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

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Abstract

Hypoxia at high elevations challenges gestational 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 limited 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 placental compartment that facilitates nutrient and gas exchange between dam and fetus. We then use layer-enriched transcriptome analyses to show that adaptive structural remodeling of the placenta is coincident with widespread changes in gene expression localized to 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 overlayed our results with genetic data from natural populations to identify the subset of placental genes that form the genetic basis of placental adaptations. Collectively, these experiments advance our understanding of placental adaptation to hypoxic environments and illuminate physiological mechanisms underlying fetal growth trajectories during a key gestational window.

Significance

Residence at high elevations is associated with increased risk for many 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 correlate 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 to the placenta and broad-scale shifts in gene expression. Our findings point towards structural phenotypes and gene expression profiles that are likely relevant to effects of hypoxia on fetal and placental development across mammals, including humans.

Main Text

Introduction

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). Reproductive outcomes are the ultimate arbiters of Darwinian fitness, and reproduction for many species is also compromised at high elevation. These effects 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 pre-eclampsia (4). However, humans with altitude-adapted ancestry (e.g., Tibetan, Quechua) experience reduced risk for these complications (5), 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. 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 (6). 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 might 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, 7–10), 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 considered outside of humans (6, 11).

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 to altitude 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 believed to be derived from lowland populations (16), which mirrors the biogeographic history of many highland human populations (i.e., lowland individuals repeatedly 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); much of the genetic background in highland mice is shared with lowland mice, which allows fine-scale resolution of genomic regions that have experienced a history of natural selection at high elevation. Finally, deer mice are a useful translational model because they share some important aspects of reproductive physiology with humans, the other well-studied example of reproductive adaptations to altitude. In addition to similarities in how maternal traits such as organ growth and blood volume expansion adjust to pregnancy, rodents also have structurally similar placentas on the macro (i.e., discoid placentas), and at a microscopic scales (hemochorial) (19, 20). Although there are some relevant differences between rodents and humans with regards to the degree of fetal cell invasion into maternal tissues and term fetal development (21), the aforementioned similarities along with tractability for physiological study make rodent systems a primary and important

model for studying the effects of hypoxia on gestational outcomes (22), and deer mice share some of this fundamental utility.

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, we predicted that (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; 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; 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.

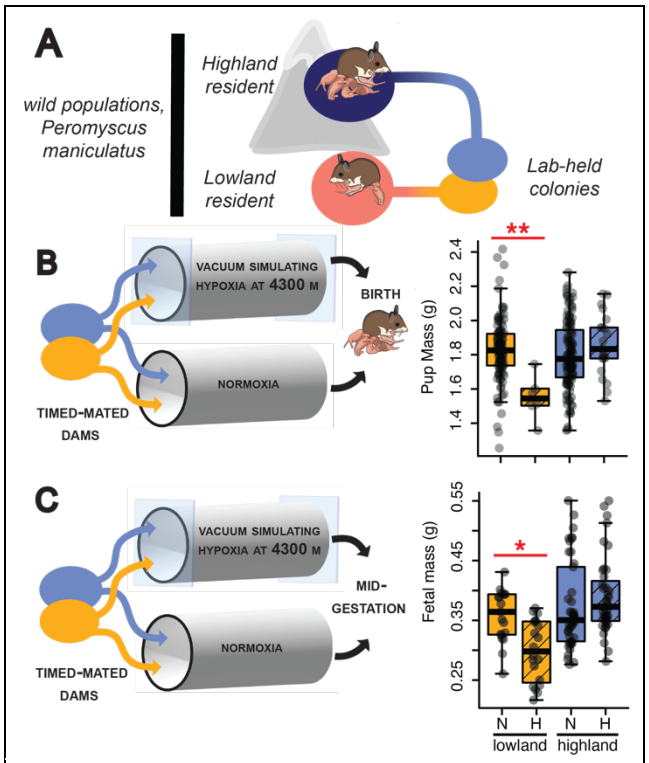

Figure 1 (A) Deer mouse populations used in experiments are derived from wild-caught populations from low and high altitude that have been maintained in captive lab colonies. (B) To test for population-specific effects of hypoxia on birth weight, mated dams from either population were held under hypoxia or normobaric normoxia until birth. On the day of birth, pups were massed. While lowland dams gave birth to smaller pups under hypoxia (**P=0.005), pups born to highland dams were not affected. (C) To test examine mid-gestation phenotypes, timed-mated dams from either population were held under hypoxia or normobaric normoxia until mid-gestation (GD 18.5-19.5). Already by this timepoint, pups from lowland dams show fetal growth restriction (*P = 0.007), whereas pups from highland dams do not. Sample sizes are provided in Table 1.

Maternal physiology does not independently explain fetal growth outcomes.

Hypoxia can impact fetal growth trajectories by altering maternal physiology, by shaping development and function of fetal tissues, or through some interaction(s) among these processes. 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 impacts 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).

If other aspects of maternal physiology, such as cardiopulmonary function, were largely responsible for fetal growth trajectories under hypoxia, maternal traits should be associated with fetal mass. Such associations could be

simple (i.e., a maternal trait correlates with fetal growth in both populations and under both treatments) or conditional (i.e., a maternal trait only correlates with fetal growth outcomes in one population or under hypoxia), and thus we examined interactions between maternal traits and population or hypoxia treatment (Table S2). We also compared variance explained by models that included maternal traits with the model using only the experimental design (i.e., Population×Hypoxia) to ask whether maternal traits explained significant variance in fetal growth, which would suggest that the maternal trait provided better predictive value.

Table 1 Sample sizes across populations and treatments.

Pop.	Treatment	Birth		Mid-pregnancy	
		Dams	Pups	Dams	Fetuses
Lowland	N	33	104	8	19
	H	3	8	10	20
Highland	N	26	119	10	20
	H	3	21	9	20

We found that several maternal cardiopulmonary traits were indeed modified by hypoxia (Figure S1, Table S1), however variation in most of these traits were unrelated to fetal mass (Table S2). Two maternal traits, left ventricle mass and dried lung mass, explained significant variation in pup mass (Table S2), but these models explained less variance in fetal growth than our null model that did not contain maternal traits. That is, while these traits did explain some variance among dams in maternal traits, the power to explain fetal growth largely overlapped with the predictive power of the null model containing population of origin and treatment.

We 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). PCA is a method used to reduce multi-dimensional data, 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 maternal physiological traits that varied in response to hypoxia and/or among populations were 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 provide predictive value with regards to fetal growth outcomes (4, 6). Furthermore, because maternal physiology interacts broadly with fetal growth and development (25, 26), it seems unlikely that maternal physiology is wholly inconsequential for fetal growth outcomes in this context. The absence of evidence for associations between maternal physiology and fetal growth in our experiments could be a function of the limited set of cardiopulmonary traits we measured in a relatively small number of dams. These patterns thus do not exclude the possibility that other traits not measured here (e.g., pulmonary function, blood pressure) may directly contribute to fetal growth outcomes in deer mice. Alternatively, and more likely in our view, is that the effect of maternal traits on fetal growth still depends on interactions with other factors, such as placental development.

Zone-specific expansion in the highlander placenta contributes to fetal growth preservation

Altered placental development has been repeatedly linked to fetal growth restriction associated with chronic gestational hypoxia (6, 27). The placenta, which is responsible for gas and nutrient exchange between the mother and fetus during gestation, is sensitive to oxygen tension during development in ways that can alter growth and organization of the tissue (28). In deer mice, 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 highland mice.

Rodent placentas are organized into three functional layers: the decidua, the junctional zone and the labyrinth zone (reviewed in (29, 30)). 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 next 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 S3). 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: $F_{1,31}$ = 7.66, P = 0.009; Effect of hypoxia: $F_{1,31}$ = 4.82, P = 0.035; Table S3). 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 in the extent observed in highlanders (e.g., Fig. 2F, Table S3). This pattern is suggestive of beneficial, but potentially insufficient plasticity in the lowlanders' responses to hypoxia.

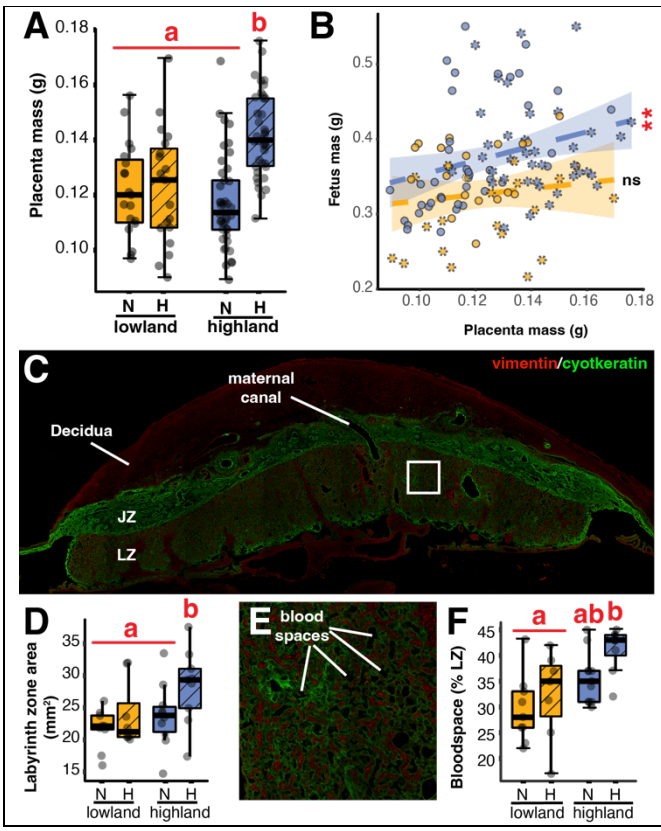

Fig. 2 Structural variation in the placenta explains fetal growth outcomes. (A) Placenta mass at mid-gestation is larger specifically in highlanders gestating under hypoxia. (B) Placental mass positively correlates with fetal mass, but only in highland deer mice. **P=0.03 (C) Compartment sizes and vascular 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.

Taken together, these results point to vascular reorganization within the labyrinth zone as a key factor mediating fetal growth under chronic gestational hypoxia.
These structural changes likely contribute to increased blood delivery and opportunity for nutrient and gas exchange within the placenta, thereby protecting fetal growth. Near term, greater blood space in the labyrinth zone and an overall increase in placental mass are similarly thought to contribute to the preservation of fetal growth and reduced oxidative stress under hypoxia in laboratory strains of mice and rats (22, 31).

In both lowland deer mice and other rodents, some placental responses to maternal hypoxia thus appear to be beneficial for fetal growth. Fetal growth restriction that persists in lowland rodents must therefore either 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 the 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 (6). However, individuals with highland ancestry display other structural changes to the vasculature of this compartment that may counteract the overall decrease in volume (6). Thus, structural differences that modify nutrient and gas exchange appear universally relevant to fetal growth outcomes under chronic gestational hypoxia, though whether the hypoxia-dependent responses to hypoxia are adaptive for fetal growth protection in lowlanders appears to differ between rodents and humans.

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 also contribute. In particular, the junctional zone has been a major focus for understanding fetal growth outcomes related to hypoxia because of its role in coordinating maternal vasculature development at the implantation site (32–36). For these reasons, expression profiles of both compartments are relevant to understanding how placental function influences fetal growth outcomes.

In order to assess the contribution of changes in gene expression within each compartment to fetal growth trajectories, we performed layer-enriched RNAseq on labyrinth zone and junctional zone/decidua tissues from lowland and highland dams, again at mid-gestation (Theiler Stage 23-24; N = 19-20 implantation sites per treatment per population; layer enrichment following (41)).

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 that fell into three categories: (i) genes with expression levels that were correlated with fetal mass, (ii) genes that were sensitive to hypoxia, and (iii) genes that were differentially expressed between populations. To test for these associations, we used a differential expression approach (dream, (42)) that uses a linear mixed modelling framework to account for non-independence among samples collected from the same dam. Few genes were differentially expressed between the sexes (18 in the labyrinth zone, and two in the junctional zone/decidua; Table S4) and including this variable did not substantially impact the number or set of genes differentially expressed in other categories of interest (Table S4). Accordingly, fetal sex was not considered in further analyses.

We first examined expression within an *a priori* gene set that included genes hypothesized to be relevant to altitude adaptation, placental physiology, and fetal growth outcomes in humans (43–51). Across its evolution, placentas have repeatedly co-opted genetic toolboxes and gene duplications from other tissues (52–54), and thus we might not expect there to be substantial overlap at the gene level between human and deer mouse gene sets that are

associated with fetal growth outcomes and hypoxia responses. However, overlap between humans and deer mice would point towards potentially universal or convergent genes and regulatory pathways involved in placental responses to hypoxia that are more likely to have shared importance across mammals and potential translational relevance.

Our *a priori* gene list contained 219 genes or gene families in humans, for which we were able to identify 253 orthologues or paralogues in deer mice. Of these, 204 were expressed in the deer mouse junctional zone/decidua and 208 were expressed within the deer mouse labyrinth zone (Table S5). Five expressed 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. We also asked whether genes identified *a priori* were more likely to be both associated with fetal growth *and* differentially expressed between populations relative to the full gene set, which could also reflect changes in expression in the placenta associated with adaptive evolution. Indeed, expression patterns of genes in our *a priori* set were more likely to be associated with fetal growth and differentially expressed between populations in both the labyrinth zone and junctional zone/decidua (labyrinth zone: P = 0.004; Junctional zone: P = 1.4E-6). Taken together, these results demonstrate that genes thought to be important in fetal growth outcomes and hypoxia adaptation in humans are also relevant to fetal growth in deer mice, and that expression of these genes has been modified in highlanders potentially as a result of local adaptation to high elevation.

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 S5). In either tissue, several genes associated with variables of interest have clear connections to hypoxia and/or placental development. Genes in the junctional zone/decidua that were associated with fetal growth include the fetal hemoglobins *Hba-x* and *Hbb-y*, which are involved in oxygen transport, *Plin2*, which is involved in trophoblast cell survival under hypoxia (55), and *F3*, a clotting factor connected to vascular signaling pathways and associated with pre-eclampsia cases (56, 57). In the labyrinth zone, we found *a priori* genes associated with treatment factors of interest that are involved in diverse processes including nutrient transport (i.e., *Slc32a2*, *Slc38a1*, *Fabp4*), vascular growth and function (i.e., *Edn1* and *Plagl1*), and trophoblast cell differentiation or survival (i.e., *Pappa2*, *Prkcz*, *Furin*, and *Mmp28*). Finally, the two most

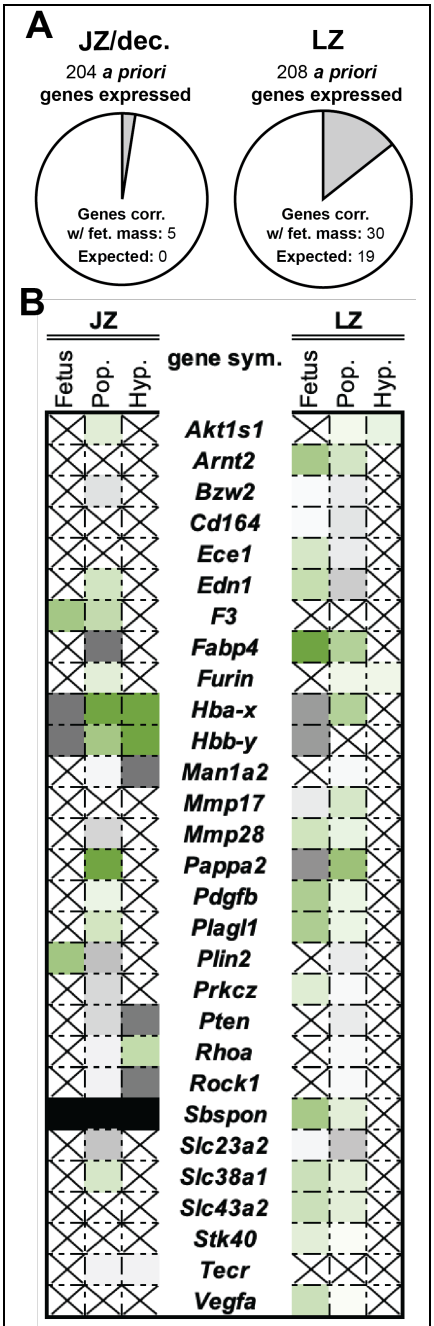

Figure 3 (A) Pie charts showing enrichment for genes significantly correlated with fetal growth. (B) Summary table showing all *a priori* genes for which at least two categories in one tissue were significant. For all columns, cells are filled in significant correlations such that green cells are positively correlated or up-regulated, and grey cells are negatively correlated or down-regulated. 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.

prominent candidates from human literature, PRKAA1 and EDNRA (43), 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 the both the labyrinth and junctional zones is constitutively down-regulated 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 functional similarities among species, concordance among genes that underlie fetal growth outcomes in humans and deer mice are suggestive of similar functional relevance and potential evolutionary convergence.

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 (58)) 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*; (58)) 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 (58). 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 (Tables S6) and three modules associated with fetal growth containing an additional 582 genes (Fig 4A,C, Tables S7). In striking contrast, we found only three genes in the junctional zone/decidua that correlated with fetal growth (Table S8), and no gene modules (Fig. 4B; Table S9). 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. It also suggests that the relative abundance of cell types in this region is likely not being altered by gestational hypoxia in our treatments. On the other hand, the pattern of extensive gene expression associations in the labyrinth zone is consistent with our histological data, in which labyrinth zone expansion was closely tied to fetal growth protection.

These associations could be explained by changes to the relative abundance of different cell types within the labyrinth zone. If there were a simple association between gene expression, labyrinth zone expansion, and fetal growth outcomes, we expected to also find extensive associations between gene expression in the labyrinth zone and mass of the placenta. However, we did not find such associations. In the labyrinth zone, only 33 genes were associated with placental mass (Table S10). Differential gene expression in the labyrinth zone associated with fetal growth is therefore unlikely to be a simple result of variation in proliferation of specific cell types within the labyrinth zone. Instead, gene expression associated with fetal growth outcomes in the labyrinth zone likely reflects true differential expression that underlies functional changes in the labyrinth zone.

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 (Table S6, S8; 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 (59). 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). Instead, overexpression of hemoglobins is associated with microvascular damage and endothelial toxicity (60, 61), and thus 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 S11). Because these terms were so broad, we also asked whether more specific enrichment categories could be associated with

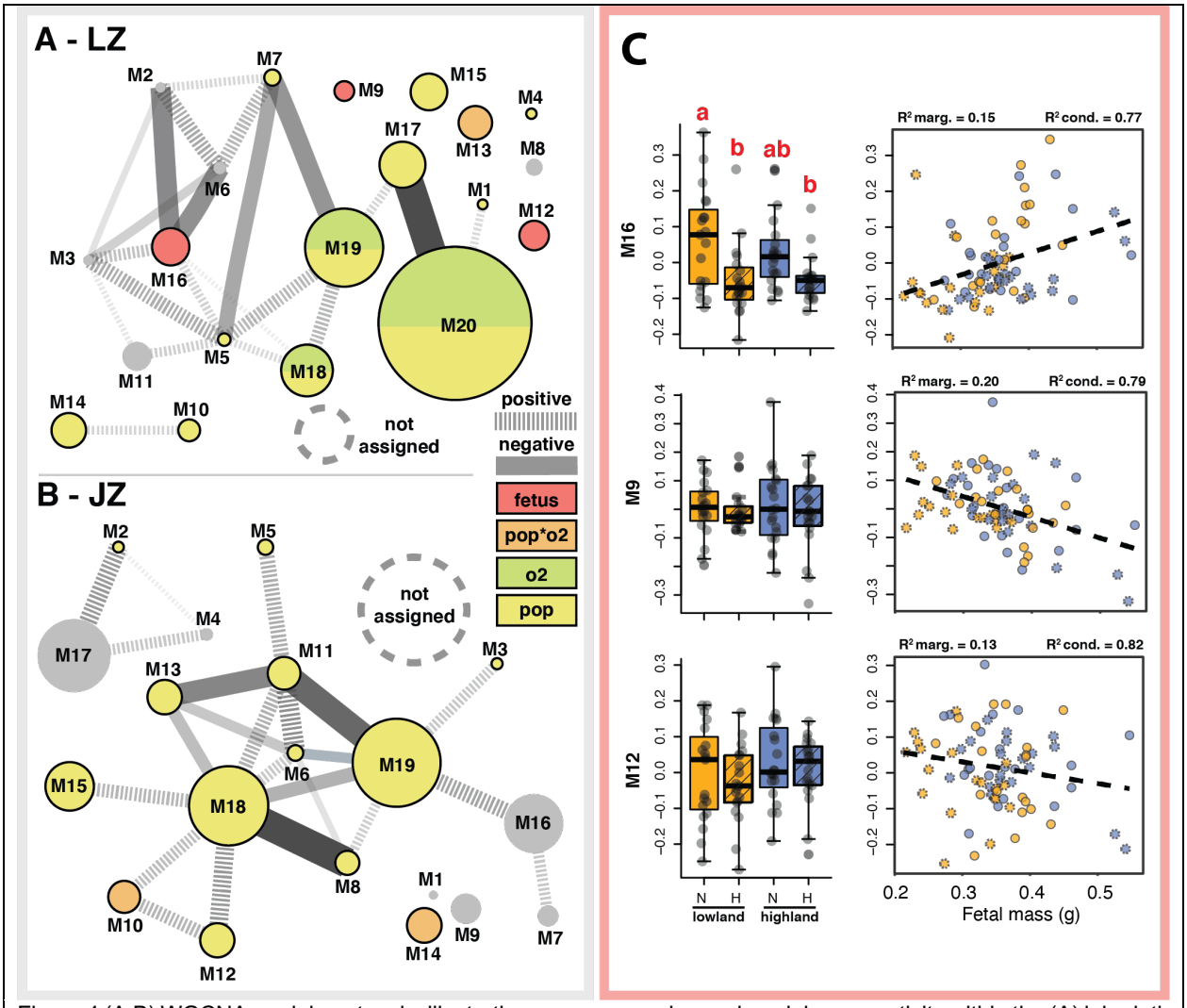

Figure 4 (A,B) WGCNA module networks illustrating gene expression and module connectivity within the (A) labyrinth zone (LZ) and (B) junctional zone (JZ). Modules (ellipses) are scaled by the number of genes in each module. Correlations between modules of >0.6 R are shown by edges connecting modules. 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.

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 S12), whereas genes with expression that was negatively correlated with fetal growth remained enriched for broad cell division and replication terms (Table S13).

We also asked whether gene modules identified through WGCNA that associated with fetal growth reflected similar functional profiles. Modules were numbered consecutively when ranked by gene content such that genes within the module increased as module number increased (i.e., M2 contains more genes than M1, which is 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 between by populations or hypoxia treatment, suggesting that these gene sets influence fetal growth but were not directly responsive to hypoxia. Instead, we focused on gene module M16, which was both correlated with fetal growth and hypoxia-responsive and contained 434 genes (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 S14). M16 also contained a number of genes involved in developmental responses of the placenta to hypoxia, including *Hif3a*, *Notch1*, and *Nrp1*(44, 62). 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 deer mice. However, because highland expression follows similar patterns without leading to fetal growth restriction, highlanders must possess some adaptations that mediate these effects on fetal growth through alternative or adjacent mechanisms.

In search of gene sets that might explain highlander protection of fetal growth, we also 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 S14). The more substantive 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 S14). It also included genes *Fosb* and *Igf2*, which are closely connected to placental growth and growth factors (63, 64) and appear in our *a priori* gene set (Table S5). 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 (65, 66), 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 have lacked a detailed understanding of placental phenotypes or even intermediate gene expression changes that occur in response to gestational hypoxia, 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, 18). Genes under selection in highland deer mice were previously identified using two metrics: (i) the Population Branch Statistic (PBS; (67), which is a measure of population differentiation similar to the population fixation index (F_{ST}), but uses an outgroup to polarize the direction of selection, and (ii) redundancy analysis, which tests for genotypic associations with elevation. Our demographically-informed selection scan identified 1037 genes as candidates for elevationally-driven selection in the same Colorado population used to source our experimental highlander mice (17, 18). Overlap between our selection targets gene set and genes associated with outcomes of interest would suggest that at least some of the genes associated with fetal outcomes harbor variants that are adaptive at high elevation. Statistical enrichment for genes under positive selection in any gene set could further highlight interacting networks of genes that are relevant to adaptive evolution in the placenta. Thus, we both looked for enrichment within gene sets as well as exploring the full set of genes under selection expressed in the labyrinth zone.

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 for genes under selection 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 tissue types, such as *Angpt1* and *Epas1*. However, the *a priori* gene set also contained genes under selection that have other well-established, specific functions connected to placental development, including several matrix metalloproteinases (70, 71) and *Flt1* (72). However, we did not find that targets of selection were overrepresented among genes correlated with fetal growth or those 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 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 dysregulation we have described within the deer mouse placenta poses some difficulties for experimental physiology; the 'best' genes for targeted manipulations 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 Methods). Although weighting based on inclusion in gene sets under selection or in our *a priori* sets increased the ranking of those genes substantially, the majority of the top 1% of ranked genes (104 of 143 genes) remain in the top 1% of regardless of whether selection and *a priori* status were considered. Furthermore, the top 0.1% of genes after weighting all fell within the top 0.2% of genes prior to weighting; thus, the weighting elevated genes of known or likely importance without swamping the majority of the biological signal in our datasets.

The 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 do find three genes that overlap with four 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). *Snx14* knockout is lethal for development and associated with differentiation pathologies in the placental syncytiotrophoblast that result in extreme underdevelopment in the labyrinth zone (73). A more nuanced role for dose-dependent effects of expression on placental development is unknown, however these knockdown experiments demonstrate a role for *Snx14* in labyrinth zone development that could explain hypoxia-dependent expansion of this zone in highland deer mice. Even less is understood about the role *Thyn1* might play in placental development and function – *Thyn1* (also known as *Thy28*) plays a potentially conserved role in apoptosis of immune cells (74–76), and *Thyn1* abundance in cell free RNA samples taken in early pregnancy is predictive for later pre-eclampsia development (77). *Thyn1* in the placenta may therefore be implicated in inflammation, however further work is necessary to ascertain a mechanistic association.

When examined in aggregate, the top 1% of genes in our 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 S16); collagen dysregulation and its metabolism in the extracellular matrix have been linked to vascular defects in the labyrinth zone (40, 78). Top genes in our weighted ranking thus again pointed to angiogenesis-related processes and their differential regulation among populations and treatments as 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 to be closely tied to how maternal hypoxia shapes fetal growth outcomes, and that highland-adapted populations preserve fetal growth under hypoxia through modifying aspects of these processes.

Because these data were generated from mid-gestation placentas, which remain understudied in rodents relative to term placentas (22), we predicted that some or many of

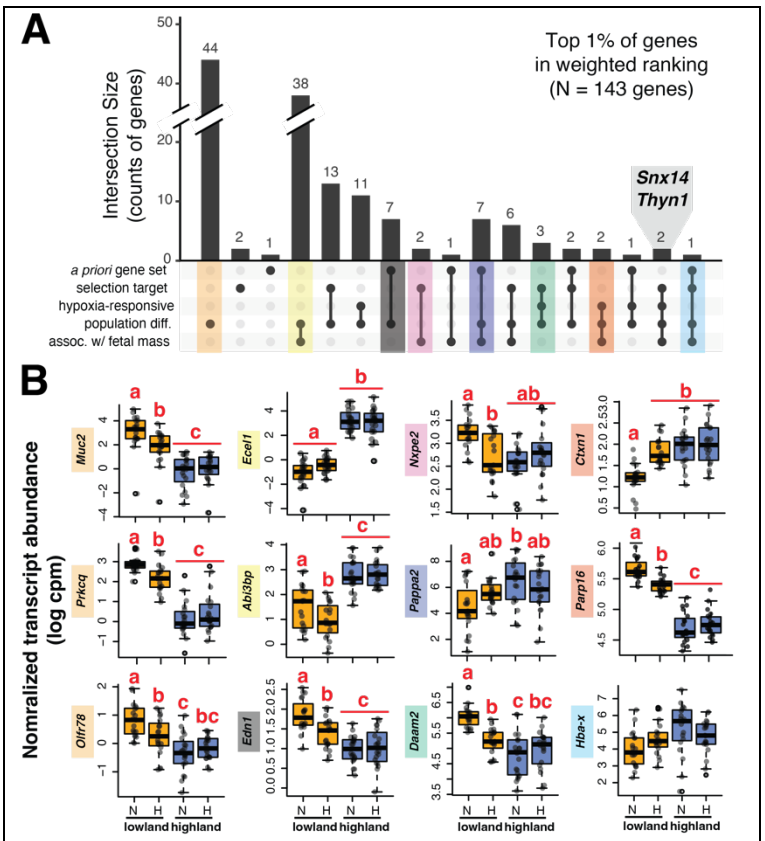

Figure 5. (A) Upset plot illustrating intersection size based only on FDR-corrected statistical significance in a differential expression framework for all genes within the top 1% of weighted rankings (N = 143). Grey insets shows genes that were significant for 4 categories but that do not appear in 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 showns 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 in the top panel genes fall in to.

our top candidates might be genes with no known or poorly understood function in the placenta. Indeed 6 of the top 12 genes (corresponding to the top 0.1% of genes; Fig. 5B, *Muc2*, *Prkcq*, *Ecel1*, *Ctxn1, Parp16* and *Nxpe2*) have no established role in placental development, hypoxia, and/or fetal growth outcomes. Furthermore, one of these genes, *Muc2,* is the top-ranked gene regardless of weighting.

As described earlier, evolution of the placenta has relied on gene duplication and co-option of pathways from other tissues, meaning that individual genes are less likely to be functionally conserved in the placenta across mammals (52, 54). Inferring the role of individual genes in placental and fetal development for non-traditional model systems may therefore benefit from a broader gene-family level view. For example, *Muc2* is a member of the mucin family of genes, some of which are secreted onto epithelial surfaces where they provide lubrication, chemical barriers, and cell signaling roles (79–81). *Muc2* expression in humans is generally limited to the gastrointestinal epithelium and neoplasms (e.g., (82, 83), 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 (84, 85) and dysregulation of placental inflammation (86, 87). 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, and it could contribute to regulation of inflammatory responses at the feto-maternal interface. Beyond this example, we should expect that greater insight about how placental development and adaptation to high altitude environments interact will depend on utilizing a comparative perspective that goes beyond considering one-to-one orthologues. Translational utility will likely similarly rely on understanding relatedness among gene families and their shared functions.

Other genes in our top 0.1% have putative roles in fetal growth or placental hypoxia responses, which affirms that mechanisms well-studied in other model systems were 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 (22, 88, 89). Perhaps most compelling, two of our top genes are intimately associated with placental development and fetal growth: *Pappa2* has been a gene of interest in fetal growth and hypoxia research for over a decade (22, 90–92), though it's causal role remains debated (93), and *Daam2* has recently been implicated in fetal growth restriction in humans (94). Serum concentrations of *Pappa2* were also correlated with elevational-variation in a measure of vascular resistance in the uterine artery of Andean women (95). Beyond this last example, neither of these genes have previously been recognized as relevant to adaptation to high-elevation in humans or other mammals. The shared basis for hypoxia responses and fetal growth is important for generalizing from deer mice across mammals and to humans specifically.

For those genes that rank highly and have known functions, we can also infer that the regulation or function of these genes can be modified in ways that may contribute to protecting fetal growth. As discussed above, instances in which expression of a gene in highland-adapted individuals differs from that in the closely-related lowlander can be evidence that natural selection has modified the expression of the gene in ways that contribute to adaptation to the new environment (96, 97). This is particularly true in cases where the regulatory response to hypoxia differs between populations, suggesting that regulation of the gene has been modified. It is also possible of course that differential expression across populations and treatments reflects effects rather than drivers of developmental trajectories shaping fetal growth outcomes. In these cases, these genes may still be useful markers of placental insufficiency or dysfunction in cases of maternal hypoxia that can be used to understand basic biology underlying development of that fetus and placenta in these contexts. Top-ranked genes from this study are therefore good candidates for more detailed study both as targets for placental evolution and translational potential.

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 have 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 altitude, can be used understand the basis of fetal growth outcomes in lowland and highland populations under hypoxia.

We find both structural and functional evidence that fetal growth under hypoxia is tied to placental 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 associated with fetal growth restriction 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 bu (a) modifying other systems that ultimately promote expansion of the placental compartment responsible for gas and nutrient exchange,(b) altering 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 show that many of these genes relevant to adaptive phenotypes in deer mice were shared with candidate genes that are the focus of on-going work in humans. However, our focus on mid-gestation has also brought to light a number of genes with previously unknown roles in placental developmental physiology and hypoxia sensitivity. A basic understanding of the genetic and physiological factors associated with gestational outcomes, as laid out here, provide opportunities to assess a variety of questions in evolutionary genetics, and to identify the mechanisms relevant to developing clinical interventions or diagnostics important for maternal-fetal health.

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 Univ. 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. Across the gestational study period, food consumption was measured, and maternal body condition was quantified 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 PBS 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 of birthweight, fetal mass outcomes, and association with maternal traits or placental mass: 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*(98). We assessed significance of fixed effects and interactions using type III sum of squares in the *car* package(99), and we performed post-hoc tests within *emmeans*(100) and *lmerTest*(101) 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: 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 Illumina 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).

RNAseq data processing and analysis: Data were trimmed for adaptor contamination and quality using Trimmomatic(102). Sequences were then aligned to the *Peromyscus maniculatus bairdii* genome (assembly HU_Pman_2.1.3) using HISAT2 (103). Read counts were determined using featureCounts in Subread (104), allowing for fractional counting of mapping reads. We also annotated and mapped 645 placenta-specific genes from *Mus* that were not annotated within the *P. maniculatus* genome (see Supplementary Information). After filtering, mapping, and feature 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(45, 51). Neither (45) nor (51) 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 2022, 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.

Aggregate rank analysis: Aggregate ranks were calculated using the following approach. First, all genes were assigned a rank (1-14,345) based on the effect size and adjusted p-value within each factor or outcome of interest (Population, hypoxia, the interaction of population and hypoxia treatment, and fetal mass), as shown in Eq. 1. By summing across these ranks (*Eq.* 2) and then examining those with the lowest summed rank score, genes that are marginally associated with many outcomes of interest can to outrank genes that may only be strongly associated with a single trait. However, we also wished to weight gene ranks based on *a priori* expectations about gene importance and what we knew about genes under selection in the deer mouse. We thus assigned weights of 1 or 0 for *a priori* and selection inclusion and weighted the summed rank based using this information as shown in Eq. 3

Equation 1

$$|logFC| \times (-log_{10}[p.val.])$$

Equation 2

$$AggRank = Rank_{fetus} + Rank_{pop.} + Rank_{hypoxia} + Rank_{popxhyp}$$

Equation 3

$$\frac{AggRank}{1 + a\ priori + selection}$$

When weighting the ranks by *a priori* and selection information, 80 of the top 100 genes were shared with the top 100 genes when ranked only using Eq.1 and Eq.2. Furthermore, 24 of the top 25 genes are shared between the two lists though their relative ranks shift.

Data availability: Data generated and analyzed as part of this study will be included in the published article online supporting files (Datasets 1-7)**,** including tables of RNAseq data for the labyrinth zone and junctional zone/decidua. Raw reads from RNAseq datasets will be made available via SRA accession.

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