Endothelin receptor B, a candidate gene from human studies at high altitude, improves cardiac tolerance to hypoxia in genetically engineered heterozygote mice

Tsering Stobdan[a], Dan Zhou[a], Eilleen Ao-leong[b], Daniel Ortiz[b], Roy Ronen[c], Iain Hartley[a], Zhuohui Gan[b], Andrew D. McCulloch[b], Vineet Bafna[d], Pedro Cabrales[b], and Gabriel G. Haddad[a,e,f,1]

[a]Department of Pediatrics, Division of Respiratory Medicine, University of California, San Diego, La Jolla, CA 92093; [b]Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093; [c]Bioinformatics and Systems Biology Graduate Program, University of California, San Diego, La Jolla, CA 92093; [d]Department of Computer Science and Engineering, University of California, San Diego, La Jolla, CA 92093; [e]Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093; and [f]Rady Children's Hospital, San Diego, CA 92123

Edited by Gregg L. Semenza, Johns Hopkins University School of Medicine, Baltimore, MD, and approved June 26, 2015 (received for review April 20, 2015)

To better understand human adaptation to stress, and in particular to hypoxia, we took advantage of one of nature's experiments at high altitude (HA) and studied Ethiopians, a population that is well-adapted to HA hypoxic stress. Using whole-genome sequencing, we discovered that *EDNRB* (*Endothelin receptor type B*) is a candidate gene involved in HA adaptation. To test whether EDNRB plays a critical role in hypoxia tolerance and adaptation, we generated *EdnrB* knockout mice and found that when *EdnrB*$^{-/+}$ heterozygote mice are treated with lower levels of oxygen (O_2), they tolerate various levels of hypoxia (even extreme hypoxia, e.g., 5% O_2) very well. For example, they maintain ejection fraction, cardiac contractility, and cardiac output in severe hypoxia. Furthermore, O_2 delivery to vital organs was significantly higher and blood lactate was lower in *EdnrB*$^{-/+}$ compared with wild type in hypoxia. Tissue hypoxia in brain, heart, and kidney was lower in *EdnrB*$^{-/+}$ mice as well. These data demonstrate that a lower level of EDNRB significantly improves cardiac performance and tissue perfusion under various levels of hypoxia. Transcriptomic profiling of left ventricles revealed three specific genes [natriuretic peptide type A (*Nppa*), sarcolipin (*Sln*), and myosin light polypeptide 4 (*Myl4*)] that were oppositely expressed ($q < 0.05$) between *EdnrB*$^{-/+}$ and wild type. Functions related to these gene networks were consistent with a better cardiac contractility and performance. We conclude that *EDNRB* plays a key role in hypoxia tolerance and that a lower level of EDNRB contributes, at least in part, to HA adaptation in humans.

Endothelin receptor type B | hypoxia | high altitude | cardiac output | lactate

Oxygen (O_2) is often referred to as a biosignature, a chemical marker in the atmosphere closely associated with life, and in a complex organism, such as humans, various physiological systems have evolved to maintain an optimal O_2 homeostasis. Arguably, humans living at high altitude (HA) for thousands of years ought to have undergone a significant level of natural selection to adjust to the challenging hypoxic condition. Human adaptation to HA hypoxia, which can be protective to tissues, can potentially be harnessed for better therapeutic modalities for sea-level diseases that involve hypoxia and ischemia in their pathogenesis. Indeed, lessons from such an "experiment in nature" can be derived from HA adaptation and can advance low-altitude medicine (1). With the advent of newer technology including next-generation sequencing (seq), this idea has recently led to intensive efforts, and a number of publications have appeared on studies of human populations living at HA (2–4). These studies also draw added significance not only to sea-level human diseases but also to more than 140 million people living at an altitude above 2,500 m, where the hypoxic condition presents a major challenge for survival (5). Although a number of laboratory methods that mimic hypoxia adaptation using model organisms (6) have been used as tools to identify causative genetic pathways (7, 8), studies in human populations living at different HA regions with distinct genetic backgrounds can provide direct insight for identifying mechanisms regulating hypoxia responses in humans.

Over the last few decades, much research has been done on humans permanently living at high altitude. Although there is still debate about the mechanisms of adaptation in HA populations, especially in relation to those populations that are best-studied (9), the Ethiopians are considered to be the best-adapted because the prevalence of chronic mountain sickness, a maladaptation syndrome to HA, has never been reported (10, 11). In our recent effort to understand the basis for adaptation to HA in humans, we analyzed the whole genome for genetic variation in HA Ethiopians (4). Using cross-population tests of selection, we searched for genomic regions with a significant loss of genetic diversity indicative of selective sweeps (4). We discovered several regions on different chromosomes that were significantly associated with HA adaptation. To elucidate the potential role of each of the individual genes in hypoxia adaptation and tolerance, we experimentally evaluated whether these genes affected hypoxia tolerance in *Drosophila* by manipulating respective orthologs in the fly. Indeed, we succeeded in functional evaluation of some of these candidate genes in flies (4). However, *Endothelin receptor B* (*EDNRB*), which is one of these significant candidate genes [chromosome (chr)13], had no ortholog in flies (4).

Significance

Although model organisms have been used to understand the underlying basis of hypoxia and how to treat it, it has always been useful to learn from natural experiments in humans, such as in high-altitude dwellers. In this study, we demonstrate that decreased *Endothelin receptor type B* (*EdnrB*), a gene associated with altitude adaptation from our human study, improves cardiac tolerance to hypoxia. We show that heterozygote *EdnrB* mice maintain higher cardiac output and peripheral perfusion and better O_2 delivery to vital organs, even in severe hypoxia. Furthermore, the transcriptome profile revealed three atria-specific genes, natriuretic peptide type A (*Nppa*), sarcolipin (*Sln*), and myosin light polypeptide 4 (*Myl4*), which were down-regulated by hypoxia and possibly have a role in improving cardiac performance during normoxia and hypoxia.

Author contributions: T.S., D.Z., P.C., and G.G.H. designed research; T.S., D.Z., E.A.-l., D.O., and P.C. performed research; D.Z., P.C., and G.G.H. contributed new reagents/analytic tools; T.S., D.Z., R.R., I.H., Z.G., A.D.M., V.B., P.C., and G.G.H. analyzed data; and T.S. and G.G.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

See Commentary on page 10080.

[1]To whom correspondence should be addressed. Email: ghaddad@ucsd.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1507486112/-/DCSupplemental.

To functionally validate and study the role of this candidate gene, we resorted to experiments in mice. Given that HA exposure has long been recognized as a cardiac stress (12) and endothelin (ET) receptor blockade with bosentan ameliorates an increase in pulmonary artery pressure (13) with hypoxia, we hypothesized that a decreased level of EDNRB would play a protective role in HA hypoxia. In addition, it appears that EdnrA-specific antagonism affects only pulmonary hypertension with no effect on cardiac performance (14). This supported the notion that EdnrB-specific antagonism of bosentan would improve cardiac performance at high altitude. Because *EdnrB* is widely expressed (15), we generated an *EdnrB* global knockout mouse model and used the heterozygous ($EdnrB^{-/+}$) to evaluate its role in cardiovascular response to various degrees of hypoxia from mild to severe. We report here, to our knowledge for the first time, a major heterozygous advantage of *EdnrB* under hypoxic conditions and its cross-talk with three specific genes that were differentially expressed in the ventricles.

Results

Genomic Analysis of *EDNRB* Reveals Fixation of SNPs in the HA Population. Earlier, we reported *EDNRB* as one of our top candidates for genes underlying hypoxia adaptation in Ethiopian highlanders (4). This HA population showed a strong signature of selection in the *EDNRB* region, with a large block of fixed SNPs that was observed at significantly lower frequencies in lowlander controls (Table S1). Upon reanalyzing the sequenced data of the region containing *EDNRB*, we noted a large block of 52 "differential" SNPs upstream of the promoter region of *EDNRB* (Fig. 1*A*), 20 of which were located near a cluster of ENCODE project (genome.ucsc.edu/ENCODE/index.html) transcription-factor binding sites (TFBSs). This region is also depicted with a stronger signal for the layered H3K4Me1 (H3 histone protein, lysine 4, monomethylation; Fig. 1*A*, pink line) histone mark across the genome as determined by ChIP-seq assay. Indirectly, H3K4Me1 is associated with enhancers and with DNA regions downstream of the transcription start site. The signal peak was observed primarily in the HUVEC (human umbilical vein endothelial cell) tissue type, represented in Fig. 1*A* as light blue peaks, implying that potential disruption of this site would be consistent with blood vessel-related phenotypes. Five of these SNPs overlapped with the ENCODE TFBSs (Table S2). This includes binding sites for transcription factors such as ATF2, FOXM1, STAT3, and MEF2A (Fig. 1*B*), which are reported to have roles in cardiac function either independently (16, 17) or in cross-talk with the endothelin system (18, 19). Also, within this cluster, the strongest signal was a 400-bp Chip-seq peak for the FOS/JUN (chr13:78503006–78503405; Fig. 1*B*) transcription factors reported as related to hypoxia and angiogenesis (20). Although we did not find any SNPs lying within this stretch of sequence, three SNPs were flanking FOS/JUN and were at 100% in the Amhara population and roughly 66% in the Oromo population, both HA populations. Furthermore, this peak appears to overlap a DNaseI-hypersensitive region, observed mainly in blood and lymphatic endothelial cells. These observations, and the fact that this region is in the 5′ region of *EDNRB* or within the first intron of one of the *EDNRB* transcripts, reference sequence (RefSeq) accession no. NM_000115 (Fig. 1*A*), makes it unlikely that the strong signature of selection in the *EDNRB* region was purely by chance. Although the SNPs in the *EdnrB* promoter region play a role (21), our rationale for hypothesizing that they render *EdnrB* hypofunctional are data obtained from the clinical use of bosentan in patients at high altitude.

Generation of *EdnrB* Heterozygous Mice. We hypothesized that decreasing functional EDNRB would be advantageous in hypoxia, and hence we knocked out this gene in a mouse model and then studied its phenotype in both normoxia and hypoxia. Using a Cre-Lox recombination system, exon 3 from $EdnrB^{flox/flox}$ was completely removed (Fig. 2*A*). A detailed strategy is provided in *SI Materials and Methods* and Fig. S1. This resulted in an out-of-frame transcript of exon 2–exon 4 splicing, making the gene nonfunctional (22). As we expected, the complete knockout of *EdnrB* ($EdnrB^{-/-}$) led to a phenotype observed in the Hirschsprung mouse model and accordingly dies at around weaning age (~21 d). Considering that the homozygous *EdnrB* mutant is lethal and believing that a partial knockout would be pharmacologically relevant, we used heterozygous ($EdnrB^{-/+}$) mice for all of our experiments (Fig. 2*B*). The body weights of $EdnrB^{-/+}$ were similar to those of age-matched littermate controls ($EdnrB^{+/+}$). Both male and female $EdnrB^{-/+}$ mice were fertile, and therefore were used as breeding pairs (Fig. 2*B*). To make sure that the gene is expressed as assumed by its genotype, we measured the expression level of *EdnrB* in left and right cardiac ventricles. Quantitative (q)RT-PCR confirmed no expression of the *EdnrB* gene in $EdnrB^{-/-}$ mice, and the ratio of expression in $EdnrB^{-/+}$ was about half (0.414-fold change) of that in $EdnrB^{+/+}$ (Fig. 2 *C* and *D*).

Better Performance of $EdnrB^{-/+}$ Under Extreme Hypoxia. To directly investigate the role of *EdnrB* in hypoxia adaptation, we examined how $EdnrB^{-/+}$ responded to 30 min of four levels of O_2 concentration compared with $EdnrB^{+/+}$ controls. Because the phenotype could be more prominent under extreme conditions, we first investigated whether severe hypoxia (5% O_2) induces hypotension, defined as mean arterial pressure (MAP) <40 mmHg. Notably, all of the $EdnrB^{-/+}$ had MAP >60 mmHg (Fig. 3*A* and Table S3), but

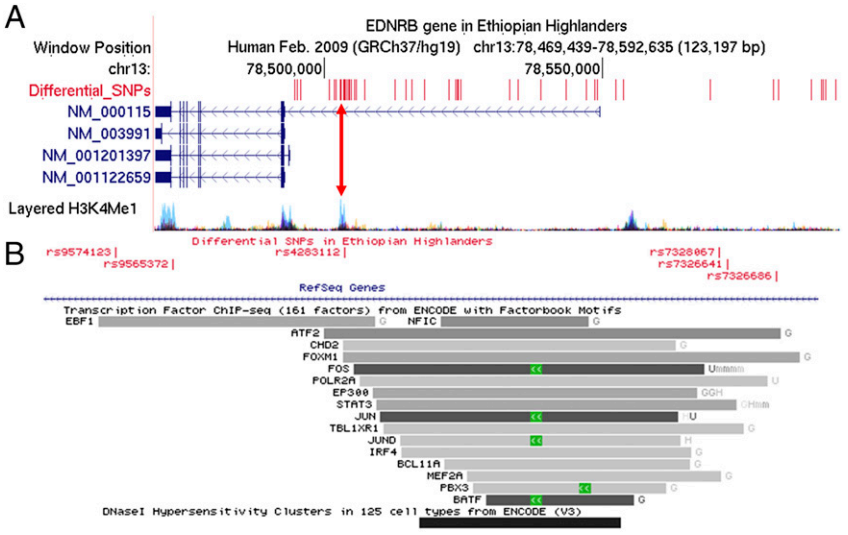

Fig. 1. *EDNRB* region in Ethiopian highlanders. (*A*) Four known transcripts of human *EDNRB* with RefSeq accession numbers. Note that *EDNRB* is transcribed from the negative strand (i.e., right to left in this figure). Overlaid above (in red) are the genomic positions of 52 SNPs deemed differential by Udpa et al. (4). These SNPs show a strong signal of frequency differentiation between the Ethiopian highlander population (Amhara) and a nearby lowlander control population (Luhya population from the 1000 Genomes project, www.1000genomes.org), indicative of strong positive selection in the region. A large number of SNPs are condensed in a region with high H3K4Me1 track [web-accessible directories of genomic data viewable on the University of California, Santa Cruz (UCSC), genome browser (the pink line overlaid is further resolved in *B*)] and its associated regulatory role. (*B*) Many of these SNPs are in the cluster of ENCODE transcription-factor binding sites. These SNPs are within the TFBSs (Table S1). This site also overlaps a DNaseI-hypersensitive region, observed mainly in blood and lymphatic endothelial cells.

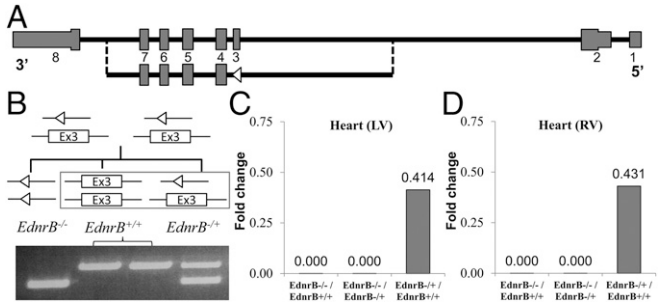

Fig. 2. Generation of *EdnrB* knockout mice confirms removal of exon 3, and its expression is reduced to approximately half in the $EdnrB^{-/+}$. (*A*) Diagram representing the complete *EdnrB* gene from exons 1–8. (*Lower*) *EdnrB* with exon 3 removed (the open triangle represents a loxP site). (*B*) Breeding strategy for generating $EdnrB^{-/+}$ and $EdnrB^{+/+}$ (box) mice used for the experiments. Lines with open triangles represent loxP sites after the removal of exon 3. The gel picture shows the three genotypes whereby the upper PCR band size of 481 bp depicts the intact exon 3 ($EdnrB^{+/+}$), whereas the lower band size of 368 bp results from the removal of the 113-bp exon 3 ($EdnrB^{-/-}$). Both bands are seen in $EdnrB^{-/+}$. (*C* and *D*) qRT-PCR from RNA isolated from heart left ventricle (LV) (*C*) and right ventricle (RV) (*D*) of $EdnrB^{-/-}$, $EdnrB^{-/+}$, and $EdnrB^{+/+}$. The *y* axis depicts the fold-change ratio.

none of the controls could maintain MAP >40 mmHg for the whole 30-min period. This suggests that $EdnrB^{-/+}$ mice are potentially more resistant to a severe hypoxic challenge. When we investigated further the differences in cardiovascular performance between $EdnrB^{-/+}$ and $EdnrB^{+/+}$, $EdnrB^{-/+}$ mice clearly had a great advantage over $EdnrB^{+/+}$, especially under extreme hypoxia (5% O_2). Cardiac output (CO) was remarkably different between the two groups (Fig. 3*B*). Whereas there was no difference in the CO between $EdnrB^{+/+}$ and $EdnrB^{-/+}$ mice at baseline and in mild hypoxia, namely at 21% and 15%, the difference was significant ($P < 0.05$) at 10% and 5% O_2, with CO being stable in the heterozygote but decreased to about half of baseline in the wild type in severe hypoxia. To further our understanding of the differences in cardiovascular function between the two groups of mice, we measured individual pressure–volume parameters. The maximum rate of pressure change (dP/dt_{max}) per ventricular end-diastolic volume (V_{ed}), which is a measure of contractility, was higher in the $EdnrB^{-/+}$ at baseline as well as at all of the subsequent hypoxia exposures (Fig. 3*C*).

Comparison of lactate levels also reveals an interesting difference: Whereas the levels were similar between the two groups at room air and 15% O_2, the difference was prominent as we gradually decreased the O_2 level. At 10% the blood lactate increased significantly in the $EdnrB^{+/+}$ compared with the $EdnrB^{-/+}$, and at 5% O_2 the levels in $EdnrB^{+/+}$ were more than double that of $EdnrB^{-/+}$ (Fig. 3*D*). There was no significant difference in the arterial O_2 partial pressure (PaO_2) between $EdnrB^{-/+}$ and $EdnrB^{+/+}$ mice. $PaCO_2$ in $EdnrB^{-/+}$ was significantly lower ($P < 0.05$) in room air but not when O_2 was lowered in inspired air. Interestingly, pH was almost identical between the two groups, except in severe hypoxia, when the blood became more acidic (a significant difference of 0.11 pH units) in the controls than in $EdnrB^{-/+}$.

Interesting observations were also noted at organ/tissue levels. When the tissues were harvested after the final 5% O_2 exposure and stained using pimonidazole dye, a hypoxia indicator (23), the relative hypoxic area (%) in brain, heart, and kidney was significantly lower in the $EdnrB^{-/+}$ compared with controls (Fig. 3*E* and Fig. S2). The difference was more significant in heart tissue. In contrast, no difference was detected in the intestine and liver.

Differential Gene Expression in $EdnrB^{-/+}$ Under Hypoxia. Transcriptome analysis of left ventricles revealed that there were more genes that were differentially expressed in the $EdnrB^{-/+}$ (Fig. 4 *A* and *B*) under hypoxia than in the controls. The baseline comparison between the normoxic $EdnrB^{+/+}$ vs. $EdnrB^{-/+}$ depicts only 34 genes differentially expressed at $q < 0.05$ [q value is the false discovery rate (FDR)-corrected P value to account for multiple testing]. The number rose to 80 in hypoxia $EdnrB^{+/+}$ vs. $EdnrB^{-/+}$, of which 12 genes were in common with the normoxia $EdnrB^{+/+}$ vs. $EdnrB^{-/+}$. However, when we compared within each group, namely $EdnrB^{+/+}$ normoxia vs. hypoxia and $EdnrB^{-/+}$ normoxia vs. hypoxia, the number of genes for which q <0.05 was 4,252. This included 342 genes (8%; Fig. 4*C*, green circles and *Inset*) that were differentially expressed only in $EdnrB^{+/+}$ and 2,207 (52%; Fig. 4*C*, mauve circles and *Inset*) in $EdnrB^{-/+}$. In addition to this, 39% of differentially expressed genes (1,659; Fig. 4*C*, blue and red circles and *Inset*) were in common in both groups. On closer examination, it was interesting to note that there were 16 genes (Fig. 4*C*, red circles) that were counterregulated by hypoxia ($q < 0.05$); that is, under hypoxia, these genes were up-regulated in $EdnrB^{+/+}$ and down-regulated in $EdnrB^{-/+}$ or vice versa. At fragments per kilobase of exon per million reads mapped (FPKMs) ≥2 there were 10 genes oppositely expressed (Fig. 4*D* and Fig. S3) and, as highlighted in Table 1, only *Pygl* (liver glycogen phosphorylase) was down-regulated in hypoxic $EdnrB^{-/+}$. The remaining nine genes were all up-regulated in $EdnrB^{-/+}$ and down-regulated in $EdnrB^{+/+}$. Of the counterregulated genes, we decided to focus on three genes, namely natriuretic peptide type A (*Nppa*), sarcolipin (*Sln*), and myosin light polypeptide 4 (*Myl4*), because their average FPKM values were >100 and because we validated these three genes (Fig. 4*E*) by RT-PCR. Interestingly, these three genes were also differentially expressed at baseline (normoxia) when $EdnrB^{+/+}$ and $EdnrB^{-/+}$ were compared. We

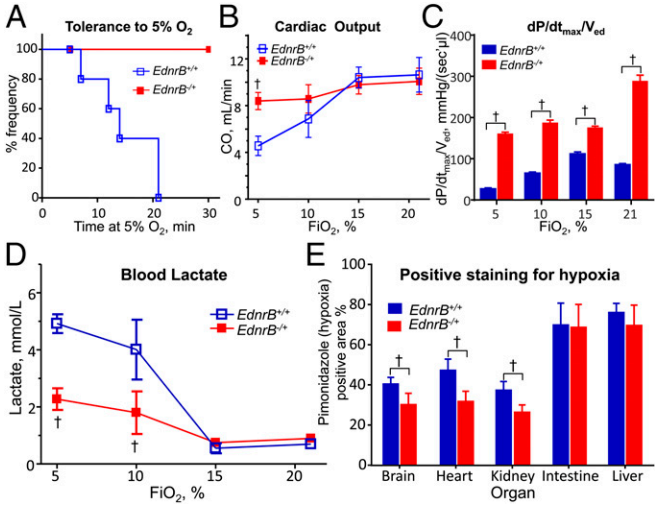

Fig. 3. Enhanced hypoxia performance of $EdnrB^{-/+}$. (*A*) Tolerance (defined as MAP >40 mmHg) of $EdnrB^{-/+}$ and $EdnrB^{+/+}$ kept in 5% O_2 for a period of 30 min. The average MAP for $EdnrB^{-/+}$ was 60.5 ± 53 mmHg at 5% O_2, and all mice from this group had MAP >40 mmHg. None of the $EdnrB^{+/+}$ littermates could maintain MAP >40 mmHg for the entire 30-min time. (*B*) Cardiac output measured in $EdnrB^{-/+}$ and $EdnrB^{+/+}$ at different O_2 concentrations (21%, 15%, 10%, and 5% O_2). The CO is similar in both groups of mice when kept under room air condition. A slight decrease in O_2 to 15% does not indicate any change in CO; however, under extreme hypoxia, namely 10% and 5% O_2, a significant drop in CO could be seen in $EdnrB^{+/+}$ but no change in $EdnrB^{-/+}$. (*C*) The $dP{\cdot}dt_{max}^{-1}{\cdot}V_{ed}^{-1}$ distinctly indicates a higher value for $EdnrB^{-/+}$ at baseline as well as during 15%, 10%, and 5% hypoxic exposures. (*D*) The blood lactate levels in $EdnrB^{-/+}$ and $EdnrB^{+/+}$ at 21% and 15% O_2 are similar in both groups. Below 15% O_2, the blood lactate levels, although increased in both groups, the increase is mild in $EdnrB^{-/+}$ (1.8 ± 0.75 mmol/L at 10%; 2.28 ± 0.38 mmol/L at 5% O_2), compared with a significant increase in $EdnrB^{+/+}$ (4.01 ± 1.05 mmol/L at 10%; 4.92 ± 0.33 mmol/L at 5% O_2). (*E*) The relative percentage hypoxic area using pimonidazole dye in brain, heart, and kidney is significantly lower in $EdnrB^{-/+}$ compared with its littermate controls ($EdnrB^{+/+}$). The difference appears more significant in heart tissue. No difference in the % hypoxic area is detected in the intestine and liver. $^{\dagger}P < 0.05$; error bar represents ± SD.

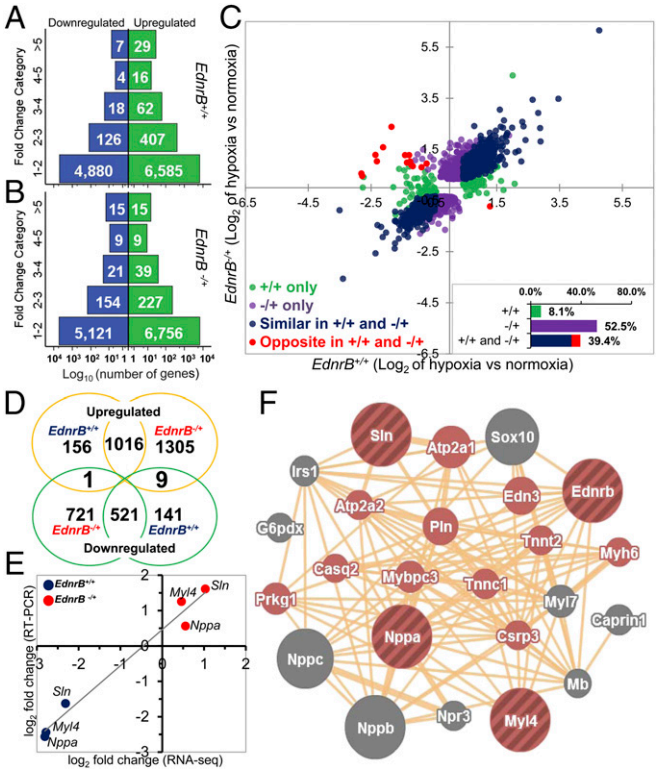

Fig. 4. Transcriptome analysis from RNA isolated from left ventricles of $EdnrB^{+/+}$ and $EdnrB^{-/+}$ mice kept in normoxia and hypoxia. (*A* and *B*) Hypoxia vs. normoxia fold change categorized as down-regulated (blue) and up-regulated (green) genes in $EdnrB^{+/+}$ (*A*) and $EdnrB^{-/+}$ (*B*) mice. The numbers within the bars represent the number of genes. (*C*) Hypoxia vs. normoxia $\log_2$ fold-change values between the genes in $EdnrB^{+/+}$ and $EdnrB^{-/+}$ plotted on the *x* and *y* axes, respectively. Only those genes for which there was a significant fold change ($q < 0.05$) in either of the groups alone or similarly and oppositely expressed in the two groups are displayed and color-coded. (*Inset*) The % genes that are differentially expressed in different groups. The bar "$^{+/+}$ and $^{-/+}$" includes similarly (blue) and oppositely (red) expressed genes. (*D*) Venn diagram depicting the number of genes in common in different groups of hypoxia treatment. The oppositely expressing genes after conditioning with different criteria for mining specific genes and pathways are 1+9 (up-regulated in $EdnrB^{+/+}$ and down-regulated in $EdnrB^{-/+}$, 1; down-regulated in $EdnrB^{+/+}$ and up-regulated in $EdnrB^{-/+}$, 9). (*E*) $\log_2$ fold-change comparison between RNA-seq and RT-PCR for the three genes (*Nppa*, *Sln*, and *Myl4*) with FPKM >100. (*F*) Network analysis of *Nppa*, *Sln*, and *Myl4* along with *EdnrB* (striped brown circles) reveals the top-scoring networks (colored circles) all related to cardiovascular function ranging from heart contraction, blood circulation, and the cGMP metabolic process to Ca^{2+} homeostasis.

then used GeneMANIA (24) to identify the final composite networks of these three oppositely expressed genes (Fig. 4*F*). The functions related to these networks were primarily related to Ca^{2+} homeostasis, regulation of ATPase activity, regulation of the force of heart contraction, and blood circulation, consistent with the better cardiovascular performance of $EdnrB^{-/+}$ under extreme hypoxia. Simultaneously, we also studied the genes closely interacting with *EdnrB*. Most importantly, the ligand Edn-1 (or ET-1) under normoxia had similar Edn-1 expression in both groups, and this was also the case at the protein level when measured in circulating plasma (2.64 ± 0.39 and 2.88 ± 0.26 pg/mL in $EdnrB^{+/+}$ and $EdnrB^{-/+}$, respectively, at room air; 2.94 ± 0.90 and 3.46 ± 1.50 pg/mL in $EdnrB^{+/+}$ and $EdnrB^{-/+}$, respectively, at 11% O_2).

Discussion

In Udpa et al. (4), we reported that *EDNRB* is one of the top candidate genes underlying hypoxia adaptation in Ethiopian highlanders. Indeed, there was a strong signature of positive selection in the DNA region containing the *EDNRB* gene, in the form of a large block of (differential) SNPs that were fixed in the highlanders but at a significantly lower frequency in nearby lowlander populations. Although none of the SNPs were in the coding regions, many of these SNPs were overlying a cluster of transcription-factor binding sites identified in ENCODE (Fig. 1 *A* and *B*), where the underlying transcription factors were reported to have roles in endothelin-mediated cardiac function (16, 18, 19). Promoter luciferase assay of the *EDNRB* gene indicated an 89-fold increased activity with an intact 1,258-bp promoter sequence and a further boost of 2.7- and 3.1-fold with overexpression of c-Jun or C/EBPb, respectively (21). Additionally, the promoter region was also found to be rich in CpG dinucleotide repeats, and methylation of this CpG-riched region reportedly regulates *EDNRB* gene expression (25). It is therefore important to note that the "differential" SNPs in the DNA region containing the *EDNRB* gene may have a regulatory role, still unexplored. Although these observations do not unequivocally characterize EDNRB to be of a higher or lower level in the Ethiopian highlanders, they do imply the presence of a regulatory connection between transcription and the level of *EDNRB*.

We believe that understanding HA adaptation (or maladaptation) is important not only for better treatment or prevention of disease at HA but also for better understanding and therapy of sea-level diseases that involve hypoxia as a major etiologic factor in pathogenesis. Here we show, to our knowledge for the first time, that indeed, *EDNRB*, a candidate gene we identified in HA adaptation, proves to be critical in protecting cardiac function in moderate to severe hypoxia at sea level. The receptor itself is one of two known endothelin receptors, the other being EDNRA. The protein encoded by *EDNRB* is a G protein-coupled receptor that activates a phosphatidylinositol/calcium second-messenger system (26). Whereas EDNRA is present on the cell membrane, EDNRB is also present on the nuclear membrane and is associated with regulation of nuclear Ca^{2+} signaling (27). The two receptors usually have opposing actions, EDNRA in vasoconstriction and EDNRB in vasodilatation. However, due to the tissue-specific vasoconstrictor role of EDNRB, the reports on this receptor are often contradictory (28). Interestingly, in humans, the *EDNRB* gene is well-known for its role in neural crest cell migration, proliferation, and differentiation, where a mutation in this gene may lead to phenotypes such as the aganglionosis of the entire gut (Hirschsprung's disease, megacolon) (29) and hearing loss (30, 31). Studies have also shown down-regulation of EDNRB in human melanoma (32, 33), colorectal cancer (25), bladder cancer (34), and renal cell carcinoma (35).

In the heart, specifically in the ventricles, the ratio of EDNRA to EDNRB density is reportedly 4:1 (36). However, studies on cardiac-specific knockout of *EdnrA* indicated that type A receptors were unnecessary in baseline cardiac function and also under stressful conditions (37). This clearly indicates a more significant role of EDNRB in cardiac tissues and would correlate with bosentan-related type A and type B receptor antagonism, improvement in cardiac performance, and microcirculatory blood flow during septic shock (38, 39). When a type A receptor-specific antagonist was administered during porcine endotoxin shock, it only antagonized pulmonary hypertension and had no

Table 1. Genes counterregulated by hypoxia

Symbol	Description [mouse genome informatic (MGI) ID]
Pygl	Liver glycogen phosphorylase [MGI:97829]
Gm1078	Predicted gene 1078 [MGI:2685924]
Mybphl	Myosin-binding protein H-like [MGI:1916003]
Myl4	Myosin, light polypeptide 4 [MGI:97267]
Sln	Sarcolipin [MGI:1913652]
Upk3b	Uroplakin 3B [MGI:2140882]
Msln	Mesothelin [MGI:1888992]
Dkk3	Dickkopf homolog 3 (*Xenopus laevis*) [MGI:1354952]
Cfd	Complement factor D (adipsin) [MGI:87931]
Nppa	Natriuretic peptide type A [MGI:97367]

effect on the deteriorated cardiac performance (14). This would suggest that the type B-specific antagonist property of bosentan was involved in improving cardiac performance. Evidence has been accumulating to implicate *EdnrB* in cardiac function, where an increased expression of vascular EDNRB in patients with ischemic heart disease (40, 41) is seemingly beneficial when the expression is reduced. In addition, pharmacological agents blocking the intracellular loop 2 of *EdnrB* also appear beneficial under hypoxic conditions (42). We demonstrate in this work that $EdnrB^{-/+}$ mice withstand various levels of hypoxia, even severe hypoxia, by maintaining cardiac performance close to that at baseline. For example, cardiac function in $EdnrB^{-/+}$ was better-maintained under moderate and severe hypoxia than in control mice, and our data (Fig. 3) are consistent with an increase in contractile force and contraction and relaxation velocities of the left ventricle (43, 44). Previous hypoxia studies on an *EdnrB*-deficient ($EdnrB^{sl/sl}$) rat model missed this very important phenotype in the heterozygote ($EdnrB^{+/sl}$), as they treated rats at 10% O_2 (45, 46). Indeed, we know from our results that the significant drop of CO in $EdnrB^{+/+}$ and sustenance of CO among $EdnrB^{-/+}$ are more profoundly visible at 5% O_2 (Fig. 3*B*). It is worth noting that a complete knockout of *EdnrB* even in specific cells may be deleterious (47).

Because $EdnrB^{-/+}$ mice maintain better cardiac performance under various levels of hypoxia, we argue that a down-regulated level of EDNRB receptor in $EdnrB^{-/+}$ mice is sufficient for normal functioning, as seen in normoxia ($P > 0.05$). However, in severe hypoxic conditions, a lower EDNRB modulates certain genes/pathways that would increase cardiac contractility, thus achieving higher CO.

Cardiovascular or metabolic parameters (such as the $dP{\cdot}dt_{max}^{-1}{\cdot}V_{ed}^{-1}$ and lactate level) started to change at a level of O_2 of ∼10–13%, altitudes that our Ethiopian subjects came from. Previous studies have also demonstrated indeed that HA dwellers have a lower chance of dying from ischemic heart disease (48), which may relate to better cardiac function. In our heterozygous mice, the remarkable cardiac contractility was associated with a higher peripheral blood flow and better O_2 delivery to vital organs (Fig. 3) especially under extreme hypoxic stress, as evidenced by a lower blood lactate level (Fig. 3*D*) and reduced hypoxic areas in vital organs (Fig. 3*E*).

To gain insight into the regulatory mechanisms that might explain the phenotypic advantage that we observed in $EdnrB^{-/+}$, we used a series of measures to shortlist specific candidate genes from the whole-genome transcriptome profile. In particular, we used a Bayesian approach, conditioning the criteria for mining specific genes and pathways. For example, because the phenotype is so different between the wild-type and the heterozygote, we were interested in focusing on genes that were statistically significant at baseline (normoxia) and during hypoxia and also on genes that changed in different directions when wild type and heterozygote were compared. As depicted, three counter-regulated genes (*Nppa*, *Sln*, and *Myl4*; $q < 0.05$) stand apart from the rest by having an average FPKM >100 and by RT-PCR validation. These three genes are differentially expressed in normoxia as well as hypoxia, and we believe that they are involved in the phenotypic difference between the $EdnrB^{+/+}$and $EdnrB^{-/+}$. Of interest, we note that these genes are expressed in the fetal heart (49, 50), possibly adding some evidence to a role in hypoxia, because gene expression of fetal transcripts can often show up during stress (51). As anticipated, the top-scoring networks for these genes were all related to cardiovascular function ranging from heart contraction, blood circulation, and the cGMP metabolic process to Ca^{2+} homeostasis (Fig. 4*F*). It is known that the endothelin system not only stimulates the secretion of *Nppa* (52) but that they also counteract each other in some cardiovascular, renal, and endocrine functions (53). Furthermore, previous studies have also demonstrated that *Nppa* secretion occurs via an EDNRB-mediated pathway involving the MAPK signaling pathway (54), and MAPK along with PI3K pathways regulate the hypoxia-induced *Nppa* secretion by controlling HIF-1α (55). Studies using *Nppa* null ($Nppa^{-/-}$) mice also provide evidence that Nppa plays an important role in pulmonary vascular adaptation to chronic hypoxia (56). Similarly, *Sln* regulates Ca^{2+} uptake through interaction with sarcoplasmic reticulum Ca^{2+} ATPase (SERCA). Like *Nppa*, *Sln* is also normally expressed at high levels in atria, and its expression is at very low/undetectable levels in ventricles (57), except in some conditions when it is increased by several-fold, such as in patients with preserved left ventricular ejection fraction and chronic isolated mitral regurgitation, where there is >10-fold increased expression of both *Nppa* and *Sln* (58). Therefore, altered *Nppa* and *Sln* expression may contribute to impaired Ca^{2+} handling, and our study indicates that their cross-talk with *EdnrB* could lead to better cardiac performance under extreme hypoxia. *Myl4*, the third prioritized gene, is also atria-specific in the adult. Interestingly, it is reexpressed in the ventricles of the diseased heart, suggesting a role in repair mechanisms (59). The up-regulation of *Myl4* in $EdnrB^{-/+}$ indicated cross-talk between the two genes (*EdnrB* and *Myl4*). This notion is supported by a recent study showing that *Myl4* in the left ventricle was differentially regulated under the protective influence of endothelin receptor inhibitors, including BQ788 (*EdnrB*-specific), which protects against doxorubicin-induced cardiomyopathy (60).

One question that can be raised is whether our results are relevant to understanding mechanisms of HA hypoxia adaptation. Although this question is difficult to answer, our observation that decreasing *EdnrB* expression (a gene we identified in HA dwellers) is beneficial for cardiovascular performance is in itself very illuminating. This is of particular interest especially because the results that we obtained are in acute hypoxia. Indeed, these data become relevant to the treatment of many more patients suffering from diseases that have acute hypoxia as part of their etiology at sea level.

In summary, we have demonstrated, for the first time to our knowledge, how an experiment in nature at high altitude in human adaptation could provide insight into disease mechanisms in acute forms involving hypoxia and ischemia at sea level. This is supported by our observation in knockout mice where $EdnrB^{-/+}$ mice were resistance to various levels of hypoxia, maintaining higher CO and peripheral perfusion and better DO_2 to vital organs, even in severe hypoxia. The idea that reducing the expression of functional EDNRB helps cells/tissues endure extreme hypoxia is appealing, and may forage novel therapy for cardiac failure in the near future. A transcriptome profile from ventricular tissue revealed three atria-specific genes, *Nppa*, *Sln*, and *Myl4*, which likely play a role in the improved cardiac performance during hypoxia in the heterozygote mouse.

Materials and Methods

Genomic Reanalysis of the Whole-Genome Seq Data. We systematically searched and compared published/publicly available data [Genotype-Tissue Expression (GTEx), Genetic European Variation in Health and Disease (GeuVadis), and ENCODE project] with our seq data (4) that may further support the regulation or disregulation of *EDNRB* [ideally via our observed SNPs as a significant expression quantitative trait loci (eQTL) for EDNRB] in human studies. See *SI Materials and Methods* for full methods.

Generation of the *EdnrB* Knockout and Its Validation. All animal care and handling were performed according to the protocols approved by the Animal Care Committee of the University of California, San Diego. We generated global knockout mice for *EdnrB*, where exon 3 of the *EdnrB* gene was completely removed, resulting in an out-of-frame transcript of exon 2–4 splicing. A schematic of the breeding strategy and its validation using RT-PCR are provided in *SI Materials and Methods* and Fig. S1*A*.

Mouse Preparation for Hypoxia Treatment. Animal handling and care followed the NIH *Guide for the Care and Use of Laboratory Animals* (61). Detailed steps are provided in *SI Materials and Methods*. Briefly, the mice were anesthetized to implant arterial catheters (PE-50) into the carotid artery and used for hypoxia treatment after a 24-h recovery. The inclusion and exclusion criteria are as mentioned in *SI Materials and Methods*. Further experimental details for the immunohistochemistry, ELISA, and statistical analysis are presented in *SI Materials and Methods*.

RNA-Seq Data Generation and Analysis. We used 500 ng of RNA with an RNA integrity number of 8 or greater to generate libraries using Illumina's TruSeq Stranded mRNA Sample Prep Kit. Details are in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Prof. Miles Epstein for providing the *EdnrB*^{flex} mice. We are grateful to Travis Smith and Orit Poulsen for technical assistance and F. Barra and C. Walser for surgical preparation of the animals. This work was supported by NIH Grants 1P01-HL098053 (to G.G.H.), P01-HL110900, R56HL123015, and R01-HL52684 (to P.C.), and NSF-IIS-1318386 (in part to V.B. and R.R.).

1. Allemann Y, Scherrer U (2010) High-altitude medicine: Important for trekkers and mountaineers, essential for progress in medicine. *Prog Cardiovasc Dis* 52(6):449–450.
2. Bigham A, et al. (2010) Identifying signatures of natural selection in Tibetan and Andean populations using dense genome scan data. *PLoS Genet* 6(9):e1001116.
3. Simonson TS, et al. (2010) Genetic evidence for high-altitude adaptation in Tibet. *Science* 329(5987):72–75.
4. Udpa N, et al. (2014) Whole genome sequencing of Ethiopian highlanders reveals conserved hypoxia tolerance genes. *Genome Biol* 15(2):R36.
5. Moore LG, Niermeyer S, Zamudio S (1998) Human adaptation to high altitude: Regional and life-cycle perspectives. *Am J Phys Anthropol* (Suppl 27):25–64.
6. Zhou D, et al. (2008) Mechanisms underlying hypoxia tolerance in *Drosophila melanogaster*: *hairy* as a metabolic switch. *PLoS Genet* 4(10):e1000221.
7. Zhou D, et al. (2011) Experimental selection of hypoxia-tolerant *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 108(6):2349–2354.
8. Zhou D, et al. (2013) Whole-genome sequencing uncovers the genetic basis of chronic mountain sickness in Andean highlanders. *Am J Hum Genet* 93(3):452–462.
9. Beall CM (2006) Andean, Tibetan, and Ethiopian patterns of adaptation to high-altitude hypoxia. *Integr Comp Biol* 46(1):18–24.
10. Beall CM, et al. (2002) An Ethiopian pattern of human adaptation to high-altitude hypoxia. *Proc Natl Acad Sci USA* 99(26):17215–17218.
11. Xing G, et al. (2008) Adaptation and mal-adaptation to ambient hypoxia; Andean, Ethiopian and Himalayan patterns. *PLoS One* 3(6):e2342.
12. Naeije R (2010) Physiological adaptation of the cardiovascular system to high altitude. *Prog Cardiovasc Dis* 52(6):456–466.
13. Kojonazarov B, et al. (2012) Bosentan reduces pulmonary artery pressure in high altitude residents. *High Alt Med Biol* 13(3):217–223.
14. Wanecek M, et al. (1999) Endothelin(A)-receptor antagonism attenuates pulmonary hypertension in porcine endotoxin shock. *Eur Respir J* 13(1):145–151.
15. Hori S, Komatsu Y, Shigemoto R, Mizuno N, Nakanishi S (1992) Distinct tissue distribution and cellular localization of two messenger ribonucleic acids encoding different subtypes of rat endothelin receptors. *Endocrinology* 130(4):1885–1895.
16. Bolte C, et al. (2012) Postnatal ablation of Foxm1 from cardiomyocytes causes late onset cardiac hypertrophy and fibrosis without exacerbating pressure overload-induced cardiac remodeling. *PLoS One* 7(11):e48713.
17. van Oort RJ, et al. (2006) MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. *Circulation* 114(4):298–308.
18. Clerk A, Sugden PH (1997) Phosphorylation c-Jun and ATF2 in ventricular myocytes by endothelin and phenylephrine. *Biochem Soc Trans* 25(2):222S.
19. Marchant D, et al. (2009) Bosentan enhances viral load via endothelin-1 receptor type-A-mediated p38 mitogen-activated protein kinase activation while improving cardiac function during coxsackievirus-induced myocarditis. *Circ Res* 104(6):813–821.
20. Tulchinsky E (2000) Fos family members: Regulation, structure and role in oncogenic transformation. *Histol Histopathol* 15(3):921–928.
21. He S, et al. (2013) Involvement of AP-1 and C/EBPβ in upregulation of endothelin B (ETB) receptor expression in a rodent model of glaucoma. *PLoS One* 8(11):e79183.
22. Druckenbrod NR, Powers PA, Bartley CR, Walker JW, Epstein ML (2008) Targeting of endothelin receptor-B to the neural crest. *Genesis* 46(8):396–400.
23. Arteel GE, Thurman RG, Raleigh JA (1998) Reductive metabolism of the hypoxia marker pimonidazole is regulated by oxygen tension independent of the pyridine nucleotide redox state. *Eur J Biochem* 253(3):743–750.
24. Warde-Farley D, et al. (2010) The GeneMANIA prediction server: Biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res* 38(Web Server issue):W214–W220.
25. Chen C, et al. (2013) Hypermethylation of EDNRB promoter contributes to the risk of colorectal cancer. *Diagn Pathol* 8:199.
26. Tykocki NR, Watts SW (2010) The interdependence of endothelin-1 and calcium: A review. *Clin Sci (Lond)* 119(9):361–372.
27. Merlen C, et al. (2013) Intracrine endothelin signaling evokes IP3-dependent increases in nucleoplasmic Ca^{2+} in adult cardiac myocytes. *J Mol Cell Cardiol* 62:189–202.
28. Schneider MP, Boesen EI, Pollock DM (2007) Contrasting actions of endothelin ET(A) and ET(B) receptors in cardiovascular disease. *Annu Rev Pharmacol Toxicol* 47: 731–759.
29. Heanue TA, Pachnis V (2007) Enteric nervous system development and Hirschsprung's disease: Advances in genetic and stem cell studies. *Nat Rev Neurosci* 8(6):466–479.
30. Lee HO, Levorse JM, Shin MK (2003) The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. *Dev Biol* 259(1):162–175.
31. Hosoda K, et al. (1994) Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* 79(7):1267–1276.
32. Eberle J, et al. (1999) Downregulation of endothelin B receptor in human melanoma cell lines parallel to differentiation genes. *J Invest Dermatol* 112(6):925–932.
33. Cruz-Muñoz W, et al. (2012) Roles for endothelin receptor B and BCL2A1 in spontaneous CNS metastasis of melanoma. *Cancer Res* 72(19):4909–4919.
34. Zuiverloon TC, et al. (2012) A methylation assay for the detection of non-muscle-invasive bladder cancer (NMIBC) recurrences in voided urine. *BJU Int* 109(6):941–948.
35. Wuttig D, et al. (2012) CD31, EDNRB and TSPAN7 are promising prognostic markers in clear-cell renal cell carcinoma revealed by genome-wide expression analyses of primary tumors and metastases. *Int J Cancer* 131(5):E693–E704.
36. Kuc RE, Maguire JJ, Davenport AP (2006) Quantification of endothelin receptor subtypes in peripheral tissues reveals downregulation of ET(A) receptors in ET(B)-deficient mice. *Exp Biol Med (Maywood)* 231(6):741–745.
37. Kedzierski RM, et al. (2003) Cardiomyocyte-specific endothelin A receptor knockout mice have normal cardiac function and an unaltered hypertrophic response to angiotensin II and isoproterenol. *Mol Cell Biol* 23(22):8226–8232.
38. Wanecek M, Weitzberg E, Alving K, Rudehill A, Oldner A (2001) Effects of the endothelin receptor antagonist bosentan on cardiac performance during porcine endotoxin shock. *Acta Anaesthesiol Scand* 45(10):1262–1270.
39. Krejci V, Hiltebrand LB, Erni D, Sigurdsson GH (2003) Endothelin receptor antagonist bosentan improves microcirculatory blood flow in splanchnic organs in septic shock. *Crit Care Med* 31(1):203–210.
40. Dimitrijevic I, et al. (2009) Increased expression of vascular endothelin type B and angiotensin type 1 receptors in patients with ischemic heart disease. *BMC Cardiovasc Disord* 9:40.
41. Dagassan PH, et al. (1996) Up-regulation of endothelin-B receptors in atherosclerotic human coronary arteries. *J Cardiovasc Pharmacol* 27(1):147–153.
42. Green DS, et al. (2013) A cell permeable peptide targeting the intracellular loop 2 of endothelin B receptor reduces pulmonary hypertension in a hypoxic rat model. *PLoS One* 8(11):e81309.
43. Meerson FZ, Pshennikova MG (1979) Effects of adaptation to high altitude hypoxia on the contractile function and adrenoreactivity of the heart. *Basic Res Cardiol* 74(2): 142–154.
44. Meerson FZ, Larionov NP, Makarova EK, Bogomolov AF (1975) [Contractile function and the effectiveness of oxygen utilization by the heart during adaptation to hypoxia]. *Kardiologiia* 15(7):70–78. Russian.
45. Ivy D, et al. (2001) Endothelin B receptor deficiency potentiates ET-1 and hypoxic pulmonary vasoconstriction. *Am J Physiol Lung Cell Mol Physiol* 280(5):L1040–L1048.
46. Ivy DD, et al. (2002) Exaggerated hypoxic pulmonary hypertension in endothelin B receptor-deficient rats. *Am J Physiol Lung Cell Mol Physiol* 282(4):L703–L712.
47. Kelland NF, et al. (2010) Endothelial ET(B) limits vascular remodelling and development of pulmonary hypertension during hypoxia. *J Vasc Res* 47(1):16–22.
48. Ezzati M, et al. (2012) Altitude, life expectancy and mortality from ischaemic heart disease, stroke, COPD and cancers: National population-based analysis of US counties. *J Epidemiol Community Health* 66(7):e17.
49. Zeller R, Bloch KD, Williams BS, Arceci RJ, Seidman CE (1987) Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. *Genes Dev* 1(7): 693–698.
50. Arnold HH, Lohse P, Seidel U, Bober E (1988) A novel human myosin alkali light chain is developmentally regulated. Expression in fetal cardiac and skeletal muscle and in adult atria. *Eur J Biochem* 178(1):53–60.
51. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H (2007) Return to the fetal gene program protects the stressed heart: A strong hypothesis. *Heart Fail Rev* 12(3-4):331–343.
52. Thibault G, Doubell AF, Garcia R, Larivière R, Schiffrin EL (1994) Endothelin-stimulated secretion of natriuretic peptides by rat atrial myocytes is mediated by endothelin A receptors. *Circ Res* 74(3):460–470.
53. Ota K, et al. (1992) Interaction of ANP with endothelin on cardiovascular, renal, and endocrine function. *Am J Physiol* 262(2 Pt 1):E135–E141.
54. Liu LP, et al. (2012) Ouabain stimulates atrial natriuretic peptide secretion via the endothelin-1/ET(B) receptor-mediated pathway in beating rabbit atria. *Life Sci* 90(19-20):793–798.
55. Zhang QL, et al. (2013) MAPK and PI3K pathways regulate hypoxia-induced atrial natriuretic peptide secretion by controlling HIF-1 alpha expression in beating rabbit atria. *Biochem Biophys Res Commun* 438(3):507–512.
56. Chen YF, et al. (2006) Atrial natriuretic peptide-dependent modulation of hypoxia-induced pulmonary vascular remodeling. *Life Sci* 79(14):1357–1365.
57. Vangheluwe P, et al. (2005) Sarcolipin and phospholamban mRNA and protein expression in cardiac and skeletal muscle of different species. *Biochem J* 389(Pt 1): 151–159.
58. Zheng J, et al. (2014) Increased sarcolipin expression and adrenergic drive in humans with preserved left ventricular ejection fraction and chronic isolated mitral regurgitation. *Circ Heart Fail* 7(1):194–202.
59. Hernandez OM, Jones M, Guzman G, Szczesna-Cordary D (2007) Myosin essential light chain in health and disease. *Am J Physiol Heart Circ Physiol* 292(4):H1643–H1654.
60. Schwebe M, et al. (2015) Protective effects of endothelin receptor A and B inhibitors against doxorubicin-induced cardiomyopathy. *Biochem Pharmacol* 94(2):109–129.
61. National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011) *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington, DC), 8th Ed.
62. Lakso M, et al. (1996) Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc Natl Acad Sci USA* 93(12):5860–5865.
63. Pacher P, Nagayama T, Mukhopadhyay P, Bátkai S, Kass DA (2008) Measurement of cardiac function using pressure-volume conductance catheter technique in mice and rats. *Nat Protoc* 3(9):1422–1434.
64. Baan J, et al. (1984) Continuous measurement of left ventricular volume in animals and humans by conductance catheter. *Circulation* 70(5):812–823.