showed that many of these genes relevant to adaptive phenotypes in deer mice are the focus of on-going work in humans or have understood roles in human placental development or responses to hypoxia. The deer mouse placenta thus shares fundamental gene networks involved in mediating hypoxia responses with human placentas. In addition to affirming deer mice as a potential translational model, these findings point to conserved or convergent gene regulatory patterns that shape adaptive evolution in divergent mammals. A basic understanding of the genetic and physiological factors associated with gestational outcomes, as laid out here, provides opportunities to assess a variety of questions in evolutionary genetics, and to identify the mechanisms relevant to developing clinical interventions or diagnostics that are important for maternal-fetal health.

Data Availability. Phenotypic and histological data generated and analyzed as part of this study will be included in the published article online supporting files (Datasets S1 and S2). Raw reads from RNAseq datasets will be made available via SRA accession.

Supporting Information Appendix (SI) included..

SI Datasets. Datasets S1 and S2 are provided with this manuscript.

Materials and Methods

Animal breeding, handling, and experimental design. Highland-adapted deer mice (P. maniculatus) were bred at University of Montana from stock trapped at the summit of Mount Evans, CO. All highland-adapted deer mice were second generation removed from the wild (F2). Lowland deer mice were purchased from the University of South Carolina stock center. Lowland deer mice (BW strain) are derived from a population trapped in Ann Arbor, MI (elevation 256 m). Pregnant dams were assigned to either hypobaric hypoxia or normobaric normoxia on day 1 of pregnancy. Animals assigned to hypobaric hypoxia were held under conditions mimicking 4300 m elevation in identical housing as the normobaric normoxia counterparts. Prior to euthanasia, we measured food consumption (relative to the starting mass of food each animal was provided with) and we quantified maternal body condition using an EchoMRI quantitative magnetic resonance system (E26-281-BHlab, Houston, TX).

Sample collection and handling. Placenta, fetal, and maternal tissue and blood samples were collected during mid-pregnancy corresponding to Theiler Stage 24 (gestational day 18.5-19.5). For each litter, whole implantation sites were collected into isopentane on dry ice for immunohistochemistry. The remaining sites were held in chilled phosphate-buffered saline for dissection. Fetuses and placentas were individually weighed before dissection and snap freezing. Details on maternal tissue handling and collection of other maternal trait data are provided in the Supplementary Information.

Statistical analyses. Pup mass at birth was evaluated using linear mixed model that included litter size, and population by treatment interaction, and maternal ID as a random effect using lme4(96). We assessed significance of fixed effects and interactions using type III sum of squares in the car package(97), and we performed post-hoc tests within emmeans(98) and lmerTest(99) packages using a Benjamini-Hochberg correction for multiple comparisons. Fetal growth in mid-pregnancy was analyzed using a similar linear mixed model including maternal ID as a random effect. Including litter size as a covariate did not improve model fit, nor was the covariate significant, and thus litter size was excluded from the analysis. Similar approaches were used to assess variation in maternal traits among populations and treatments, to test for correlations between maternal traits and fetal growth outcomes, and to test for an effect of placental mass on fetal size. Further information can be found in the Supplementary Information.

Placental histology. Frozen implantation sites (N = 1 per dam per experimental group) were cryosectioned at 10 um and midline sections (identified by the presence of the maternal canal) were slide mounted for imaging. Sections were fixed with 4% paraformaldehyde, permeabilized using methanol, and blocked using 10% normal goat serum in PBS (Vector Laboratories S-1000). Sections were incubated over night with mouse anti-vimentin (Sigma-Aldrich V6630) followed by 1-h incubation with anti-mouse Alexafluor 568 (Invitrogen A11031). Sections were then incubated for 2-h with pan-cytokeratin antibody conjugated to FITC (Sigma-Aldrich F3418, lot: 088M4797V) followed by DAPI to visualize nuclei. Immunostained sections were cover slipped with Fluoromount-G and stored at 4C until imaging on a Zeiss Laser-Scanning Microscope 880 at 10X. Quantification was performed using FIJI (ImageJ 2.0.0-rc-69/1.52p). Comparisons among populations and treatments were carried out in R 4.0.5 using lmer(), lmerTest(), and emmeans(). Measures falling more than 3 SDs beyond the mean were excluded as erroneous.

RNAseq data generation and analysis. Tissue was homogenized in TriReagent (T9424, Sigma Life Sciences) using a Qiagen TissueLyser, and RNA was extracted using a hybrid TriReagent – RNeasy spin column method. Following TriReagent phase separation, the aqueous phase was used as input to an RNeasy column (Qiagen 74106), after which the manufacturer's protocol was followed. Stranded, RNA libraries were then prepared by Oregon Health Science University and sequenced using 150 bp PE IIlumina NovaSeqS4. We generated an average of 50.7M paired-end raw reads for our junctional zone/decidual samples (Range: 27.1 - 67.1M) and an average of 53.8M paired-end raw reads for our labyrinth zone samples (Range: 31.3 - 78.8M). Data were trimmed for adaptor contamination and quality using Trimmomatic (100). Sequences were then aligned to the Peromyscus maniculatus bairdii genome (assembly HU_Pman_2.1.3) using HISAT2 (101). Read counts were determined using featureCounts in Subread (102), allowing for fractional counting of mapping reads. We also annotated and mapped reads to 182 placenta-specific genes from Mus that were not annotated within the P. maniculatus genome (see Supplementary Information and Datasets for full details). After filtering, mapping, and feature

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assignment, our analysis included an average of 29.5M reads from our junctional zone/decidua samples (Range: 16.2 - 39.3M), and an average of 31.3M reads from our labyrinth zone samples (Range: 17.2 – 47.4M). Further details on analyses can be found in the Supplementary Information.

GO-enrichment analyses. We cross-referenced P. maniculatus gene IDs with Mus gene IDs via Ensembl before running GO analyses. P. maniculatus genes without Mus orthologues could not be included in GO analyses, leaving us with 13632 genes in the LZ and 13269 genes in the JZ/Dec. for analysis.

***a priori* dataset generation and analysis.** We compiled *a priori* genes of interest from the literature, including genes hypothesized to be relevant to altitude adaptation and protection of fetal growth in humans as well as genes with empirical evidence for differential expression among lowland and highland human populations in the placenta (39, 45). Neither (39) nor (45) associate gene expression with fetal growth outcomes, but instead focus on genes are differentially expressed between highlanders (Tibetans or Andeans) and lowlanders and hypoxia sensitivity. From Wu et al.51, we selected only the top 10% of genes that were differentially expressed between highlanders and lowlanders (approximately 100 genes). We asked whether *a priori* genes were more likely to be correlated with fetal growth or other factors of interest than the full dataset by comparing the proportion of genes within each with a nominal p-value of < 0.05 using a Fisher's exact test.

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1. JF Storz, GR Scott, ZA Cheviron, Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. *J. Exp. Biol.* **213**, 4125–4136 (2010) PMID: 21112992.
2. JF Storz, ZA Cheviron, Physiological genomics of adaptation to high-altitude hypoxia. *Annu. review animal biosciences* **9**, 149 (2021) publisher: NIH Public Access.
3. JB West, *High Life: A History of High-Altitude Physiology and Medicine*. (Springer), (2013) Google-Books-ID: o0rhBwAAQBAJ.
4. LG Moore, Hypoxia and reproductive health: Reproductive challenges at high altitude: fertility, pregnancy and neonatal well-being. *Reproduction* **161**, F81–F90 (2021) publisher: Bioscientifica Ltd section: Reproduction.
5. K Wilsterman, ZA Cheviron, Fetal growth, high altitude, and evolutionary adaptation: a new perspective. *Am. J. Physiol. Integr. Comp. Physiol.* **321**, R279–R294 (2021).
6. CE Robertson, K Wilsterman, Developmental and reproductive physiology of small mammals at high altitude: challenges and evolutionary innovations. *J. Exp. Biol.* **223** (2020) number: 24 publisher: The Company of Biologists Ltd section: Review.
7. CG Julian, MJ Wilson, LG Moore, Evolutionary adaptation to high altitude: a view from in utero. *Am. journal human biology : official journal Hum. Biol. Counc.* **21**, 614–622 (2009) PMID: 19367578 PMCID: PMC2850611.
8. J Friedrich, P Wiener, Selection signatures for high-altitude adaptation in ruminants. *Animal Genet.* **51**, 157–165 (2020).
9. ME Pamenter, JE Hall, Y Tanabe, TS Simonson, Cross-species insights into genomic adaptations to hypoxia. *Front. Genet.* **11** (2020) [Online; accessed 2022-09-26].
10. JF Storz, Hemoglobin function and physiological adaptation to hypoxia in high-altitude mammals. *J. Mammal.* **88**, 24–31 (2007).
11. DD Wu, et al., Convergent genomic signatures of high-altitude adaptation among domestic mammals. *Natl. Sci. Rev.* **7**, 952–963 (2020).
12. NL Bedford, HE Hoekstra, Peromyscus mice as a model for studying natural variation. *eLife* **4**, e06813 (2015).
13. JF Storz, ZA Cheviron, GB McClelland, GR Scott, Evolution of physiological performance capacities and environmental adaptation: insights from high-elevation deer mice (peromyscus maniculatus). *J. Mammal.* **100**, 910–922 (2019) publisher: Oxford Academic.
14. JF Storz, ZA Cheviron, Functional genomic insights into regulatory mechanisms of high-altitude adaptation. *Adv. experimental medicine biology* **903**, 113–128 (2016) PMID: 27343092 PMCID: PMC5482796.
15. JF Storz, et al., The molecular basis of high-altitude adaptation in deer mice. *PLoS Genet.* **3** (2007) number: 3 PMID: 17397259 PMCID: PMC1839143.
16. C Natarajan, et al., Intraspecific polymorphism, interspecific divergence, and the origins of function-altering mutations in deer mouse hemoglobin. *Mol. Biol. Evol.* **32**, 978–997 (2015).
17. RM Schweizer, et al., Broad concordance in the spatial distribution of adaptive and neutral genetic variation across an elevational gradient in deer mice. *Mol. Biol. Evol.* **38**, 4286–4300 (2021).
18. RM Schweizer, et al., Physiological and genomic evidence that selection on the transcription factor epas1 has altered cardiovascular function in high-altitude deer mice. *PLOS Genet.* **15**, e1008420 (2019) number: 11 publisher: Public Library of Science.
19. AM Carter, Animal models of human placentation–a review. *Placenta* **28 Suppl A**, S41–47 (2007) PMID: 17196252.
20. E Siragher, AN Sferruzzi-Perri, Placental hypoxia: What have we learnt from small animal models? *Placenta* **113**, 29–47 (2021) PMID: 34074553.
21. PL Grigsby, Animal models to study placental development and function throughout normal and dysfunctional human pregnancy. *Semin. reproductive medicine* **34**, 11–16 (2016) number: 1 PMID: 26752715 PMCID: PMC4799492.
22. MJ Soares, KM Varberg, K Iqbal, Hemochorial placentation: development, function, and adaptations†. *Biol. Reproduction* **99**, 196–211 (2018).
23. H Abdi, LJ Williams, Principal component analysis. *WIREs Comput. Stat.* **2**, 433–459 (2010).
24. T Schreiber, Processing of physiological data in *Nonlinear analysis of physiological data*. (Springer), p. 7–21 (1998).
25. PD Gluckman, MA Hanson, Maternal constraint of fetal growth and its consequences. *Semin. Fetal Neonatal Medicine* **9**, 419–425 (2004).
26. PD Gluckman, MA Hanson, The developmental origins of health and disease in *Early life origins of health and disease*. (Springer), p. 1–7 (2006).
27. F Mecacci, et al., Fetal growth restriction: Does an integrated maternal hemodynamic-placental model fit better? *Reproductive Sci.* **28**, 2422–2435 (2021).
28. J Schulkin, ML Power, *The Evolution of the Human Placenta*. (Johns Hopkins University Press, Baltimore), (2012) DOI: 10.1353/book.18851.
29. L Woods, V Perez-Garcia, M Hemberger, Regulation of placental development and its impact on fetal growth—new insights from mouse models. *Front. Endocrinol.* **9** (2018) publisher: Frontiers.
30. H Matheson, JHW Veerbeek, DS Charnock-Jones, GJ Burton, HW Yung, Morphological and molecular changes in the murine placenta exposed to normobaric hypoxia throughout pregnancy. *The J. Physiol.* **594**, 1371–1388 (2015) number: 5.
31. I Brosens, et al., The enigmatic uterine junctional zone: the missing link between reproductive disorders and major obstetrical disorders? *Hum. Reproduction (Oxford, England)* **25**, 569–574 (2010) number: 3 PMID: 20085913.
32. P Bu, SMK Alam, P Dhakal, JL Vivian, MJ Soares, A prolactin family paralog regulates placental adaptations to a physiological stressor. *Biol. Reproduction* **94** (2016) number: 5 PMID: 26985002 PMCID: PMC4939737.
33. GJ Burton, T Cindrova-Davies, HW Yung, E Jauniaux, Hypoxia and reproductive health: Oxygen and development of the human placenta. *Reproduction (Cambridge, England)* **161**, F53–F65 (2021) PMID: 32438347.
34. D Chakraborty, MAK Rumi, T Konno, MJ Soares, Natural killer cells direct hemochorial placentation by regulating hypoxia-inducible factor dependent trophoblast lineage decisions. *Proc. Natl. Acad. Sci.* **108**, 16295–16300 (2011) number: 39 publisher: National Academy of Sciences section: Biological Sciences PMID: 21900602.
35. CA Ducsay, et al., Gestational hypoxia and developmental plasticity. *Physiol. Rev.* **98**, 1241–1334 (2018).
36. D Qu, A McDonald, KJ Whiteley, SA Bainbridge, SL Adamson, Layer-enriched tissue dissection of the mouse placenta in late gestation in *The Guide to Investigation of Mouse Pregnancy*, eds. BA Croy, AT Yamada, FJ DeMayo, SL Adamson. (Academic Press, Boston), pp. 529–535 (2014) DOI: 10.1016/B978-0-12-394445-0.00044-8.
37. GE Hoffman, P Roussos, Dream: powerful differential expression analysis for repeated measures designs. *Bioinformatics* **37**, 192–201 (2021).
38. AW Bigham, et al., Maternal prkaa1 and ednra genotypes are associated with birth weight, and prkaa1 with uterine artery diameter and metabolic homeostasis at high altitude. *Physiol. Genomics* **46**, 687–697 (2014) number: 18 PMID: 25225183 PMCID: PMC4166715.
39. WE Gundling, et al., Dna methylation explains a subset of placental gene expression differences based on ancestry and altitude. *bioRxiv* p. 261974 (2018).
40. LG Moore, Human genetic adaptation to high altitudes: Current status and future prospects. *Quat. Int.* **461**, 4–13 (2017) PMID: 29375239 PMCID: PMC5784843.
41. LG Moore, SM Charles, CG Julian, Humans at high altitude: hypoxia and fetal growth. *Respir. physiology neurobiology* **178**, 181–190 (2011) number: 1 PMID: 21536153 PMCID: PMC3146554.
42. LG Moore, et al., Maternal adaptation to high-altitude pregnancy: an experiment of nature–a review. *Placenta* **25A**, S60–71 (2004) PMID: 15033310.
43. MCT van Patot, G Ebensperger, M Gassmann, AJ Llanos, The hypoxic placenta. *High Alt. Medicine Biol.* **13**, 176–184 (2012) PMID: 22994517.
44. OR Vaughan, et al., Effect of high altitude on human placental amino acid transport. *J. Appl. Physiol.* **128**, 127–133 (2019) number: 1 publisher: American Physiological Society.
45. D Wu, et al., How placenta promotes the successful reproduction in high-altitude populations: A transcriptome comparison between adaptation and acclimatization. *Mol. Biol. Evol.* **39**, msac120 (2022).
46. S Zamudio, The placenta at high altitude. *High Alt. Medicine Biol.* **4**, 171–191 (2003) PMID:

DRAFT

12855050.
47. A Dupressoir, et al., A pair of co-opted retroviral envelope syncytin genes is required for formation of the two-layered murine placental syncytiotrophoblast. *Proc. Natl. Acad. Sci.* **108**, E1164–E1173 (2011) publisher: Proceedings of the National Academy of Sciences.
48. CS Foster, et al., Different genes are recruited during convergent evolution of pregnancy and the placenta. *Mol. Biol. Evol.* **39**, msac077 (2022).
49. K Knox, JC Baker, Genomic evolution of the placenta using co-option and duplication and divergence. *Genome Res.* **18**, 695–705 (2008) PMID: 18340042 PMCID: PMC2336813.
50. I Bildirici, et al., Plin2 is essential for trophoblastic lipid droplet accumulation and cell survival during hypoxia. *Endocrinology* **159**, 3937–3949 (2018) PMID: 30351430 PMCID: PMC6240902.
51. P Ayala-Ramírez, et al., Expression of tissue factor and thrombomodulin in the placentas of pregnancies affected by early-onset and late-onset preeclampsia. *J. Obstet. Gynaecol. Res.* **47**, 2307–2317 (2021).
52. P Ayala-Ramírez, et al., Increased tissue factor and thrombomodulin expression and histopathological changes in placentas of pregnancies with preeclampsia. *J. Neonatal-Perinatal Medicine* **9**, 31–39 (2016) publisher: IOS Press.
53. S Lager, TL Powell, Regulation of nutrient transport across the placenta. *J. Pregnancy* **2012**, e179827 (2012) publisher: Hindawi.
54. HB Schiöth, S Roshanbin, MGA Hägglund, R Fredriksson, Evolutionary origin of amino acid transporter families slc32, slc36 and slc38 and physiological, pathological and therapeutic aspects. *Mol. Aspects Medicine* **34**, 571–585 (2013).
55. RR Starks, et al., Transcription factor plagl1 is associated with angiogenic gene expression in the placenta. *Int. J. Mol. Sci.* **21**, 8317 (2020) PMID: 33171905 PMCID: PMC7664191.
56. LR Stow, ME Jacobs, CS Wingo, BD Cain, Endothelin-1 gene regulation. *The FASEB J.* **25**, 16–28 (2011).
57. LG Thaete, T Jilling, S Synowiec, S Khan, MG Neerhof, Expression of endothelin 1 and its receptors in the hypoxic pregnant rat. *Biol. reproduction* **77**, 526–532 (2007) PMID: 17554077 PMCID: PMC1989130.
58. M Rasmussen, et al., Rna profiles reveal signatures of future health and disease in pregnancy. *Nature* **601**, 422–427 (2022) number: 7893 publisher: Nature Publishing Group.
59. S Shaha, et al., Human placenta and trophoblasts simultaneously express three isoforms of atypical protein kinase-c. *Placenta* **119**, 39–43 (2022).
60. S Sifakis, et al., Placental expression of pappa, pappa-2 and plac-1 in pregnacies is associated with fgr. *Mol. Medicine Reports* **17**, 6435–6440 (2018) publisher: Spandidos Publications.
61. PK Wagner, A Otomo, JK Christians, Regulation of pregnancy-associated plasma protein a2 (pappa2) in a human placental trophoblast cell line (bewo). *Reproductive Biol. Endocrinol.* **9**, 48 (2011).
62. Z Zhou, et al., The proprotein convertase furin is required for trophoblast syncytialization. *Cell Death Dis.* **4**, e593–e593 (2013) number: 4 publisher: Nature Publishing Group.
63. JY Zhu, ZJ Pang, Yh Yu, Regulation of trophoblast invasion: The role of matrix metalloproteinases. *Rev. Obstet. Gynecol.* **5**, e137–e143 (2012) PMID: 23483768 PMCID: PMC3594863.
64. P Langfelder, S Horvath, Wgcna: an r package for weighted correlation network analysis. *BMC bioinformatics* **9**, 559 (2008) PMID: 19114008 PMCID: PMC2631488.
65. AN Chesnutt, Physiology of normal pregnancy. *Critical Care Clin.* **20**, 609–615 (2004) publisher: Elsevier PMID: 15388191.
66. M Centlow, et al., Placental expression profiling in preeclampsia: local overproduction of hemoglobin may drive pathological changes. *Fertility sterility* **90**, 1834–1843 (2008) PMID: 18166190 PMCID: PMC2628488.
67. K May, et al., Perfusion of human placenta with hemoglobin introduces preeclampsia-like injuries that are prevented by 1-microglobulin. *Placenta* **32**, 323–332 (2011).
68. DIR Holmes, I Zachary, Placental growth factor induces fosb and c-fos gene expression via flt-1 receptors. *FEBS Lett.* **557**, 93–98 (2004).
69. V Sitras, R Paulssen, J Leirvik, årtun, G Acharya, Placental gene expression profile in intrauterine growth restriction due to placental insufficiency. *Reproductive Sci.* **16**, 701–711 (2009).
70. F Colleoni, et al., Suppression of mitochondrial electron transport chain function in the hypoxic human placenta: A role for mirna-210 and protein synthesis inhibition. *PLOS ONE* **8**, e55194 (2013) publisher: Public Library of Science.
71. HW Yung, M Cox, M Tissot van Patot, GJ Burton, Evidence of endoplasmic reticulum stress and protein synthesis inhibition in the placenta of non-native women at high altitude. *The FASEB J.* **26**, 1970–1981 (2012) publisher: Federation of American Societies for Experimental Biology.
72. X Yi, et al., Sequencing of 50 human exomes reveals adaptation to high altitude. *Science* **329**, 75–78 (2010) number: 5987 PMID: 20595611.
73. J Chen, RA Khalil, Chapter four - matrix metalloproteinases in normal pregnancy and preeclampsia in *Progress in Molecular Biology and Translational Science*, Matrix Metalloproteinases and Tissue Remodeling in Health and Disease: Target Tissues and Therapy, ed. RA Khalil. (Academic Press) Vol. 148, pp. 87–165 (2017) DOI: 10.1016/bs.pmbts.2017.04.001.
74. J Jebbink, et al., Expression of placental flt1 transcript variants relates to both gestational hypertensive disease and fetal growth. *Hypertension* **58**, 70–76 (2011) publisher: American Heart Association.
75. D Bryant, et al., Diverse species-specific phenotypic consequences of loss of function sorting nexin 14 mutations. *Sci. Reports* **10**, 13763 (2020) number: 1 publisher: Nature Publishing Group.
76. MN Moufarrej, et al., Early prediction of preeclampsia in pregnancy with cell-free rna. *Nature* **602**, 689–694 (2022).
77. T Fujita, F Kitaura, H Fujii, A critical role of the thy28-myh9 axis in b cell-specific expression of the pax5 gene in chicken b cells. *PLOS ONE* **10**, e0116579 (2015) publisher: Public Library of Science.
78. F Kitaura, et al., Normal b cell development and pax5 expression in thy28/thyn1-deficient mice. *PLOS ONE* **14**, e0220199 (2019) publisher: Public Library of Science.
79. H Toyota, XZ Jiang, H Asakura, J Mizuguchi, Thy28 partially prevents apoptosis induction following engagement of membrane immunoglobulin in wehi-231 b lymphoma cells. *Cell. Mol. Biol. Lett.* **17**, 36–48 (2012) publisher: De Gruyter.
80. BV Natale, et al., Reduced uteroplacental perfusion pressure (rupp) causes altered trophoblast differentiation and pericyte reduction in the mouse placenta labyrinth. *Sci. Reports* **8**, 17162 (2018) number: 1 publisher: Nature Publishing Group.
81. BV Natale, et al., 9-tetrahydrocannabinol exposure during rat pregnancy leads to symmetrical fetal growth restriction and labyrinth-specific vascular defects in the placenta. *Sci. reports* **10**, 1–15 (2020) publisher: Nature Publishing Group.
82. A Corfield, Eukaryotic protein glycosylation: a primer for histochemists and cell biologists. *Histochem. Cell Biol.* **147**, 119–147 (2017) PMID: 28012131 PMCID: PMC5306191.
83. AP Corfield, Mucins: a biologically relevant glycan barrier in mucosal protection. *Biochimica Et Biophys. Acta* **1850**, 236–252 (2015) PMID: 24821013.
84. N Moniaux, F Escande, N Porchet, JP Aubert, SK Batra, Structural organization and classification of the human mucin genes. *Front. Biosci. A J. Virtual Libr.* **6**, D1192–1206 (2001) PMID: 11578969.
85. A Allen, DA Hutton, JP Pearson, The muc2 gene product: a human intestinal mucin. *The Int. J. Biochem. Cell Biol.* **30**, 797–801 (1998).
86. S Yonezawa, M Goto, N Yamada, M Higashi, M Nomoto, Expression profiles of muc1, muc2, and muc4 mucins in human neoplasms and their relationship with biological behavior. *PROTEOMICS* **8**, 3329–3341 (2008).
87. MK Shyu, et al., Muc1 expression is increased during human placental development and suppresses trophoblast-like cell invasion in vitro1. *Biol. Reproduction* **79**, 233–239 (2008).
88. MK Shyu, et al., Mucin 15 is expressed in human placenta and suppresses invasion of trophoblast-like cells in vitro. *Hum. Reproduction* **22**, 2723–2732 (2007).
89. G Laskarin, SS Medancic, A Redzovic, D Duric, D Rukavina, Specific decidual cd14+ cells hamper cognate nk cell proliferation and cytolytic mediator expression after mucin 1 treatment in vitro. *J. Reproductive Immunol.* **95**, 36–45 (2012).
90. C Scholz, et al., Association of placental inflammation with fetomaternal hemorrhage and loss of placental mucin-1. *Arch. Gynecol. Obstet.* **285**, 605–612 (2012).
91. J Nevalainen, S Skarp, ER Savolainen, M Ryynänen, J Järvenpää, Intrauterine growth restriction and placental gene expression in severe preeclampsia, comparing early-onset and late-onset forms. *J. Perinat. Medicine* **45**, 869–877 (2017) publisher: De Gruyter.
92. N de Alwis, et al., Daam2 is elevated in the circulation and placenta in pregnancies complicated by fetal growth restriction and is regulated by hypoxia. *Sci. Reports* **11**, 5540 (2021) number: 1 publisher: Nature Publishing Group.
93. K Macintire, et al., Pappa2 is increased in severe early onset pre-eclampsia and upregulated with hypoxia. *Reproduction, Fertility Dev.* **26**, 351 (2014).
94. AW Kramer, LM Lamale-Smith, VD Winn, Differential expression of human placental papp-a2 over gestation and in preeclampsia. *Placenta* **37**, 19–25 (2016).
95. LM Lamale-Smith, et al., Uteroplacental ischemia is associated with increased papp-a2. *Reproductive Sci.* **27**, 529–536 (2020).
96. D Bates, M Mächler, B Bolker, S Walker, Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67** (2015) number: 1.
97. J Fox, S Weisberg, *An R companion to applied regression*. (Sage publications), (2018).
98. RV Lenth, emmeans: Estimated marginal means, aka least-squares means (2021) Programmers:n25039.
99. A Kuznetsova, PB Brockhoff, RHB Christensen, lmertest package: Tests in linear mixed effects models. *J. Stat. Softw.* **82** (2017) number: 13.
100. AM Bolger, M Lohse, B Usadel, Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
101. D Kim, JM Paggi, C Park, C Bennett, SL Salzberg, Graph-based genome alignment and genotyping with hisat2 and hisat-genotype. *Nat. biotechnology* **37**, 907–915 (2019) PMID: 31375807 PMCID: PMC7605509.
102. Y Liao, GK Smyth, W Shi, featurecounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).

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