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Gene expression of the liver in response to chronic hypoxia

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Abstract

Hypoxia is an important ecological, evolutionary, and biomedical stressor. While physiological acclimatization of mammals to hypoxia is well studied, the variation in gene expression that underlies acclimatization is not well studied. We acclimatized inbred mice for 32 days to hypoxic conditions that simulated altitudes of 1400, 3000, and 4500 m. We used oligonucleotide microarrays to measure changes in steady-state abundance of mRNA in the livers of these mice. Mice exposed to more severe hypoxia (simulated altitude of 4500 m) were smaller in mass and had higher hematocrit than mice exposed to less severe hypoxia. ANOVA and false discovery rate tests indicated that 580 genes were significantly differentially expressed in response to chronic hypoxia. Few of these 580 genes have previously been reported to respond to hypoxia. In contrast, many of these 580 genes belonged to same functional groups typically respond to acute hypoxia. That is, both chronic and acute hypoxia elicit changes in transcript abundance for genes involved in angiogenesis, glycolysis, lipid metabolism, carbohydrate metabolism, and protein amino acid phosphorylation, but the particular genes affected by the two types of hypoxia were mostly different. Numerous genes affecting the immune system were differentially expressed in response to chronic hypoxia, which supports recently proposed hypotheses that link immune function and hypoxia. Furthermore, our results discovered novel elevated mRNA abundance of genes involved in hematopoiesis and oxygen transport not reported previously, but consistent with extreme hematocrits found in hypoxic mice.

Keywords: microarray, hematopoiesis, leptin receptor, *Mus musculus*, immune system

THE SURVIVAL OF AN ORGANISM often depends on its ability to acclimatize to environmental stressors. Acclimatization results from remodeling of the organism's physiological and anatomical phenotype in an attempt to accommodate an environmental stressor. A major mechanism underlying physiological remodeling is the regulation of genes and their products (84). Hence, physiologists are increasingly utilizing genomic tools to investigate physiological change. In particular, high-throughput studies of steady-state mRNA expression with DNA microarrays have proven useful in generating hypotheses concerning the mechanisms of acclimatization (34, 35).

Acclimatization and adaptation of mammals to high altitude hypoxia [reduced partial pressure of oxygen (P_{O_2})] have received much attention due to its biomedical and ecological relevance (46, 51, 70, 72–74, 79, 92, 93, 103). Mammals experiencing chronic hypoxia undertake diverse responses aimed at maintaining oxygen delivery despite a reduced driving force (partial pressure gradient) for delivering oxygen. These responses include:

increased hemoglobin production with a concomitant increase in hematocrit and blood oxygen carrying capacity; increased production of 2,3 bisphosphoglycerate, an allosteric effector of hemoglobin-oxygen binding affinity; pulmonary vasoconstriction; increased lung and liver mass; increased left ventricular mass, which leads to increased stroke volume; increased tidal volume and ventilation rate; increased capillary density; and anorexia and subsequent weight loss (1, 6, 12, 33, 64, 87). In addition, while the above physiological changes are taking place, many mammals alter their metabolism to decrease their demand for oxygen. These metabolic alterations may include increased anaerobic glycolysis and glucose utilization, decreased whole animal metabolic rate, and decreased body temperature (32, 45, 86, 90).

While much is known about the physiological acclimatization to high-altitude hypoxia, few studies have focused on the underlying molecular genetic changes. Most studies of the effects of hypoxia on gene and protein expression have focused on responses of cells to acute hypoxia (seconds to hours of exposure). Acute responses to cellular hypoxia include changes in a number of transcription factors, such as AP-1, NF-κB, p53, Erg-1, and the Myc family of proteins. These transcription factors respond to a variety of stressors, and they are essential for regulating cell development, apoptosis, cell proliferation and differentiation, and inflammation (20, 54). Hypoxia-inducible factor (HIF) is the best understood of the transcription factors that responds to hypoxia. HIF is a heterodimer of HIF-1β and one of three tissue-specific, hypoxia-regulated dimers, HIF-1α, HIF-2α, or HIF-3α. The HIF-α subunits rapidly accumulate during hypoxic conditions, dimerize with HIF-1β, and induce the transcription of >70 hypoxia-responsive genes (40, 42, 52). Many of these genes are associated with physiological changes observed in response to hypoxia (81). For example, in response to hypoxia, HIF causes upregulation of *1*) erythropoietin (EPO), which is involved in erythropoiesis (increasing red blood cell production), *2*) transferrin, a key to iron transport and metabolism, *3*) genes associated with angiogenesis, such as vascular endothelial growth factor (VEGF), and *4*) genes associated with many metabolic substrates, such as GAPDH and pyruvate kinase, which increase glucose uptake and anaerobic glycolysis (11, 85). Indeed, a deficiency of HIF-1α, one of the HIF subunits, dramatically inhibits long-term physiological responses to hypoxia (107). Thus, the HIF pathway plays an important role in both acute responses and physiological acclimation to hypoxia. However, the relationship between acute and chronic hypoxic responses remains unexplored.

A limitation of many studies investigating genomic responses to hypoxia is that they are often conducted using in vitro cell lines. Focusing on the cellular response to hypoxia removes the cells from their normal environment of the tissue within the organism. In recent years, increased attention has been placed on the effect of hypoxia on tissues in vivo (21, 25, 47). From the few studies that have employed multiple in vivo tissues, it appears that changes in gene expression are likely to vary with how the tissues experience hypoxia and with their metabolic role within the organism (8, 36, 80, 94, 105). Whereas previous studies have focused primarily on acute or intermittent hypoxia, none have compared genome-wide gene expression of mammals acclimated to constant hypoxia for extended periods of time (i.e., weeks) with unacclimated controls.

Herein we document changes in steady-state mRNA expression in the livers of house mice acclimated to normobaric hypoxia for 32 days. Much of what we know of the cellular response to hypoxia was established with hepatocyte cell lines (4, 27, 33, 86, 102), and the liver is known to alter is gene expression profile in response to acute hypoxia in vivo (21, 52). The response of the liver to hypoxia is not surprising given that the liver plays a central role in regulating energy balance, substrate metabolism, detoxification, and a small role in erythropoiesis (23). All of these processes are affected by hypoxia, and they may be critical for acclimatizing to hypoxia. For example, acute hypoxia stimulates cells to switch from oxidative phosphorylation to anaerobic glycolysis, while perhaps decreasing fatty acid utilization. Increased gluconeogenesis performed by the liver can supply these hypoxic tissues with the glucose that they require (16) while increasing lipid storage of circulating fatty acids (13, 76). In contrast, during chronic hypoxia liver substrate preference appears to switch from glucose and anaerobic glycolysis toward fatty acid metabolism (68). Under normal circumstances, the liver produces small amounts of EPO compared with the kidney, but under extreme hypoxia, production of EPO by the liver increases substantially

(75). Patients suffering from chronic obstructive pulmonary disease have decreased drug clearance ability, and this response has been linked to the decreased expression of various cytochrome P450 isoforms by the hypoxic liver (28). Finally, the liver is one of the few tissues that expresses all three HIF isoforms (HIF-1α, HIF-2α, and HIF-3α), which suggests that it is important in responding to hypoxic conditions (76). Because the liver is a key mediator of metabolic process and because it plays an important role in acclimatization to hypoxic, we focused our study on the liver (52).

MATERIALS AND METHODS

Treatments and animal handling. We obtained 36 male mice of the inbred strain C57BL/6 from Charles River Laboratories (Hollister, CA). After acclimating to our vivarium in Reno, Nevada, for 1 wk, the 10 wk old mice were randomly assigned to one of three simulated altitude treatments. The treatments approximated the P_{O_2} found at 1,400 m (18.0 kPa), 3,000 m (14.5 kPa), and 4,500 m (11.5 kPa). The P_{O_2} were achieved by adding appropriate amounts of nitrogen to air using thermal mass flow controllers, and supplying this mixed air to each of three 150 l environmental chambers. Each treatment consisted of one chamber with 12 mice. Reno, NV, is located at 1,400 m above sea level, and the ambient barometric pressures averages 86.1 kPa. The 1,400 m treatment received ambient air. The 3,000 m group received air with 15.3% oxygen, and the 4,500 m group received air with 11.8% oxygen. Because animals inside the environmental chamber consume oxygen, the concentration of oxygen leaving the environmental chambers (i.e., the excurrent concentration) will be lower than the concentration entering the chamber (incurrent concentration). The faster the flow rate through the chamber is, the lower the oxygen concentration difference will be between the inlet and outlet air. Hence, faster flow rates are good for minimizing the oxygen concentration difference, but they also require more nitrogen and more frequent changing of the gas cylinders providing the nitrogen. As a compromise between these two constraints, we calculated the flow rate necessary to keep the incurrent and excurrent concentration within 1% O_2 using the formula $\dot{V}_{O_2} = FR_i (O_2C_i - O_e)/(1 - O_2C_e)$, where $\dot{V}_{O_2}$ = oxygen consumed, FR = flow rate, O_2C = fractional oxygen concentration (e.g., O_2C for ambient air ≈0.2095), the subscripts i and e stand for incurrent and excurrent, respectively (106). To be conservative, we estimated total $\dot{V}_{O_2}$ in the chamber as 24 times the average daily oxygen consumption reported by Jackson Laboratories (Bar Harbor, Maine, http://www.jax.org) for adult male C57BL/6 mice. That is, we calculated the flow rate needed to keep the incurrent and excurrent oxygen concentrations within 1% O_2 even if the $\dot{V}_{O_2}$ of the 12 mice per chamber was double the normal $\dot{V}_{O_2}$. Based on this calculation, we estimated that a flow rate of ~2.5 l/min was needed, although the flow delivered to each chamber ranged from 2.5 to 2.8 l/min. Periodic checks over the course of the experiment indicated that the average oxygen concentrations of excurrent air were 20.2, 14.5, and 11.2% for the 1,400, 3,000, and 4,500 m treatments, respectively. These values compare with average incurrent concentrations of 20.9, 15.3, and 11.8%, respectively. We acknowledge that imperfect mixing may have resulted in some variation in P_{O_2} within each chamber and that the slight pressure differential needed to ensure airflow through each chamber may have resulted in achieved P_{O_2}s that were slightly different than those that would be calculated from our data on oxygen concentration and ambient barometric pressure.

Within each 150 l chamber, the 12 mice were individually housed in 30 × 12 × 8 cm standard rodent cages and provided with food and water ad libitum for 32 days. The chambers were maintained in a room with 12 h light-12 h dark cycle and at ~21°C (i.e., room temperature that reflected ambient building condition, not a precisely regulated thermal environment). Data from other related experiments indicate that temperatures within the chamber did not vary significantly from the ambient temperature of the room. The chambers were opened briefly on *days 9*, *17*, *24*, and *30* to change cages, add food and water, and weigh the mice.

After 32 days, 2 days after the final body mass measurements, we removed the mice, took two blood samples from the infraorbital sinus with microhematocrit tubes, and killed the mice via cervical dislocation. We immediately harvested the liver and preserved a small tissue sample in RNALater (Ambion). The time span from the chamber

opening to tissue preservation was <30 min. Within 3 h of collecting the blood samples we centrifuged the microhematocrit tubes. Hematocrit was measured as the percentage of red blood cells in the column of blood. The two samples for each mouse were averaged before subsequent analyses were performed. Variations in body mass and hematocrit were analyzed with a one-way ANOVA followed by the Ryan-Einot-Gabriel-Welsch multiple-range test for pair-wise tests among the means of the three groups (104). All procedures outlined above were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

Liver samples were stored at −80°C until the time of RNA purification. After homogenizing 30 mg of liver tissue using a liquid nitrogen cooled hammer and QiaShredder vials (Qiagen,), we used a Qiagen RNA Extraction Minikit (Qiagen) to purify RNA from the tissue samples following the procedures outlined in the Qiagen RNA Extraction Minikit instruction manual. The quality and quantity of the RNA were evaluated using an ABI Bioanalyzer (ABI Systems), and all samples were found to be of acceptable quality for microarray hybridization.

Microarray experiment. For the purpose of minimizing individual variation, RNA samples obtained from mice were pooled. Each pooled sample contained RNA from four mice. With 12 mice in each treatment, this design resulted in three microarrays per treatment, and a total of nine microarrays in the experiment. Because of the documented reliability of Affymetrix products, we used the Affymetrix GeneChip Mouse Genome 430 2.0 Array (22, 57). The RNA sample preparations and microarray hybridization procedures were carried out by the University of Nevada Genomics Center according to the procedures set forth by Affymetrix. Aside from the microarray platform used, these procedures are identical to that described in Grimplet et al. (38).

GeneChip data processing and analysis. The Affymetrix GeneChip arrays were first inspected using a series of quality control steps. Images of all arrays were examined, and no obvious scratches or areas of spatial variation were observed. A visual inspection of the distributions of raw perfect match (PM) probe values for the nine arrays showed one outlying array (the third replicate of the 3,000 m experiment). Digestion curves describing trends in RNA degradation between the 5′ end and the 3′ end of each probe set were examined, showing a notable outlying trend in the same outlying array, with irregular degradation between the sixth and eleventh probe pairs. The three Affymetrix quality control metrics for noise and background of this array were greater than two standard deviations away from the mean rates across all nine arrays, which led us to discard this array from further study.

For the 22,226 probe sets that were detected in at least one of the eight remaining arrays, raw intensity values were processed and normalized by RMA (robust multiarray average) (48). Specifically, expression values were computed from raw *CEL* files by first applying the RMA model of probe-specific correction of PM probes. These corrected probe values were then normalized via quantile normalization, and a median polish was applied to compute one expression measure from all probe values. Resulting RMA expression values were $\log_2$-transformed. The RMA expression values and raw microarray data have been submitted to the Gene Expression Omnibus database (series no. GSE15891).

Distributions of expression values processed via RMA of the eight arrays were similar with no apparent outlying arrays. Pearson correlation coefficients and Spearman rank coefficients were computed on the RMA expression values for each set of biological replicates, with all coefficients ranging between 0.992 and 0.997. A principal components analysis showed a clear separation between the three experimental conditions, with >99% of the variability of the experiment attributed to differences between expression measures of the highest elevation and the two lower elevations (Fig. 1).

To determine whether genes were differentially expressed between the three simulated altitudes, an ANOVA was performed on the RMA expression values. Data were fit to a simple linear model for a one-way ANOVA, ANOVA was performed on each probe set using the linear model, and contrasts were based on differences between the three experimental conditions (57). A multiple testing correction method [false discovery rate (FDR)] was applied to the P values of the F-statistic (6). Genes identified with an adjusted P value $P < 0.05$ were extracted for further inspection and analysis. The z-scored values (expression values normalized across all eight

arrays) of these probe sets were clustered using a simple hierarchical clustering procedure with the Pearson correlation coefficient as distance metric and the average agglomerative method.

The genes associated to the resulting list of 580 differentially expressed probe sets were compared with previous studies on hypoxia gene expression, and a list of genes common to previous studies was compiled. Gene annotation was gathered from the Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx). Genes were categorized by their biological functions as classified by Gene Ontology (GO) (3), and these categories were analyzed using a series of Fisher's exact tests to determine whether functional categories of the selected genes were over- or underrepresented. GO functional groups were also compared with other investigations in hypoxia induced gene expression. Gene Set Enrichment Analysis (GSEA) (63) was performed as recommended by the GSEA User's Guide (http://www.broad.mit.edu/gsea/doc/GSEAUserGuideFrame.html) on the pathways including at least 15 probe sets from the significant gene list (Supplementary Table S1[1]). Pathways were defined by the Broad Institute's Molecular Signatures Database C2 collection of curated gene sets from online pathway databases, PubMed, and domain experts. One thousand trials were performed, in which gene set labels were randomly permuted to estimate *P* values for each pathway. The *P* values were adjusted using the FDR method, where the FDR is the estimated probability that a gene set represents a false positive finding. In the case of small sample sizes, the recommended FDR threshold is 5%. Thus, we considered only those pathways with an FDR of at most 0.05.

Confirmatory real-time qRT-PCR. We selected six genes showing significant differential expression between simulated altitudes to verify the microarray experiment. β-Actin (NM_007393) was chosen as our endogenous control gene. Previously published primer pairs for each of these seven genes were selected from Primerbank (Table 1). The efficacy of each primer pair was verified prior to performing the qRT-PCR experiment. The source RNA used for amplification was derived from the same samples used for microarray hybridization. Aliquots of the same 36 RNA samples were pooled in an identical manner to that used for microarray hybridization such that we had nine samples representing the three hypoxia treatments. These nine pooled samples were then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA).

For each gene, qRT-PCR was performed in duplicate on each cDNA sample. Genes were amplified using 1 ng of total cDNA, 150 nM of each primer (forward and reverse), and the iTaq SYBR Green Supermix with ROX reagents per manufacturer's direction (Bio-Rad). PCR plates were designed such that two genes and the endogenous control gene (β-actin) along with the corresponding standard curves were run on each plate. Amplifications were performed using an ABI 7000 PRISM Sequence Detection System (Applied Biosystems, Foster City, CA) through 40 cycles 95°C for 3 s and 58°C for 30 s. We analyzed the data using comparative cycle threshold method ($\Delta\Delta C_T$ method). The ΔC_T was calculated as $C_{T\ target} - C_{T\ \beta\text{-actin}}$. The $\Delta\Delta C_T$ was $\Delta C_{T\ 4,500} - \Delta C_{T\ 1,400}$ for each gene. The fold difference was expressed as $2^{-\Delta\Delta CT}$ (60).

RESULTS

Physiological responses. After 4 wk of exposure to hypoxia, the body masses of mice differed significantly ($P < 0.001$) among the three treatments. Mice from the two most hypoxic treatments (3,000 and 4,500 m) were significantly ($P < 0.05$) smaller in mass than mice from the 1,400 m treatment. This difference appeared to have occurred during the first week of exposure to hypoxia. Data for the initial starting masses are unavailable. However, because these mice are from an inbred strain and because they were randomly assigned to treatments, it is unlikely that the starting masses were significantly different. As illustrated in Fig. 2, variation in body mass was established within the first week by either a loss of body mass or a slower growth rate of hypoxic mice. The growth rates thereafter appear to be comparable among the treatments.

Whether the body mass of the 3,000 and 4,500 m mice differed depended on the inclusion of two potential outlier mice. During the course of the experiment, two of the mice that were in the 4,500 m chamber were much smaller

(>3.5 g) than the other mice in that group. If those mice are excluded from the body mass analyses, then the mice in the 3,000 and 4,500 m chambers are not significantly different from each other (mean masses of 24.6 and 23.9 g, respectively), but both those groups of mice are significantly different in mass ($P < 0.05$) from the 1,400 m group (mean mass of 26.6 g, Fig. 2). If the two unusually small mice (16.9 and 19.1 g) from the 4,500 m are included in the analyses, the residuals from the analysis of variance deviate significantly from a normal distribution, which might cast doubt on that analysis. However, the nominal *P* values from the analyses including all 36 mice suggest that the body mass of all three groups differed significantly ($P < 0.05$) from one another. Figure 2 depicts the mean body mass over the 4 wk with the two outlier mice excluded from the data set.

Hematocrit differed significantly ($P < 0.001$) among the three treatments. Mice housed in the 1,400 m chamber had a mean hematocrit of 56.2% red blood cells (RBC), the 3,000 m treatment had a mean hematocrit of 60.6% RBC, and mice in the 4,500 m treatment had an incredibly high mean hematocrit of 80.7% RBC (Fig. 3).

Microarray results. Of the 45,101 probe sets on the array, 22,226 probe sets were detected in at least one of the eight useable arrays. Upon the application of ANOVA and the control of the FDR, 580 probe sets showing significant ($P < 0.05$ after multiple testing correction) differential expression across the three experimental conditions. Of these probe sets, 455 showed an increase in expression in the 4,500 m treatment compared with the 1,400 m treatment, with the remaining 125 having a decrease in expression in 4,500 m treatment vs. the 1,400 m treatment. A heat map representation of gene expression patterns revealed a trend of distinct differences between the high and low elevation, with ~85% of genes showing intermediate transcript abundances in the 3,000 m group (Fig. 4). Because of this trend, we focused further investigation on the comparison between 1,400 and 4,500 m groups.

To determine whether a specific functional group was overrepresented in the set of 580 probe sets that showed significant differences in expression, a series of Fisher's exact tests was performed on 21 GO functional categories, each of which contained at least five probe sets. After a multiple testing correction, only one category other than the “Undefined Function” category showed a significant difference (decrease) in the percentage of occurrence of functionally assigned probe sets among all probe sets in the analysis ($n = 14,107$), and those in our list of interest ($n = 389$). This category, defined as “transcription,” indicated that genes involved in transcription are underrepresented in hypoxic mice (4,500 vs. 1,400 m). Other than this group, the lack of under- or overrepresentation of a particular functional group may be due to the small number of probe sets with significant differences in expression.

Independent of the GO functional analysis above, we compiled a list of biological functional groups with more than five representative probe sets that show significant differential expression between the 1,400 and 4,500 m treatments. These functional groups included those associated with the immune response, angiogenesis and blood vessel development, oxygen transport, oxidation reduction (electron transport), glycolysis, protein amino acid phosphorylation, carbohydrate metabolic process, fatty acid and lipid metabolic processes, and other metabolic processes (Table 2). While the representations of these functional groups were not statistically significant in our selected subset of interest, many of these functional groups have previously been reported to be important to responses to hypoxia.

In addition to using GO term-based functional analyses, we used GSEA to investigate the over- and underrepresentation of functional groups within the genes of interest (95). GSEA is a method that tests whether gene sets, rather than individual genes or probe sets, are associated with experimental conditions or classes in a given study. Definitions of gene sets are based on published and curated information on biochemical pathways. The GSEA method yielded several pathways having a statically significant (FDR value of <0.05) association with the upregulation in the 4,500 m group vs. the 1,400 m group. Of particular interest was the Hematopoietic Stem Cell and Adult Progenitors gene set, which is a set of genes found to be expressed during hematopoietic stem cell and hematopoietic progenitor cell development (49). The list of genes belonging to this gene set show increased

expression in the 4,500 m treatment and is presented in Table 3.

Based on previous studies of how gene expression responds to acute or cellular hypoxia, we compiled a list of genes that we suspected would be affected by chronic systemic hypoxia. Many of these genes are regulated by the HIF pathway. However, few of these genes appeared to be significantly differentially expressed in this experiment. Indeed, in our list of 580 probe sets, only seven are known to be regulated by HIF (Table 4). When we compared our data for genes not regulated by HIF with data from a study looking at effects of acute hypoxia on hepatocytes (89), we found three genes in common with two genes showing concordant responses to hypoxia and one gene being expressed in the opposite direction in response to hypoxia. Furthermore, only a handful of the 580 genes that were differentially expressed in our experiment have previously been reported to respond to hypoxia in any study (Table 5).

Several genes unique to hypoxia studies were expressed >1.5-$\log_2$-fold when comparing 4,500 and 1,400 m simulated altitudes. A probe set representing leptin receptor showed the most dramatic difference in expression with a $\log_2$-fold increase of 3.05 in 4,500 m mice compared with 1,400 m mice. Two other probe sets representing the leptin receptor also showed significant differences in expression with $\log_2$-fold changes of 2.93 and 0.807, respectively. Consistent with the hematocrit results, a transcript representing α-globin showed a 1.97-$\log_2$-fold increase in expression in the 4,500 m vs. the 1,400 m mice. Two other probe sets representing α-globin and one probe set representing β-globin also showed significant differences in gene expression, though none over 1.5-$\log_2$-fold. Several genes encoding three families of cytochrome p450 and several genes involved in the G protein-coupled receptor protein signaling pathway were among the genes with >1.5-$\log_2$-fold changes (Table 6).

As mentioned earlier, relatively few genes showed decreased transcript abundance in the hypoxic conditions compared with genes that showed increased transcript abundance. Table 7 lists 25 genes that had a decreased $\log_2$-fold change of >1.0 in the 4,500 m treatment vs. the 1,400 m treatment. These genes represent a diverse set of functions, without any readily discernable pattern.

Confirmatory qRT-PCR. To validate the expression profiles obtained from the Affymetrix GeneChip Mouse 430 expression set array, quantitative real-time PCR was performed on six target genes and one endogenous control gene. Each of these genes was covered by at least one probe set that appeared in our list of the 20 most differentially expressed transcripts and had known biological function. We compared transcript abundance between the 4,500 and 1,400 m for all six genes. We performed a linear regression analysis to measure the relationship between the PCR data and the microarray data, which resulted in a very strong correlation of 0.935, and a P value of $P = 0.006$. This result indicates a high correlation and confirmation of our microarray results (Fig. 5).

DISCUSSION

Hypoxia is a significant physiological stressor associated with high altitude environments. Although most mammals have limited evolutionary history with hypoxic environments, mammals display various characteristic physiological responses when acclimatizing to high altitudes. This study utilized DNA microarrays *1*) to help understand the gene expression changes in the liver that may underlie physiological responses following chronic exposure and *2*) to identify other potential physiological effects of hypoxia that may have been overlooked previously by acute studies.

The experiment was conducted at 1,400 m, not sea level. While there are some effects of this altitude on physiology (for instance, a hematocrit of 56.2 as opposed to 52% as is average for sea level C57BL/6 mice), this elevation is not considered to be extreme. The Po_2 found in Reno is not expected to elicit many of the characteristic responses to high altitude that we typically see >2,000 m (103). That being said, it is important to appreciate that the variation in gene expression we report is not a comparison of hypoxia vs. normoxia, but of varying degrees of hypoxia, from mild to extreme.

Based on the observed differences in body mass and hematocrit among our treatments, the hypoxic conditions were effective in eliciting significant physiological responses. However, the mRNA expression in the liver after 32 days of hypoxia was different from expression patterns reported in studies of acute hypoxia studies (37, 89, 99). While several previous studies reported similar numbers of genes with differential expression, most of the genes in this experiment exhibited increased transcript abundance, but most studies of acute or cellular hypoxia report a general trend of decreased transcript abundance (89). Thus, it appears that after more prolonged periods of hypoxia, relative steady-state transcript abundance changes from a trend of being reduced to a trend of being increased. This result is consistent with data collected from murine hearts (25).

Few genes known to be regulated by HIF are included among the 580 genes that were significantly differential expressed between our high- and low-altitude treatments. This result indicates that, after 32 days, the previously described stress pathways, and in particular the HIF pathway, are no longer significant factors in regulating and maintaining physiological acclimation to hypoxia. Indeed, other studies have shown that HIF-1α abundance peaks after 4–5 h in the liver then decreases back to normal over the course of several hours (94). These findings indicate that, while HIF is an important transcription factor in regulating the initial responses to acute hypoxia, it is not as important in the maintenance of acclimatization to chronic hypoxia stress, at least in the liver. There are two nonmutually exclusive explanations for this result. One is that as animals physiologically acclimate to hypoxic environments, their tissues return to a state of oxygen homeostasis and various stress responses and the HIF pathways are no longer activated. The second is that as hypoxic exposure moves from an acute to chronic state, other genetic pathways and transcription factors are activated that regulate the specific processes associated with maintaining the acclimatized state.

Few genes that are typically reported to be responsive to hypoxia showed differential expression in our study. A possible explanation for this discrepancy is that we studied an organ in vivo, whereas most other studies have been in vitro. However, the patterns of gene expression reported here are different than those reported in in vivo studies of murine livers exposed to either acute systemic or intermittent hypoxia (21, 56). Both of those studies of murine livers reported significant changes in lipid biosynthesis and sterol metabolism. While some genes related to these processes are differentially expressed in this study, there are not as many, nor are they the same genes. This finding indicates that chronic hypoxia induces different gene expression patterns in liver in vivo than either acute or intermittent hypoxia. Indeed, recent findings confirm that intermittent and chronic hypoxia stimulate different gene expression patterns in cardiac tissue (25, 47).

The most dramatic result of this study was the large increase in hematocrit in the 4,500 m mice. While modest increases in hematocrit are generally seen as being beneficial acclimations to hypoxia (10), an increase to 80.7% RBC is extreme and much greater than expected. A hematocrit this high leads to viscous blood and can put a significant strain on the heart. Consistent with this response was the increased expression of both α-globin and β-globin, the two protein subunits of hemoglobin. However, what is unclear is why these genes are expressed in the liver. There are two possible explanations for this phenomenon. One is the presence of immature erythrocytes within the liver tissue, which would express both the α-globin and β-globin mRNAs. Indeed, polycythemia at the level reported here is often accompanied by increased circulation of immature erythrocytes. However, it is unclear whether there would be sufficient numbers of immature erythrocytes in the liver to elicit this response. An alternative explanation is that portions of the liver were converted to hematopoietic tissue to support the massive production of RBCs. This hypothesis is supported by the overrepresentation of differentially expressed genes belonging to the hematopoietic stem cell gene set, as indicated by the GSEA. The phenomenon of extramedullary hematopoiesis is well documented in cases of pathological anemia, such as severe thalassemia or myelofibrosis (62, 63), and it can occur when anemia is induced artificially (77). However, there is little, if any, information on this phenomenon in relation to systemic hypoxia or exposure to high altitude environments. Therefore, the incidence of extramedullary hematopoiesis and its consequences for high-altitude acclimation have yet to be seriously considered. This result highlights the dynamic nature of the liver and the importance of in vivo studies to

chronic hypoxia. The result deserves additional study. While we were unable to conduct additional confirmatory histological studies to confirm that extramedullary hematopoiesis took place in the liver, it is one possible explanation for our results.

The transcript showing the most dramatic increase in expression in the 4,500 m treatment vs. the 1,400 m treatment encoded a leptin receptor. This result is intriguing given the known responsiveness of the cytokine leptin to hypoxic stimulus. Circulating levels of leptin, a pleiotropic cytokine released by fat cells, are increased by acute hypoxia, and transcription appears to be regulated by HIF-1 (39, 61, 87, 88). However, in contrast, humans exposed to chronic high-altitude hypoxia had decreased levels of circulating leptin (108). It is possible that regulation of leptin receptor is another important mechanism of regulating the leptin pathway. Indeed, leptin receptor mRNA abundance in the liver increases in response to short-term fasting and increased circulating leptin (17).

While our results indicate that the leptin pathway may be an important response to hypoxia, the pleiotropic nature of this pathway makes it difficult to interpret the physiological significance of increased leptin receptor mRNA abundance in the liver. The leptin pathway is best known for its inhibitory effect on appetite, and an obvious hypothesis would be to link the leptin pathway with hypoxia-induced anorexia. However, despite increased expression during hypoxia, leptin does not appear to regulate hypoxia-induced anorexia (88, 98). Leptin signaling also has a significant positive effect on organismal metabolic rate and body temperature (2, 31, 59, 69, 91). In contrast, acute hypoxia typically leads to decreased metabolic rate and body temperature, at least in small mammals (12, 29, 32, 64, 65, 90). While we did not measure metabolic rate or body temperature in our mice, it seems likely that they became hypometabolic and anapyrexic when initially exposed to hypoxia (41). However, the effects of prolonged hypoxic exposure on metabolism are less obvious as there are comparatively fewer data on the effects of chronic hypoxia. Nonetheless, we hypothesize that at least in some strains and species, hypometabolism and anapyrexia may be transient responses to hypoxia (1, 67, 78). As an animal acclimates to hypoxia and attempts to regain oxygen homeostasis via increased oxygen delivery to its tissues, it seems plausible that metabolic rate would return toward normal. Perhaps leptin and its receptors help regulate this process? Furthermore, responses to hypoxia can be species specific (40, 42). For example, compared with effects on metabolic rates of rats, hamsters may be less affected by hypoxia and mice may be more affected (30, 41). Accordingly hypoxia-induced changes in gene expression may also be species specific, and as for any study of a single species of mammal, our results may not be generally applicable to other mammals. We therefore conclude that the effects of chronic hypoxia on the leptin pathway and its relationship to body temperature and metabolism is an area in need of further research.

Other physiological processes that are affected by leptin include glucose and fatty acid metabolism, hematopoiesis, and immune function (2, 31, 100). These physiological processes also appear to be important in acclimation to hypoxia. Previous studies have demonstrated that fatty acid metabolism is enhanced in rats exposed to chronic hypobaric hypoxia (68), and indeed several genes related to fatty acid metabolism were differentially expressed in our experiment. While it is unknown whether fatty acid metabolism was increased in our mice, it is possible that altering fatty acid metabolism is important for acclimating to hypoxia and that the leptin pathway plays a role in this response. Another important function of leptin is its role in hematopoiesis. Leptin receptor can be expressed by hematopoietic stem cells, and indeed, the leptin pathway is an important progenitor of hematopoiesis (7, 26). The dramatic increased expression of leptin receptor in our 4,500 m liver samples may be another indication that extramedullary hematopoiesis is taking place.

We sorted transcripts significantly affected by hypoxia into their functional classes (Table 2). These biological functional groups include transcripts involved in angiogenesis, oxidation reduction, carbohydrate metabolism, fatty acid and lipid metabolism, glycolysis, and protein amino acid phosphorylation. Many of these functional groups typically respond to acute or intermittent hypoxia, and thus these results are not surprising (37, 56, 89). However, the particular genes representing these functional groups are not typical of previous studies of hypoxia. This result

indicates that these biological functions are important in acclimation to hypoxia but that the particular transcripts that are involved in adjusting to hypoxia vary temporally with duration of hypoxic exposure.

The metabolic activities of the liver, which often generate carbon dioxide, also play an important role in acid-base homeostasis. This process is important to hypoxia acclimatization in that the hypoxic ventilatory response (HVR) typically leads to respiratory alkalosis. While increased ventilation remains during long periods of acclimatization (71), the pH of the blood typically returns close to normal via metabolic and blood regulation of bicarbonate (1, 67, 103). However, because we did not measure either ventilatory rate or blood pH, it is impossible to say whether alterations in the above transcript abundances could be contributing to maintenance of acid-base homeostasis or be affected by the slightly higher blood pH typically found in mammals acclimatized to hypoxia. To add to the complexity, two transcripts that have been implicated in maintaining acid-base balance had decreased expression in the 4,500 m mice: aquaporin 8 ($\log_2$-fold change of −1.09) and carbonic anhydrase 14 ($\log_2$-fold change of −0.95) (44, 50, 58). So while we are unable to draw direct conclusions regarding the relationship between liver transcript abundance and acid-base balance in response to HVR, we encourage the reader to keep this process in mind.

In this study we found an abundance of differentially expressed genes related to the immune system. Unlike the functional classes described above, the immune system is not typically viewed as important in responding to chronic, systemic hypoxia. However, there is increasing evidence that hypoxia may alter the immune system. For example, several studies of humans in high-altitude environments indicate perturbations of the immune system, particularly with respect to changes in circulating leukocytes (5, 14, 15, 24). Numerous explanations have been proposed to explain why sojourners to high altitude experience immune changes, including changes in diet, stress, etc. (5). More recent evidence indicates that hypoxia in and of itself has an influence on the circulating immune system. In particular, hypoxia has a proinflammatory effect on macrophages, neutrophils and other leukocytes and an anti-inflammatory effect on some lymphocytes (9, 18, 55, 66, 97, 101). Furthermore, there is growing evidence that HIF is a key regulator of many immunological processes (19, 43, 109). Yet again, the particular genes regulated by HIF pathway are not differentially expressed in our study. This result is similar to that for the other biological functional groups reported above. Namely, the immune system appears to respond to both acute and chronic hypoxia, but the particular transcripts involved in the immune systems responses vary with length of exposure to hypoxia.

The liver is a dynamic organ that not only is composed of hepatocytes but also houses many immune cells, blood cells, and, perhaps in this case, hematopoietic tissue. Unfortunately, it is not possible to distinguish which responses in gene expression are attributable to which cell types or processes. This may be a major source of variation between this study and other reports of gene expression in response to hypoxia. However, this study indicates that many of the biological functional groups regulated in response to acute hypoxia are also important in maintaining acclimation to chronic systemic hypoxia. Therefore, we hypothesize that regulatory pathways, and thus the representative genes expressed from a functional group, change with time and exposure to systemic hypoxia. So, while one set of genes achieves initial acclimatization to hypoxia, different but related groups of genes are likely involved in maintaining acclimatization. Understanding these differences will be important to better understanding the physiological effects of hypoxia and acclimatization to high altitudes.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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Footnotes

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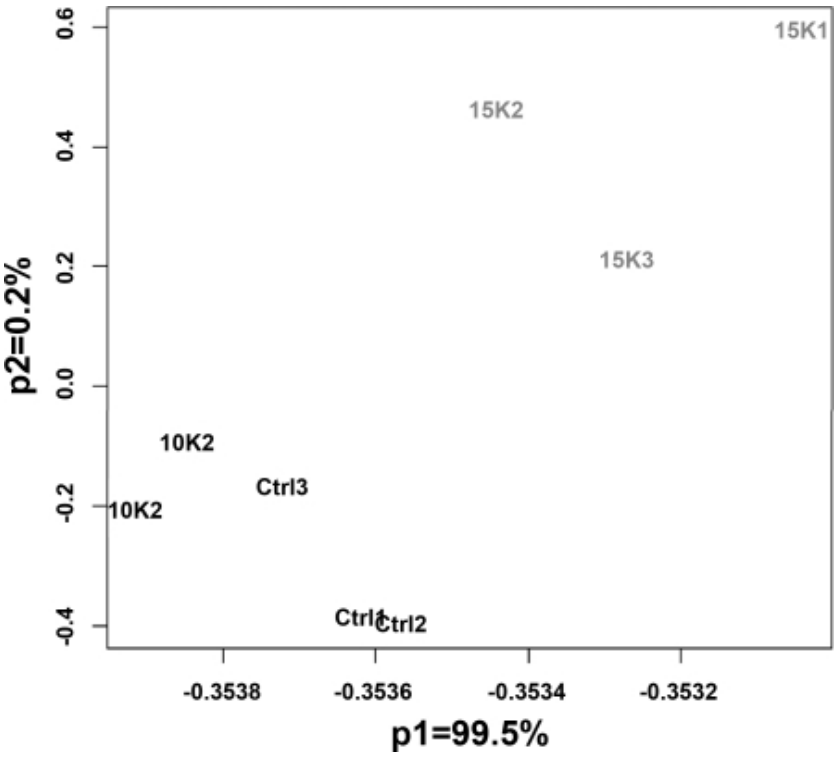
Figures and Tables

Fig. 1.



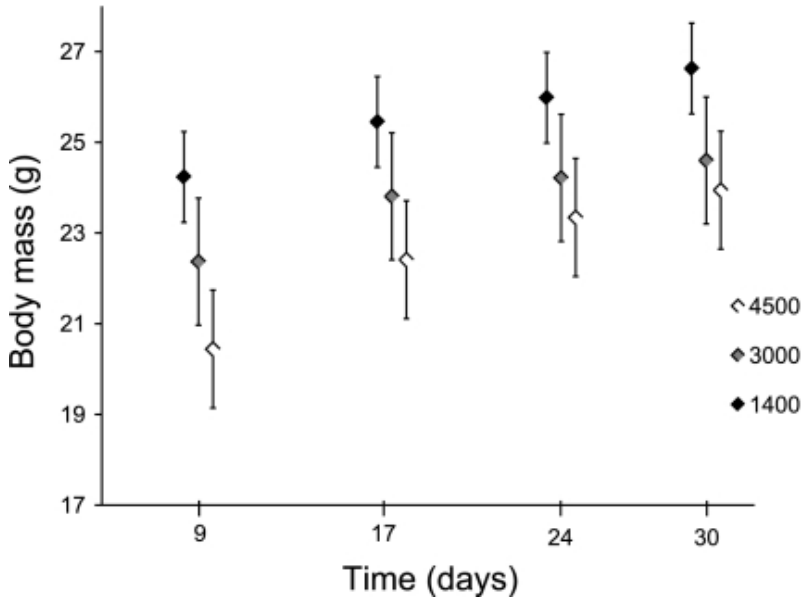
Principal component analysis of the 8 Affymetrix arrays. Note that 99.5% of the experimental variation is associated with the first principal component, and it indicates the separation between the highest altitude group and the other 2 groups.

Table 1.

Selected genes and primers used for qRT-PCR

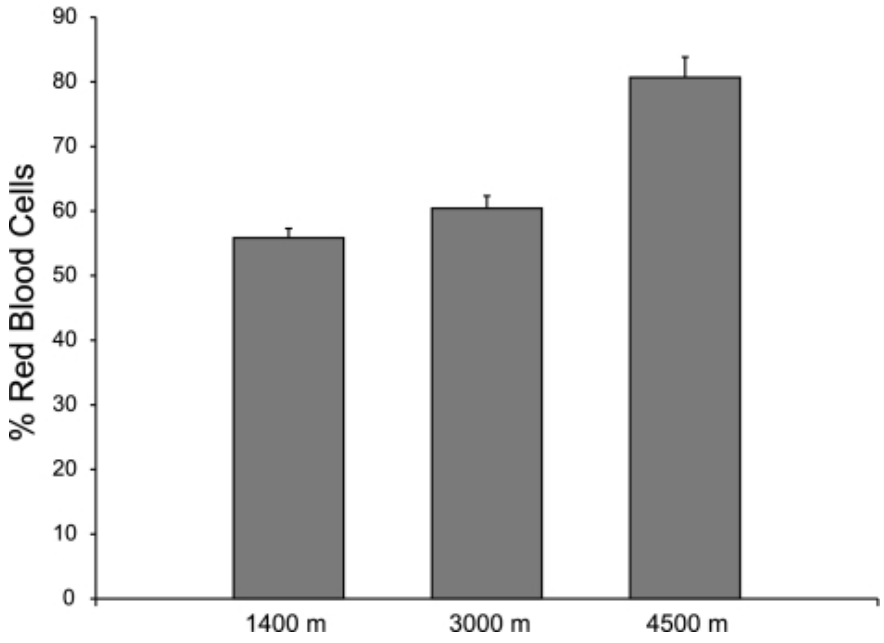
Ref Sequence ID	Common Gene Name	Forward Primer Sequence	Reverse Primer Sequence
NM_007468	apolipoprotein A-IV	CAACAGGCTGAAGGCTACGAT	CGATTTTTGCGGAGACCTTGG
NM_010704	leptin receptor	GTCTTCGGGGTTGTGAATGTC	ACCTAAGGGTGGATCGGGTTT
NM_009063	regulator of G protein signaling 5	ATGGATTTGCCAGCTTCAAAAGT	GAAGTGGTCAATGTTCACCTCTT
NM_008218	hemoglobin-α	CACCACCAAGACCTACTTTCC	CAGTGGCTCAGGAGCTTGA
NM_024406	fatty acid binding protein 4	AAGGTGAAGAGCATCATAACCCT	TCACGCCTTTCATAACACATTCC
NM_007822	cytochrome P450 4,a,14	GTCTCTCGGGGAGCAATATACG	ACCAATCCAGGGAGCAAAGAA
NM_007393	β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Fig. 2.



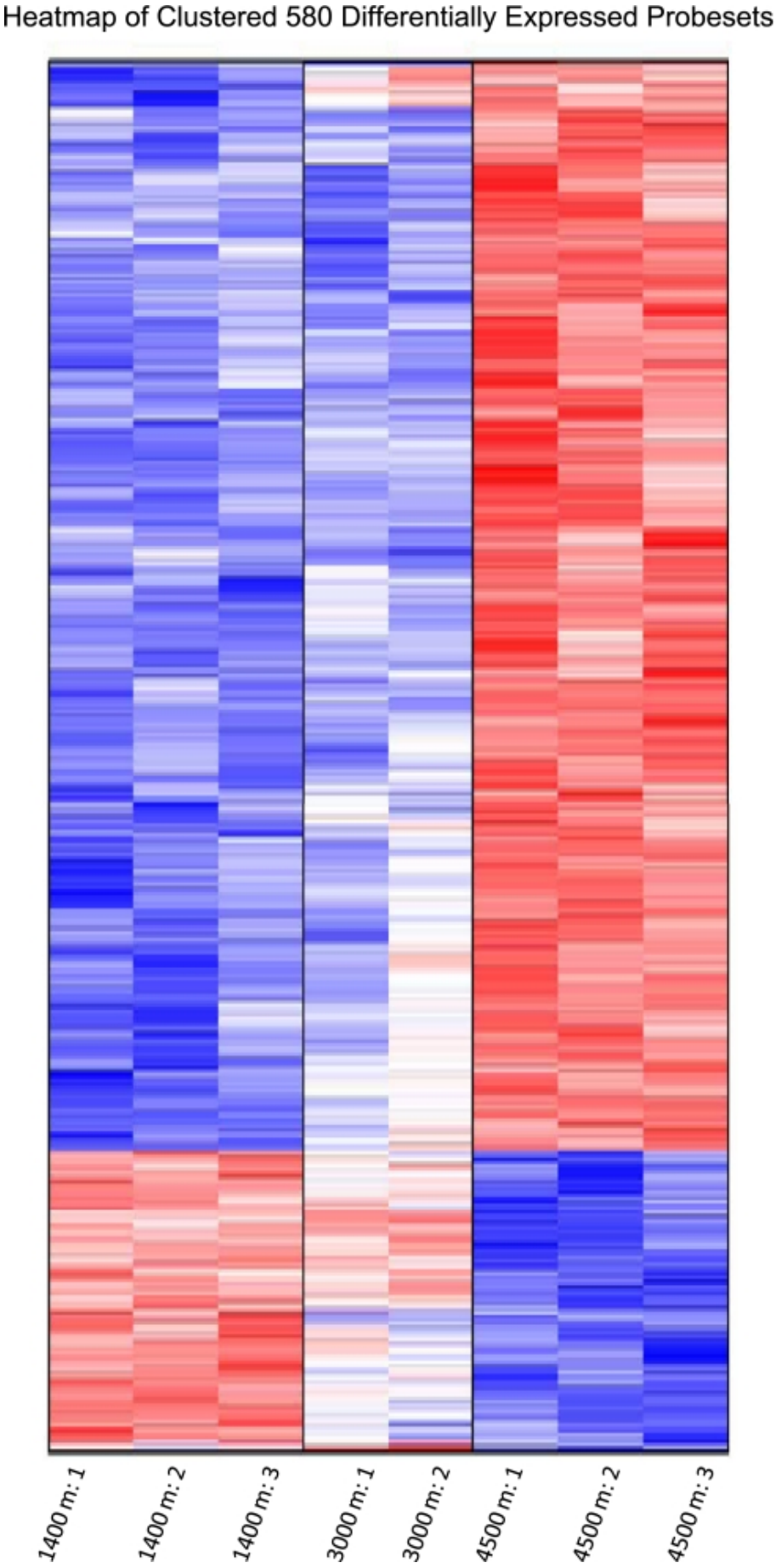
Mean body mass of mice over 30 days of residence in hypoxia chambers simulating 3 altitudes. By *day 9*, 4,500 m mice were significantly smaller (20.38 ± 1.20 g) than 3,000 m mice (22.37 ± 1.35 g), and both 4,500 m and 3,000 m mice were significantly smaller than 1,400 m mice (24.24 ± 1.35 g) ($P < 0.001$). This pattern remained until the end of the experiment, but the differences between the 3,000 and 4,500 m experiments were no longer significant. After 30 days, mice in the 4,500 m (23.9 ± 0.91 g) and 3,000 m (24.6 ± 1.40 g) chambers were significantly smaller than mice in the 1,400 m chamber (26.6 ± 1.34 g) ($P < 0.001$).

Fig. 3.



Mean hematocrits (Hct) of mice after 32 days in hypoxia chambers. All Hct are significantly different ($P < 0.001$) with the 4,500 m mice having a mean hematocrit of 80.9% RBC (± 3.4%), the 3,000 m group having a mean Hct of 60.6% RBC (± 1.9%), and the 1,400 m group having a Hct of 56.1% (± 1.4%). RBC, red blood cell.

Fig. 4.



Heat map of 580 differentially expressed probe sets. Values are normalized across the 8 arrays and clustered via a simple hierarchical clustering procedure.

Table 2.

Genes differentially expressed by chronic hypoxia arranged in functional classes

Functional Class	Common Gene Name	Probe Set ID	Log_2-fold Change	*P* Value
Immune System[*]				
	apolipoprotein A-IV	1417761_at	1.801	0.012
	apolipoprotein A-IV	1436504_x_at	1.415	0.005
	ATP-binding cassette, subfamily C (CFTR/MRP), member 9	1420408_a_at	1.252	0.040
	vascular cell adhesion molecule 1	1448162_at	1.247	0.010
	CD24a antigen	1448182_a_at	0.994	0.032
	CD38 antigen	1450136_at	0.980	0.020
	serine (or cysteine) peptidase inhibitor, clade A, member 3G	1424923_at	0.861	0.012
	C-type lectin domain family 4, member n	1425951_a_at	0.833	0.011
	CD68 antigen	1449164_at	0.833	0.019
	eosinophil-associated, ribonuclease A family, member 1	1422411_s_at	0.794	0.002
	cytotoxic T lymphocyte-associated protein 2α	1448471_a_at	0.779	0.011
	defensin-β1	1419491_at	0.751	0.019
	CD55 antigen	1460242_at	0.711	0.010
	CD163 antigen	1419144_at	0.706	0.035
	interleukin 10 receptor, β	1419455_at	0.704	0.004
	CD24a antigen	1416034_at	0.698	0.018
	BCL2/adenovirus E1B interacting protein 3-like	1448525_a_at	0.695	0.032
	Fc receptor, IgG, low affinity III	1448620_at	0.692	0.011
	C-type lectin domain family 4, member n	1419627_s_at	0.684	0.013
	interleukin 13 receptor, α1	1427164_at	0.662	0.015
	collectin subfamily member 10	1457871_at	0.661	0.009
	CD48 antigen	1427301_at	0.558	0.023
	leukocyte immunoglobulin-like receptor, subfamily B, member 4	1460258_at	0.558	0.011
	interleukin 6 signal transducer	1460295_s_at	0.557	0.019
	eosinophil-associated, ribonuclease A family, member 2	1449846_at	0.551	0.035
	attractin	1421166_at	0.517	0.034
	serine (or cysteine) peptidase inhibitor, clade A, member 3K	1424923_at	0.501	0.035
	interleukin 6 signal transducer	1452843_at	0.480	0.028
	ATP-binding cassette, subfamily C (CFTR/MRP), member 9	1435751_at	0.409	0.009

Angiogenesis and Blood Vessel Development†				
	heme oxygenase (decycling) 1	1448239_at	1.959	0.022
	connective tissue growth factor	1416953_at	1.385	0.013
	ELK3, member of ETS oncogene family	1448797_at	0.774	0.026
	forkhead box O1	1416981_at	0.615	0.046
	vascular endothelial growth factor B	1451803_a_at	0.606	0.024
	endomucin	1425582_a_at	0.600	0.020
	EGF-like domain 7	1451428_x_at	0.573	0.018
	platelet-derived growth factor, α	1418711_at	0.571	0.031
	matrix metallopeptidase 14 (membrane-inserted)	1448383_at	0.515	0.021
	endoglin	1432176_a_at	0.493	0.050
	reticulon 4	1421116_a_at	0.467	0.027
	cadherin 5	1433956_at	0.416	0.035
Electron Transport				
	cytochrome P450, family 4, subfamily a, polypeptide 14	1423257_at	1.970	0.009
	cytochrome P450, family 17, subfamily a, polypeptide 1	1417017_at	1.800	0.005
	cytochrome P450, family 39, subfamily a, polypeptide 1	1418780_at	1.576	0.002
	flavin containing monooxygenase 2	1453435_a_at	1.485	0.006
	flavin containing monooxygenase 2	1435459_at	1.449	0.007
	P450 (cytochrome) oxidoreductase	1416933_at	0.808	0.012
	STEAP family member 4	1425829_a_at	0.672	0.039
	cytochrome P450, family 4, subfamily a, polypeptide 10	1424853_s_at	0.648	0.019
	ERO1-like (*S. cerevisiae*)	1419030_at	0.647	0.040
	cytochrome b-245, β-polypeptide	1422978_at	0.641	0.043
	glutaredoxin	1416593_at	0.617	0.009
	cytochrome P450, family 2, subfamily c, polypeptide 38	1452501_at	0.569	0.012
	cathepsin B	1417491_at	0.562	0.022
	glutaredoxin	1416592_at	0.499	0.022
Oxygen Transport				
	hemoglobin-α, adult chain 1	1417714_x_at	1.865	0.000
	hemoglobin-α, adult chain 1	1428361_x_at	0.641	0.019
	hemoglobin, β-adult major chain	1417184_s_at	0.593	0.015
	hemoglobin-α, adult chain 1	1452757_s_at	0.536	0.020
Protein Amino Acid Phosphorylation				

	wee 1 homolog (*S. pombe*)	1416774_at	1.426	0.025
	Rap guanine nucleotide exchange factor (GEF) 4	1421622_a_at	1.159	0.015
	wee 1 homolog (*S. pombe*)	1416773_at	0.818	0.015
	endothelial-specific receptor tyrosine kinase	1418788_at	0.745	0.013
	serum/glucocorticoid-regulated kinase 3	1420918_at	0.739	0.031
	protein kinase C, η	1422079_at	0.655	0.033
	transient receptor potential cation channel, subfamily M, member 7	1416800_at	0.639	0.038
	serine/threonine kinase 3 (Ste20, yeast homolog)	1418513_at	0.523	0.034
	cyclin-dependent kinase 4	1422440_at	0.506	0.035
	tyrosine kinase receptor 1	1416238_at	0.502	0.026
	protein kinase N2	1437296_at	0.484	0.033
	receptor (TNFRSF)-interacting serine-threonine kinase 1	1419508_at	0.392	0.040
Metabolic Processes Glycolysis				
	pyruvate kinase, muscle	1417308_at	0.750	0.016
	2,3-bisphosphoglycerate mutase	1448119_at	0.731	0.016
	2,3-bisphosphoglycerate mutase	1415864_at	0.629	0.016
	aldolase 1, A isoform ///	1439375_x_at	0.494	0.041
	aldolase 1, A isoform	1434799_x_at	0.482	0.024
	pyruvate dehydrogenase E1 α1	1449137_at	0.463	0.040
Metabolism‡				
	leptin receptor	1456156_at	3.051	0.002
	leptin receptor	1425644_at	2.934	0.001
	CD38 antigen	1450136_at	0.980	0.002
	ATPase, class VI, type 11C	1442367_at	0.935	0.006
	ATPase, class VI, type 11C	1442367_at	0.935	0.006
	ectonucleotide pyrophosphatase/phosphodiesterase 1	1419276_at	0.838	0.030
	leptin receptor	1425875_a_at	0.807	0.013
	RIKEN cDNA A230097K15 gene	1454799_at	0.676	0.032
	carbonyl reductase 1	1460196_at	0.656	0.009
	glutathione *S*-transferase, mu 3	1427473_at	0.633	0.013
	retinol dehydrogenase 9	1427963_s_at	0.624	0.022
	phosphoribosyl pyrophosphate amidotransferase	1452831_s_at	0.589	0.018
	arsenic (+3 oxidation state) methyltransferase	1431980_a_at	0.548	0.013
	dehydrogenase/reductase (SDR family) member 7	1426440_at	0.495	0.017
	NAD kinase	1416249_at	0.455	0.029
	RIKEN cDNA A530057A03 gene	1456208_at	0.453	0.043
Carbohydrate and Glucose				

Metabolism[§]				
	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1416432_at	1.673	0.021
	glucokinase	1419146_a_at	0.979	0.009
	glucokinase	1419146_a_at	0.979	0.009
	glucokinase	1425303_at	0.849	0.010
	glucokinase	1425303_at	0.849	0.010
	preimplantation protein 4	1429639_at	0.784	0.046
	protein phosphatase 1, regulatory (inhibitor) subunit 2	1417341_a_at	0.624	0.024
	preimplantation protein 4	1429144_at	0.566	0.028
	pyruvate dehydrogenase kinase, isoenzyme 1	1423748_at	0.509	0.037
	membrane interacting protein of RGS16	1418444_a_at	0.489	0.035
	N-acetylneuraminate pyruvate lyase	1424265_at	0.340	0.039
Fatty Acid and Lipid Metabolic Process[a]				
	cytochrome P450, family 39, subfamily a, polypeptide 1	1418780_at	1.576	0.002
	lipin 1	1426516_a_at	1.280	0.009
	lipin 1	1418288_at	1.262	0.007
	very low density lipoprotein receptor	1434465_x_at	1.134	0.007
	N-acylsphingosine amidohydrolase 3-like	1451355_at	0.994	0.003
	ELOVL family member 6, elongation of long chain fatty acids (yeast)	1417404_at	0.967	0.016
	ELOVL family member 6, elongation of long chain fatty acids (yeast)	1417403_at	0.844	0.024
	acyl-CoA synthetase long-chain family member 4	1427595_at	0.807	0.031
	cytochrome P450, family 4, subfamily a, polypeptide 10	1424853_s_at	0.648	0.019
	platelet-activating factor acetylhydrolase, isoform 1b, α2 subunit	1422793_at	0.632	0.026
	acetyl-coenzyme A carboxylase-α	1427595_at	0.450	0.045

[*]Containing Gene Ontology (GO) Biological Functional designations immune response, inflammatory response, acute phase response, defense response, chemotaxis.
[†]Containing GO Biological Functional designations angiogenesis, blood vessel development, blood vessel maturation.
[‡]Containing GO Biological Functional designations metabolic process, regulation of metabolic process, one-carbon metabolic process).
[§]Containing GO Biological Functional designations carbohydrate metabolism, glucose metabolic process.
[a]Containing GO Biological Functional terms for fatty acid metabolism or lipid metabolism.

Table 3.

List of genes belonging to the Hematopoietic Stem Cell and Progenitors gene set that show increased expression in 4,500 m treatment

Common Gene Name	Probe Set ID	Log_2-fold Change	Adjusted *P* Value
Ubiquitin-specific peptidase 2	1417168_a_at	1.643	0.013
Regulator of G protein signaling 3	1425701_a_at	1.509	0.019
C-type lectin domain family 14, member a	1419467_at	1.273	0.001
Coiled-coil domain containing 85B	1435589_at	1.112	0.002
Rap guanine nucleotide exchange factor (GEF) 5	1455137_at	1.110	0.009
EGF, latrophilin seven transmembrane domain containing 1	1418059_at	1.095	0.002
Peptidylprolyl isomerase C	1416498_at	0.983	0.005
ELOVL family member 6, elongation of long chain fatty acids (yeast)	1417403_at	0.967	0.016
Ankyrin repeat domain 37	1436538_at	0.948	0.007
Rho-related BTB domain containing 1	1429206_at	0.921	0.019
Tissue factor pathway inhibitor	1452432_at	0.842	0.020
Ectonucleotide pyrophosphatase/phosphodiesterase 1	1419276_at	0.838	0.030
FYVE, RhoGEF and PH domain containing 5	1460578_at	0.760	0.040
Endothelial-specific receptor tyrosine kinase	1418788_at	0.745	0.013
Cyclin D2	1434745_at	0.693	0.010
Programmed cell death 6 interacting protein	1426184_a_at	0.692	0.021
Coiled-coil domain containing 80	1424186_at	0.674	0.009
Protein kinase C, η	1422079_at	0.655	0.033
Gap junction membrane channel protein α1	1438945_x_at	0.616	0.048
Vascular endothelial growth factor B	1451803_a_at	0.606	0.024
MAM domain containing 2	1453152_at	0.540	0.015
Thymosin, β10	1436902_x_at	0.521	0.022
Dynamin 1-like	1452638_s_at	0.415	0.037
N-acetylneuraminate pyruvate lyase	1424265_at	0.340	0.039
Aldehyde dehydrogenase 1 family, member L1	1424400_a_at	−0.411	0.036
ATP-binding cassette, subfamily B (MDR/TAP), member 11	1449817_at	−0.438	0.020
Endothelial cell growth factor 1 (platelet-derived)	1432181_s_at	−0.499	0.023

Table 4.

Transcripts known to be regulated by HIF-1α that show significant differences in abundance between 1,400 and 4,500 m treatments after 32 days

Common Name	Probe Set ID	Log_2-fold Change	*P* Value
6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1416432_at	1.67	0.021
Heme oxygenase (decycling) 1	1448239_at	0.97	0.022
Lectin, galactose binding, soluble 3	1426808_at	0.83	0.015

Pyruvate kinase, muscle	1417308_at	0.75	0.016
Insulin-like growth factor binding protein 3	1458268_s_at	0.54	0.029
Aldolase 1, A isoform	1434799_x_at	0.48	0.024
Presenilin 2	1425869_a_at	−0.68	0.009

HIF, hypoxia-inducible factor.

Table 5.

Transcripts showing differential expression in response to chronic hypoxia that have previously been reported to respond to hypoxic stimulus

Common Gene Name	Probe Set ID	Log_2-fold Change	*P* Value	Reference No.
Cytochrome P450, family 17, subfamily a, polypeptide 1	1417017_at	1.800	0.005	(56)
CD24a antigen	1416034_at	0.994	0.011	(83)
Heat shock protein 110	1423566_a_at	0.772	0.009	(89)
CD24a antigen	1448182_a_at	0.70	0.019	(82)
Superoxide dismutase 3, extracellular	1417633_at	0.657	0.012	(96)
ATPase, Na^+/K^+ transporting, β1 polypeptide	1439036_a_at	0.585	0.033	(53)
Caveolin, caveolae protein 1	1449145_a_at	0.541	0.036	(82)
Pyruvate dehydrogenase kinase, isoenzyme 1	1423748_at	0.509	0.037	(89)
Caspase 1	1449265_at	0.450	0.027	(110)
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	1425631_at	−0.937	0.044	(89)
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	1433691_at	−1.096	0.013	(89)

Table 6.

List of genes showing >1.5-log_2-fold increased transcript abundance in response to chronic hypoxia

Common Name	Probe Set ID	Log_2-fold Change	Adjusted *P* Value
Leptin receptor	1456156_at	3.05	0.002
Leptin receptor	1425644_at	2.93	0.001
Regulator of G protein signaling 16	1426037_a_at	2.26	0.024
Regulator of G protein signaling 5	1420941_at	2.24	0.000
Fatty acid binding protein 4, adipocyte	1417023_a_at	2.21	0.000
G protein-coupled receptor 98	1425314_at	2.19	0.000
Regulator of G protein signaling 16	1451452_a_at	2.07	0.027
—	1417466_at	2.01	0.000
Cytochrome P450, family 4, subfamily a, polypeptide 14	1423257_at	1.97	0.009
Hemoglobin-α, adult chain 1	1417714_x_at	1.87	0.000
Fatty acid binding protein 4, adipocyte	1451263_a_at	1.86	0.004
Synuclein-α	1436853_a_at	1.86	0.000

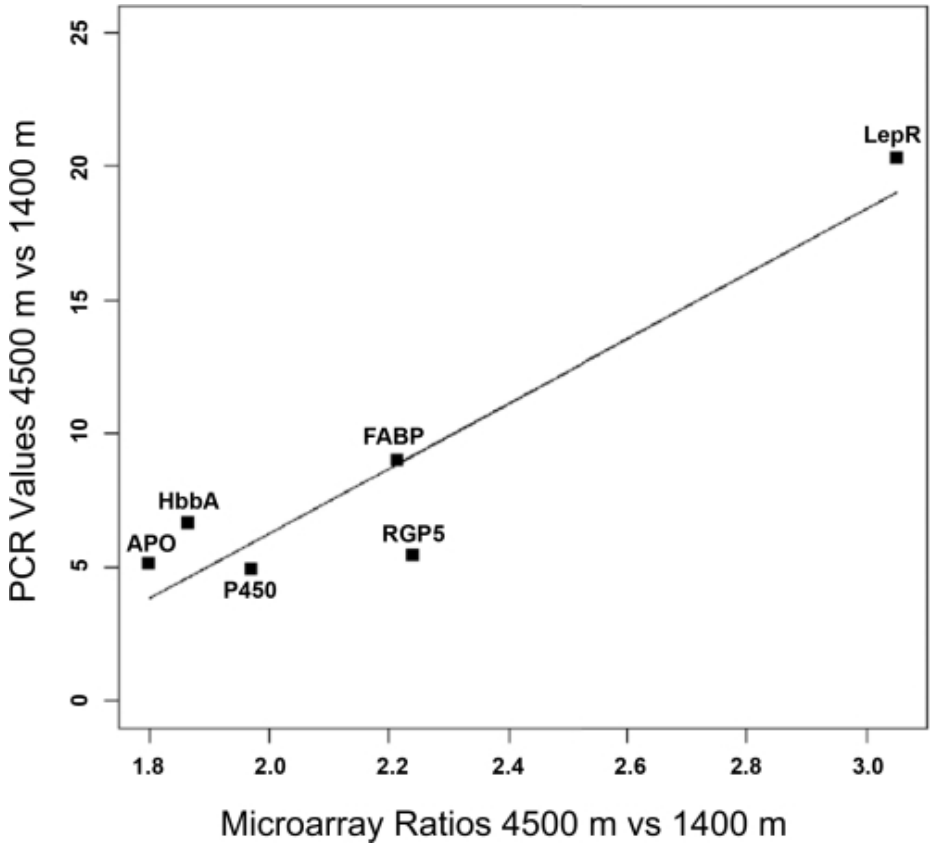
Predicted gene, ENSMUSG00000073738	1439816_at	1.84	0.000
Regulator of G protein signaling 5	1420940_x_at	1.84	0.000
Apolipoprotein A-IV	1417761_at	1.80	0.005
Cytochrome P450, family 17, subfamily a, polypeptide 1	1417017_at	1.80	0.005
ATP-binding cassette, subfamily D (ALD), member 2	1438431_at	1.76	0.005
Major facilitator superfamily domain containing 2	1428223_at	1.73	0.019
ATP-binding cassette, subfamily D (ALD), member 2	1419748_at	1.71	0.008
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1416432_at	1.67	0.021
Ubiquitin-specific peptidase 2	1417168_a_at	1.64	0.013
Von Willebrand factor homolog	1435386_at	1.63	0.004
Synuclein-α	1418493_a_at	1.58	0.010
Cytochrome P450, family 39, subfamily a, polypeptide 1	1418780_at	1.58	0.002
—	1417466_at	1.57	0.000
Regulator of G protein signaling 3	1425701_a_at	1.51	0.019

Table 7.

List of genes showing >1.0-$\log_2$-fold decreased in transcript abundance in response to chronic hypoxia

Common Gene Name	Probe Set ID	Log_2-fold Change	*P* Value
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427351_s_at	−1.60	0.028
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427329_a_at	−1.58	0.030
Tubulin, β2a	1427347_s_at	−1.57	0.009
Macrophage activation 2 like	1438676_at	−1.46	0.005
Macrophage activation 2 like	1447927_at	−1.44	0.003
Oligodendrocyte transcription factor 1	1416149_at	−1.28	0.007
Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	1420722_at	−1.27	0.002
UDP glucuronosyltransferase 2 family, polypeptide B38	1423397_at	−1.25	0.011
Early growth response 1	1417065_at	−1.22	0.011
RIKEN cDNA 2310043N10 gene	1428083_at	−1.11	0.031
RIKEN cDNA 1810046K07 gene	1453547_at	−1.10	0.003
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	1433691_at	−1.10	0.013
Aquaporin 8	1417828_at	−1.10	0.001
cDNA sequence BC015286	1457619_at	−1.04	0.011
Carboxylesterase 2	1424245_at	−1.02	0.001

Fig. 5.



Confirmatory real-time qRT-PCR of 6 transcripts. Comparison between the expression values from the Affymetrix GeneChip Mouse Expression Set 430 and expression values from quantitative real-time PCR. LepR, leptin receptor; FABP, fatty acid binding protein; RGP5, regulator of G protein signaling 5; P450, cytochrome P450 family 4, subfamily a, protein 14; HbbA, hemoglobin-α; APO, apolipoprotein.