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Human Genetic Adaptation to High Altitudes: Current Status and Future Prospects

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Abstract

The question of whether human populations have adapted genetically to high altitude has been of interest since studies began there in the early 1900s. Initially there was debate as to whether genetic adaptation to high altitude has taken place based, in part, on disciplinary orientation and the sources of evidence being considered. Studies centered on short-term responses, termed acclimatization, and the developmental changes occurring across lifetimes. A paradigm shift occurred with the advent of single nucleotide polymorphism (SNP) technologies and statistical methods for detecting evidence of natural selection, resulting in an exponential rise in the number of publications reporting genetic adaptation. Reviewed here are the various kinds of evidence by which adaptation to high altitude has been assessed and which have led to widespread acceptance of the idea that genetic adaptation to high altitude has occurred. While methodological and other challenges remain for determining the specific gene or genes involved and the physiological mechanisms by which they are exerting their effects, considerable progress has been realized as shown by recent studies in Tibetans, Andeans and Ethiopians. Further advances are anticipated with the advent of new statistical methods, whole-genome sequencing and other molecular techniques for finer-scale genetic mapping, and greater intradisciplinary and interdisciplinary collaboration to identify the functional consequences of the genes or gene regions implicated and the time scales involved.

Keywords

AMPK; evolution; fetal growth; HIF; pregnancy

HAS GENETIC ADAPTATION TO HIGH ALTITUDE OCCURED?

There has been a longstanding debate as to whether genetic adaptation to high altitude has occurred. Joseph Barcroft (1872–1947), an early leader in high-altitude physiology (see, for example (Physiol, 2016)), was of the opinion that it had not taken place. He stated “The

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acclimatised man is not the man who has attained to bodily and mental powers as great in Cerro de Pasco [Peru, elev 4300 m] as he would have in Cambridge (whether that town be situated in Massachusetts or in England). Such a man does not exist. All dwellers of high altitudes are persons of impaired physical and mental powers” (Barcroft, 1925). This view was contested by another pioneer, Carlos Monge M (1884–1970), who stated “Biologically we have shown that the Andean carries in his organism the hereditary and ancestral soma which permits life at the very great altitudes that mark certain large inhabited areas of South America” (Monge-M., 1948). The debate continues (Kayser, 2013a, b; Léon-Velarde and Villafuerte, 2013a, b), with arguments stemming from differences in disciplinary orientation and the sources of evidence being considered.

Disciplinary orientation influences the way in which the term *adaptation* has been interpreted. Physiologists generally use the term to refer to any physiological response regardless of whether the response is likely to be beneficial or otherwise affect the chance(s) of being able to live or reproduce, *i.e.*, reproductive success, whereas evolutionary biologists or geneticists restrict its usage to those responses likely to influence reproductive success. Barcroft followed the first usage by, for example, referring to the physiological characteristics of both high-altitude newcomers and natives without recognizing that some of the newcomers might have been suffering from the usually non-life threatening, but nonetheless discomforting, symptoms of acute mountain sickness (West, 2016). Persons using *adaptation* in an evolutionary context employ it in the latter sense; for example, the evolutionary biologist Theodosius Dobzhansky defined it as “any feature of structure, function or behavior that enables the organism to live and reproduce in a given environment” (Dobzhansky, 1968). Usage here is confined to this latter sense.

There are at least five sources of evidence by which adaptation to high altitude may be assessed. One is simply the presence of populations that have long resided at high altitude, conventionally defined as > 2500 m (8250 ft) as that is the altitude at which arterial O₂ saturation begins to fall. Of the four major high-altitude regions of the world (Figure 1), Native Americans resided seasonally at high altitude for millennia but permanent populations, largely of European origin, only for the past 150 years and hence not the longer time horizons required for genetic adaptation¹. Of note, there are no permanently inhabited communities at altitudes >2500 m in Europe. Archeological evidence for human occupation on the Andean Altiplano extends back as far as ~14,000 years (Rademaker et al., 2014), ~30,000 years on the Himalayan Plateau (Jeong et al., 2014; Malassé and Gaillard, 2011), and from 500 to ~70,000 years in the Ethiopian Highlands (Huerta-Sanchez et al., 2013), although the permanence of such residence continues to be debated (Capriles and Albarracín-Jordan, 2013; Rademaker et al., 2016) and year-round residence was probably not possible until plant and animal domestication was complete (Aldenderfer, 1999; Dong et al., 2016; Prates et al., 2013). However, while the presence of populations at high altitude is useful (Beall, 2007), the information provided is limited since residence alone cannot

¹The question as to how much time is required for genetic adaptation depends on the numbers of genes involved and the strength of natural selection operating on the gene or genes in question. While its answer is outside the scope of this review, it is generally assumed that ~8 – 10,000 years of high-altitude residence is required based on the amount of time during which natural selection likely operated on other human biological traits such as hemoglobin S or lactase deficiency (Flint, J., Harding, R.M., Boyce, A.J., Clegg, J.B., 1998. The population genetics of the haemoglobinopathies. *Baillieres Clin Haematol* 11, 1–51).

distinguish between degrees of adaptation nor can it provide information about the specific genes or physiological mechanisms involved.

A second source of evidence is the physiological response(s) being considered. Since O₂ is relatively insoluble, in vertebrates it must be actively transported via the O₂ transport system (Figure 2). Therefore, physiological responses to the reduced O₂ availability or hypoxia present at high altitude can be directly measured as the change in each O₂ transport component. Studies by pioneering high-altitude physiologists such as Bruce Dill, Jack Reeves, Bob Grover and others focused on exercise performance in young, healthy men (Dill et al., 1931; Reeves and Grover, 2001) for which a convenient measure is the maximum level of oxygen consumption (VO₂ max, i.e., that at which there is no further rise in VO₂ during an at least 30 sec increase in workload). While VO₂ max has the advantage of being an overall measure of the operation of the O₂ transport system (Figure 2), organisms seldom operate at maximal levels of exertion and therefore the relevance of VO₂ max for the ability to live and/or reproduce is unclear. Thus more functional measures are recommended. Among these are the altitude-associated reduction in birth weight (Haas et al., 1980; Moore, 1990), increased frequency of preeclampsia (Moore et al., 1982; Palmer et al., 1999), increased infant mortality and morbidity (Beall et al., 2004; Moore et al., 2001a; Wiley, 1994), slowed childhood and adolescent growth (Beall et al., 1977; Frisancho, 1969; Greksa and Haas, 1982), elevated pulmonary artery pressure (Ppa) (Banchero et al., 1966; Cruz-Jibaja et al., 1964; Groves et al., 1993; Hoit et al., 2011), or the prevalence of the maladaptive syndrome known as chronic mountain sickness (CMS) (Léon-Velarde et al., 2005; Moore et al., 1998a) as such conditions vary among highland populations and can interfere with the ability to live and/or reproduce in the high-altitude environment.

A third source of evidence is the time period over which the physiological response(s) to high altitude is (are) being assessed. Those responses that occur in newcomers within minutes to days or months, termed acclimatization (Figure 3), include an almost immediate increase in ventilation (V_E) and contraction of plasma volume that serve to increase the partial pressure of O₂ in the arterial blood (pO₂) and hemoglobin (Hb) concentration respectively, and therefore help offset the fall in arterial SaO₂ saturation (SaO₂) and O₂ content due to the reduced partial pressure of O₂ in the inspired air. The plateau increase in V_E requires several days and the increase in Hb concentration weeks to months to be complete. Cardiac output rises slightly due to increases in heart rate but is not fully maintained during maximal exercise and hence limits maximal exercise performance for reasons that remain unclear (Kayser, 2013a). Blood flow distribution is also altered, with high-extraction organs such as the brain and the heart receiving a greater proportion of the cardiac output than low-extraction organs such as muscles and the viscera (Hultgren and Grover, 1968).

The physiological changes that occur over lifetimes are termed developmental responses. The clearest such one is an increase in lung volume, which raises the alveolar surface area over which O₂ diffusion can take place and acts, in turn, to narrow the alveolar to arterial O₂ gradient and improve SaO₂, particularly during exercise (Kiyamu et al., 2015). Increases in lung volume occur universally in persons (or experimental animals) raised at high altitude, being seen in the short-term residents of Colorado (DeGraff et al., 1970; Johnson et al.,

1985) as well as the multigenerational residents of the Andes or Himalayas (Droma et al., 1991; Frisancho, 1969), although some features such as chest size and shape appear specific to Andeans (Frisancho, 1983). Other features such as greater uterine artery blood flow during pregnancy and heavier birth weights in Andean than shorter-term residents of high altitude do not appear to be subject to developmental control (Julian et al., 2011; Weinstein and Haas, 1977).

After controlling for developmental factors, differences between natives and newcomers and among native high-altitude populations in physiological responses to high altitude can be interpreted as evidence of genetic adaptation to high altitude. While such comparisons are complicated by methodological differences and variation in the newcomer groups with which the high-altitude natives are compared, natives generally resemble acclimatized newcomers in terms of having higher levels of V_E , SAO_2 , Hb and cardiac output than persons at sea level (Figure 3). The Andeans' increased lung O_2 diffusion, as reviewed above, is a developmental response that is largely recapitulated in persons without multigenerational residence at high altitude but who themselves were born and raised at high altitude. Unknown is whether the Tibetans' improved tissue O_2 diffusion and utilization (Holden et al., 1995; Simonson et al., 2015) also occur with lifelong residence. With respect to comparisons among native high-altitude populations, the relatively small number of studies in Ethiopian highlanders makes three-way comparisons difficult but several comprehensive reviews of Andeans and Tibetans have been published (Beall et al., 1990; Bigham and Lee, 2014; Gilbert-Kawai et al., 2014; Holden et al., 1995; Moore, 2001; Moore et al., 1998b; Simonson, 2015; Simonson et al., 2015; Winslow et al., 1989). To summarize, Tibetans compared to Andeans have higher V_E at rest and during exercise, marginally lower lung volumes and diffusing capacities, similar levels of SAO_2 , lower Hb concentrations, less hypoxic pulmonary vasoconstriction and hence lower Ppa pressures (Figure 4B), and perhaps better preservation of cardiac function, exercise performance, O_2 diffusion to the muscle and tissue O_2 utilization. Both Tibetans and Andeans demonstrate greater uterine artery and, in the case of Tibetans, forearm blood flow than acclimatized newcomers (Erzurum et al., 2007; Julian et al., 2009; Moore et al., 2001b). Thus, while there is a continuing need for additional studies, differences between natives and newcomers and among long-resident high-altitude populations support the likelihood that genetic adaptation has occurred.

Especially useful have been comparisons between natives and newcomers in physiological factors affecting functional responses to residence at high altitude at the various time points in the life cycle (see Figure 4). For example, since residence at high altitude exerts one of the most powerful depressant effects on fetal growth and thereby reduces infant birth weight (Jensen and Moore, 1997) and since size (or weight) at birth is one of the most powerful predictors of infant mortality (Karn et al., 1951; Williams et al., 1982), factors that preserve normal birth weights at high altitude can be expected to be adaptive. Consistent with such an expectation, data collected in nearly 306,000 infants from all studies in which birth weights were recorded at both low (~sea level) and high altitude (~3600 m) reveal that the altitude-associated reduction in infant birth weight in long-term, Andean and Tibetan populations is approximately half that seen in shorter-term, European or Han high-altitude residents (Figure 4A). Better fetal growth stems, at least in part, from greater maternal uterine artery

blood flow during pregnancy (Julian et al., 2009; Moore et al., 2001b) and likely contributes to improved infant or childhood survival under comparable socioeconomic or health care conditions (Moore et al., 2001a). Offspring survival also appears to be enhanced for those babies born to Tibetan women with a gene variant associated with higher SaO₂ (Beall et al., 2004). Other traits such as resting Ppa and the prevalence of CMS also vary among high-altitude populations (Figure 4B, 4C) in ways that may be functionally important. That is, the Tibetans' higher V_E and lower Hb would be expected to be protective against the lower SaO₂ and elevated Hb levels defining CMS; indeed, the prevalence of CMS is markedly less in Tibetan than Andean or North American high-altitude residents (Figure 4C). Additionally, their lower Ppa would be expected to reduce the risk of developing pulmonary hypertension and right heart failure often present in terminal cases of CMS.

The fourth and most recent kind of evidence regarding genetic adaptation to high altitude comes from genome scans. These employ single nucleotide polymorphisms (SNPs) and gene-chip technologies to compare genetic variation in high- vs. low-altitude populations, and then use statistical tests to identify those regions where natural selection has taken place in the highland groups. Such statistical tests derive from the observation made more than 30 years ago that two of the four forces driving genetic evolution, gene flow and natural selection (but not mutation or genetic drift), have directional effects. Since gene flow would affect all alleles or loci in the same way whereas natural selection would be expected to act on specific gene regions, Lewontin and Krakauer proposed that natural selection could be measured as the heterogeneity between genetic loci, termed the F value (Lewontin and Krakauer, 1973). In the intervening years various tests have been developed for detecting different kinds of natural selection producing, for example, increased variation, decreased variation, or increased linkage disequilibrium (i.e., the nonrandom association of alleles usually due to being situated on the same chromosome; for review see (Zhang et al., 2013)).

CURRENT STATUS

Being able to perform genome scans at relatively low cost has constituted a breakthrough for detecting genetic adaptation to high altitude. As a result, there has been an exponential rise in the number of reports of genetic adaptation to high altitude (Figure 5). Ours was the first such study (Moore et al., 2004); searching the US National Library of Medicine's PubMed database with the term "genetic adaptation to high altitude" reveals a total of 319 publications over the past 40 years. The largest number of studies has been conducted in Tibetan, Sherpa and other Himalayan populations (Beall et al., 2010; Bigham et al., 2010; Huerta-Sanchez et al., 2014; Ji et al., 2012; Peng et al., 2011; Simonson et al., 2010; Wang et al., 2011; Wilkins et al., 2014; Xu et al., 2011; Yi et al., 2010). A lesser but still considerable number of studies has been performed in Andeans (Bigham et al., 2008; Bigham et al., 2009; Eichstaedt et al., 2015; Eichstaedt et al., 2014; Moore et al., 2004; Rupert et al., 2003; Valverde et al., 2015; Wang et al., 2010; Zhou et al., 2013) and fewer, but nonetheless important ones, being carried out in Ethiopian highlanders (Alkorta-Aranburu et al., 2012; Huerta-Sanchez et al., 2013; Scheinfeldt et al., 2012).

Of the gene regions identified, many reside in the Hypoxia-Inducible Factor (HIF) pathway, which is the master regulator of the transcriptional response to hypoxia in metazoans

(Semenza, 1999). HIF-1 is a basic transcription factor composed of two subunits, HIF-1alpha and HIF-1beta/ARNT. There are three HIF-alpha subunits (-1alpha, -2alpha, and -3alpha), with HIF-1alpha being expressed ubiquitously and -2alpha and -3alpha having more limited distributions. All three are O₂ dependent and instantly degraded under normoxic conditions via ubiquitination steps involving von Hippel-Lindau (VHL) and several prolyl hydroxylase enzymes (PHD1, 2 and 3). But HIF-alpha degradation is blocked under hypoxic conditions due to inhibition of the PHDs since they use O₂ as a substrate. As a result, HIF-alpha initiates transcription in the currently more than 100 genes possessing a hypoxic response element in their promoter region. Among these are genes involved in angiogenesis (*VEGF*, *PDGF*, *FLT*), erythropoiesis (*EPO*), cell death (*TGFA*, *TNF*, *MMP*), inflammation (*IL6*), vasoreactivity (*EDN*, *NOS*, *PRKAA1*), glucose metabolism (*IGFBP*, *PRKAA1*) as well as other processes affecting the various steps of the O₂ transport system (Greer et al., 2012).

The more than 300 publications concerning genetic adaptation to high altitude have not yet produced a consensus as to what gene or genes are primarily involved. To illustrate this, a recent review identified 60 gene regions as being acted upon by natural selection at high altitude (Table 1) with one-third being common to Andeans and Tibetans and approximately the same number found in only one population or the other (Bigham and Lee, 2014). While many of the genes in Table 1 are in the HIF-pathway, others are not and available evidence suggests that natural selection has not acted on the HIF-pathway as a whole but rather on selected targets (Bigham et al., 2010).

A pressing need is to identify the functional consequences of the genes or gene regions implicated by SNP or whole-genome sequencing studies. Knowing the functional consequences of genetic variation is not a problem confined to studies at high altitude; as an expert recently concluded “the number of identified loci that have been subsequently connected in a meaningful way to phenotypic or fitness level changes is limited” (Jensen et al., 2016). Considerable progress is being realized by studies at high altitude as is illustrated by the examples drawn from Tibetan and Andean studies below.

Tibetan studies

Several groups have shown that putatively adaptive variants in the *EPAS1* and *EGLN1* gene regions in Tibetans are associated with lower Hb levels (Beall et al., 2010; Simonson et al., 2010; Yi et al., 2010). Similar findings were reported for the *EPAS1* locus in Ethiopians (Scheinfeldt et al., 2012). Of interest the Tibetans' *EPAS1* variant appears to have been introgressed from the archaic Denisovans (Huerta-Sanchez et al., 2014), indicating a very long time horizon for at least some of the gene regions involved in adaptation to high altitude.

Functional variants in *EPAS1* or other selected-for gene regions in Tibetans have not been identified, but Lorenzo and colleagues performed detailed gene-sequencing studies of protein-coding regions that identified a missense variant in the *EGLN1* gene (that codes for PHD2) that was more common in Tibetans and tightly linked to another missense variant (Lorenzo et al., 2014). Their kinetic studies in transfected Hep3B hepatoma cells showed that the selected-for PHD2 double mutant was more efficient at hydroxylating HIF-1alpha

subunits at lower O₂ tensions than wild-type PHD2, and that cells expressing the variant had decreased HIF-2alpha protein levels and a trend toward reduced HIF-1alpha protein as well. The authors concluded that the selected-for variants were more active in down-regulating HIF subunits than wild-type PHD2. However, whether these study findings help to explain the Tibetans' lower Hb levels was unclear since the relationship of the selected-for *EGLN1* variant to Hb levels varied according to homozygous *vs.* heterozygous genotype and study conditions. Specifically, erythroid progenitor cells from Tibetans homozygous for the selected-for variants were hypersensitive to erythropoietin whereas heterozygous Tibetans were hyposensitive, and HIF-regulated gene expression was increased in erythroid progenitor cells cultured under normoxic study conditions but decreased in both homozygotes and heterozygotes under the 1% or 5% O₂ hypoxic conditions similar to those present in the bone marrow *in vivo*.

However another group (Song et al., 2014) found that the Tibetan variants potentiated, not depressed, HIF-alpha signaling. Specifically, they showed that transfection of HEK293 cells with the Tibetan PHD2 variant disrupted the recruitment of tumor suppressor protein p53 to the heat-shock protein 90 (HSP 90) pathway, and impaired the ability to degrade HIF-alpha. They speculated that such a potentiation of HIF-alpha signaling may have beneficial effects in terms of stimulating V_E and nitric oxide (NO) production but that other gene variants, specifically those in the *EPAS1* region, blunted the HIF-2alpha response so as to reduce sensitivity to erythropoietin and lower Hb levels. Thus further Tibetan tissue or cellular studies are needed to determine whether Tibetan gene variants augment or depress HIF-alpha signaling.

Additional work is also required to determine whether selection is acting on Hb levels *per se* or other targets since Hb is but one element in the O₂ transport chain, and not only O₂ delivery but also metabolic factors influence cellular energy supply (Figure 2). Helpful in this regard is a recent study showing that healthy Tibetan men with lower Hb levels had greater VO₂ max as the result of greater cardiac output and muscle O₂ diffusion capacity, although such relationships were not specific to Tibetans but also observed among Han residents of high altitude exhibiting a limited range of Hb concentrations (Simonson et al., 2015). Raising the possibility that differences in Hb levels during adulthood reflect earlier lifetime events, Julian and co-workers showed that young Andean men with modestly-elevated Hb had more often been hypoxic *in utero* or during neonatal life (Julian et al., 2015), and showed early signs of pulmonary vascular dysfunction and intermittent hypoxia during sleep (Julian et al., 2013). Regarding the possible involvement of metabolic factors, Ge and coworkers showed that Tibetans with *vs.* without the selected-for *EPAS1* and peroxisome proliferator-activated receptor alpha (*PPARA*) haplotypes had increased lactate and serum free fatty acid concentrations respectively (Ge et al., 2012).

Andean studies

Given the importance of birth weight for neonatal survival and that Andeans are relatively protected from altitude-associated reductions in birth weight, we asked whether any of the gene regions identified as having been acted upon by natural selection in Andeans were related to birth weight at high altitude (Bigham et al., 2014). Of 63 SNPs in 16 gene regions

identified as having been acted upon by natural selection, several SNPs in the *PRKAA1* and *EDNRA* gene regions were associated with birth weight such that women with the SNP allele present at greater frequency in Andeans than Europeans had heavier birth-weight infants at high (but not low) altitude. Because our and others' studies had shown that preservation of a normal rise in uterine artery blood flow, due chiefly to greater uterine artery diameter, was a contributor to Andean protection from altitude-associated reductions in birth weight (Julian et al., 2009; Zamudio et al., 2007), we also examined the relationship of the *PRKAA1* and *EDNRA* SNPs to uterine artery diameter. We found that the *PRKAA1* but not the *EDNRA* gene-region SNPs were associated with larger uterine artery diameters at high altitude.

To explore the mechanisms by which variation in the *PRKAA1* gene region may have been acting, we compared the gene-expression patterns in peripheral blood mononuclear cells (PBMCs) from women with vs. without the selected-for *PRKAA1* SNP genotype. PBMCs were utilized for convenience but also because, as among the most transcriptionally active cells in the body (Bull et al., 2004), they are likely to reflect systemic processes involved in maternal vascular and metabolic responses to pregnancy. We focused on the mTOR pathway for three (3) reasons: (1) *PRKAA1* codes for the alpha-1 catalytic domain of AMPK, a key regulator of the mTOR pathway; (2) mTOR genes were over-represented in our selected-for gene regions (Bigham et al., 2014); and (3) inhibition of the mTOR pathway has been implicated in fetal growth restriction resulting from preeclampsia or residence at high altitude in Colorado (Yung et al., 2008; Yung et al., 2012). Twelve mTOR pathway genes were differentially expressed in women with vs. without the selected-for *PRKAA1* genotype, which is highly significant ($P=7.51e-09$) and suggests that AMPK activation may influence fetal growth.

Since AMPK is a key regulator of metabolic homeostasis (Hardie et al., 2012), we also measured serum or plasma levels of various substrates or metabolic products, and compared the levels present during pregnancy in healthy women with vs. without the selected-for *PRKAA1* gene region SNP. Unfortunately, like the Ge report (Ge et al., 2012), it was not possible to perform such measurements in fasted individuals but all samples were collected at a similar (mid-morning) time of day. Results suggested that Andean women with the protective TT genotype had greater insulin resistance (IR), lipolysis, and fat oxidation as indicated by their higher homeostatic model assessment (HOMA)-IR, free fatty acids and beta-hydroxybutyrate values (Table 2). Given their higher birth weights, perhaps the greater IR helped drive more glucose to the fetus to fuel fetal growth.

Vascular factors may also be involved insofar as AMPK also affects vascular growth and function (Ford and Rush, 2011; Schulz et al., 2009; Wang and Proud, 2006). To determine whether uterine vessels during pregnancy were affected, we used a mouse model in which uterine arteries could be isolated from near-term animals and studied *ex vivo* using wire myography. We found that AMPK activation vasodilated uterine arteries and did so via both NO-dependent and -independent mechanisms. Further, chronic hypoxia sufficient to restrict fetal growth increased the vasodilator actions of AMPK activation in opposing phenylephrine-induced vasoconstriction in the isolated uterine arteries (Skeffington et al., 2016). While further study is needed to determine the metabolic as well as vascular

mechanisms by which variants in the *PRKAA1* gene region may be acting, the possibility exists that AMPK activation is playing a key role in matching uterine artery blood flow with the energy supply needed to fuel fetal growth.

In sum, while considerable progress has been realized, existing data are unclear as to the physiological effects of the gene variants identified in Tibetans or Andeans to date. Nor can we yet determine whether these groups have employed an adaptive strategy that, for example, decreases the relative importance of the energetically-expensive activation of the HIF-pathway vs. improves O₂ utilization or increases the availability of other fuel sources.

CHALLENGES FOR STUDIES OF GENETIC ADAPTATION TO HIGH ALTITUDE

The high-altitude environment is ideally situated for studying genetic adaptation given the ubiquity of the hypoxic stress. As reviewed above, more than a century of research has identified the relevant O₂ transport components (Figures 2 and 3), and genome scans have implicated multiple gene regions. However, several challenges remain for definitively determining the specific genes involved.

The first set of challenges stems from the nature of SNPs. As noted above, the availability of relatively low-cost gene-chips containing hundreds of thousands of SNPs provided a breakthrough for being able to detect gene regions that had been acted upon by natural selection. However, SNPs are merely signposts for tagging signals of natural selection and hence may not be the actual variant under selection. For example, the SNP may be at a considerable distance from the gene of interest. Further the choice of SNPs available is limited by ascertainment bias given that most SNPs were discovered in Europeans, who are not a long-resident high-altitude population. Moreover, most SNPs are in non-coding regions given that such regions are more variable but, as a result, the regions identified are without known function. For this reason, it is more appropriate to speak of gene regions, rather than actual genes, as having been targeted by natural selection in SNP studies.

Secondly, the statistical tests and analytical strategies themselves are subject to limitations. For example, it is difficult to control for the possibility of finding false positives due to changes in population size, background selection, or the genetic architecture of the trait in question (Jensen et al., 2016). Given that typically a very large number of gene regions is being queried and a much smaller number of subjects studied, there is an inherent loss of power unless the gene region(s) is (are) specified in advance. Therefore, most studies focus on distinct or candidate gene regions, which adds to heterogeneity in study results. Comparisons among geographic regions to assess whether convergent evolution² or parallel evolution³ has taken place are also limited by the necessity of correcting for multiple tests. Finally, practical limitations stem from variation among the individuals being sampled, even

²Independent evolution of a common phenotype based on similar environmental pressures, in this case hypoxia.

³Lineages deriving from a common ancestor and evolving in similar ways over time as the result of common developmental or environmental selection pressures.

if drawn from the same geographic region, or the choice of low-altitude populations with which to compare the high-altitude groups.

A third set of challenges pertains to the uncertainty surrounding the adaptive benefit of the particular O₂ transport trait in question. V_E, SaO₂, Hb or blood flow are each but one component of the O₂ transport system and hence whether the values observed have adaptive value and the mechanisms involved are difficult to determine.

FUTURE DIRECTIONS

Several developments are likely to aid in our ability to overcome the above challenges and enable determination of the specific genes responsible for genetic adaptation to high altitude. These involve methodological advances in the fields of statistics, genetics and molecular biology as well as efforts to promote even greater intradisciplinary and interdisciplinary collaboration.

New statistical methods are being developed to overcome limitations of current tests. For example, Foll and co-workers developed a novel approach for assessing positive selection for advantageous alleles (as opposed to negative selection against deleterious mutations) using a hierarchical Bayesian model and Monte Carlo simulations (Foll et al., 2014). Demonstrating its utility, they applied their model to the SNP data that we and Brutsaert had collected in 49 Andeans and 49 Tibetan residents of high altitude (3600 m) (Bigham et al., 2010). While the original report only identified a single gene region, *EGLN1* (PHD2), Foll and co-workers were able to identify 25 regions under selection in both high-altitude groups. These 25 genes were clustered in two pathways – fatty acid omega oxidation and interferon gamma signaling – suggesting attractive targets for follow-up studies that could, for example, test whether such pathways are altered in persons with vs. without the selected-for genotype.

Other methodological developments are also occurring or becoming increasingly available. Perhaps the most important of these is the increasing availability of relatively low-cost, next-generation whole-genome sequencing. Given that even large numbers of SNPs probe only a small fraction of the human genome and the other limitations of SNP studies mentioned above, whole-genome sequencing is required to determine the actual genes that have been subject to the strongest selection pressure (Cirulli and Goldstein, 2010). For example, whole-genome sequencing in an Andean study of susceptibility to CMS implicated two genes, an erythropoiesis regulator SUMO1/sentrin specific peptidase 1 (*SENPI*) and an oncogene acidic nuclear phosphoprotein 32 family member D (*ANP32D*), neither of which were detected despite using nearly one million SNPs in our Andean report (Bigham et al., 2010; Zhou et al., 2013). However, even though costs for genome sequencing have come down, budgetary limitations still require investigators to choose between sequencing more individuals at a low depth of coverage or a smaller sample with a high depth of coverage, both of which (for different reasons) limit the information that can be derived. Other kinds of tests can be applied to help overcome such limitations. For example, principal component analysis can be used to reduce multiple co-varying traits to a single variable, SNP panels used to genotype large numbers of individuals for that trait, and then other methods (*e.g.*,

restriction fragment length polymorphism, *in situ* fluorescent hybridization, specifically-designed primer sets) to supplement coverage of important candidate regions (Staiger et al., 2016).

Greater intradisciplinary and interdisciplinary collaboration involving archeologists, biological anthropologists, and other life scientists is needed to establish not only the genetic relationships between ancient and modern high-altitude residents but also whether temporal changes have occurred in the frequencies of the selected-for genes. An example of one such study is that of Fehren-Schmitz and colleagues (Fehren-Schmitz and Georges, 2016) in which they used ancient DNA (aDNA) from 150 highland and coastal Peruvians from ~8500–560 BP to genotype three loci in the *NOS3* and *EGLN1* gene regions. These loci were chosen because *NOS3* codes for endothelial NO synthase (eNOS), an important vasodilator, and higher levels of exhaled NO have been reported in high-altitude Andeans or Tibetans compared with a low-altitude USA population (Beall et al., 2001). *EGLN1* was chosen because it was one of the gene regions identified in our prior study of Andeans and Tibetans (Bigham et al., 2010). As these authors note, *NOS3* has not been identified as having been acted upon by natural selection (Table 1) but one group found that a *NOS3* variant associated with susceptibility to acute mountain sickness was less common in Andeans (Wang et al., 2010). Comparisons of aDNA and contemporary Peruvian data from the 1000 Genomes Project indicated that the frequency of the *NOS3* rs1799983 G allele increased over time in the highland region while the *EGLN1* rs176792 G allele decreased, but neither differed from levels present in the ancient coastal populations. The other *EGLN1* SNP sampled, rs1769813, was also similar in the ancient highland and coastal groups. The authors concluded that considerable population mobility likely occurred between the lowland and highland regions but information was limited concerning the possible changes in gene frequencies over time. The Fehren-Schmitz study demonstrates the feasibility of comparing ancient and modern genetic variants but also the need for identifying genes with demonstrated relevance for physiological adaptation. Whole-genome sequencing of aDNA, rather than reliance on specific SNPs or short mitochondrial or nuclear fragment sequences, will also improve the ability to detect directional selection (Malaspina, 2016). Further, the comparison of mitochondrial and nuclear genomes will be informative about sex-specific patterns of migration and admixture (Shapiro and Hofreiter, 2014).

Finally, greater interdisciplinary collaboration is needed for the effective use of the methodological advances reviewed above. Toward such ends are efforts to employ novel study designs (Frise and Robbins, 2015), combine field-based with laboratory research using transfection and other molecular biology techniques (Bigham et al., 2014; Lorenzo et al., 2014; Song et al., 2014), and the pooling of databases to overcome limitations of statistical power.

SUMMARY AND CONCLUSIONS

Considerable evidence exists to indicate that genetic adaptation to high altitude has occurred in Andean, Tibetan and likely also Ethiopian populations. Such evidence derives from the many thousands of years during which such populations have resided at high altitudes, the physiological and functional differences between multigenerational *vs.* shorter-term

residents of high altitude, the variation in physiological responses to high altitude among multigenerational populations, and the identification of multiple gene regions as having been acted upon by natural selection.

Despite having shown that genetic adaptation to high altitude has occurred and the progress realized for identifying the gene regions likely involved, our knowledge of the specific genes and the physiological mechanisms by which they may be acting remains incomplete. Required will be advances in statistical methodologies so as to overcome, for example, limitations affecting the ability to compare signals of natural selection across multiple geographic regions. Other advances may be anticipated by finer-scale genome sequencing and the use of a broader range of genetic and molecular biology techniques for identifying the specific genes involved. Finally, greater interdisciplinary collaboration is required to perform the requisite physiological and molecular biology studies for determining the physiological mechanisms by which such gene variants may be acting. Archeologists have an important role to play by generating aDNA from the early inhabitants of high-altitude regions for determining not only the precise relationships between ancient and modern residents of high altitude but also with which to directly test for changes in allele frequencies in the selected-for (or against) genes. Since budgetary and sample size constraints will limit what can be accomplished within a single study, efforts to pool data from multiple studies are to be especially encouraged.

The benefits to be derived from studies of genetic adaptation to high altitude are important not only for the more than 140 million residents of high altitude but also more generally since diseases affecting the operation of the O₂ transport system are among the leading causes of mortality and morbidity throughout the world. Studies at high altitude are therefore ideally positioned for identifying genetic factors that influence susceptibility to such diseases as well as for determining the causal mechanisms and therapeutic targets by which such diseases can be more effectively treated and perhaps even ultimately prevented.

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Figure 1. High-altitude (>2500 m or 8250 ft) regions of the world where human populations permanently reside and approximate numbers of residents (Jarvis et al., 2008; Moore et al., 1998b).

Figure 2.

The O_2 transport system is comprised of two pumps (the lungs and the heart) and two diffusion steps. The lungs bring in O_2 from the atmosphere and the heart pumps it via the blood throughout the organism. O_2 diffuses across the alveolar membrane to enter the blood where it is reversibly bound to hemoglobin, and then diffuses from the blood into cells where it is consumed in the mitochondria to generate chemical energy in the form of adenosine triphosphate (ATP).

Figure 3. Physiological adaptation increases with time. The changes in the various components of the O_2 transport system that occur within minutes to days or months are termed acclimatization, those occurring during the period of growth and development are referred to as developmental responses, and those taking place across generations as genetic adaptation. Newcomers are acclimatized European or other persons of low-altitude origin and Natives are Andeans or Tibetans (see (Beall, 2007; Gilbert-Kawai et al., 2014; Moore et al., 1998b) for review). Abbreviations: V_E = ventilation, SAO_2 = arterial O_2 saturation, Hb = hemoglobin concentration, C.O. = cardiac output.

Figure 4.

Panel A summarizes birth weight data from a total of 305,935 infants from 15 studies in which birth weights were collected by the same investigator at both low (sea level) and high altitude (~3600 m) and five (5) in which birth weights collected at one altitude are compared to the group value at the other altitude. Lines connect the low- and high-altitude data obtained in a single study and points show mean values (with bars showing the standard error of the mean) at either low or high altitude. Panel B shows resting pulmonary arterial pressures in response to decreasing arterial pO₂ as summarized for young male residents of European descent residing at high altitudes in North America and for young Andean men. Values are also shown from studies in young Tibetan men and, as extrapolated, young male Ethiopian high-altitude residents. Panel C presents data for the prevalence of chronic mountain sickness (CMS) as reported for persons of various ancestry groups residing at the altitudes shown. Figure is adapted from (Niermeyer et al., 2015) where the original references may be found.

Figure 5.

The number of studies identified using the search term “genetic adaptation to high altitude” in the National Library of Medicine’s PubMed data base has risen exponentially. Shown are the number of studies over 5-year intervals from 1975 through 2015.

Table 1

Candidate genes identified in Andeans and/or Tibetans

<u>19 genes common to Andeans and Tibetans:</u>				
<i>ADRA1B</i>	<i>EGLN1</i>	<i>IL1B</i>	<i>NOS2</i>	<i>TNC</i>
<i>COPSS5</i>	<i>IGFBP1</i>	<i>IL6</i>	<i>NRP1</i>	<i>TNF</i>
<i>EDN1</i>	<i>IGFBP2</i>	<i>MDM2</i>	<i>NRP2</i>	<i>VEGFA</i>
<i>EDNRA</i>	<i>IL1A</i>	<i>NOS1</i>	<i>POLR2A</i>	
<u>20 genes in Andeans only:</u>				
<i>ARNT2</i>	<i>EDNRB</i>	<i>MTOR</i>	<i>PRKAA1</i>	<i>SNAIL3</i>
<i>ATP1A1</i>	<i>EGLN2</i>	<i>NOS1</i>	<i>PRKAA2</i>	<i>SPRY2</i>
<i>CDH1</i>	<i>ELF2</i>	<i>NOTCH1</i>	<i>PSMC3</i>	<i>TF</i>
<i>CXCR4</i>	<i>KCNMA1</i>	<i>PIK3CA</i>	<i>SATB1</i>	<i>TGFA</i>
<u>21 genes in Tibetans only:</u>				
<i>ARNT</i>	<i>EPAS1</i>	<i>HIF1A</i>	<i>PIK3CB</i>	<i>VEGFC</i>
<i>ANGPTL4</i>	<i>EPO</i>	<i>HMOX2</i>	<i>PIK3CG</i>	
<i>CASR</i>	<i>FLT1</i>	<i>MMP2</i>	<i>PKLR</i>	
<i>EGLN3</i>	<i>HBB</i>	<i>PDGF2</i>	<i>PPARA</i>	
<i>EP300</i>	<i>HBG2</i>	<i>PGF</i>	<i>RBX1</i>	

Adapted from (Bigham and Lee, 2014)

Abbreviations: *ADRA1B*, adrenoceptor alpha 1B; *ANGPTL4*, angiopoietin like 4; *ARNT*, aryl hydrocarbon receptor nuclear translocator 1 or 2; *ATP1A1*, ATPase Na⁺/K⁺ transporting subunit alpha 1; *CASR*, calcium sensing receptor; *CDH1*, cadherin 1; *CXCR4*, C-X-C motif chemokine receptor 4; *EDN1*, endothelin 1; *EDNR*, endothelin receptor type A or B; *EGLN*, egl-9 family hypoxia inducible factor 1, 2 or 3; *ELF2*, E74 like ETS transcription factor 2; *EP300*, E1A binding protein p300; *EPAS1*, endothelial PAS domain protein 1; *EPO*, erythropoietin; *FLT1*, fms related tyrosine kinase 1; *HB*, hemoglobin subunit beta or gamma 2; *HMOX2*, heme oxygenase 2; *HIF1A*, hypoxia inducible factor 1 alpha subunit; *IGFBP*, insulin like growth factor binding protein 1 or 2; *IL1*, interleukin 1 alpha or beta; *IL6*, interleukin 6; *KCNMA1*, potassium calcium-activated channel subfamily M alpha 1; *MDM2*, MDM2 proto-oncogene; *MMP2*, matrix metalloproteinase 2; *MTOR*, mechanistic target of rapamycin; *NOS*, nitric oxide synthase 1 or 2; *NOTCH1*, notch 1; *NRP*, neuropilin 1 or 2; *PDGF2*, platelet derived growth factor subunit B; *PGF*, placental growth factor; *PIK3C*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, beta or gamma; *PKLR*, pyruvate kinase, liver and RBC; *POLR2A*, polymerase (RNA) II subunit A; *PPARA*, peroxisome proliferator activated receptor alpha; *PRKAA*, protein kinase AMP-activated catalytic subunit alpha 1 or 2; *PSMC3*, proteasome 26S subunit, ATPase 3; *RBX1*, ring-box 1; *SATB1*, SATB homeobox 1; *SNAIL3*, snail family zinc finger 3; *SPRY2*, sprouty RTK signaling antagonist 2; *TF*, transferrin; *TGFA*, transforming growth factor alpha; *TNC*, tenascin C; *TNF*, tumor necrosis factor; *VEGF*, vascular endothelial growth factor A or C.

Table 2

Metabolic phenotypes associated with genetic selection in Andeans

Variable	<i>PRKAA1</i> rs1345778 genotype		P value
	Not selected-for (GG/GT)	Selected-for (TT)	
n	22	26	
Infant birth weight, gm	2770±106	3120±70	<0.01
Glucose, mg/dl	76.4±4.4	65.5±3.3	ns
Insulin, µU/ml	15.3±3.4	21.5±5.7	<0.01
HOMA-IR	3.2±0.9	4.0±1.3	ns (0.051)
Lactate, nmol/µl	1.20±0.07	1.07±0.07	ns
Triglycerides, mg/dl	172.2±17.4	174.3±17.1	ns
Cholesterol, µg/µl	2.2±0.1	2.1±0.1	ns
FFA, mM	0.13±0.01	0.17±0.02	<0.01
β-hydroxybutyrate, mM	0.12±0.01	0.17±0.04	<0.01

Values are mean ± standard error of the mean.

Abbreviations: HOMA-IR, homeostatic model assessment insulin resistance ((glucose/insulin) × 405); FFA, free fatty acids.