ADAPTATION TO ENVIRONMENTAL STRESS IN THE HUMAN PLACENTA

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Molecular and Integrative Physiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

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ABSTRACT

The placenta mediates fetal growth by acting as a substrate for nutrient exchange between the mother and the fetus. Environmental factors such as altitude have been shown to result in reduced maternal-fetal exchange leading to reduced birth weight. To determine if differences in gene expression maybe contributing to physiological differences between individuals at high and low altitude, we tested for differences in gene expression and DNA methylation between Andean and European placental samples from La Paz (~4,000m) and Santa Cruz, Bolivia (~400m). Among the ancestry-associated differentially expressed genes, were genes involved in inflammation and placental specific pro-angiogenic macrophages, Hofbauer cells, contributing to increased capillary growth seen among Andeans residing at high altitude. Among the altitude-associated differentially expressed genes, we saw decreased expression of genes associated with the activator protein 1 (AP-1) transcription factor pathway and increased expression of genes involved in cytotrophoblast fusion including the gene dysferlin (*DYSF*). Upon closer examination we noticed that *DYSF* had a variant (rs10166384;G/A) at a methylation site with 3 levels of DNA methylation corresponding to individual genotypes. We tested for natural selection by sequencing a ~2.5kb fragment from 90 samples and performing Tajima's D test across the sample groups. We found that balancing selection (Tajima's D=2.37) was acting on a ~2.5kb fragment around our variant of interest among Andeans regardless of altitude. This suggests that balancing selection acting on dysferlin maybe altering DNA methylation patterns. Also, preservation of both the A and G alleles may aide Andeans in moving between altitudes.

ACKNOWLEDGEMENTS

I would like to thank the past and present members of the Wildman and Uddin lab for all the helpful scientific discussion and advice during the duration of my degree. I would like to thank my advisor Dr. Derek Wildman for his support and advice during the course of my PhD. Secondly; I would like to thank Dr. James Schulte II for introducing me to bioinformatics and genomic research early in my career. Also I would like to thank my committee at the University of Illinois at Urbana Champaign; Dr. Milan Bagchi, Dr. Kathryn Clancy, and Dr. Lori Raetzman for their valuable input and discussion about my project. I would also like to thank my former committee from Wayne State University consisting of Dr. Francesca Luca, Dr. Roger Pique-Regi, and Dr. D. Randall Armant for their suggestions and guidance in the early phases of this project. Also, I would like to thank Dr. Adi Tarca from Wayne State University for his help with learning the bioinformatics approaches necessary to complete this project. I would like to thank our collaborators Dr. Stacy Zamudio, Dr. Nicholas Islley, Dr. Christopher Kuzawa, and Dr. Julienne Rutherford for their expertise and guidance at many different points of this project. Lastly, I would like to thank my family and friends for supporting and encouraging me during the past 7 years especially my parents, William and Christine Gundling without whom none of this would be possible.

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CHAPTER 1: A REVIEW OF INTER- AND INTRA-SPECIFIC VARIATION IN THE EUTHERIAN PLACENTA

Abstract:

The placenta is one of the most morphologically variable mammalian organs. Four major characteristics are typically discussed when comparing the placentas of different eutherian species: placental shape, maternal-fetal interdigitation, intimacy of the maternal-fetal interface, and the pattern of maternal-fetal blood flow. Here we describe the evolution of three of these features as well as other key aspects of eutherian placentation. In addition to inter-specific anatomical variation, there is also variation in placental anatomy and function within a single species. Much of this intra-specific variation occurs in response to different environmental conditions such as altitude and poor maternal nutrition. Examinations of variation in the placenta from both intra- and inter-species perspectives elucidate different aspects of placental function and dysfunction at the maternal-fetal interface. Comparisons within species identify candidate mechanisms that are activated in response to environmental stressors ultimately contributing to the etiology of obstetrical syndromes such as preeclampsia. Comparisons above the species level identify the evolutionary lineages on which the potential for the development of obstetrical syndromes emerged.

Keywords: variation, placenta, adaptation[1]

[1]Published as : **Gundling W.E. Jr.,** Wildman D.E. A review of inter- and intra- specific variation in the eutherian placenta, *Philosophical Transactions of the Royal Society B*, 2015 Mar 5;370(1663).

Introduction:

The placenta is the conduit between the mother and fetus and directs fetal growth and development by acting as a source of nutrient exchange (1). The placenta mediates the transfer of nutrients including oxygen, amino acids, lipids, and glucose from the mother to the fetus and provides a method for fetal waste excretion by way of the mother (2). While these functions are conserved among placental mammals (i.e. eutherians), the morphology of the placenta is not (3). Here we discuss inter-specific placental variation in terms of how major characteristics of placental morphology differ within the eutherian mammals. Specifically, we focus on the shape of the placenta, the intimacy of the maternal-fetal interface, and the interdigitation pattern of the trophoblast in relation to the maternal tissue (4). We then discuss how these characteristics can shed light on multifactorial obstetrical syndromes associated with human placental dysfunction. Lastly, we examine how environmental stressors such as hypoxia and poor nutrition can drive placental plasticity, and how adaptations to these stressors can provide insight into the etiology of obstetrical syndromes (5-7).

Placental Variation in Eutheria:

The placenta is arguably one of the most variable organs in the animal kingdom. There are many characteristics that can be used when comparing the placentas of different species. Here we focus on three of the four major and most variable characteristics (placental shape, intimacy of maternal-fetal interface, and placental interdigitation). The fourth major feature involves the arrangement of the maternal and fetal bloodstreams (4). This feature partially determines the physiological diffusion efficiency of the various molecules exchanged between mother and fetus (2).

Unfortunately, this feature has not been described in enough mammalian species to accurately reconstruct its evolutionary history. Humans have a relatively inefficient multimillions exchange system (4). The other three major features of the placenta are better described across mammalian diversity. The placental shape describes the pattern that makes contact with the uterine wall where nutrient exchange occurs. The maternal-fetal interface describes the type of barrier that separates the maternal and fetal tissue. Placental interdigitation refers to how the trophoblast and maternal blood supply are interwoven. These, as well as other characteristics can be used to study different aspects of placental and fetal development.

There are five commonly described placental shapes that are found among eutherians (4). The most common placental shape is the discoid placenta where the placental tissue makes contact with the uterine wall in the shape of a single disc. This shape was present in the most recent common ancestor of placental mammals (3, 8, 9). The zonary placenta was the first shape to diverge from the ancestral discoid placenta and is present in some afrotherians such as the members of Sirenia and the African elephants as well as the carnivores of the laurasiatherian clade (3, 9-12). Another evolutionarily derived placental shape is the diffuse placenta present in some primates and most of the cetartiodactyls (i.e. cetaceans and artiodactyls) except bovines (3, 4). The diffuse placenta has a large surface area for contact between the fetus and the uterine wall allowing a high surface area for maternal-fetal nutrient exchange. Bovines have a cotyledonary placental shape which is characterized by many “polka dot” like attachments to the uterine wall (4). Among primates, there are three different placental shapes, the diffuse placenta of the strepsirrhines, the discoid placenta present in

humans, other apes, some catarrhines and platyrrhines, and the bidiscoid placenta (i.e. the placenta attaches to the uterine wall as two discs that share an umbilical cord). In this case, one disc attaches on the anterior and the other disc attaches to the posterior wall of the uterus (4). The bidiscoid placental shape is found in a subset of anthropoid primates including some macaques and marmosets (3). The different placental shapes among eutherians indicates varying amounts of contact between the mother and the fetus, with the diffuse placenta typically having relatively the most and the discoid having the least surface area for contact and nutrient exchange (13).

Another way to compare and classify placentas of different species is through looking at differences in their interdigitation pattern, or the way the fetal tissue interacts with the maternal tissue (14). There are five different types of placental interdigitation observed in eutherians (labyrinthine, villous, trabecular, lamellar, and folded) (4). The most recent common ancestor of placental mammals is inferred to have had a labyrinthine pattern of interdigitation (3). This pattern is present in most placental mammals including all afrotherians, Glires (rodents, rabbits and allies), soricid shrews, moles, hedgehogs and bats (3, 15, 16). One characteristic of the labyrinthine interdigitation pattern is web-like channels containing either maternal or fetal blood (4). When xenarthrans (e.g. sloths, armadillos, anteaters) diverged from the other placental mammals they evolved a trabecular interdigitation pattern, in which globular folds of trophoblast terminate in branching villi like tubes known as trabeculae (3, 17). Three different types of placental interdigitation are present in Euarchontoglires. Rodents, lagomorphs, scandentians, and dermopterans have a labyrinthine interdigitation pattern. Primates, either have a trabecular interdigitation pattern, seen in tarsiers and

platyrrhines, or a villous interdigitation pattern seen in catarrhines and strepsirrhines (Figure 1.1a). In catarrhines, fetal villi project into the maternal endometrium (18)(Figure 1.1b). One feature that distinguishes platyrrhine trabeculae is the presence of prominent maternal channels lined with endothelium (19). Within Laurasiatheria, perissodactyls and artiodactyls have a villous interdigitation pattern, with the exception of pigs, that have a folded interdigitation pattern, i.e. fetal tissue folding in and out of maternal tissue. Some carnivores have a more complex folding pattern known as a lamellar interdigitation. Two crown laurasiatherian orders maintain the ancestral labyrinthine interdigitation, and these are Soricomorpha and Chiroptera (4).

One of the most studied characteristics that can be used to classify eutherian placentas is the type of interface between the fetal tissue and the maternal blood (4). They range in levels of intimacy, i.e. the degree of separation or number of layers separating the fetal and maternal tissues. The least intimate is the epitheliochorial placenta where there is a layer of uterine epithelial cells apposed to a layer of trophoblast cells, limiting interaction between the maternal blood and fetal tissue (4)(Figure 1.2a). This type of barrier has evolved independently multiple times during eutherian evolution, and it is a derived character state (13)(Figure 1.3). The second most invasive barrier is the endotheliochorial interface where, after implantation, the uterine epithelium is degraded leaving only the maternal endothelium adjacent to the trophoblast (Figure 1.2b). The endotheliochorial interface is found in some afrotherians (elephants and aardvarks), some Euarchontoglires (tree shrews), and some laurasiatherians (moles, most carnivores and some chiropterans) (13). The most intimate form of maternal-fetal interface is the hemochorial barrier, in which both the

maternal epithelial cells and maternal endothelial cells are degraded leaving only the trophoblast cells in direct contact with the maternal blood (Figure 1.2c). This is the most widespread type of interface present in all four of the major eutherian superordinal clades (i.e. Xenarthra, Afrotheria, Laurasiatheria, and Euarchontoglires). The hemochorial interface has also been inferred to represent the ancestral state of extant placental mammals (3, 8)(Figure 1.3). The hemochorial arrangement has also been described to vary in the number of trophoblast layers that surround the syncytium (20). The hemomonochorial placenta has a layer of syncytium not surrounded by cytotrophoblast, hemodichorial placentas have a layer of cytotrophoblast on the fetal side of the syncytium; and lastly, the hemotrichorial placenta syncytium is surrounded by a layer of cytotrophoblast on the maternal and fetal sides (4, 20). A hemochorial state requires the tolerance of the maternal immune system towards paternal antigens since the placental tissue expresses both maternal and paternal antigens (21). If immune tolerance is disrupted, problems with placental function may occur (22). The great variation in placenta morphology across the eutherian mammals makes it more complicated to design animal models to study human diseases associated with placental dysfunction.

Comparative placental transcriptomics:

Sequencing technology allows us to identify genes that are differentially expressed across the different eutherian mammals. One study involving the placenta transcriptome used next generation sequence technology to sequence the placenta transcriptome of the African elephant (*Loxodonta africana)* and compared it to previously published transcriptome data from humans, mice, cows, platypuses, and

chickens. Roughly 3,000 genes were expressed in the placentas of all sampled eutherians. The list of these genes is overrepresented by genes associated with many annotations including 128 genes related to stress, (e.g. corticosteroid signaling). One class of corticosteroid, glucocorticoids, has increased expression in cases of reduced fetal growth (23, 24)**.** The conservation of corticosteroid signaling gene expression in the placenta suggests an ancient origin of the mechanism of response to stress across eutherian mammals. Other studies have used placenta transcriptomes of several equines to study parent of origin effects and genetic imprinting, where the fetus inherits genes from either the mother or the father (25, 26). One study compared placental transcriptomes of horses, donkeys, and their crosses (mules and hinnies) and found that a majority of the genes that were significantly different were paternally expressed, showing that the father's size has some influence in determining the size of the fetus (25).

Placental transcriptomics has also identified novel splice variants that are processed differently in various eutherian species. Prolactin (*Prl*) for example, a gene involved in regulating lactation in placental mammals, has many alternative transcripts (27). Placental mammals such as dogs and armadillos show no evidence of a myometrial *Prl* transcript; while humans, some New World monkeys, mice, and elephants all express *Prl* transcripts; however, each species expresses different splice variants with alternative transcription start sites (28). Another gene that has multiple alternative transcripts across the placental mammals is the gene encoding aromatase (*CYP19*). Aromatase converts androgens into estrogens, and thus plays a role in regulating placental growth (29-31). There is a placenta specific *CYP19* gene isoform in

bovines, ovines, and humans, but not rodents (29, 32, 33). The human *CYP19* isoform contains a long terminal repeat (LTR) that functions to localize *CYP19* to the syncytiotrophoblast (29, 34). This LTR is most likely an endogenous retrovirus. This retrovirus was integrated into a promoter region, and therefore effects transcription of the gene. Endogenous retrovirus integration may have played a role in the evolution of the primate placenta (29). Other human genes that play a role in placental development and function and contain endogenous retroviruses include the syncytins (encoded by *ERVW-1* and *ERVFRD-1)*. Syncytin-1 plays a role in cell fusion during trophoblast formation and is expressed throughout pregnancy. The main role of syncytin-2 is the suppression of the maternal immune system but unlike syncytin-1, expression declines as pregnancy progresses (29, 35). It has been shown that down regulation of syncytin-1 is associated with preeclampsia (36). Interestingly, syncytin genes have evolved independently over the course of eutherian evolution (37). Mice for example, also express a pair of syncytins, syncytin-A and syncytin-B. Like syncytin-2, syncytin-B has immunosuppressive properties not seen in syncytin-1 or syncytin-A, both of which play a role in cell fusion during syncytiotrophoblast and trophoblast giant cell formation respectively (37). The blind mole rat placenta expresses *SynA* and *SynB*, but these genes are not present in the naked mole rat genome (38). The human syncytin genes have trophoblast specific enhancers that contain binding sites for transcription factors such as glial cell missing 1 (*GCM1*), that induce the expression of the syncytins (39). *GCM1* is a transcription factor that is specifically expressed in trophoblast cells and has been shown to increase syncytin-1 when over expressed in trophoblast cell lines (40,

41). Other genes that have been shown to have placental specific promoters or enhancers include rat placental lactogenlactose 2 (*rPLII*) and *AP-2* (42, 43).

There are other larger gene families that have placenta specific members (29). One example is the human leukocyte antigen (*HLA*) gene family. *HLA-g* is a placenta specific immune system gene that plays a role in trophoblast invasion (44). Another example of a eutherian specific gene expressed in placenta that acts differently in various eutherian species is placenta specific 1 (*PLAC1)* (45). This gene is mainly expressed in the placenta but may also be expressed in the testis (29, 46). Orthologs of *PLAC1* are found only in placental mammals (29, 45, 46). *PLAC1* is expressed all throughout gestation in humans but only during e7.5 through e14.5 in rodents (47, 48). Galectins are another gene family with placenta specific members that play a role in the maternal-fetal immune tolerance and implantation (49). All members of this family are expressed at the maternal-fetal interface (50). The placenta-specific galectins are located in a cluster on chromosome 19. This chromosome contains many placenta specific genes including the pregnancy specific glycoproteins and a cluster of placenta specific microRNAs (51, 52).

Animal models for placental dysfunction:

Due to the wide variation among placental mammals, it is challenging to develop animal models of obstetrical syndromes associated with placental dysfunction (53, 54). While some mouse models have been used to study obstetrical syndromes associated with placental dysfunction such as implantation failure, miscarriages, and preeclampsia, the results may not be directly relevant to the human condition (54-57). One difficulty to overcome with mouse models of obstetrical syndromes is the relatively superficial

invasion of the fetal tissue into the maternal decidua (21). Human placentas typically have a deeper invasion into the maternal myometrium than mice and since preeclampsia is characterized by relatively shallow trophoblast invasion (i.e. the typical condition in mice); studies of reduced placental invasiveness may be difficult to model using mice (58). Also, the cellular makeup of the murine placenta is different than the human placenta. Mice have polyploid trophoblast giant cells. Like human cytotrophoblast cells these giant cells regulate uterine implantation. Mice also have a layer of spongiotrophoblast similar to the human extravillous trophoblast (59). Sheep have also been used to study placental dysfunction (60). Sheep pregnancy has been used to model human intra-uterine growth restriction by inducing maternal hypothermia, removing of endometrial caruncles to limit placental growth, limiting blood supply through the restriction of uterine blood flow, and maternal over nutrition (60). All of these methods result in altered oxygen and nutrient transfer leading to a decrease in fetal growth (60). Guinea pigs have also been used to model human placentation because they share features with humans (54). Guinea pigs have smaller litter sizes, longer gestation, and deeper trophoblast invasion than other rodents (54, 61). Guinea pig models have been developed to study placental transfer and intra-uterine growth restriction (54, 62, 63).

Preeclampsia is an example of an obstetrical syndrome related to shallow trophoblast invasion (64, 65). In preeclampsia, the spiral arteries that supply maternal blood to the developing fetus are poorly remodeled due to decreased trophoblast invasion; therefore, blood supply to the fetus may be limited (66-68). Some studies have identified non-human primate models that can be used to study placental invasiveness

especially in those with a bidiscoid placenta such as the rhesus monkey (69, 70). Preeclampsia is typically thought to be a human specific syndrome (69, 71). However, recent work has challenged this view, primarily due to the fact that placental invasiveness is also quite deep in the closest relatives of humans, chimpanzees and gorillas (72-74). Thus it is likely that the potential for preeclampsia due to dysregulation of placental invasiveness predated the most recent common ancestor of humans, chimpanzees, and gorillas during the Miocene. A recent study supporting this hypothesis, examined the evolution of orthologous protein coding gene sequences and found evidence for adaptive evolution (i.e. positive selection as measured through comparison of nonsynonymous and synonymous substitution rates) in genes associated with the more invasive phenotype seen on the evolutionary lineage leading to the most recent common ancestor of these three species (75). In particular, genes associated with immune function, regulation of blood vessel size, and hypertension were enriched among the adaptively evolving genes. These findings suggest that the molecular mechanisms for the induction of preeclampsia were in place long before the evolution of modern humans.

Natural human models for studying placental dysfunction:

While animal models can be useful for studying specific symptoms associated with the complex diseases involving placental dysfunction, some researchers have begun using environmentally variable, natural human models to study the genetic and morphological differences associated with placental dysfunction (6, 76, 77). The human placenta brings practical advantages to biomedical studies as it is a temporary organ that like the fetus, i.e. the child, is delivered (4). Some human populations that have

been used to study placental morphology in regard to placental dysfunction are high altitude natives as well as multigenerational populations who experienced periods of poor nutrition in at least one generation (5, 6).

Populations native to high altitude, such as native Andeans and Tibetans, are candidates to study how hypoxic conditions affect the placenta and contribute to complex obstetrical syndromes such as intra-uterine growth restriction and preeclampsia (5, 77). These populations also allow for a case-control type experimental design in which high altitude native populations are used as the case populations and natives in low altitude regions can be used as the control populations (76). The natives of the Tibetan Plateau and Andes Mountains have been exposed to hypoxic conditions for roughly 20,000 and 10,000 years respectively (78, 79). Infant cohorts exposed to the hypoxic conditions of high altitude during gestation experience lower than average birth weights when compared to their sea-level counterparts (80). The exceptions to this pattern are infant cohorts born to mothers who have an ancestral pattern of high altitude hypoxia. These infants exhibit birth weights comparable to those at sea level suggesting some level of adaptation to the hypoxic environment (79). Morphological studies of placentas of high altitude dwelling individuals show an increased capillary surface area and greater placental weight. These features combine to increase the total area for oxygen exchange between the mother and fetus (81). Physiological studies have shown that Andeans and Tibetans are different at high altitude with Tibetans being more adapted to high altitude with attributes more similar to sea level populations (78). Genetic studies have shown that differences in the genetics between Andean and Tibetans resulted in convergent phenotypic evolution between high and low altitude

populations (79, 82, 83). Recently, a genome-wide study compared two populations native to high altitude for several millennia: 1) the native inhabitants of the Tibetan Plateau and 2) the Native inhabitants of the Andean Altiplano of South America. These populations have been exposed to chronic hypoxia, using low altitude individuals as control populations. In 2010, Bigham et. al. were able to identify gene regions and single nucleotide polymorphisms (SNPs) that show evidence of positive selection (i.e. adaptive evolution). Of the dozens of genes identified, one deserves special attention. *EGLN1*, encodes hypoxia-inducible factor prolyl hydroxylase 2, a protein that regulates the expression of HIF-1a, the transcription factor responsible for the hypoxic response (84). *EGLN1* shows evidence for positive selection at 25 SNPs in Andeans and 28 SNPs in Tibetans (83). This finding suggests there is a genetic basis for the adaptation to hypoxia in these populations, and future work should test how these positively selected SNPs effect the oxygen exchange between the mother and fetus via the placenta. This is important since recent migrants to high altitude tend to have offspring with relatively low birth weights compared to their native counterparts who have evolved adaptions to hypoxia (85).

Another class of human populations that have shed light on placental plasticity are those in which pregnant woman were exposed periods of low nutrient availability during all or part of their pregnancy. One of the first studies using famine as a mediator of fetal growth and development examined samples from a Dutch population whom had suffered through the famine during the second World War (6). Famine studies have shown that those women who were exposed to famine conditions during their third trimester had offspring with low fetal birth weights as they experienced limited nutrient

availability (6, 86). Those who experienced famine conditions during their first trimester produced offspring with heavier placentas. This was thought to be an adaptive mechanism to allow for increased surface area for nutrient exchange (7).

Epigenetic studies have also examined placental gene expression and placental methylation differences in famine populations and those individuals with low birth weights. They found differential expression in genes associated with fetal growth as well as differential methylation in those growth related genes (87-89). One study examined differential methylation along insulin-growth factor 2 (*IGF2*)(89), a gene known to promote fetal growth (90). This study used whole blood from 60 individuals conceived during the Dutch famine and compared them to their control, same sex, siblings born before or conceived after the famine (89). The study describes five methylation sites along the promoter region of *IGF2* and found decreased methylation across all sites in the famine-exposed individuals when compared to the control siblings. This finding suggests that during times of low nutrition, genes such as *IGF2* can be differentially methylated and potentially provide a link between maternal nutrition and fetal gene expression (89). These results add another layer of placental adaptation in which during times of low nutritional availability, the placenta responds by either expanding in size to increase surface area of nutrient exchange or repressing genes associated with fetal growth in order to protect the mother.

Low birth weight as a result of poor maternal nutrition not only affects the conceptus during fetal development but also impacts their adult life (91). According to the Developmental Origins of Health and Disease (DOHaD) paradigm, low fetal birth weight can lead to complications such as diabetes, obesity and renal malfunction later

in life (92). This has been hypothesized to be an adaptive mechanism, as the fetus has become accustomed to the low nutrient environment in the womb. Later, if the child is exposed to a healthy, more enriched diet after birth, their body reacts differently than would children who had sufficient nutrients during fetal development. This reaction to a more enriched diet after birth can result in poor outcomes later in life including obesity and obesity related conditions (93).

Parent - Offspring Conflict:

Robert Trivers pioneered investigations on the conflict between the parents and offspring. Trivers defined parental investment as, “any investment by the parent in an individual offspring that increases the offspring’s chance of surviving (and hence reproductive success) at the cost of the parent’s ability to invest in other offspring” (page 139) (94). In this manner, the survivability of future offspring is affected if the current offspring takes an excess amount of maternal resources limiting the amount of resources available to the offspring’s future siblings (95).

David Haig extended the idea of parental-offspring conflict by adding that there is also conflict between the mother and the fetus. In this view, the fetus aims to extract the maximum amount of maternal nutrients (thus engendering conflict), but the mother and the fetus must also cooperate during a normal pregnancy (96, 97). Trophoblast invasion is an example of a balance between maternal-fetal conflict and cooperation. During a normal human pregnancy, the trophoblast invades into the maternal myometrium and extracts nutrients from maternal blood (98). Conflict arises because while the mother wants to limit trophoblast invasion, the placenta invades deeply thereby extracting more nutrients (98, 99). Cooperation is necessary to balance

invasion. Optimal placenta invasion is deep enough into the maternal tissue ensuring the fetus can absorb enough nutrients but shallow enough to protect the mother from syndromes such as placenta accreta (98). One obstetrical syndrome that is related to the improper balance of conflict and cooperation is preeclampsia. Conflict theory suggests that fetal actions need to be distinguished from maternal responses in preeclampsia (98, 99).

General conflict between the mother and the fetus can be mediated by genetic processes such as gene imprinting. Imprinted genes are those genes in which only one parental allele is expressed, while the other allele is silenced through mechanisms such as DNA methylation (100, 101). Conflict theory predicts that maternally expressed imprinted genes restrict fetal growth by retaining maternal resources. In contrast, paternally expressed imprinted genes promote fetal growth and maximize resource extraction (102, 103). Despite the conflict arising from genomic imprinting, cooperation between the mother and fetus is important in regard to nutrient exchange and fetal size. If the mother fails provide the fetus with enough resources, the fetus will have a low birth weight, which leads to complications later in their adult life, and possibly reduced reproductive success (91, 92).

Cooperation can also be observed in the maternal immune system because it tolerates non-self paternal antigens expressed in the placenta, which is considered a semi-allograft (18, 104). Obstetrical syndromes such as preeclampsia have been posited to result from excessive conflict between the mother and the fetus as well as a breakdown in maternal-fetal cooperation (105). This balance of cooperation and conflict is heightened at the human maternal-fetal interface because the intimate hemochorial

placenta has more contact with maternal blood (and by extension paternally expressed alleles), than in less intimate endotheliochorial and epitheliochorial placentas (18).

Conclusions:

The placenta varies across eutherian species and this variation should be considered when developing animal models to study human diseases associated with placental dysfunction. While many animal models have mimicked several of the symptoms associated with placental dysfunction, it is unsure how applicable any of these results would be to the human condition (21). One possible approach would be to examine naturally occurring conditions that in humans are associated with obstetrical syndromes. For example, species with less intimate maternal fetal interfaces (e.g. artiodactyls, lemurs) might be appropriate models for preeclampsia, because the syndrome is characterized by less placenta invasion (75). Primate models, including natural examples of environmental stress in humans are promising because factors such as hypoxia or nutritional stress modify the placenta, and therefore provide insight into complex obstetrical syndromes associated with placental dysfunction.

Acknowledgements:

We would like to acknowledge our funding provided by the National Institutes of Health through grant 1R211HD068954-01. Also, we would like to thank Stacy Zamudio, Nick Illsley, Christopher Kuzawa, and Julienne Rutherford for their contribution through useful discussion related to this topic. Lastly we would like to thank Eric Barrington, Graham Burton, and Ashley Moffett for organizing the excellent meeting entitled *Human Evolution: brain, birth weight and the immune system* and this issue of *The Philosophical Transactions of the Royal Society B*.

Figures:

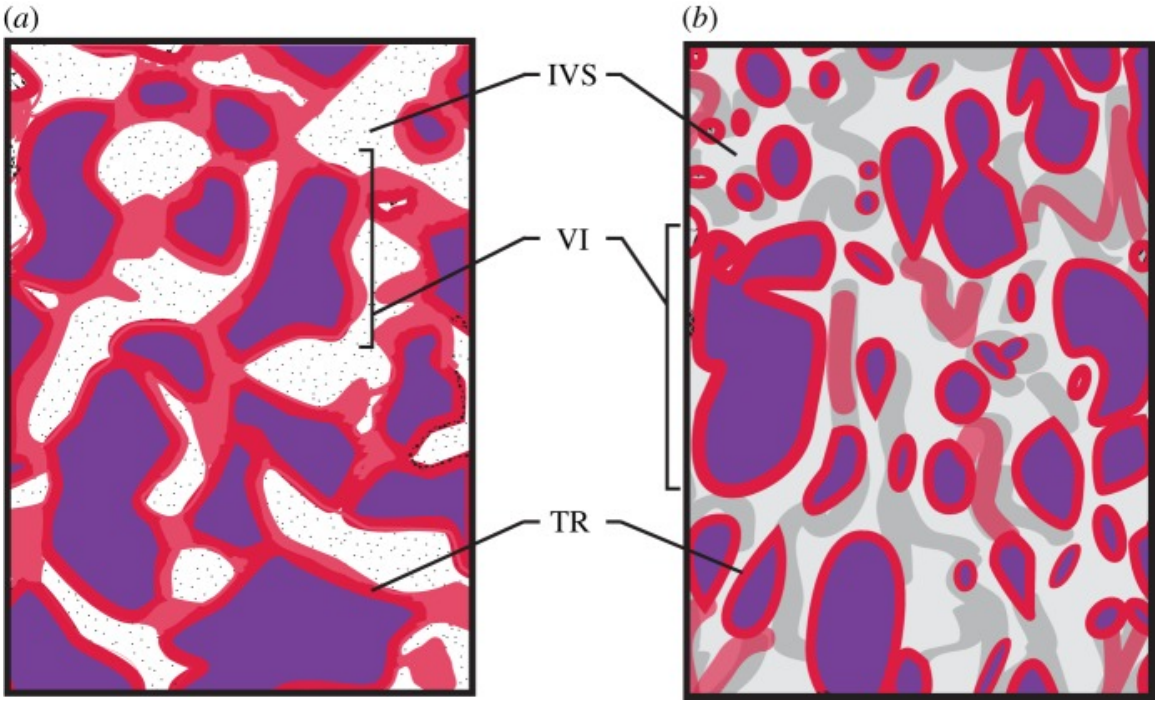


Figure 1.1: Placental Interdigitation across primates:
Cross-sectional view of two types of maternal-fetal interdigitation found in primates: trabecular (a) and villous (b). The trabecular interdigitation pattern is characterized as branching globular folds that terminate in villi and this is seen in tarsiers and platyrrhines. The fetal trophoblast cells (red) surround the fetal villi (purple), which contain fetal blood (purple) separating the fetal blood from the maternal blood located in the intervillous space. The villous interdigitation pattern is found in catarrhines and strepsirrhines, and this is characterized by a branching villi pattern (4). TR= trophoblast cells, VI= villi, and IVS = maternal intervillous space. Modified from (18)

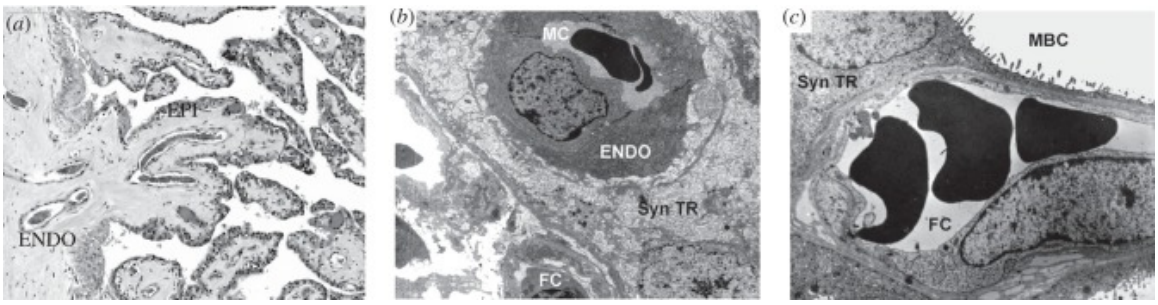


Figure 1.2: Three types for interface between the mother and the fetus
Histological examples of the three different levels maternal fetal interface seen across the eutherians. A is section of placental tissue from a giraffe which has an epitheliochorial interface where the mother and the fetus is separated by layer of uterine epithelial cells (EPI) and maternal endothelial cells (ENDO). B is an example of an endotheliochorial placenta of a three toed sloth originally from (12). This type of placenta has one layer of maternal; endothelial cells (ENDO) separating the maternal blood from the fetal trophoblast. C is an example of a hemochorial placenta of a degu, a rodent from (106). The hemochorial placenta is the most invasive maternal-fetal interface with fetal trophoblast in direct contact maternal blood. ENDO: Fetal Endothelial Cells, EPI- Maternal Epithelial Cells. Syn TR: Syncytiotrophoblast, FC: Fetal Capillary, MBC: Maternal Blood Channel.

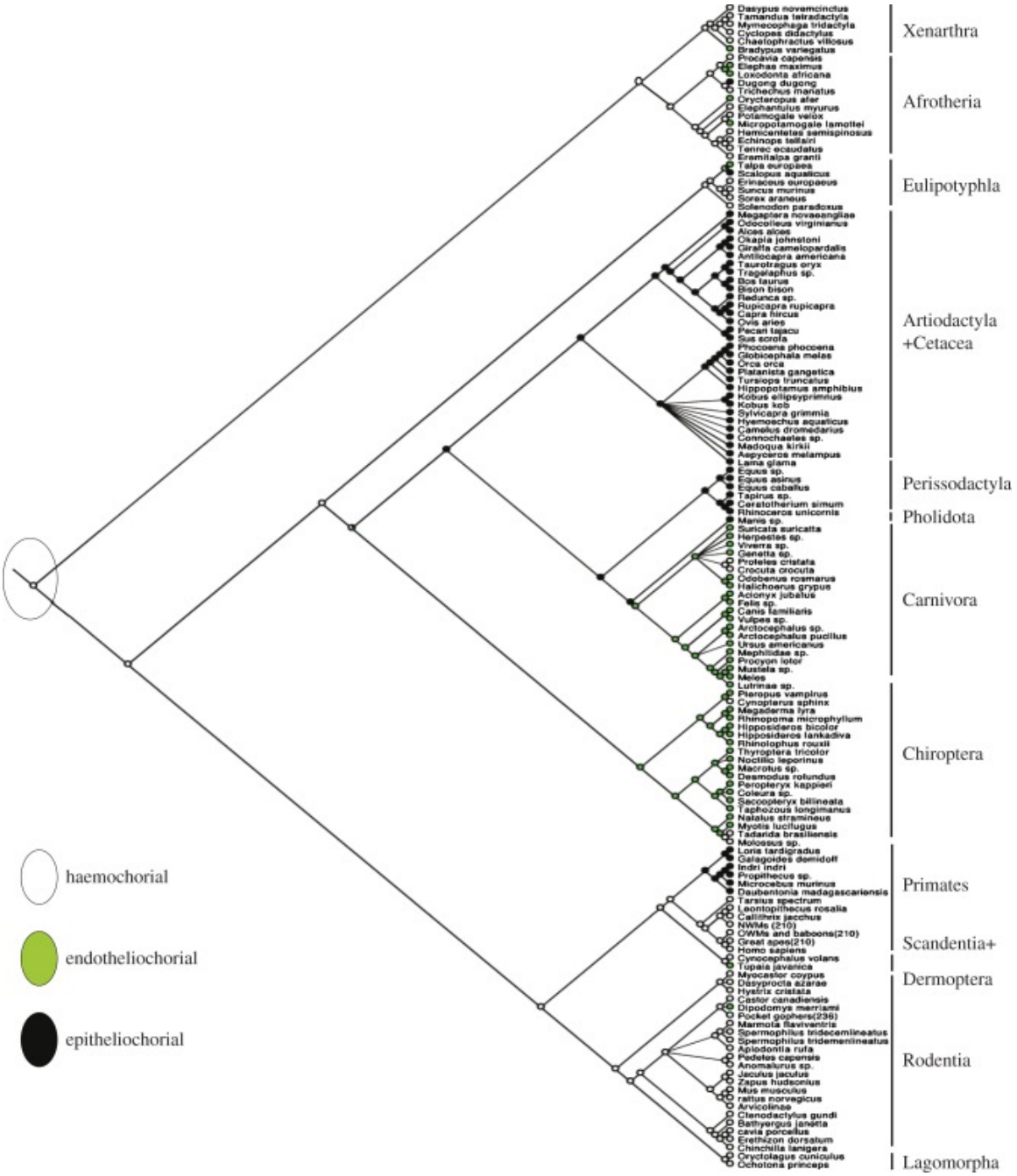


Figure 1.3: Evolution of the maternal fetal interface
Maximum parsimony phylogenetic reconstruction of the maternal-fetal interface of 141 mammals through parsimony analysis showing the root placental mammal having a hemochorial placenta (white) with epitheliochorial (black) and endotheliochorial (green) as divergent states(3, 8, 107-110). Tree topology taken from (111)

CHAPTER 2: INTRODUCTION TO THE DISSERTATION

Understanding how human physiological variation is affected by adaptation is a major goal of biology. An extreme environment in which humans show evidence of adaptation is high altitude. Some of the factors that affect the high altitude environment are decreased temperature, increased ultraviolet radiation, and decreased partial pressure of oxygen. One population that has adapted to the high altitude environment are the long-term inhabitants of the South American Altiplano (79). These native Andean populations, which have resided at high altitude for 10,000 years, have altered physiological parameters such as decreased pulmonary oxygen pressure, elevated hemoglobin concentrations, elevated pulmonary arterial pressure and, decreased resting ventilation when compared to their sea-level dwelling counterparts (112-115). Despite these differences in adult physiology, the Andeans give birth to children with birth weights similar to what is seen in birth weight in sea-level populations (79). When the Spanish settlers arrived in the Andes roughly 450 years ago, they did not have the adapted physiological toolkit that affords protection to the high altitude environment that the natives had and thus experienced decreased reproductive success (116).

European mothers that move to a high altitude environment during their lifetime and later become pregnant experience a two to four fold increase in obstetrical syndromes such as preeclampsia and intrauterine growth restriction (IUGR) (117). This observation stands in contrast to long-term inhabitants of high altitude such as Native Andeans who do not show such a marked risk increase for obstetrical syndromes. Additionally, reduced fetal growth is associated with the development of metabolic disorders later in life such as type II diabetes mellitus (91). The placenta plays a major

role in fetal growth as it acts both as a barrier, and as mediator for maternal-fetal nutrient transport (1). Therefore, there is some evidence that adaptations in pregnancy have occurred in Native Andean residents in high altitude. While there have been many studies that have examined morphological and physiological differences between high and low altitude populations, to our knowledge, little has been done to identify the genetic and epigenetic factors related to the Andean placental adaptation to high altitude.

To study adaptation to high altitude, we examined healthy placental tissue from individuals residing in Santa Cruz and La Paz, Bolivia, located at 400m and 3600m, respectively. At each altitude, we studied populations of 1) Aymaran speaking (AS) Andean and 2) European descent who have resided at high altitude for at most 10,000 and 450 years, respectively. The rationale for selecting these populations was that Europeans living at high altitude have significantly lower birth and placental weights when compared to AS Andeans living at high altitude, demonstrating that AS Andeans have potentially adapted to the high altitude environment through 10,000 years of habitation. Through examining genetic and epigenetic signals we expect to be able to identify pathways that may contribute mechanistically to the placental adaptation to high altitude. We measured placental gene expression patterns and test for differential gene expression to determine what biological processes are involved in placental adaptation at high altitude. We identified candidate genes that are differentially expressed between Andeans and European descendants at both low (Santa Cruz, Bolivia) and high altitude (La Paz, Bolivia). Once regions of differential expression were determined, we attempted to identify mechanisms responsible for the differential expression including

DNA methylation within regulatory regions.

The central goal of the proposed research was to determine which ***genetic and epigenetic factors contribute to the Andean adaptation to the environmental stress acting on the placenta at high altitude.*** To accomplish this, we conducted the following specific objectives:

Specific Objective 1: Test for differences in gene expression and DNA methylation that contribute to the placental adaptation to high altitude seen in Andean populations.

Hypothesis: Gene expression differences and DNA methylation contribute to the response or adaptation to high altitude stress acting on the placenta.

Specific Objective 2: Test if natural selection influenced placental gene expression and DNA methylation differences between individuals of Andean and European descent residing at high altitude.

Hypothesis: We predicted there would be methylation differences associated with differentially expressed genes involved in fetal growth related pathways between high and low altitude populations that are affected by natural selection induced by the high altitude environment.

CHAPTER 3: DNA METHYLATION EXPLAINS ROUGHLY ONE THIRD OF PLACENTAL GENE EXPRESSION DIFFERENCES BASED ON ANCESTRY AND ALTITUDE

Abstract:

Objectives: The most pronounced effect of high altitude (>2700m) on reproductive outcomes is reduced birth weight. Indigenous Bolivians (Andean Native Americans) residing for generations at high altitudes have higher birth weights relative to more recent migrants of primarily European ancestry. Previous research demonstrated that the placenta is a key contributor to the preservation of Andean birth weight at high altitude. Our current research investigated how gene expression and epigenetics contribute to the conservation of birth weight at high altitude by examining mRNA expression and DNA methylation differences between placentas of Andeans and those of European ancestry residing at high and low altitude.

Methods: Genome-wide mRNA expression and DNA methylation of villous placenta tissue was quantified utilizing microarray technology. Subjects were of Andean and European ancestry and resident at high (3600m) or low (400m) altitudes. Differentially expressed genes (DEGs) associated with altitude or ancestry were identified (FDR<0.1, |fold change|>1.25). To predict which DEGs could be regulated by methylation we tested for correlation between gene expression and methylation values.

Results: 70 DEGs associated with altitude (n=36) or ancestry (n=35) were identified. Altitude-associated DEGs included members of the AP-1 transcription factor family. [2]

Published as: Gundling WE, Pantham P, Illsley NP, Echalar L, Zamudio S, Wildman DE. DNA Methylation Explains a Subset of Placental Gene Expression Differences Based on Ancestry and Altitude. bioRxiv. 2018.[2]

Ancestry-associated DEGs were implicated in inflammatory pathways and associated with pro-angiogenic macrophages. More ancestry-associated DEGs correlated significantly (n=17) (FDR<0.1) with promoter or gene body methylation (p=0.0242) when compared to altitude associated DEGs (n=8).

Conclusions: Compared to altitude-associated DEGs, methylation regulates more ancestry-associated DEGs, potentially allowing for rapid modification in the expression of inflammatory genes to attract pro-angiogenic macrophages as a means of promoting placental capillary growth in Andeans, regardless of altitude.

Introduction:

The placenta mediates fetal growth by orchestrating nutrient and gas exchange between the mother and the fetus (118). We have shown there is altered substrate delivery from placenta to fetus under conditions of chronic maternal hypoxemia (lowered arterial oxygen tension due to high-altitude residence). High altitude environments contributes to reduced birth weights in humans, with a gradient of reduction in birth weight that corresponds to the evolutionary duration of population exposure to high altitude (119-121). At ≥3000 meters, Tibetans and Andeans have higher birth weights than more recent migrants such as European and Han (Ethnic Chinese) (120, 122). The Andeans, with a population history of ~10,000 years at high altitude have birth weights >200 grams higher than Europeans at ≥3000 meters (119, 120, 123, 124). This suggests a relatively rapid adaptation to the high altitude environment in terms of the promotion of favorable reproductive outcomes among Andeans. Recent analyses have shown Tibetan and Andean populations have undergone natural selection to favor variants of genes associated with the hypoxia response, oxidative metabolism, and

possibly regulation of vascular tone (83, 121, 125). However, prior work looking at gene expression in relation to altered physiological function has only looked at gene expression differences in leukocytes so in order to begin to related gene expression to physiological function we need to look at expression patterns in a reproductive tissue such as the placenta. We hope that by identifying differential gene expression of genes previously associated with physiological processes associated with the placenta such as angiogenesis and immune response, we can begin to identify how these processes may be contributing to the conservation of birth weight seen in the native Andeans residing at high altitude.

Reduced birth weight in a population subjected to environmental stress is said to be disadvantageous because reduced birth weight is associated with increased fetal/neonatal morbidity and mortality (126). Lowered birth weight is also associated with an increased risk for chronic diseases later in life, including cardiovascular and metabolic diseases (127, 128). Hence placental adaptations that protect against growth restriction not only benefit offspring survival, but also population-wide health in later life. The accumulated data suggests that at high altitude, there is diminished vascular elasticity, consistent with the reduced uterine arterial and fetal umbilical venous blood flow we and others have reported in high-altitude residents (128-132).

The greater fetal growth in Andean Native Americans is associated with higher uterine arterial and umbilical venous blood flows compared to more recent migrants to high altitude (129, 130). Our prior work found no relationship between oxygen-associated variables such as maternal and fetal PO_2, arterial oxygen content and birth outcomes. Instead, placental weight was the strongest correlate of fetal size at birth,

accounting for 35% of the variation in birth weight in both Andean and European women at high altitude (130). Also, greater blood flow from the umbilical artery was seen in individuals of Andean descent residing at high altitude compared to their European counterparts residing in the same location as well as increased oxygen delivery which may also aid in protecting the Andeans against altitude associated reduced fetal growth (133). Along with higher umbilical blood flow between Andeans and Europeans residing at high altitude where was a decrease in the anti-angiogenic factor fms-like tyrosine kinase (sFlt-1) suggesting that increased angiogenesis may also be aiding in the increased birth-weights seen among the native Andeans at high altitude (134). Moreover, for any given placental weight, Andean neonates were larger than Europeans, indicative of greater placental efficiency among Andeans. For these reasons we proposed that the ancestry differences we have reported are due to changes in placental function (130, 135). This was the stimulus for the present study, in which we hypothesize that differences in placental gene expression contribute to the greater growth of the Andean fetus at high altitude.

Previously, other studies have examined the role of DNA methylation in hypertensive pregnancies utilizing DNA extracted from peripheral blood mononuclear cells(136-138). To our knowledge, global gene expression and DNA methylation in placental tissue has never been examined relative to ancestry, nor to an environmental challenge such as high vs. low altitude. It is important to compare DNA methylation and gene expression in the same tissue because both DNA methylation and gene expression patterns are tissue specific. While we understand that methylation in different parts of the genome can affect gene expression in other regions, having trans-

effects, we are focusing on local (cis) methylation as it is more understood. Our approach was to compare the gene expression signatures of placentas from indigenous Andeans to those of more recent migrants, most of whom are the descendants of European colonists residing at high and low altitude in Bolivia (<500 years). The ancestry-associated DEGs may provide insight into how the Andean placenta has adapted to protect the fetus from altitude-associated reduction in birth weight. Altitude-associated DEGs may provide new biomarkers for, or even point towards therapeutic options for hypoxia-related placental pathologies such as preeclampsia and idiopathic intrauterine growth restriction.

We also investigated how DNA methylation correlates with gene expression in the altitude or ancestry-associated DEGs. We reasoned that the ~10,000 year exposure of Andeans to the high altitude environment is generally considered long enough for evolution by random mutation and subsequent selection. Adaptions including lactase persistence have occurred in less time with lactase persistence developing in roughly 9000 years(139). The Andean plateau, while long, is narrow, and undoubtedly prehistoric populations moved between altitudes(140). DNA methylation may explain how gene expression has been altered relatively rapidly in a temporary manner(141). An environmentally induced methylation event may allow for the alteration in gene expression after the germ cell sequence has been established(142). This allows for a more temporary and less “costly” method of adaptation in gene expression(142).

Methods:

Subjects

The data reported here are derived from a subset of pregnancies studied in La Paz, Bolivia (altitude ~3600 m) and Santa Cruz, Bolivia (altitude ~400 m). The original studies were designed to examine maternal and fetal blood flow, oxygen delivery, and consumption in a four-way cross-sectional design comparing indigenous Andeans versus European migrants residing at high and low altitude (129). All women gave written informed consent to protocols approved by the Bolivian National Bioethics Committee, the San Andreas Mayor University, Instituto Boliviano de Biologia de Altura Consejo Tecnico, and the USA Institutional IRBs approved by the New Jersey Medical School.

Women completed an interview and questionnaire in which they identified the birthplace and residence history of themselves, their parents, their grandparents, and those of the baby's father and his recent ancestors. Three generation residential history in the Altiplano region, Andean surnames, self-identification as, and fluency in Aymara or Quechua initially defined our Andean samples from both high and low altitude. Women who self-identified as Spanish or European, reported no known Andean ancestry, and who documented three generations of low altitude residence were provisionally assigned to the European group from both high and low altitudes. Ancestry was evaluated using 133 Ancestry Informative Markers (AIMs) to confirm self-identified ancestry and quantify admixture (130, 143, 144). The Andean women in this study had biogeographic ancestry profiles that were on average 86% Native American (95% Confidence Interval (CI) 76-92%), and 9% European (95% CI 6-20%). The European

sample population had an average of 63% European ancestry (95% CI 54-68%) and 31% Native American (non-Andean) ancestry (95% CI 22-43%). Low levels of sub-Saharan African (2-6%) and East Asian admixture (3-5%) were detected in both ancestry groups. There was no significant difference between altitudes in the measures of geographic ancestry, i.e. Andean individuals at low vs. high altitude did not differ in their proportional admixture, nor did those of European ancestry between the low and high altitude sites. Maternal inclusion criteria were conception, gestation and birth at altitude of residence, singleton pregnancy, a healthy mother age 18-45 with no known chronic disease, a normal glucose tolerance test, and the absence of any known pregnancy complications.

Placental Sampling/Morphometrics

All placentas were obtained from elective, non-laboring term cesarean deliveries without supplemental oxygen. Placentas were obtained immediately after delivery. The sampling strategy was designed to ensure multiple portions of the placenta were sampled, but also that RNA was collected as quickly as possible. Therefore, a semi-random sampling strategy was used, in which the placenta was visualized as roughly circular and divided into four quadrants. One full-depth sample from approximately the center of each quadrant was excised, and the basal and chorionic plates were removed, and the core villous tissue minced. Tissue from all four quadrants was thoroughly mixed and 200 mg aliquots were flash frozen in liquid nitrogen for later RNA/DNA isolation. Each RNA/DNA aliquot, therefore, contained tissue from four regions of the placenta and was flash frozen in liquid nitrogen less than 30 minutes following delivery. For placental morphometric studies, eight full depth sections were collected (two from each

quadrant), formalin fixed and paraffin-embedded. Five of these samples were used for morphometric analyses following the methods originally described by Weibel et al (145, 146), and as applied by Jackson and Mayhew (147-149) and Burton et. al.(150). Morphometric studies were overseen by DCM in consultation with Dr. Graham Burton of Cambridge University. The fetal membranes and umbilical cord were trimmed and the placentas were weighed. Placental volume was measured by water displacement, and correction for tissue shrinkage was based on maternal erythrocyte diameters (151). While complete morphometric analyses were undertaken of 20-30 placentas from each of the 4 low and high altitude groups, only data pertaining to vascular variables is considered here in relation to our microarray results.

Nucleotide Isolation and Expression Microarray

RNA was isolated from the core villous tissue of 45 placentas (n=10 Andeans from high altitude, n=12 Andeans born at high altitude but who migrated to low altitude, n=11 European individuals born at low altitude but who migrated to high altitude, and n=12 Europeans individuals born and residing at low altitude). The concentration and optical density of each RNA sample was measured using a NanoDrop spectrometer (Thermo-Fisher, Waltham MA). The quality of the RNA was further examined by calculating the RNA integrity number (RIN) using a Bioanalyzer (Agilent, Santa Clara CA). RIN score for all samples used for microarray array analysis ranged from 7.3 to 4.6. One μg of RNA per sample was supplied to the Applied Genomics Technology Center at Wayne State University (Detroit, MI). The RNA was converted to cDNA and then hybridized to Illumina HumanHT-12v4 Expression BeadChips (Illumina, San Diego, CA) containing 47,231 probe sequences with each probe sequence replicated on 20-30

beads using Illumina's standard protocol. The average fluorescence intensity value for each probe set was calculated using the Illumina GenomeStudio software (Illumina, San Diego, CA). The probe sequences target more than 31,000 annotated genes. Samples were plated in random order across the different microarray chips on the same day to minimize batch effects. Intensity values for annotated genes were $\log_2$ transformed and quantile normalized using the Preprocesscore package from Bioconductor software collection in R (152). Probes located on the X- and Y-chromosomes were excluded because of sex-based dosage-dependent effects on gene expression. To compensate for our small sample size, we calculated the variance of each of the remaining probes and sorted them from highest to lowest variance and used the top 5% most variable probes leaving 1,523 probes to be used for further analysis (153). All raw files, non-normalized intensity values as well as quantile normalized and log-transformed files have been uploaded to the GEO database (GSE100988).

Determination of Differentially Expression Genes

We tested for differential expression using a two-way analysis of covariance (ANCOVA) with the model, probe expression in proportion to altitude + fetal sex + gestational age or probe expression in proportion to ancestry + fetal sex + gestational age as well as testing for probe expression in proportion to the interaction between altitude and ancestry (Supplementary Table 3.1). P-values were generated from each independent variable and the false discovery rate (FDR) was estimated using the Benjamini-Hochberg (BH) method to adjust for multiple hypothesis testing (154). A probe sequence was considered to be significantly differentially expressed if it had an FDR<0.1 and an absolute fold change≥1.25.

Validation of Microarray Data

We conducted qRT-PCR using Taqman gene expression assays (Invitrogen, Carlsbad, CA) to validate the microarray results. 500ng of RNA from each sample was reverse transcribed to cDNA using the Superscript III First-Strand Synthesis system (Invitrogen, Carlsbad, CA). qRT-PCR was performed on the StepOnePlus real-time PCR machine (Applied Biosystems, Foster City, CA) with Taqman Fast Advanced Master Mix (Invitrogen, Carlsbad, CA) and fast cycling conditions. We tested six genes that were differentially expressed due altitude or ancestry and involved in inflammation and the AP-1 transcription factor pathway with qRT-PCR: lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1,* Taqman probe Hs00272659_m1*),* stanniocalcin 1 (*STC1,* Taqman probe Hs00292993_m1)*,* fos proto-oncogene (*FOS,* Taqman probe Hs99999140_m1)*,* arachidonate 5-lipoxygenase-activating protein (*ALOX5AP,* Taqman probe Hs00233463_m1)*,* nicotinate phosphoribosyltransferase (*NAPRT,* Taqman probe Hs00292993_m1)*,* and phospholipase A2, group 2A (*PLA2G2A,* Taqman probe Hs00179898_m1). For each gene tested, samples were randomized and run in triplicate, incorporating a multiplexed 18S ribosomal RNA endogenous control (*18S*, Taqman probe Hs99999901_s1) in each well. The Ct values for each sample and gene were analyzed using the $2^{-\Delta\Delta Ct}$ method (155). The qRT-PCR intensity value (Ct) for each gene was correlated with the intensity value yielded by the gene expression microarray to validate results from the gene expression microarray.

Pathway Analyses

KEGG pathway enrichment analyses were performed separately on the altitude- and ancestry-associated DEGs using the GOstats package provided by Bioconductor

(156). We used a hypergeometric test to determine which pathways were significantly over-represented within our lists of altitude- and ancestry-associated DEGs. A pathway was considered to be significantly over-represented if it had an FDR<0.05.

Methylation Detection and Quantification

We isolated DNA from the same villous placental tissues of the 45 individuals analyzed for gene expression. The DNA samples were bisulfite converted using the EZ DNA methylation kit protocol (Zymo Research, Irvine, CA) and hybridized to the Illumina HM 450k methylation microarray (Illumina, San Diego, CA) and scanned using the Illumina iScan Array Scanning System (Illumina, San Diego, CA) at the Applied Genomic Technology Center at Wayne State University (Detroit, MI). Fluorescence intensity values (i.e. β values) were calculated using the Illumina GenomeStudio software (Illumina, San Diego, CA), and a final report containing information for each probe was generated and preprocessed with the IMA (Illumina Methylation Analyzer) R package (157). After preprocessing, we used beta-mixture quantile normalization (BMIQ) to normalize type 1 and type 2 probe intensities (158). β values were logit transformed and quantile-normalized into M values to reduce the heteroscedasticity of the distribution across our samples (159). To restrict our analysis to determine which differentially expressed genes may be regulated by DNA methylation, which could potentially play a regulatory role in differential gene expression, we only analyzed probes located in the promoter or gene body of altitude- or ancestry-associated DEGs. Those genes that had a significant correlation between gene expression and cis-DNA methylation may suggest that the differences in gene expression. We defined the promoter region as the area located within 1.5kb upstream of the transcription start site

(TSS) through the 5' untranslated region (UTR). We considered probes to be in the gene body if they were located downstream of the 5'UTR and upstream of the 3'UTR. By only analyzing methylation sites within gene bodies and promoter regions of differentially expressed genes we were able to avoid further interaction between DNA methylation and ancestry. To avoid further confounding, zero of the methylation sites analyzed in this study were associated with our ancestry informative markers, which were all located in non-coding regions.

Correlation between Gene Expression and Methylation

Pearson's correlation test was used for the evaluation of correlation between the $\log_2$ intensity RNA abundance from gene expression microarrays and the M values of individual methylation probes within the promoter or gene body region of the identified DEGs in 42 samples for which we had both gene expression and DNA methylation data. Samples were excluded if we were unable to extract DNA with high enough quality for the methylation array. A correlation between gene expression intensity and M-value was considered to be statistically significant if the Pearson correlation test had an FDR<0.1. FDR was estimated for ancestry- and altitude-associated DEGs separately, similar to how it was estimated in the 2-way ANCOVA for gene expression.

Results:

Subjects / Placental Morphometrics

The mothers in this study were similar to those of the larger parent study. Andean women were older and had higher gravidity and parity (Table 3.1). Non-pregnant Body Mass Index (BMI) was similar across all four groups, as was gestational age at delivery, placental weight and volume (Table 3.1). Andean women had heavier

birth weights than European women, and less reduction in birth weight at high altitude. Because birth weights were lower, despite similar placental size, altitude reduced placental efficiency, reflected in the placenta:birth weight ratio (Table 3.1). Morphometric analyses of the placentas revealed greater vascularization in Andean vs. European placentas at high altitude as seen through greater capillary surface area and capillary volume (Table 3.1). Of the 45 placentas included in our microarray and methylation experiments, 42 had been used in prior studies where morphological data was collected (130). Relative to their low-altitude counterparts Andean subjects had an altitude-associated increase in capillary surface area, length and volume of 32%, 28% and 75% respectively (Table 3.1). These changes are greater than those in the total sample of low and high-altitude Andean placentae, in which these differences were 25%, 15% and 38% respectively. Among Europeans there was a 0%, 9% and 4% increase in capillary surface area, length and volume respectively. Again, these data differ somewhat from the larger sample of European placentas analyzed (6%, <1%, 23%). However, it must be remembered that the subset of subjects chosen for the genetic analyses represented the highest degree of Native American ancestry among Andeans and of European ancestry among the subjects of low-altitude origin.

Placental Gene Expression

In total, we identified 70 DEGs. Thirty-five genes (42 probe sequences) were differentially expressed between individuals of Andean and European descent (Figure 3.2, Table 3.2) and 36 genes (36 probe sequences) were differentially expressed between individuals residing at high and low altitude (Figure 3.1, Table 3.3). One gene, Carboxypeptidase, Vitellogenic Like (*CPVL*) was differentially expressed associated

with both ancestry and altitude. There were no significant differences between fetal sexes and across gestational ages among the genes used to test for differential expression (Supplementary Table 3.1). We tested six DEGs (*LYVE1*, *STC1*, *FOS*, *ALOX5AP*, *NAPRT1*, and *PLA2G2A*) using qRT-PCR and all showed a significant correlation with microarray expression, however after doing differential gene expression analysis were able to validate all but *PLA2G2A* validating the microarray analysis (Supplementary Table 3.2). Of the 34 ancestry-associated DEGs, ten had higher expression in European versus Andean placentas and 24 had higher expression in the Andean placentas (Table 3.2, Figure 3.2) (*CD163*) and lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1*) (160).

Among the 36 altitude-associated DEGs, 24 DEGs had higher expression levels in placentas of individuals residing at high altitude compared to those residing at low altitude, whereas 12 DEGs were more highly expressed in placentas of individuals residing at low altitude (Table 3.3, Figure 3.1). DEGs with decreased expression in high altitude placentas compared to low altitude placentas, include fos (*FOS*), fosb (*FOSB*), and jun (*JUN)* proto-oncogenes, which form part of the activator protein 1 (AP-1) transcription factor family. The AP-1 transcription factor family plays a role in a variety of processes including angiogenesis, cell proliferation, and cytotrophoblast differentiation/fusion. Carboxypeptidase, vitellogenic–like (*CPVL*) was both an altitude-associated and an ancestry-associated DEG, demonstrating decreased expression at high altitude and increased expression in Andean placentas. When we compared the gene expression patterns of the native Andeans at high altitude to the gene expression patterns of native Andeans residing at low altitude we found one gene (BTBD16) was

differentially expressed, and also shows higher expression in preeclampsia compared to healthy tissue(161). We found zero of our altitude associated DEGs were differentially expressed between Europeans residing at high altitude and Europeans residing at low altitude.

To test for enriched KEGG pathways amongst the DEGs, over-representation analysis was conducted using GOstats (156). Enriched pathways (FDR<0.05) among altitude-associated DEGs include osteoclast differentiation (KEGGID: hsa04380) and rheumatoid arthritis (KEGGID: hsa05323); each involves DEGs within the activator protein 1 (AP-1) transcription factor family including *JUN, FOS, FOSB,* and early growth response 1 (*EGR1*). Enriched pathways among ancestry-associated DEGs included rheumatoid arthritis (KEGGID: hsa05323), allograft rejection (KEGGID: hsa05330), and graft-versus-host disease (KEGGID: hsa05332). These pathways include DEGs that are involved in inflammation and angiogenesis including *HLA-A*, *HLA-DRB1*, *CCL2,* and *CCL3L3*.

Correlation of DNA Methylation with Gene Expression

1,065 Illumina HM450k methylation microarray probes, each designed to measure methylation at a specific CpG site, were located within the promoter or gene body region of the 69 ancestry- and altitude-associated DEGs. 121 methylation sites correlated significantly with gene expression levels when corrected for multiple hypothesis testing (FDR<0.1) (Supplementary Table 3.3). 106 methylation sites had a significant correlation with expression levels of 17 ancestry-associated DEGs while 15 methylation sites correlated significantly with expression levels of 8 altitude-associated DEGs (Supplementary Table 3.3, Supplementary Figure 3.2). Seventy-two percent

(28/39) of the methylation probes located in a promoter region had a negative correlation with gene expression. Among these 39 probes, eighty-nine percent (8/9) of the probes among altitude associated DEGs had a negative correlation while sixty-six percent (20/30) of the probes among ancestry-associated DEGs had the same negative correlation. A negative correlation suggests that higher methylation values were associated with lower gene expression and vice versa. Some ancestry-associated DEGs that had a significant negative correlation between gene expression and promoter DNA methylation included the cell surface markers *HLA-DRB1*, *HLA-A*, *CPVL*, and *LYVE1*. Altitude-associated DEGs showing a negative correlation (i.e. Increased DNA methylation being associated with decreased gene expression and vice versa) included genes involved in the response to oxidative stress and inflammation such as gliomedin (*GLDN*), TYRO protein tyrosine kinase binding protein (*TYROBP*), dysferlin (*DYSF*), and *CPVL*.

In addition to promoter region methylation, 69% (58/84) of the methylation probes that correlated significantly within gene bodies showed a pattern of negative correlation with gene expression. We also calculated correlation coefficients within CpG Islands located within gene bodies (i.e. >200 bp sequence within a gene body with > 60% CpGs). Ninety-seven percent (38/39) CpG sites within CpG islands located within gene bodies showed a negative correlation with gene expression. The genes that were differentially expression between Andeans and Europeans had more methylation sites with in regulatory regions with significant correlations between gene expression and DNA methylation than the altitude-associated DEGs (Fisher's exact p=0.0242, Table 3.4).

Discussion:

We present here a genome scale analysis of mRNA expression and DNA methylation in villous placental tissue from 45 Bolivian individuals of primarily Andean or European ancestry from high versus low altitude. We have shown that (1) a total of 69 genes were differentially expressed in the placenta in association with either altitude or ancestry, and (2) that epigenetic regulation of gene expression was more commonly associated with ancestry (Andean vs. European) as opposed to the environmental stressor of interest (altitude); and (3) how the DEGs might relate to the structural differences observed in the placentas themselves, which confer fetal growth advantages of Andean relative to European high-altitude inhabitants.

Ancestry-Associated Differentially Expressed Genes

Of the 34 ancestry-associated DEGs, 24 were more highly expressed in Andean compared to European placentas (Table 3.2, Figure 3.2, Supplementary Table 3.1). Among these ancestry-associated DEGs are several genes associated with inflammation including two inflammatory chemokines, c-c motif chemokine ligand 2 (*CCL2*) and c-c motif chemokine ligand 3 like 3 (*CCL3L3*). The ancestry-associated increase in expression of inflammatory chemokine ligands, *CCL2* and *CCL3L3,* would be expected to attract increased numbers of immune cells to the placenta (e.g. natural killer cells, macrophages, and monocytes), important in normal invasion and remodeling of the maternal-fetal interface (162). Previous work has shown increased pro-inflammatory cytokines such as interleukin 1-beta in the circulation of pregnant women at high altitude regardless of ancestry(163, 164).

In addition to these inflammatory genes, several markers of fetally derived pro-

angiogenic macrophages (Hofbauer cells), including CD163 molecule (*CD163*), lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1*), and major histocompatibility complex class 1 A (*HLA-A*), displayed higher expression levels in Andeans compared to Europeans. Hofbauer cells, i.e. placental macrophages, can secrete pro-angiogenic factors, which may contribute to the enhanced vascularity of the high-altitude Andean placenta and contribute to enhanced nutrient and gas exchange (165). We have shown that the placenta engages in increased glucose metabolism under high altitude conditions, and regulated by HIF-1 alpha (166, 167). HIF-1 alpha regulates expression of chemokines, including *CCL2* and *CCL3L3, which* may result in increased angiogenesis (168). We therefore speculate that the genes we have identified related to the innate immune response may be permissive for the greater angiogenesis that we and others have observed in Andean placentas at high altitude (163).

We tested for differential expression between those of Andean descent and those of European descent residing at high altitude and found that thirteen of the ancestry associated differentially expressed genes were differentially expressed between Andeans and Europeans residing at high altitude. Among those thirteen genes are *CD163*, *LYVE1*, *CPVL*, *STC1,* Coagulation Factor XIII A Chain (*F13A1),* LY6/PLAUR Domain Containing 3 (*LYPD3),* are all involved in angiogenesis, whether it be through recruitment of Hofbauer cells, or promoting invasion of the blood vessels. In contrast, there are zero genes that are differentially expressed between Andeans and Europeans residing at low altitude. This suggests that increased angiogenesis may be one of the factors that may be allowing native Andeans to healthier birth weights at high altitude compared to their European counter parts.

Andean women had a robust, altitude-associated increase in placental capillary surface area, length and volume, despite general similarity in placental weight and volume across the 4 groups. Increases in these functional parameters were 19-71% greater in Andean than European placentas for the same vascular variables. Functionally, one would expect greater capillary surface area and length to be associated with increased maternal-fetal nutrient transport and gas diffusion, whilst volume might be expected to relate to blood flow. We have no direct measures of diffusion, although extensive analysis of fetal blood gas parameters and oxygen delivery suggested the Andean fetus is advantaged relative to the European migrants (129). At the same time, and in these same pregnancies, we showed greater umbilical venous blood flow in the Andean vs. European fetus at high altitude, which may be related to their greater capillary volume (129). These data indicate a pronounced Andean advantage in the placental structural response to the chronic hypoxia of high altitude, centered on angiogenesis, and consistent with many of the ancestry-associated DEGs. We tested for correlation between gene expression levels ancestry associated DEGs and capillary volume and found that two genes had nominally significant correlations ($p < 0.01$). *NAPRT* has a nominal p-value of 0.004 and a Pearson correlation (r) of -0.435. This suggests that as expression levels of NAPRT decreases, resulting in decreased NAD+ and increased NADH, capillary volume increases (169). *ATP2C2* has a nominal p-value of 0.0095 and has an R-value of 0.40 suggesting that increased expression of ATP2C2 is associated with increased capillary volume.

Altitude-Associated Differentially Expressed Genes

Of the 36 altitude-associated DEGs, 10 DEGs had lower expression levels at high altitude compared to low altitude (Table 3.3). Three of those genes, fos proto-oncogene (*FOS),* fosb proto-oncogene (*FOSB*), and jun proto-oncogene (*JUN*) had the greatest negative fold-change in high altitude placentas. These genes are part of the activator protein 1 (AP-1) transcription factor complex. The AP-1 complex is a family of transcription factors formed by the dimerization of products from the *FOS* and *JUN* gene families (170). AP-1 transcription factors regulate the expression of genes involved in cell proliferation, cell death, and cellular transformation (171). This is consistent with molecular analyses addressing regulation of the cell cycle and apoptosis under in vivo (preeclampsia, IUGR, altitude) and in vitro low oxygen conditions (172-175). Previous studies have found an increase in cytotrophoblasts in high altitude placenta (176, 177), interpreted as hypoxia-induced proliferation. However, molecular data supports the excess cytotrophoblast cells are there due to a reduced rate of cytotrophoblast fusion into the syncytium. In essence syncytialization is delayed or slowed and apoptotic stimuli are diminished in the high altitude placenta.

Decreased expression levels of genes forming the AP-1 transcription factor complex may indicate that there is less AP-1 transcription factor available to bind to its target genes. The reduction in expression levels of four AP-1 target genes at high altitude supports the idea that there may be less availability of the AP-1 transcription factor. These four genes include early growth response 1 (*EGR1*), olfactomedin like 3 (*OLFML3)*, regulator of G protein signaling (*RGS1)* and immediate early response 3 (*IER3)* all of which have been shown to be involved in cell proliferation, suggesting that

decreased expression may result in reduced cell proliferation (178-181). The reduced cell proliferation may also contribute to reduced cytotrophoblast fusion into the syncytium (182, 183).

Prior studies of gene expression in Tibetans and Andeans implicate the HIF pathway as a critical target of natural selection, and hence these pathways were examined carefully in this study (79, 184). The altitude associated DEGs include genes involved in the response to hypoxia and the induction of the hypoxia-inducible factor (HIF) pathway. DEGs associated with hypoxia and the HIF pathway show higher gene expression among residents of high vs. low altitude. The DEGs associated or induced by HIF induction with higher expression at high altitude include family with sequence similarity 46 member C (*FAM46C*), solute carrier family 4 member 1 (Diego blood group) (*SLC4A1*), and alpha hemoglobin stabilizing protein (*AHSP*) (Figure 3.1, Table 3.3) (185-187). We have previously shown increased HIF-1 alpha gene expression, protein levels, and increases in the products of HIF's target genes in placentas of non-indigenous peoples residing at high altitude (188, 189). Placental gene expression for HIF-1 alpha (*HIF1A*), HIF-2 alpha (*EPAS1*), and HIF-3 alpha (*HIF3A*) in this study demonstrated low total variance ($\sigma^2 < 0.1186$) and therefore were not subject to genome wide hypothesis testing. To determine if the three hypoxia inducible factors are differentially expressed regardless of low variance we tested these probes using a two-way ANCOVA. There was greater expression of the HIF-3 alpha gene product among Andeans compared to Europeans regardless of altitude (p=0.01). We also found that there was greater HIF-1 alpha gene expression among Europeans vs. Andeans residing at high altitude (interaction p-value = 0.02). It is unlikely that the HIF-1 and HIF-3s

nominal p-value would hold up to the multiple hypothesis testing required in genome wide hypothesis testing, but this observation illustrates an interesting point. Several of the HIF-3alpha isoforms can attenuate HIF-1alpha binding to HREs in target genes (190, 191) and thereby modulate HIF-1alpha-dependent gene transcription. Studies of gene expression from leukocytes of Tibetans and Andeans indicate natural selection has favored variants of genes in the HIF-pathway that would be expected to lower the pO_2 at which a HIF response is stimulated. This appears counter-intuitive unless one posits that this lowered threshold for stimulation of a HIF response contributes to maintenance of sea-level values for some physiological parameters, despite the high altitude environment, which is as good a measure of adaptation as any other (79, 184, 192). That HIF-3alpha can attenuate HIF-1alpha target gene transcription and is elevated in placentas of Andeans regardless of altitude suggests it may act to further 'fine-tune' HIF-1alpha responses. Given that vascularity in low altitude Europeans and Andeans is similar, these differences in HIF-related gene expression at high altitude begs the question of whether the increased HIF-3alpha and relatively lower, but still elevated, HIF-1alpha levels plays a role in the Andean advantage in placental vascular response to high altitude hypoxia (191) .

Correlation between Genes that were Differentially Expressed Associated with Altitude or Ancestry and Methylation Patterns

There were more ancestry-associated DEGs (n=17) that correlated with DNA methylation than altitude-associated DEGs (n=8, Table 3.4, Supplementary Table 3.3). This significant difference (Fisher's exact p = 0.0242) supports our hypothesis that methylation plays an important role in placental adaptation to high altitude. DNA

methylation changes can be introduced within a population at a faster rate than gene expression differences due to evolutionary selection for extant or new sequence polymorphisms (193). Placental DNA methylation would be favored over just sequence variation in a subset of genes as a readily modifiable way to alter gene expression without inducing a permanent genetic change in the germ cell line. An example of this would be if a sequence variant occurred at a CpG site that would change the CpG site, which is most likely to be methylated to a CpA. The G/A variant would there for affect DNA methylation. However change in sequence would take generations to become fixed where as DNA methylation would not take quite as long. This has been recognized in the obstetric literature (194). It is one additional way in which phenotypic plasticity, which is amply well documented among Andeans in quantifiable traits such as chest dimensions and lung volumes, is favored over genetic changes that would be permanent.

It is well known that hypermethylation in the promoter region of genes typically results in lower gene expression resulting in an inverse correlation between DNA methylation and gene expression (195). The inverse correlation is likely due to DNA methylation blocking the binding of transcription factors to their respective binding sites (196). We have shown that among our DEGs, 72% (28/39) of methylation probes located within promoter regions had methylation values that were inversely correlated with gene expression with the remaining 28% (11/39) of promoter methylation sites having a positive correlation. We also found negative correlations between gene expression levels and DNA methylation within 97% (37/38) of the methylation probes located within CpG islands of gene bodies. These findings suggest that these CpG

islands may be acting as alternative promoters since CpG islands are typically located within promoter regions (197, 198). Transcription at alternative promoters may lead to isoforms that are unique to either an ancestral group or individuals exposed to an environment such as high altitude (199, 200). The use of intragenic CpG islands as tissue specific alternative promoters has been among cells that are part of the hematopoietic steam cell lineages as different cell types have different methylation patterns within CpG islands (200).

In contrast to DNA methylation in promoters and CpG islands within gene bodies (i.e. introns and exons), increased DNA methylation within non-CpG island gene bodies is increasingly becoming associated with increased mRNA abundance and a positive correlation between gene expression and DNA methylation within the gene body (201). However, our results show only 57% (25/43) of the methylation sites within the gene body, excluding those within CpG islands, had methylation values that correlated positively with gene expression. All of the 25 DNA methylation sites that had a positive correlation between gene body DNA methylation and gene expression were associated with ancestry associated DEGs. This suggests that 43% of non-CpG island gene body DNA methylation may be acting in a similar fashion to promoter region methylation (195). The genes we found that had decreased promoter methylation and consequently higher gene expression among Andeans, are associated with inflammation and pro-angiogenic macrophages. Those ancestry-associated DEGs with decreased promoter methylation include carboxypeptidase, vitellogenic-like (*CPVL*), lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1*), prolyl 3-hydroxylase 2 (*LEPREL1*) and N-acetylated alpha-linked acidic dipeptidase 2 (*NAALAD2*) (202-206). This may contribute

to the increased angiogenesis in Andeans compared with Europeans placentas at high altitude. Gene expression differences associated with altitude may be less dependent on epigenetic mechanisms and more dependent on the availability of transcription factors (e.g. AP-1 binding), or sequence variation favored by natural selection because we found less altitude associated DEGs correlated with DNA methylation. Seven altitude-associated DEGs had expression levels that correlated significantly with DNA methylation including genes involved in cellular and membrane repair such as gliomedin (*GLDN*) and dysferlin (*DYSF*). Both genes have a negative correlation between gene expression values and promoter DNA methylation suggesting that those residing at high altitude have decreased promoter methylation being associated with the increased gene expression compared to the low altitude populations.

This is the first study, to our knowledge, that investigates how placental gene expression differs among distinct populations of different biogeographic ancestry, which gene expression differences may be regulated by DNA methylation, and how these mechanisms may differ in the setting of reduced oxygen tension. It is also the first to consider these differences in relation to structural/functional differences in the target organ. It differs from prior studies that have identified polymorphisms found in DNA from leukocytes, which are likely unrepresentative of tissue or cell-specific gene expression patterns (79, 83). The gene variants identified in those studies have not yet been examined in a functional context nor have pre- or post-translational modifications to the proteins encoded by the genes of interest been tested. Moreover, DEGs based on functional tissues of impact on reproductive outcomes are likely not reflected in DEGs derived from hematopoietic cells, which have a biological turnover of 3 months, and are

more likely to reflect short-term, rather than long term, reproductively important outcomes. The study reported here provides insight into how DNA methylation may be regulating gene expression in the placental tissue of Andeans, which, unlike Tibetans, may have significant exposure to low altitude environments through culture and commerce. It suggests that Andean placentas may be adapting to the high altitude environment, seen by a preservation in birth weight, through epigenetic modifications as opposed to sequence variation. This work supports the well known idea that ancestry has a great impact on DNA methylation because the ancestral background has been shaped by the different environments, which the different ancestries were exposed to. For example there are ancestry specific DNA methylation sites between Europeans and African Americans that may affect how they metabolism vitamin D which include areas on various tumor suppressor genes that may be passed from the mother to the child (207). Also in a study comparing cancer patients they found ancestry specific DNA methylation within intergenic regions between individuals of African, European, and Asian ancestry (208).

A major limitation of this study is the small sample size, which restricts the power of our analysis by limiting the number of probes that would be significant after adjusting for multiple hypothesis testing. To account for this, we focused our analysis on only the top 5% most variable probes. Recent literature on altitude-associated genetic variation revealed polymorphisms in genes related to the hypoxia-inducible factor nuclear transcription family of genes (i.e. *HIF1A*, *EPAS1*, *HIF3A*) between high and low altitude populations (79, 209). The fact that we did not see differential expression in the same genes in which leukocyte DNA polymorphisms were identified may be associated with

the fact that the placenta of adapted individuals at high altitude receives the same amount of oxygen as the low altitude placenta, albeit at markedly lowered blood flow (129). Other limitations include the range of RIN scores used in this study, which may affect the ability of the respective cDNA to hybridize to the microarray chip affecting expression levels. Studies asking whether there has been natural selection of genetic polymorphisms or novel sequence variants in high altitude populations are limited to leukocytes. In our study, HIF-related genes did not achieve the level of statistical significance required for further exploration. Larger sample sizes may remedy this. Finally, ancestry differences may have affected hybridization, as the microarray chips were designed using primarily European sequences.

Conclusion:

We have identified differences in gene expression within the term human placenta in response to two different factors, altitude (high vs. low) and biogeographic ancestry (Andean vs. European). We have attempted to link the gene expression changes to differences in placental structure and consequently birth weights at high altitude. The approach is warranted because of the strong correlation between placental size and structure with pregnancy outcomes such as birth weight, preeclampsia and IUGR (210-213). We found that the ancestry-related expression differences are related to inflammatory processes and angiogenesis. By contrast, the altitude-related expression differences are more-closely linked to changes in the AP-1 transcription network. These may be linked to decreased cytotrophoblast turnover (and/or impaired syncytialization).

Lastly, we have shown that DNA methylation appears to regulate the ancestry-associated DEGs more than the altitude-associated DEGs. Taken together, the results suggest that the phenotypic plasticity conferred by methylation over sequence variation in genetic polymorphisms is a preferred evolutionary strategy. Epigenetic changes, because they can be environmentally induced and presumably absent or reversed in an alternative environment, permit greater flexibility in the face of stressful/changeable environments. These data also support that organ-specific or target tissue evaluation of gene expression is required in the assessment of genetic adaptation to environmental stress.

Acknowledgements

The authors would like to Acknowledge Dr. L Raetzman, Dr. M. Bagchi, and Dr. K. Clancy from the University of Illinois Urbana-Champaign for their advice and guidance while preparing the manuscript. Also Dr. A. Tarca from Wayne State University for his guidance during the initial data analysis. Lastly they would like to thank the people of Bolivia for being supportive of this project.

Figures:

Figure 3.1 Heat map of Z-scores for each altitude-associated differentially expressed gene

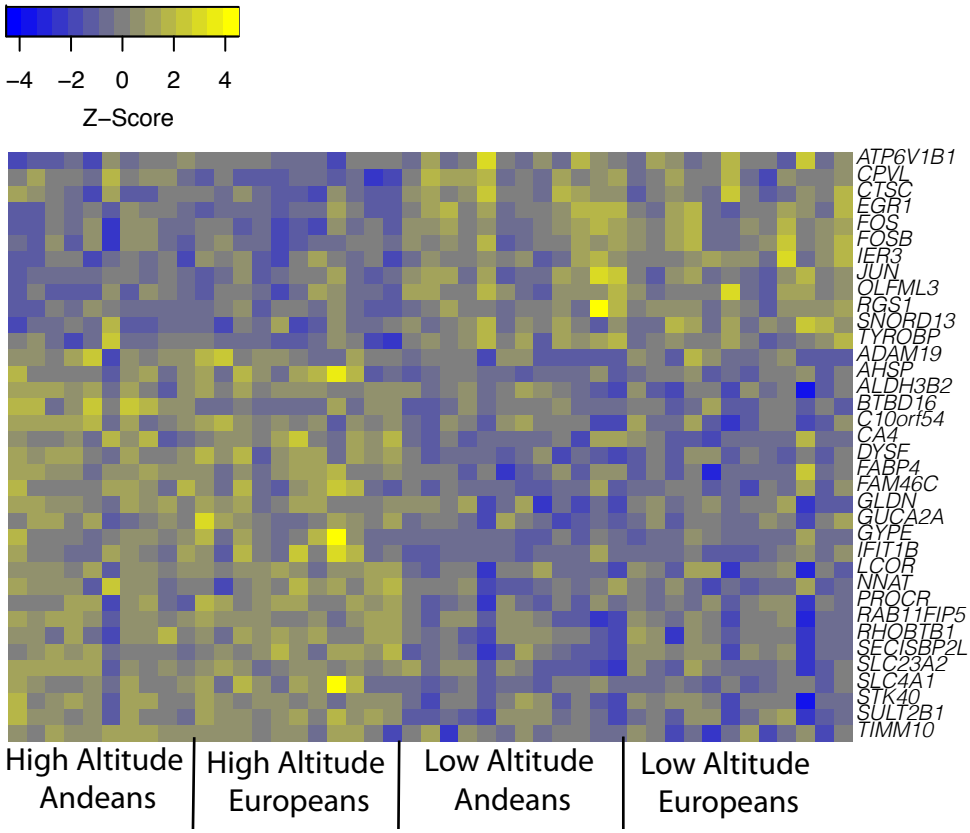


Figure 3.1: Heat map of Z-scores for each altitude-associated differentially expressed gene: Z-scores calculated from gene expression of each altitude-associated differentially expressed gene. The blue pixels represent negative z-scores (i.e. expression level for that sample is less than the mean expression) and the yellow pixels represent samples with positive z-scores (i.e. expression levels for that sample is greater than the mean expression level). A gene was considered to be differentially expressed with a FDR<0.1 and an absolute fold change≥1.25 between individuals residing at high and low altitude.

Figure 3.2 Heat map of Z-scores for each ancestry-associated differentially expressed gene

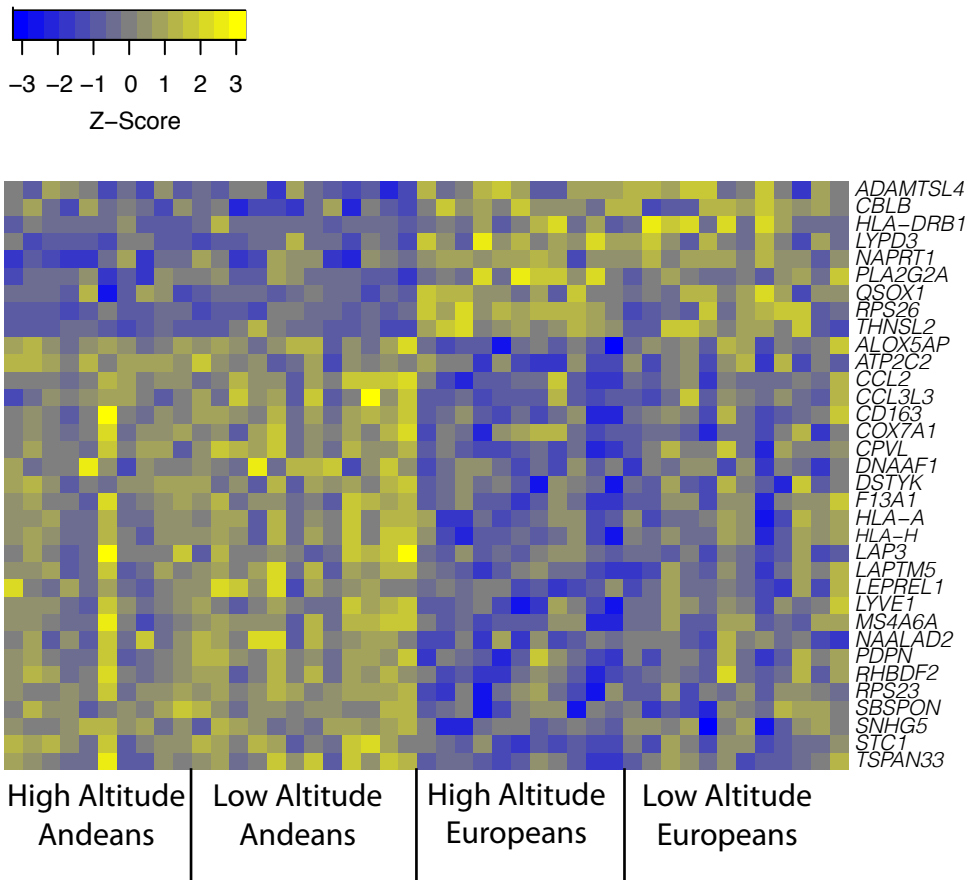


Figure 3.2: Heat map of Z-scores for each ancestry-associated differentially expressed genes
Z-scores calculated from gene expression of each ancestry-associated differentially expressed gene. The blue pixels represent negative z-scores (I.e. expression level for that sample is less than the mean expression) and the yellow pixels represent samples with positive z-scores (i.e. expression levels for that sample is greater than the mean expression level). A gene was considered to be differentially expressed with a FDR<0.1 and an absolute fold change≥1.25 between individuals of Andean and European descent.

Tables:

Table 3.1: Clinical data for placenta samples. A 2-way ANOVA was used with altitude and ancestry as the independent variables. An interaction term with P < 0.05 indicates that the phenotype associated with the combination of altitude and ancestry differs significantly between biogeographic ancestry groups. For example, the increase in placental capillary volume due to altitude is greater among Andeans than Europeans, hence the interaction term is significant.

	High Altitude Andean	High Altitude European	Low Altitude Andean	Low Altitude Auropean	Altitude P-value	**A**ncestry p-value	Interaction P-value
N	10	11	12	12			
Maternal age	32.6±5.2	30.8±3.1	29.3±5.4	25.9±5.9	0.006	0.046	0.802
Pre-pregnant BMI	23.9±2.9	23.9±3.0	26.2±3.5	22.8±4.3	0.953	0.032	0.437
Gravidity	4.4±3.7	1.6±0.9	3.8±2.5	2.3±1.3	0.900	0.004	0.400
Parity	1.9±1.7	0.5±0.5	2.3±2.5	1.0±1.0	0.330	0.009+	0.843
Neonatal Sex	M=6, F=4	M=4, F=7	M=7, F=5	M= 5, F= 7	0.889	0.178	0.413

Table 3.1 (cont.)

Birth Weight(g)	3444±43	3026±27	3766±22	3621±38	<0.001	0.009	0.174
Gestational Age (wks.)	38.7±1.4	38.3±1.1	39.0±0.7	38.4±0.9	0.585	0.094	0.838
Placental Weight (g)	549±102	500±87	493±82	476±84	0.146	0.240	0.552
Placental volume (cm^3)	556±115	500±87	463±97	446±76	0.015	0.219	0.497
Birth to Placental weight ratio	6.4±0.7	6.2±0.9	7.8±1.3	7.9±1.1	<0.001	0.691	0.797
Capillary length (km)	564±245	441±161	506±142	454±146	0.654	0.336	0.139
Capillary surface area (m^2)	16.7±4.3	12.2±2.6	13.5±3.3	13.2±3.8	0.302	0.053	0.068
Capillary volume (cm^3)	53.1±15.4	33.1±10.8	37.8±7.9	31.0±11.2	0.003	0.006	0.016

Table 3.2: Fold change of Illumina HT12-v4 gene expression microarray probes that are significantly differentially expressed (FDR > 0.1) as a function of ancestry

Probe ID	Gene Symbol	Fold Change	FDR
ILMN_1710752	*NAPRT*	-2.03	0.011
ILMN_1740586	*PLA2G2A*	-1.7	0.072
ILMN_2173294	*THNSL2*	-1.65	0.001
ILMN_3248833	*RPS26*	-1.64	0.001
ILMN_3299955	*RPS26*	-1.56	0.001
ILMN_1739605	*LYPD3*	-1.56	0.001
ILMN_2209027	*RPS26*	-1.54	0.001
ILMN_2310703	*RPS26*	-1.52	0.004
ILMN_1715169	*HLA-DRB1*	-1.48	0.092
ILMN_1685580	*CBLB*	-1.47	0.011
ILMN_2411282	*QSOX1*	-1.42	0.011
ILMN_1687035	*ADAMTSL4*	-1.32	0.035
ILMN_1762294	*ADAMTSL4*	-1.3	0.053
ILMN_1691717	*RHBDF2*	1.26	0.051
ILMN_1749403	*TSPAN33*	1.27	0.035
ILMN_1772359	*LAPTM5*	1.28	0.071
ILMN_1733270	*CD163*	1.28	0.064
ILMN_1717163	*F13A1*	1.29	0.094
ILMN_1683792	*LAP3*	1.29	0.054
ILMN_1721035	*MS4A6A*	1.31	0.094
ILMN_1785413	*ATP2C2*	1.31	0.047
ILMN_2352023	*DSTYK*	1.32	0.094
ILMN_1657373	*LEPREL1*	1.33	0.071
ILMN_1808114	*LYVE1*	1.34	0.099
ILMN_1740024	*NAALAD2*	1.35	0.051
ILMN_1670490	*PDPN*	1.36	0.094
ILMN_2203950	*HLA-A*	1.38	0.094
ILMN_2200659	*SNHG5*	1.38	0.088
ILMN_1808245	*SBSPON*	1.38	0.072
ILMN_1662419	*COX7A1*	1.39	0.072
ILMN_1682928	*CPVL*	1.39	0.015
ILMN_2379599	*CD163*	1.4	0.027
ILMN_2130441	*HLA-H*	1.4	0.086
ILMN_2186806	*HLA-A*	1.41	0.060
ILMN_1722622	*CD163*	1.41	0.035
ILMN_2400759	*CPVL*	1.42	0.011
ILMN_1797875	*ALOX5AP*	1.42	0.094
ILMN_1758164	*STC1*	1.43	0.004
ILMN_1772459	*RPS23*	1.44	0.010

Table 3.2 (cont.)			
ILMN_1776967	*DNAAF1*	1.46	0.089
ILMN_2105573	*CCL3L3*	1.53	0.099
ILMN_1720048	*CCL2*	1.56	0.035

Table 3.3: Fold change of Illumina HT12-v4 gene expression microarray probes that are significantly differentially expressed (FDR < 0.1) associated with altitude.

Probe ID	Gene Symbol	Fold Change	FDR
ILMN_1669523	*FOS*	-2.04	0.045
ILMN_1762899	*EGR1*	-1.9	0.055
ILMN_1751607	*FOSB*	-1.63	0.080
ILMN_1806023	*JUN*	-1.56	0.068
ILMN_1892403	*SNORD13*	-1.47	0.068
ILMN_2242463	*CTSC*	-1.35	0.098
ILMN_1778977	*TYROBP*	-1.31	0.084
ILMN_1727532	*OLFML3*	-1.3	0.069
ILMN_1682928	*CPVL*	-1.3	0.075
ILMN_1656011	*RGS1*	-1.28	0.069
ILMN_1682717	*IER3*	-1.27	0.075
ILMN_1812073	*ATP6V1B1*	-1.25	0.075
ILMN_2075927	*STK40*	1.26	0.068
ILMN_1727589	*SULT2B1*	1.29	0.055
ILMN_1763749	*GUCA2A*	1.29	0.075
ILMN_1689817	*LCOR*	1.29	0.098
ILMN_1772809	*SLC4A1*	1.29	0.055
ILMN_1677684	*BTBD16*	1.3	0.080
ILMN_1713751	*ADAM19*	1.3	0.069
ILMN_2205963	*C10orf54*	1.31	0.068
ILMN_1784333	*SECISBP2L*	1.32	0.055
ILMN_2376502	*RHOBTB1*	1.32	0.070
ILMN_1765332	*TIMM10*	1.33	0.069
ILMN_1695187	*GYPE*	1.34	0.068
ILMN_1746578	*SLC23A2*	1.35	0.055
ILMN_1717262	*PROCR*	1.35	0.069
ILMN_1695157	*CA4*	1.37	0.069
ILMN_1810420	*DYSF*	1.38	0.001
ILMN_1740604	*RAB11FIP5*	1.4	0.069
ILMN_1763666	*ALDH3B2*	1.42	0.067
ILMN_1759155	*IFIT1B*	1.42	0.068
ILMN_1707734	*NNAT*	1.43	0.075
ILMN_1704376	*GLDN*	1.44	0.075
ILMN_1696512	*AHSP*	1.52	0.068
ILMN_1713266	*FAM46C*	1.57	0.068
ILMN_1773006	*FABP4*	1.67	0.068

Table 3.4: Table of the number of genes that may be regulated by DNA methylation or not regulated by DNA methylation among altitude and ancestry associated differentially expressed genes.

	Ancestry	Altitude
Regulated by DNA methylation	17	8
Not Regulated by DNA methylation	17	28

Supplement List

Supplementary Table 3.1: Tables of differentially expressed genes associated with altitude and/or ancestry.

For supplementary table 3.1 please use the provided link:

https://www.biorxiv.org/highwire/filestream/113157/field_highwire_adjunct_files/0/261974-1.xlsx

Supplementary Table 3.2: Validation of gene expression microarray by qRT-PCR

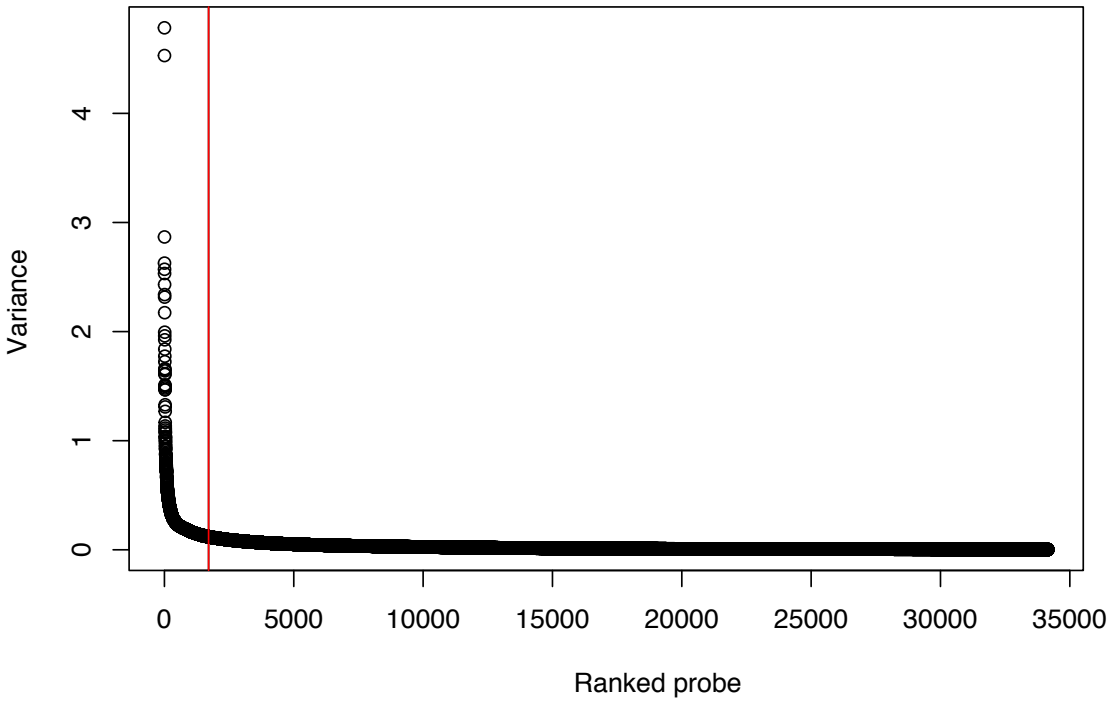
Gene	TaqMan Probe	Pearson Correlation	Correlation p value	Ancestry or Altitude DEG	T- Test p-value
FOS	Hs99999140_m1	0.75	<0.0001	Altitude	0.0043
NAPRT	Hs00292993_m1	0.56	0.0004	Ancestry	0.043
PLA2G2A	Hs00179898_m1	0.51	0.0012	Ancestry	0.91
LYVE1	Hs00272659_m1	0.47	0.003	Ancestry	0.095
STC1	Hs00174970_m1	0.46	0.004	Ancestry	0.099
ALOX5AP	Hs00233463_m1	0.42	0.0125	Ancestry	0.043

Supplementary Table 3.3: Results of the Pearson's correlation test between DNA methylation and gene expression

For supplementary table 3.3 please use the provided link:

https://www.biorxiv.org/highwire/filestream/113157/field_highwire_adjunct_files/1/261974-2.xlsx

Supplementary Figure 3.1: Scatterplot of variance of Illumina HT12v4 gene expression microarray probes



Supplementary Figure 3.1: Scatterplot of variance of Illumina HT12v4 gene expression microarray probes

Supplementary Figure 3.2: Correlation of gene expression with methylation values of differentially expressed genes

For supplementary Figure 3.2 please use the provided link:

https://www.biorxiv.org/highwire/filestream/113157/field_highwire_adjunct_files/4/261974-5.jpg

Supplementary Figure 3.2: Differentially expressed genes with at least one methylation site that significantly correlates with gene expression values sorted by its location with the right most sites being located on chromosome 1. The green circles represent CpG sites located within the gene body. The orange circle represents CpG sites located within the promoter region. The purple circles represent CpG sites that are located within CpG islands of a differentially expressed gene.

CHAPTER 4: ANCESTRY DEPENDENT REGULATION OF PLACENTAL DYSFERLIN AT HIGH ALTITUDE

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Abstract:

The placenta mediates fetal growth by acting as a substrate for nutrient exchange between the mother and the fetus. Environmental factors such as altitude have been shown to result in reduced maternal-fetal exchange leading to reduced birth weight. To determine if differences in gene expression maybe contributing to physiological differences between individuals at high and low altitude, we tested for differences in DNA methylation between Andean and European placental samples from La Paz (~3,600m) and Santa Cruz, Bolivia (~400m). Among altitude associated differentially expressed genes, we previously noticed that one gene, dysferlin (*DYSF*) had higher expression among residents at high altitude compared to low altitude. *DYSF* had a variant (rs10166384;G/A) at a methylation site and we observed 3 levels of DNA

methylation that correspond to individual genotypes (A/A < A/G < G/G methylation). We tested for natural selection by sequencing a ~2.5 kb fragment that includes the above *DYSF* variant from 90 Bolivian samples and compare with 1000 genome data. We found that balancing selection (Tajima's D=2.37) was acting on this fragment among Andeans regardless of altitude. This suggests that balancing selection acting on dysferlin is altering DNA methylation patterns. Also, preservation of both the Adenine (A) and Guanine (G) alleles may aide Andeans in moving from high to low altitude and vise versa.

Introduction:

Natural selection is one of the mechanisms that has been shown to contribute to differences between populations at both the genotypic and phenotypic level (214). The two major types of natural selection that cause changes in allele frequencies are directional selection and balancing selection. A site under directional selection has one allele frequency that is greater than the other suggesting the more frequent allele may be more beneficial than the less frequent allele (215). One example of directional selection, specifically positive selection, is seen in relation to lactase persistence among individuals residing in arid regions (139); the authors showed the three major variants within the lactase gene show positive selection that favor better absorption of nutrients and water from milk in regions where water is less available. A site experiencing balancing selection has two alleles of roughly equal frequency suggesting both alleles may be beneficial (216). A well-known example of balancing selection is seen in those individuals who are heterozygotes for the sickle cell allele associated with hemoglobin beta (217, 218). Those individuals that are heterozygotes have the

healthy disk shape cell but also show some resistance toward malaria (217). In opposition to natural selection, if the change in allele frequency occurred randomly in a population, that population is experiencing genetic drift (219). One common instance where genetic drift occurs is when a population migrates, typically reducing in size (220). This reduction in population size can cause a shift in allele frequency, not related to natural selection.

The high altitude environment has been shown to act as a selective pressure resulting in variant changes in different regions of the human genome potentially leading to physiological adaptations. Early studies among Andeans residing at high altitude found signatures of positive selection in genes associated with the response to hypoxia including endothelial PAS domain protein 1 and Egl-9 family hypoxia inducible factor 1 (221). In addition to pervious studies, our prior work found genes associated with cell fusion and proliferation were differentially expressed between individuals residing at high and low altitude many of which showed differences in DNA methylation. In this prior study, we found 8 out of 36 altitude associated differentially expressed genes had significant correlation between gene expression and DNA methylation (222). One of the altitude-associated differentially expressed genes that showed differences in DNA methylation is dysferlin (*DYSF*). *DYSF* is a large gene comprised of roughly 230 Kb with more than 50 coding exons. Within the third intron of the gene dysferlin, there is a single nucleotide polymorphism (SNP) that could change a CpG site to a CpA, which is less likely to be methylated (223). *DYSF* encodes a membrane bound protein associated with calcium regulated membrane repair (224). *DYSF* plays an important role in the formation of syncytial tissues, multinucleated cells

formed by the fusion of mononucleated cells (225-227) (Figure 4.1). In the placenta, dysferlin is expressed in the apical membrane of the multi-nucleated syncytiotrophoblast and is induced when the mononucleated cytotrophoblast fuse during syncytiotrophoblast formation and repair (228).

The goal of this work was to determine if natural selection, induced by the high altitude environment, was acting on dysferlin leading to a change in allele frequency affecting DNA methylation and gene expression. To do this we tested if the population was in accordance with Hardy-Weinberg equilibrium and distinguished between selection and genetic drift by estimating Tajima's D. We found that those individuals residing at high altitude show a signature of balancing selection suggesting that two alleles may be beneficial.

Materials and Methods:

Samples

Term placentas from 96 Bolivian individuals were collected from La Paz, Bolivia (3,600m) and Santa Cruz, Bolivia (400m). The placentas were from healthy individuals undiagnosed with preeclampsia or intrauterine growth restrictions and were delivered through caesarian section. All women gave written informed consent to protocols approved by the Bolivian National Bioethics Committee, the San Andreas Mayor University, Instituto Boliviano de Biologia de Altura Consejo Tecnico, and the USA Institutional IRBs from the University of New Jersey Medical School.

Identification of Differentially Methylated Sites Associated with Differential Gene Expression

DNA was extracted from the villous tissue of term placenta and used for DNA methylation analysis and SNP genotyping. DNA methylation data was generated using the HM450k methylation microarray chips using previously described methods (222). Validation was performed using 350 ng of placental DNA from 8 individuals from our sample groups as well as high and low methylation controls (Zymo Research, Irvine, CA) were bisulfite converted using the Zymo EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). The samples chosen were those that had methylation microarray data and provided an even representation of each genotype. Primers developed for this study were designed using the PyroMark Q24 Assay Design Software 2.0 (Qiagen, Hilden, Germany)(Supplementary Table 4.1). The desired fragment was 60 bp containing three different CpG sites and amplified using the Qiagen PyroMark PCR master mix and manufacture suggested protocol (Supplementary Table 4.1). Pyrosequencing was performed on the PyroMarkQ24 Advanced using a sequencing primer (Supplementary Table 4.1) as well as the standard PyroMarkQ24 Advanced reagents and protocol.

Single Nucleotide Polymorphism Genotyping

We genotyped SNP rs10166384 within dysferlin (*DYSF*) using DNA from term placentas of 96 individuals (48 Andean, 48 European) collected from La Paz, Bolivia (3,600m) and Santa Cruz, Bolivia (400m) including the 45 individuals from our prior study (222). We genotyped SNP rs10166384 in 96 individuals using a TaqMan SNP genotyping assay (TaqMan assay C__387100_10). This probe was 50 nucleotides long

with rs10166384 (A/G) being located at the 26th base. The adenine was labeled with VIC and the Guanine was labeled with FAM. The assay was run on an ABI 7500 (Applied Biosystems, CA). The run conditions used were those standard to the TaqMan SNP genotyping assay provided in the ABI 7500 manual. We then calculated the allele frequencies of the A and G allele for each of the four sample populations measured by the Taqman genotyping. Allele frequencies were calculated for each sample group to determine if the environment of high altitude provided a selective pressure on this site. We then tested to see if the proportion of the three genotypes were what one would expect for a population within Hardy-Weinberg equilibrium. When a population is within Hardy-Weinberg equilibrium it is assumed to have no selection, no mutation, no migration, a large population size, and random mating. χ^2 analysis was performed to determine if the proportion of genotypes in each of the four sample groups were significantly out of Hardy-Weinberg equilibrium ($p<0.05$).

Detecting Natural Selection

To determine if natural selection was acting on our SNP of interest (rs10166384) we amplified a ~2500 base pair region using polymerase chain reaction from DNA of 96 individuals (Supplementary Table 4.1). PCR products were purified using the QIAquick pcr purification kit (Qiagen, Hilden, Germany). Between 30 and 50 µg of each PCR product was sequenced at the UIUC core sequencing center (Urbana, IL). The sequences generated from each primer were aligned using Sequencher (Gene Codes, Ann Arbor, MI). The complete sequences from each sample were then aligned to each other using the ClustalW (229) algorithm implemented in MEGA version 6.06 (230). Once sequences were aligned, a variant call format file (vcf file) was generated using

SNP-sites (231). We calculated allele frequencies for each polymorphism, determined if each subpopulation was in Hardy-Weinberg equilibrium, and calculated Tajima's D for all SNPs with a minor allele > 0.05 using vcftools version 0.1.13 (232, 233).

Results:

Through analyzing DNA methylation patterns of genes we previously found to be differentially expressed (222), we found a probe (cg09829645) within the third intron of dysferlin that had three distinct levels of DNA methylation (Figure 4.2a). One group showed hypomethylation, low levels of DNA methylation (M=-5.01±0.67), one group was hypermethylated, high levels of DNA methylation (M=1.97±0.36), and one group was hemimethylated with median DNA methylation levels (M=-0.11±0.49). This probe was located in the same position as a polymorphism (rs10166384),which can either, be a Guanine (G) or an Adenine (A) nucleotide. After genotyping this site in 96 individuals of Andean and European descent from high and low altitude, we found that the levels of DNA methylation tended to correspond to the three different genotypes possible at rs10166384 (Figure 4.2b). Those that were homozygous for the A allele (A/A) had on average the lowest levels of DNA methylation (M=-4.21±1.96), those that there homozygous for the G allele (G/G) had on average the highest levels of DNA methylation (M=1.41±0.97), and those that were heterozygous (A/G) had methylation values that fell in between the two homozygous groups ((M=-0.51±2.06) (Figure 4.2b). The genotype specific DNA methylation was confirmed through pyrosequencing a region containing rs10166384 in a subset of individuals for which we have methylation microarray data for probe cg09829645 (Supplementary Figure 4.1)(222).

Individuals residing at high altitude were more likely to have the A allele and those residing at low altitude were more likely to have the G allele. The allele frequencies varied depending on ancestry as well. Europeans residing at high altitude had an A allele frequency of 0.64 while Europeans residing at low altitude had an A allele frequency of 0.38 (Table 4.1). Europeans residing at high altitude had a greater number of individuals that were homozygous for the A allele compared to Europeans residing at low altitude (Table 4.1). We noticed that those of primarily European descent had a negative correlation between the A allele frequency and the percentage of Native American admixture, previously estimated in Zamudio et. al. (130). Europeans residing at high altitude had on average 26% Native American admixture and an A allele frequency of 0.64 while low altitude Europeans had more native admixture (~33%) and a lower frequency of the A allele of 0.38 (Table 4.1). Among Native Andeans, there was a less drastic change between high and low altitude Andeans with A allele frequencies of 0.54 and 0.46 respectively. There was also much less admixture among the Andean populations compared to the European populations. The Andeans residing at high altitude had ~3% European admixture and those Andeans residing at low altitude had ~5% European admixture.

We tested to see if natural selection induced by the high altitude environment was contributing to the change in allele frequency at the location of SNP rs10166384 causing a shift in DNA methylation. We tested to see if any of our four sample groups were out of Hardy-Weinberg equilibrium suggesting that the proportion of the genotypes are different then would be expected for a population not being influenced by selective pressure. The group of Andeans residing at high altitude was the only sample group

that was significantly out of Hardy-Weinberg equilibrium ($\chi^2 = 5.916$, $p = 0.015$). The high altitude Andeans had fewer heterozygotes then a population in accordance with Hardy-Weinberg equilibrium (Table 4.2). While the high altitude Andeans were the only population that was significantly out of Hardy-Weinberg equilibrium, the high altitude Europeans were only nominally significant for being out of Hardy-Weinberg equilibrium ($\chi^2 = 3.711$, $p = 0.054$), also having fewer heterozygotes then would be expected for a population in Hardy-Weinberg equilibrium. Lastly, both Andeans and Europeans residing at low altitude had genotype frequencies that were in accordance with Hardy-Weinberg equilibrium.

We then determined the if change in genotype proportions was influenced by directional selection, balancing selection, or random genetic drift by calculating Tajima's on a 2.5kb region with six polymorphisms with had minor allele frequencies > 0.05 (233). We found that the Andeans regardless of altitude had the highest estimated Tajima's D value of 2.35 suggesting balancing selection or roughly equal frequencies of each alleles. Also, the Europeans regardless of altitude had a Tajima's D of 1.86 also suggesting balancing selection.

Discussion:

Nucleotide Adaptation in Dysferlin

The variant rs10166384 (G/A) is located directly downstream of a cytosine causing a CpG site to potentially become a CpA site reducing the likelihood of DNA methylation within our four sample populations (234). rs10166384 is located within a TEAD4 transcription factor binding site, involved in the formation of syncytial tissues including the syncytiotrophoblast, the outer layer of the placental villi (Figure 4.1)(235). Increased

DNA methylation along transcription factor binding sites limits the binding of the TEAD4 transcription factor to its binding site resulting in decreased *DYSF* gene expression. We found that the A homozygotes had the lowest level of DNA methylation (Figure 4.2b). The G homozygotes had the highest levels of DNA methylation and likely decreased *DYSF* gene expression. The heterozygotes, having both an A and G allele, had methylation levels between the homozygous groups (Figure 4.2b). Reduced *DYSF* expression may result in reduced cytotrophoblast fusion and less effective syncytiotrophoblast fusion at high altitude (236). Several pregnancy complications where hypoxia is a contributing factor such as preeclampsia and intrauterine growth restriction show decreased *DYSF* gene expression and problems with syncytiotrophoblast formation (237).

We compared allele frequencies across our four sample populations (high altitude Andeans, high altitude Europeans, low altitude Andeans, and low altitude Europeans) and found the populations with the highest A allele frequencies were of the high altitude European ancestry (A allele frequency =0.64). The higher A allele frequency corresponds with a decrease in methylation compared to low altitude Europeans (ΔM =-2.62). Lower DNA methylation may contribute to the higher *DYSF* gene expression within the high altitude European population compared to the low altitude European population. The A allele is also more abundant among those individuals residing in Spain who were genotyped as part of the 1000 genomes project, suggesting that the A allele is the ancestral European allele. The high frequency of A alleles among Europeans residing at high altitude could be related to the fact that this study only examined individuals whom experienced healthy pregnancies. The A allele may be

more beneficial for the Europeans residing at high altitude because it may be linked to more effective DYSF transcription and more effecting cytotrophoblast fusion and syncytiotrophoblast repair increasing the chance of a healthy pregnancy. We predict that if we were to genotype Europeans residing at high altitude who experienced problematic pregnancies we would see an increased G allele frequencies potentially related to less effective syncytiotrophoblast repair.

We compared allele frequencies of our sample populations to those of East Asian, European, and South American descent from the 1000 genome project (238) and found that allele frequencies of our Bolivian samples had allele frequencies that lie in between those of the East Asian and European 1000 genomes populations (Figure 4.3). We chose to look at the east Asian populations because they are the ancestral population for the Native Andeans as the east Asians crossed over the Bering strait land bridge being the first people to settle in Americas roughly 12,000 years ago (239). The East Asians that were part of the 1000 genomes project had G as the major allele for SNP rs10166384 with an average G allele frequency of 0.74. Apart from the East Asians, the European samples from the 1000 genomes had the A as the major allele at this site with an A allele frequency of 0.68. The fact that both the Andeans and Europeans residing in Bolivia had intermediate allele frequencies, having roughly equal allele frequencies of both the A and G allele suggests that there has been East Asian and European admixture resulting in the Andeans gaining more of the European A allele and the Europeans gaining more of the East Asian G allele (Figure 4.3). This suggests that the more generations Europeans have resided at high altitude the greater the chance that they will inherit native admixture resulting in an increase in the frequency of G allele, the

allele most frequent among East Asians, compared to the Europeans from the 1000 genomes project causing them to become significantly out of Hardy-Weinberg equilibrium.

We found that the Native Americans residing at high altitude had genotype proportions that were significantly out of Hardy-Weinberg equilibrium (p=0.015). This suggests that over the ~12,000 years the Andeans have resided at high altitude the selective pressure of the environment has caused a shift in genotype proportions. This shift at rs10166384 may potentially allow the Andeans to utilize DNA methylation to alter *DYSF* expression as they shift between high and low altitude. We noticed that Europeans residing at high altitude are nominally out of Hardy-Weinberg equilibrium (p=0.054). This indicates that the selective pressure of high altitude may also be resulting in sequence level change at site rs10166384, however the Europeans have not resided at high altitude for enough generations to cause the population to be significantly out of Hardy-Weinberg equilibrium. We predict that after a few more generations at high altitude, rs10166384 will be significantly out of Hardy-Weinberg equilibrium in the high altitude European population. Those of European ancestry residing in Bolivia who had healthy pregnancies had a higher frequency of the A allele compared to the G allele resulting in decreased methylation through less CpG sites. The decreased methylation may lead to more effective TEAD4 binding and more effective transcription leading to increased *DYSF* expression. The increased expression of *DYSF* allows for better syncytiotrophoblast repair and fusion in response to the damage induced by the high altitude environment.

Allele frequencies for the Andean and European populations regardless of ancestry had A and G allele frequencies of 0.5 suggesting that balancing selection is acting on and around variant rs10166384. This is supported by the positive Tajima's D value of 2.37 and 1.85 across Andean and European populations respectively. A significantly positive Tajima's D ($D > 1.80$) indicates balancing selection suggesting that both the A and G allele maybe beneficial in the high altitude environment (233). Those possessing a G allele are more likely to be methylated limiting the binding of the TEAD4 transcription factor resulting in decreased gene expression. Methylation at this site however may allow for more transient regulation of dysferlin allowing for a smoother transition between high and low altitude. Those that have the A allele maybe regulating gene expression by limiting the ability of this CpG site to be methylated potentially resulting in higher gene expression. Higher dysferlin gene expression may result in more effective syncytiotrophoblast repair in the high altitude environment increasing the likelihood of a successful pregnancy. The preservation of both the A and G alleles among native Andeans regardless of altitude suggests that both the ability to be methylated and regulating of transcription factor binding may aide the Andeans in giving births children with healthy birth weights regardless of what altitude they reside in at the time.

Conclusion:

In this work, we identified a case where ancestry dependent natural selection may alter DNA methylation by changing the nucleotide sequence. In the gene dysferlin, we noticed a variant (rs10166384) located within a transcription factor-binding site that can alter dysferlin gene expression. Those individuals with the A allele whom reside at high

altitude are likely to have decreased DNA methylation and higher dysferlin gene expression. The higher dysferlin gene expression is likely to contribute to more effective syncytiotrophoblast repair in response to the high altitude environment, which may lead to more successful pregnancies at high altitude. Those with the G allele are more likely to have DNA methylation along the transcription factor-binding site and decreased gene expression. This may be advantageous among those of Andean ancestry because it may allow them a way to easily modify gene expression as they move between high and low altitude. As native Andeans move to high altitude the DNA methylation may be removed increasing dysferlin gene expression allowing for increased membrane repair compared to low altitude.

Figures:

Figure 4.1: Drawing of a villous cross-section of a human placenta

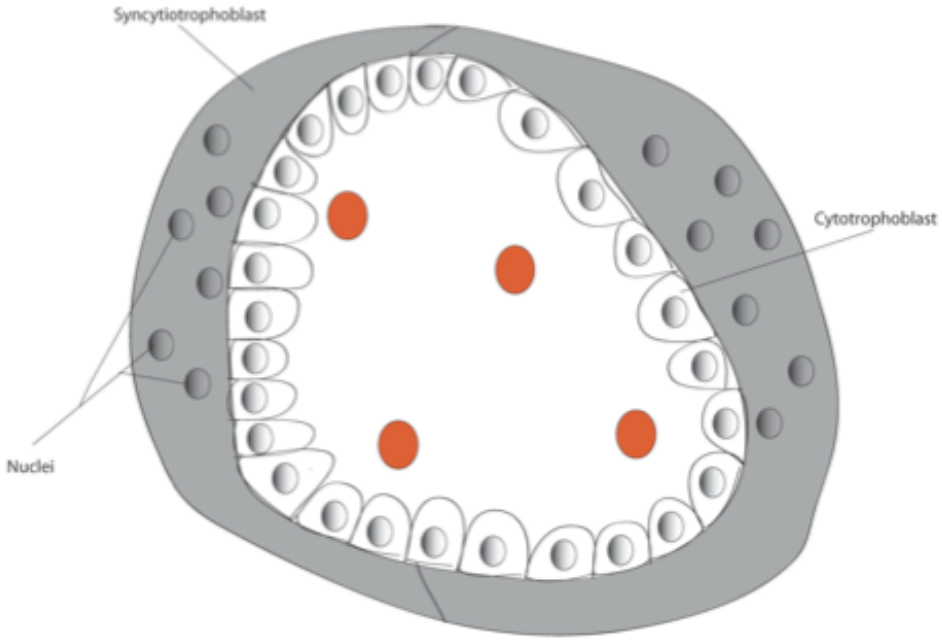


Figure 4.1: Figure 4.1 is a schematic of a cross-section of the villous tissue of the human placenta. The outer layer comprised of multi-nucleated syncytiotrophoblast. The second layer is comprised of mononucleated cytotrophoblast, which fuse to form the syncytiotrophoblast. Within the center are fetal capillaries shown in red.

Figure 4.2: Estimated genotypes based on methylation.

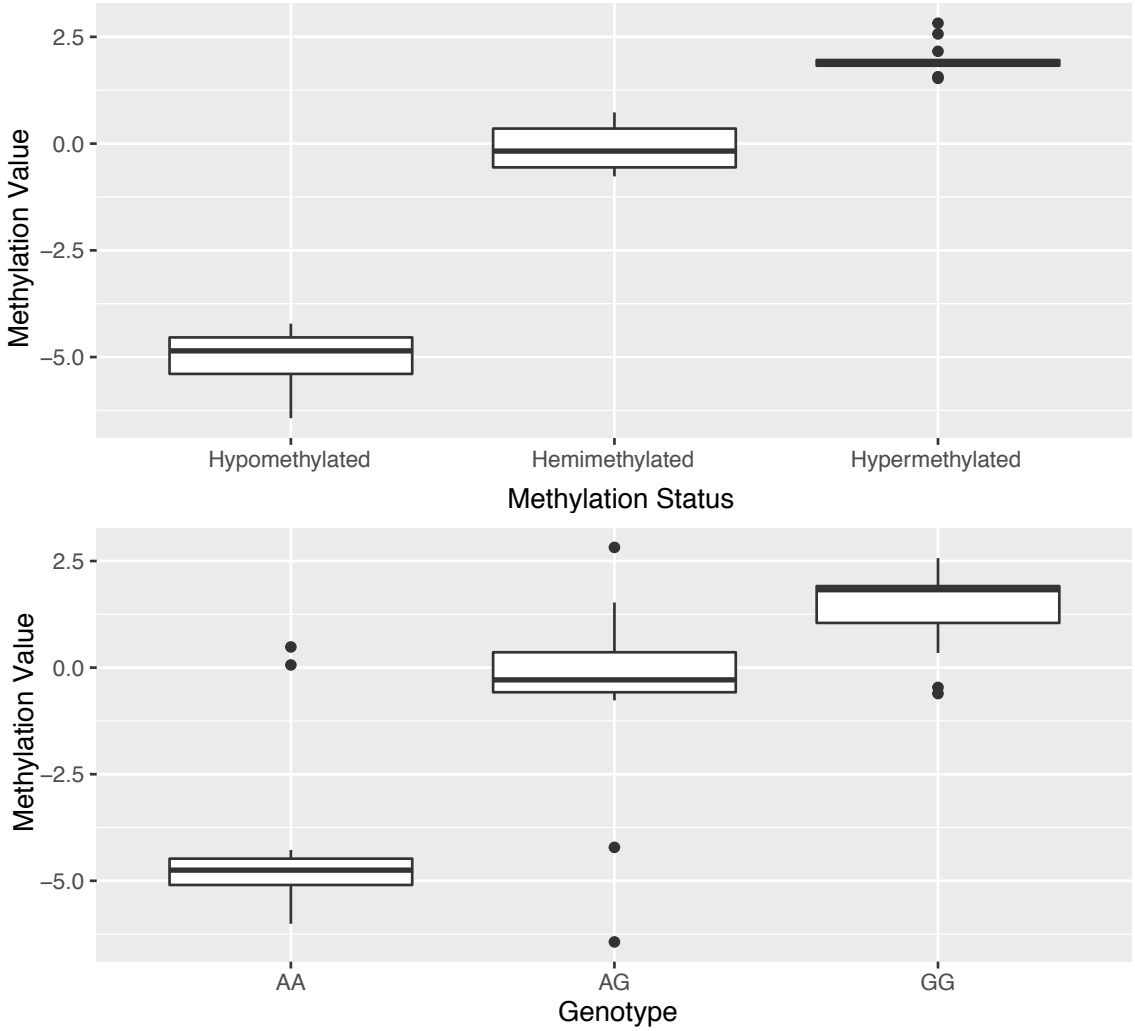


Figure 4.2: Prediction and confirmation of genotype using DNA methylation status at methylation probe cg09829645 which is in the same location as variant rs10166384. Figure 4.2a shows three groups with different levels of DNA methylation at probe cg09829645, one group is hypomethylated, one group is hemimethylated, and one group is hypermethylated. Figure 4.2b shows the average methylation for the individual genotypes that are possible at the rs10166384 variant. Those with the AA genotype tend to have low levels of methylation, those with GG genotype tend to have high levels of DNA methylation and those with the AG genotype tend to have methylation levels that fall in between the two homozygous groups.

Figure 4.3: Comparisons of Andean Allele Frequency to 1000 Genomes Data

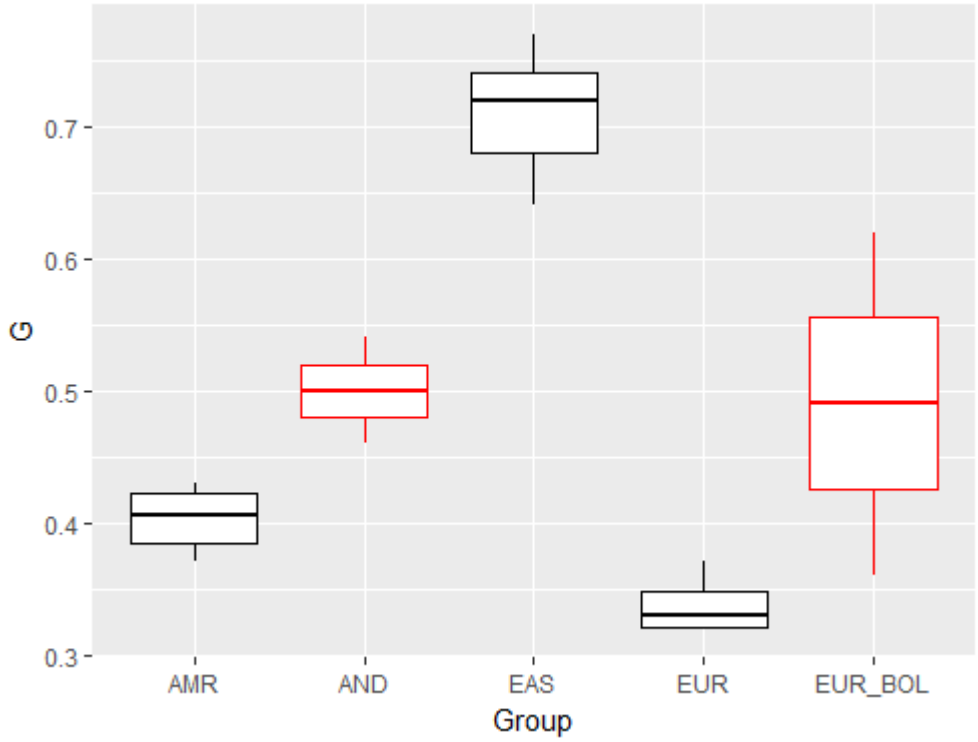


Figure 4.3: Figure 4.3 shows the comparison of the G allele frequency among our Andean sample populations (red) to example 1000 genomes populations (black). AMR = Americans from the 1000 genomes project, AND = Native Andeans from Bolivia, EAS = East Asians from the 1000 genomes project, EUR = Europeans from the 1000 genomes project, and EUR_BOL = Europeans from Bolivia.

Tables:

Table 4.1: Allele frequency and genotype proportions for site rs10166384 among the Bolivian samples as well as East Asian and European populations from the 1000 genomes project

Population	A Frequency	G Frequency	AA Frequency	AG Frequency	GG Frequency
CHB	0.28	0.72	0.08	0.60	0.32
LE	0.38	0.62	0.10	0.36	0.54
L	0.42	0.58	0.15	0.54	0.31
LA	0.46	0.54	0.22	0.48	0.30
A	0.50	0.50	0.28	0.45	0.27
E	0.50	0.50	0.32	0.36	0.32
HA	0.54	0.46	0.42	0.25	0.33
H	0.59	0.41	0.46	0.26	0.28
HE	0.64	0.36	0.49	0.39	0.12
IBS	0.68	0.32	0.50	0.27	0.23

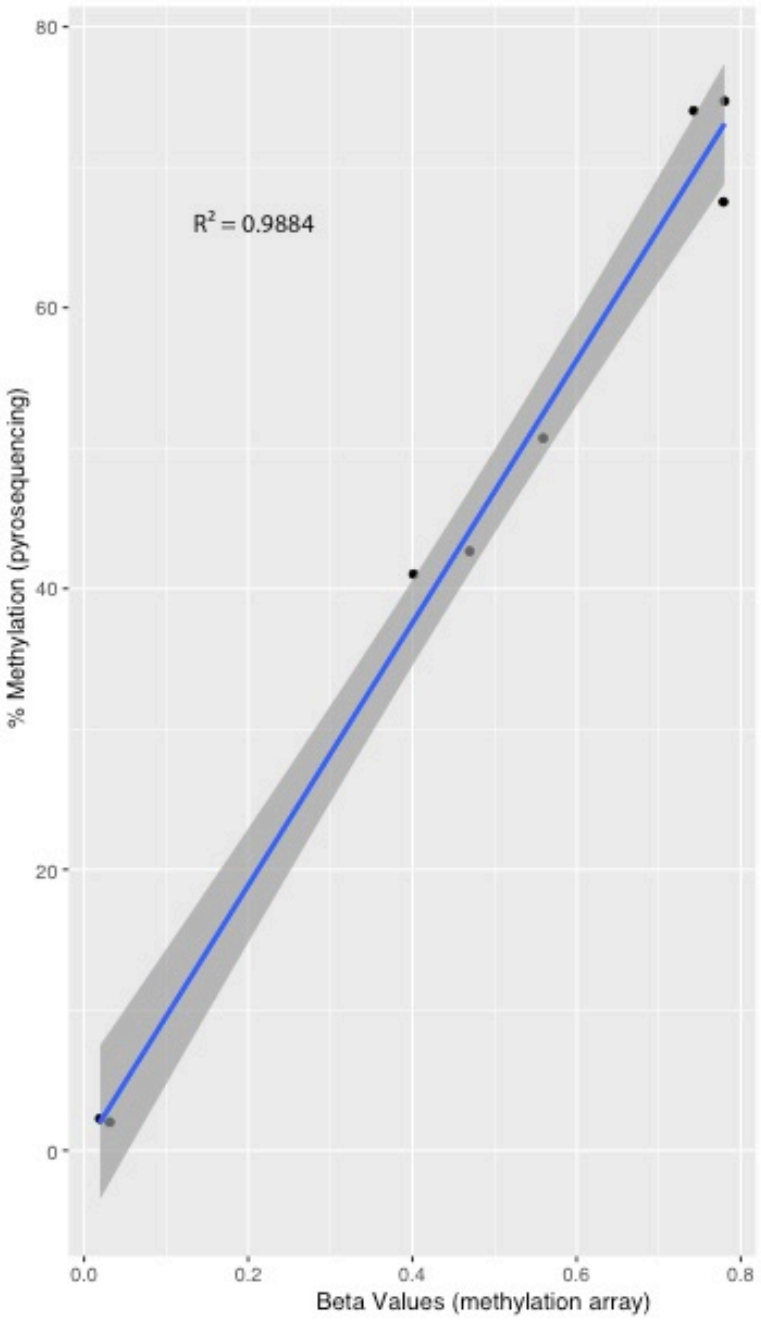
Table 4.1: Allele and genotype frequencies site rs10166384 for the Bolivian sample populations and representative east Asian (CHB) and European (IBS) populations, LE = Low Altitude Europeans, L = Low Altitude, LA = Low Altitude Andeans, A = Andeans, E = Europeans, HA = High Altitude Andeans, H = High Altitude, HE = High Altitude Europeans, IBS = Iberian Spanish.

Supplementary Table 4.1: Primers used for PCR, Sanger sequencing ,and pyrosequencing

Primer Name	Primer Sequence(5'-3')	Annealing Temperature (°C)	Amplicon length(bp)
DYSF_F1	ATGCCATCCCTGCATAGTGT	56.6	851
DYSF_R1	AGCCTCCTGTGCCATCTTAG		
DYSF_F3	CCTGCTGGTAAGTGAGGAGT	56	2051
DYSF_R4	GGCCAGCTAACCATAGAAAA		
DYSF_F5	CCCCAAACCTCCATGATTTA	53	675
DYSF_R5	CATAATCCCCATGTGCTGTG		
DYSF_PyroF1	GGTTTGGGGATTGGTTAGTT	54	110
DYSF_PyroR1-Biotin	Biotin-TCCCCTATCCTAAACACCATAAA		
Sequencing Primer DYSF_Pyro_S1	GTTTGAATAATTTTTGTGGG		

Supplementary Table 4.1: Table of primers used for PCR, Sanger sequencing and pyrosequencing. Primers DYSF_F1, DYSF_R1, DYSF_F3, DYSF_R4, DYSF_F5, and DYSF_R5 were primer sets used for the detection of natural selection. Primers DYSF_PyroF1 and DYSF_PyroR1-Biotin were primers used on bisulfite converted DNA to generate the amplicon used for the vaiidation of the methylation array. Primer DYSF_Pyro_S1 was the sequencing primer used as part of the pyrosequencing protocol.

Supplementary Figure 4.1: Correlation of 450k methylation array with pyrosequencing



Supplementary Figure 4.1: Correlation of 450k methylation with pyrosequencing Correlation of beta values generated using the Illumina 450k methylation microarray (x-axis) and percent methylation calculated from the pyrosequencing analysis. The gray band surrounding the blue regression line represents the 95% confidence interval.

CHAPTER 5: DISCUSSION

In this dissertation I (along with my colleagues) discussed how placental gene expression differences among Andeans and Europeans may contribute to physiological differences seen among Andeans and Europeans residing at high altitude. In chapter two I discussed how increased expression of genes associated with placental macrophages (i.e. Hofbauer cells), may have contributed to increased capillary surface area among native Andeans residing at high altitude compared to Europeans residing in the same location (222). Along with the gene expression differences between Andean and Europeans, we noticed that those residing at high altitude regardless of ancestry, had decreased expression of genes associated with the AP-1 transcription factor family including *JUN* and *FOS*. The decrease expression of genes involved in the AP-1 transcription factor family and its targets suggests decreased cell fusion and less cell proliferation (240). We also tested for correlation between gene expression and DNA methylation to determine if DNA methylation explained a greater subset of differential gene expression among the altitude or ancestry associated differentially expressed genes. We found that more ancestry associated differentially expressed genes had a significant correlation between gene expression values and DNA methylation values. The more ancestry associated genes with a significant correlation between gene expression and DNA methylation suggests that the Andeans may be using DNA methylation as a temporary method to alter gene expression as they move between high and low altitude. In chapter three we discussed the altitude associated differentially expressed gene dysferlin and how it has a variant (rs10166384) that may alter DNA methylation and possibly gene expression. We found that there is a signature of

balancing selection at site rs10166384 within dysferlin. This suggests that both the major and minor allele may both be beneficial among Andeans and Europeans residing at high altitude.

In chapter two we found that the Native Andeans had higher placental gene expression values among genes associated with inflammation and Hofbauer cells compared to Europeans. This suggests that the Andeans are able to use the inflammation induced by the high altitude environment to recruit Hofbauer cells to induce angiogenesis, a phenomenon that has been shown to occur in vitro (241). The induced angiogenesis may lead to increase capillary surface area and better diffusion of nutrients across the placentas of high altitude Andeans. Another potential mechanism that may contribute to increased angiogenesis and increased capillary growth is recruitment of maternal uterine natural killer cells (242). Uterine natural killer cells are attracted to sites of inflammation through secretion of chemokine CCL2 to induce angiogenesis resulting in capillary growth, a mechanism similar angiogenesis induced by Hofbauer cells (243). However, through our gene expression analysis we did not see differential expression of genetic markers associated with natural killer cells such as *CD56* and *CD16 (244, 245)*. Along with lack of differential gene expression, we found that gene expression microarray probes associated with *CD56* and *CD16* had low gene expression values. Low gene expression values suggests that little RNA from uterine natural killer cells was mixed with the placental RNA samples. One potential explanation for lack of differentially expressed natural killer cell markers is that during the sample processing the goal was to remove as much as the maternal tissue as possible to analyze of the fetal DNA and RNA. This achieved by removing the decidual membrane

and washing off the maternal blood. Through removing maternal tissue we would have removed a majority of the uterine natural killer cells and any genetic markers associated with them.

Due to the small sample size (n=45) we are only able to detect relatively large differences in gene expression so we focused on the most variable probes, which would likely have the greatest expression differences. It would be beneficial to increase the sample size to increase power. Along with increasing the power it would be useful to collect placentas from other populations with long-term inhabitance at high altitude such as those residing at the Tibetan plateau and the Ethiopian highlands. Prior work has looked at how the reduction in birth weight at high altitude changes across ancestral groups (122). Moore et al. found that those of Tibetan ancestry had less of a reduction in birth weight as they move between altitudes, compared to those of Han descent who reside at low altitude. Adapted individuals such as Tibetan and Andean individuals had birth weight reductions of 250g and 300 grams as they move from low altitude to high altitude respectively (~4000m). This is different compared to non-adapted individuals including Europeans residing in Bolivian and Han Chinese residing in Beijing, China who have reductions in birth weight of roughly 450 grams and 500 grams respectively (122). If we were to compare differences in placental gene expression between individuals of Han and Tibetan descent and those of Andean and European descent, we would expect more differences between the Han and Tibetan individuals as the Tibetan individuals have resided at high altitude for more generations then those of Andean descent and have been exposed to slightly different environments (79, 246).

While decreased partial pressure of oxygen is one of the defining characteristics of the high altitude environment, there are other factors that contribute to the adverse environment. Individuals residing at high altitude also experience decreased air temperature, increased ultraviolet (UV) radiation and a less dramatic shift in seasonal weather compared to individuals residing at low altitude (247, 248). Residents residing at high altitude have developed cultural and physiological ways of adapting to the different factors affecting the high altitude environment. To adapt to the decreased temperatures, Andeans residing at high altitude have developed increase blood flow to the extremities to increase warmth and oxygen transport compared to Europeans residing at high altitude and individuals residing at low altitude regardless of ancestry (248). Also, residents at high altitude have darker skin found to be related to increased vascularity meaning the color change to blood flow is increasing skin reflectance compared to residents of low altitude (249). The idea that adaptations to both decreased temperature and increased UV radiation may be related to increased blood flow suggests that increased blood flow in the placenta seen earlier in life may be similar to other physiological adaptions.

In chapter three, we discussed how within the gene dysferlin (*DYSF*), there is a SNP (rs10166384) that has the ability to affect methylation at a transcription factor binding site for TEAD4. TEAD4 is a transcription factor that affects cell fusion and has been studied primarily in syncytial tissue, including skeletal muscle tissue and the formation of the syncytiotrophoblast (250). We predict that having the G allele at rs101066384 will lead to increased methylation within the TEAD4 transcription factor binding site reducing TEAD4s binding affinity resulting in reduced transcription of *DYSF*.

The reduced expression would lead to less efficient cytotrophoblast fusion in the placenta. Within C2C12 muscle cells, the lack of TEAD4 leads to shorter muscle cells, with 20% fewer nuclei compared to wild-type cells, similar to mouse primary myocytes when inhibited by short hairpin RNA targeting the TEAD4 transcription factor(235, 251). Also, those mice that lack the TEAD4 transcription factor show slowed skeletal muscle regeneration, a process similar to syncytiotrophoblast fusion (251, 252). Lastly in chapter 3 we focused on those individuals who gave birth without pregnancy complications and saw an increased A allele frequency in non-adapted individuals residing at high altitude. This leads us to predict that there would be a higher frequency of the G allele in those individuals who experienced pregnancy complications at high altitude. This would lead us to suggest rs10166384 as a potential marker for pregnancy complications at high altitude.

Placental gene expression and other physiological responses to high altitude would also likely vary among species depending on the type of placenta that the individual animals have. For example, sheep have an epitheliochorial placenta, meaning that there is a layer of epithelial cells separating the maternal and fetal blood as well as a cotyledonary shape meaning that the placenta attaches to the uterine wall at many different points (253, 254). Parraguez et al. found that the there is more contact between the placenta and the uterine wall as evident through a 70 percent increase in cotyledon diameter in sheep residing at high altitude compared to those residing at low altitude with average cotyledon diameters of 2.70 cm and 1.59 cm respectively (255). This finding suggests that sheep born to the high altitude environment cope with the adverse environment by increasing the surface area of contact between the fetal

placenta and maternal uterine wall increasing the amount of exchange (255). Another animal used to study adaptation to high altitude is the yak (*Bos grunniens*). Qiu et al. compared the genome of the domestic yak to the genome of the domestic cow. They found positive selection in the yak in genes related to blood vessel size and regulation of angiogenesis suggesting that like in humans there are differences in angiogenesis and vascularization between the adapted yak species and the non-adapted domestic cow (256). An ideal animal that could be used as a non-human model organism to study adaptation to high altitude would be the guinea pig (*Cavia porcellus).* Like humans, guinea pigs have a hemochorial placenta which invades into the uterus more extensive then other rodents (257). Also, guinea pigs are native to the Andean altiplano making them good candidates to study high altitude adaption(258). Rockwell et al. noticed that guinea pigs born at high altitude weighed less then those born at low altitude with guinea pigs residing at high altitude having an average birth weight of 60.4 grams and those born at low altitude having an average birth weight of 74.5 grams (259). Also, studies have shown that guinea pigs are at greater risk of hypertension when pregnant at high altitude compared to non-pregnant individuals, with hypertension during pregnancy being a common symptom of preeclampsia in humans (260).

In summary, this dissertation provides new information about how altitude effects gene expression and methylation in placentas of individuals of both European and Andean descent. It also provides evidence about how sequence variation effects DNA methylation as well in these same individuals. Because the placenta is an organ that mediates fetal growth, the adaptations we have described in the placenta may prevent common obstetrical syndromes such as preeclampsia and fetal growth restriction. The

evolutionary approach we have taken in these studies provides a framework for studying adaptations in human pregnancy. In the future it will be important to sample more individuals and more populations and gestational time points in order to gain a comprehensive few of placental adaptations. These present studies will also benefit from future mechanistic physiological studies *in vitro* and *in vivo*.

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