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Comparative SAGE Analysis of the Response to Hypoxia in Human Pulmonary and Aortic Endothelial Cells

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Abstract

We have utilized Serial Analysis of Gene Expression (SAGE) to analyze the temporal response of human pulmonary artery endothelial cells (HPAECs) to short-term chronic hypoxia at the level of transcription. Primary cultures of HPAECs were exposed to 1% O_2 hypoxia for 8 and 24 hours and compared to identical same passage cells cultured under standard (5% CO_2 95% air) conditions. Hierarchical clustering of significant hypoxia-responsive genes identified temporal changes in the expressions of a number of well-described gene families including those encoding proteins involved in thrombosis, stress response, apoptosis, angiogenesis and cell proliferation. These experiments build upon previously published data describing the transcriptomic response of human aortic endothelial cells (HAECs) obtained from the same donor and cultured under identical conditions and we have thus taken advantage of the immortality of SAGE data to make direct comparisons between these two data sets. This approach revealed comprehensive information relating to the similarities and differences at the level of mRNA expression between HAECs and HPAECs. For example, we found differences in the cell type-specific response to hypoxia amongst genes encoding cytoskeletal factors, including paxillin, and proteins involved in metabolic energy production, the response to oxidative stress and vasoreactivity (e.g. endothelin 1). These efforts contribute to the expanding collection of publicly available SAGE data and provide a foundation upon which to base further efforts to understand the characteristics of the vascular response to hypoxia in the pulmonary circulation relative to systemic vasculature.

Introduction

Hypoxia is an important pathological stimulus that has profound effects on the vasculature. These include alteration of vascular tone, coagulant function, redox homeostasis, endothelial permeability, and cell proliferation. Significantly, the response to hypoxia is different in distinct vascular beds. Hypoxia elicits systemic vasodilation yet causes acute pulmonary vasoconstriction, which if sustained leads to profound remodeling of the pulmonary vasculature and culminates in structural-based increases in pulmonary vascular resistance and subsequent development of pulmonary hypertension (30, 33, 36).

Although the focus of intense research for nearly a century, the mechanisms underlying these differential vascular responses to hypoxia remain unclear (55). It has, however, been previously noted that fundamental molecular differences exist between the response of pulmonary and systemic vascular cells to hypoxia (19) but little information exists regarding the differences in genome-wide response to hypoxic stress between pulmonary and systemic vascular endothelial cells.

Despite the intense interest in the cellular response to hypoxia, particularly in the context of vascular biology, there have been few systematic attempts to document the transcriptional response to hypoxia in primary vascular endothelial cells. We have previously utilized Serial Analysis of Gene Expression (SAGE) to determine the temporal response to short-term chronic hypoxia in primary cultures of human aortic endothelial cells (HAECs) (40). The goals of this current study were to expand this database of hypoxia-responsive vascular gene expression by comprehensively characterizing the temporal response of human pulmonary artery endothelial

cells (HPAECs) grown under identical conditions and to directly compare these two data sets by taking advantage of the fact that SAGE provides immortal data. Novel statistical tools were thus used to identify similarities and differences between the transcriptomic response of HPAECs and HAECs to either 8 hours or 24 hours exposure to hypoxia (1% O_2).

Materials and Methods

Primary Endothelial Cell Culture and RNA Isolation

For SAGE and RTPCR/Northern data in Figures 2 and 3, human pulmonary artery endothelial cells (HPAEC) derived from a female donor (52 years old) were obtained at passage 4 from Clonetics (Clonetics, San Diego, CA). These cells were from the same donor as those previously used to assess the response to short-term chronic hypoxia in ECs derived from the systemic circulation (40) to minimize the potential for detecting genome-specific expression differences. This donor had a history of heart disease and type-2 diabetes and was a non-smoker. It is not known whether the donor was hypertensive. For the endothelin RTPCR (Figure 4), human HPAEC's and human aortic endothelial cells (HAEC's) were derived from a second female donor (5 years old). These were obtained at passage 3 from Clonetics (Clonetics, San Diego, CA). This donor had no known pathology. Cells were maintained in growth medium EBM-2 supplemented with EGM-2 (Clonetics), in a humidified incubator containing an atmosphere of 5% CO_2 and 95% air at 37^{o}C. Cells were split twice and grown to 90% confluence, then exposed to hypoxia (1%O_2, 5% CO_2, balanced N_2) for 8h and 24 h in a tightly sealed modular chamber (Billups-Rothenberg, DelMax, CA). Subsequently, total RNA from normoxic and

hypoxic treated endothelial cells was isolated by the Trizol reagent method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

SAGE

20 μg of total RNA was used to construct each SAGE library, as previously described (44) with some minor modifications (40). In brief, double stranded cDNA was synthesized from mRNA bound to oligo (dT) magnetic beads (Dynal Biotech, Lake Success, NY), using Superscript-II reverse transcriptase (Invitrogen). The cDNAs were cleaved with *NlaIII* (anchoring enzyme) and the most-3' terminal cDNA fragments were captured with magnetic beads and divided into two pools. Each pool was ligated to 5' biotinylated linker A or B (51), containing recognition site for the tagging enzyme BsmFI. After ligation, the beads were washed and the SAGE tags released from both pools by digestion with *BsmF*. Tags were blunted at their 3'ends and combined to form the 104 bp ditags-linker products, which were then amplified by PCR. The amplified ditags-linkers were redigested with *NlaIII* to remove the linkers and the ditags (26 bp) were isolated by gel electrophoresis and purified through Spin X tubes (VWR, West Chester, PA) and concatemerized by self-ligation. Concatemers with sizes between 500-2500 bp were obtained by gel purification and cloned into the *SphI* site of vector pZero (Invitrogen) and transformed into *Escherichia coli* strain DH10B (Invitrogen) by electroporation. For each library, about 1,200 colonies were random picked and plasmids with concatemer inserts were cycle sequenced with Big Dye terminator chemistry (Big Dye version 1, Applied Biosystems, Foster City, CA) and analyzed on a 3700 Applied Biosystems DNA sequencer (Applied Biosystems).

SAGE Data Analysis

The sequence file generated by the automated sequencer was analyzed using the SAGE 2000 Software (version 4.12; kindly provided by K.W. Kinzler and colleagues). After elimination of linker sequences and the duplicate ditags, the software was used to extract tags from the sequence file and create a report of the sequence and the occurrence of each of the transcript tags. Tags were matched to gene database entries using the Cancer Genome Anatomy Project SAGEGenie databse (http://cgap.nci.nih.gov/SAGE). Each specific transcript abundance was then determined by its unique tag count. Tag counts were normalized to 30,000 for each library.

Distribution of the Counting of a Tag

The analysis of SAGE data assumes that the distribution of tag counts follows a binomial distribution. Given a SAGE library of size *n*, the count of a type of tag *t* has a binomial distribution with parameters (*n,p*), where *p* is the relative frequency of tag *t*, or ideally, the gene represented by tag *t* in the original tissue/cell population (9).

Test for Differentially Expressed Genes in HPAEC Alone

Suppose we have *s* SAGE libraries. Let n_i be the size of the *i*th library, and X_i the counting of tag *t* in the *i*th library. Pearson's χ^2 statistic is defined as:

$$T = \sum_{i=1}^{s} (X_i - n_i \hat{p})^2 \left(\frac{1}{n_i \hat{p}} + \frac{1}{n_i (1 - \hat{p})} \right)$$

where $\hat{p} = \sum_{i=1}^{s} X_i \Big/ \sum_{i=1}^{s} n_i$

Asymptotically, under the null hypothesis that t is not differentially expressed, T has a χ^2_{s-1} distribution. Simulation studies show that for SAGE data, the asymptotic distribution is a good approximation to the exact distribution of T (under the null hypothesis). In this paper, we use the following level 5% test: *A tag t is differentially expressed if the T statistic for this tag is greater than the 95% quantile of the* χ^2_{s-1} *distribution.*

Control of the False Discovery Rate

Because we are testing the expression levels of thousands of tags simultaneously, we need to control the false discovery rate (FDR), i.e., among the tags claimed to be differentially expressed, the (average) percentage of the tags that actually are not differentially expressed. We use Benjamini and Hochberg's *Linear Step Up Multiple Comparison Procedure* (BH procedure) (4). The BH procedure first sorts the p-values of the test statistics $p_{(1)} \leq \ldots \leq p_{(k)}$ in ascending order, where k is the number of tests. To keep the average false discovery rate below a given level α we search for the largest i such that $p_{(i)} \leq \alpha\, i / k$, and reject all the null hypotheses whose p-values are smaller than $p_{(i)}$. Using this procedure, all the tags whose T statistics are greater than the $1-p_{(i)}$ quantile of the χ^2_{s-1} distribution will be considered differentially expressed. We apply the BH procedure only to the tags that are at least moderately expressed in one library, because we know in advance that a tag barely expressed in both the libraries is not likely to be differentially expressed. Genes that would not be considered differentially expressed when FDR is controlled at 5%, but would be considered differentially expressed without FDR control, were included in cases where they match genes of potential biological significance.

Test for Differentially Expressed Genes Between HPAEC Versus HAEC

The following test was used to identify genes that display different patterns of expression over the time course in the two groups of libraries. Let p_1, p_2, p_3, q_1, q_2, q_3 be the true concentration levels of gene G in the 3 pulmonary and 3 aortic tissues respectively. Pearson's χ^2 statistic can be used to test the null hypothesis that there is a constant r such that $p_i = rq_i$ for i = 1, 2, 3. The test statistic is:

$$T_2 = \sum_{i=1}^{3} (x_i - m_i \hat{p}_i)^2 \left(\frac{1}{m_i \hat{p}_i} + \frac{1}{m_i (1 - \hat{p}_i)} \right) + \sum_{i=1}^{3} (y_i - n_i \hat{r} \hat{p}_i)^2 \left(\frac{1}{n_i \hat{r} \hat{p}_i} + \frac{1}{n_i (1 - \hat{r} \hat{p}_i)} \right)$$

where $\hat{p}_i$ and $\hat{r}$ are maximum likelihood estimates of p_i and r under the null hypothesis obtained using iterative method. We chose a significance level of 5%, and accepted the alternative hypothesis that a gene's expression level changes over the time course of 24 hours following different patterns in the two groups of libraries if the T_2 statistic for this gene is greater than 5.99, the 95% quantile of the χ_2^2 distribution. Table 2 lists the genes whose T_2 statistic is greater than or equal to 5.99.

Real -Time Quantitative RT-PCR

Total RNAs were purified by the RNeasy® Mini Kit (Qiagen, Valencia, CA). Residual genomic DNA was eliminated by the DNA-free kit (Ambion, Austin, TX) according to the manufacturer's protocol, and quantified by spectrophotometry (Beckman DU 640). The optimal reverse transcription (RT) was carried out in 100-µl volumes as described (10) and two RNA inputs (100

and 400ng). No-reverse transcriptase controls were carried out with 400 ng of RNA. Quantitative PCR was performed on this cDNA on the ABI 7700 Sequence Detection Instrument (Applied Biosystems) using TaqMan^{R} MGB probes. QRT-PCR was carried out for four genes that were identified as being hypoxia-inducible genes in HPAEC by SAGE analyses. PCR primers and probe were ordered from Applied Biosystems (MMP2:Hs00234422_m1, SERPINE1:Hs00167155_m1, CAV:Hs00184697_m1, MET:Hs00228845_m1, CTGF:Hs00170014_ml). PCR amplification of cDNA derived from HPAECs (n=2) was performed in duplicate in 50-µl volumes as described (10) with the optimal primer and probe concentrations used for each gene (300nM for primer, 100 nM for probe). Gene expressions were measured relative to the endogenous reference gene, human ß-glucuronidase (*ß-GUS*), using the comparative C_T method described previously (10).

Results

We sequenced a total of 95,623 tags from SAGE libraries derived from primary cultures of human pulmonary artery endothelial cells grown under standard conditions (5% CO_2, 95% air) or exposed to 8 hours hypoxia and 24 hours hypoxia (1% O_2) respectively. Full data sets are available at the Gene Expression Omnibus (GEO: http://www.ncbi.nlm.nih.gov/geo/).

Identification of Differentially Expressed Tags

We identified 342 tags whose expressions varied significantly between the three experimental conditions. Within these 342 tags, 324 matched human Unigene clusters, 41 matched ESTs or other uncharacterized cDNA clones, 18 had no match to any UniGene entry and the remaining 283 tags matched known genes. The entire list of 346 differentially expressed tags, their database matches (if any) and relevant gene function is shown in supplemental data Table s1 (online supplemental data).

Hierarchical Clustering to Identify Genes Whose Expression Patterns are Similarly Affected by Hypoxia

We next performed hierarchical clustering analysis to identify clusters of genes whose expressions vary in a similar fashion following exposure to hypoxia. We identified 9 major clusters (1-9) of genes and these can be broadly defined as follows. Cluster 1 includes genes whose expressions are moderately increased or unchanged within 8 hours and then increased between 8 and 24 hours. Cluster 2 includes genes whose expressions are decreased between 0 and 8 hours and then moderately reduced or unchanged between 8 and 24 hours. Cluster 3 includes genes whose expressions are dramatically increased between 0 and 8 hours and then

dramatically decreased back to (or just above) baseline between 8 and 24 hours. Cluster 4 contains genes that are relatively unchanged between 0 and 8 hours and then dramatically decreased between 8 and 24 hours. Cluster 5 contains genes that are increased between 0 and 8 hours and then decreased to below baseline between 8 and 24 hours. Cluster 6 contains genes whose expressions are moderately decreased between 0 and 8 hours and then increased between 8 and 24 hours. Cluster 7 contains genes whose expressions are dramatically reduced between 0 and 8 hours and then dramatically increased between 8 and 24 hours. Cluster 8 contains genes whose expressions are slightly reduced between 0 and 8 hours and then further reduced between 8 and 24 hours. Finally, cluster 9 contains genes that are increased between 0 and 8 hours and then relatively unchanged or moderately increased between 8 and 24 hours. These data are summarized in Table s1 and examples of cluster-specific gene expression patterns are shown in Figure 1.

Functional Characteristics of Hypoxia-Responsive Genes in HPAECs

We utilized the Expression Analysis Systematic Explorer (EASE) (20) to match differentially expressed genes to gene ontology (GO) terms using the Biological Process category. EASE is able to perform "theme discovery", defined as the identification of terms or phrases that describe statistically significant genes in a list of genes (or in our case a cluster) with respect to the number of genes described by the term or phrase in the population of genes (the entire list of differentially expressed genes) from which the list derived.

Using EASE, we found a number of statistically significant enriched biological themes in specific clusters of genes (Table 1). Significance is based upon EASE scores of <0.05 (20). For

example, cluster 8 is enriched for genes encoding proteins involved in cell growth. The fact that the expressions of genes in cluster 8 are dramatically reduced by hypoxia in our data strongly suggests a coordinated reduction in cell cycle progression, which is consistent with our previous findings in aortic endothelial cells (40). Cluster 5 contains a significant number of genes involved in the response to oxidative stress, specifically periredoxin 1 and 6, which is consistent with previous observations (26, 41) but notably distinct from previous results in HAECs (40). Cluster 6 contains an overrepresentation of genes involved in cell adhesion/integrin mediated signaling, cluster 1 contains genes that encode proteins involved in cell communication and signal transduction and cluster 2 contains a preponderance of genes involved in protein and RNA biosynthesis.

Hypoxia Elicits a Prothrombotic Endothelial Phenotype

Genes involved in promoting thrombosis are increased by exposure to hypoxia in clusters 1 (SERPINE1, VWF) and 3 (PROCR) and there is a corresponding and dramatic reduction between 0h and 8h in the expression of TFPI, which is involved in the inhibition of coagulation. Notably, TFPI is then dramatically increased between 8h and 24h, which suggests the possibility of a homeostatic feedback loop to regulate thrombotic potential in endothelial cells. Similar results were previously observed in HAECs (40).

Hypoxia Causes a Coordinated Elevation in Stress Response Genes

We also found a clear and coordinated increase in the expressions of a number of genes that encode stress response proteins. These data are consistent with previously reported observations in HAECs (40). The majority of these were co-expressed in cluster 3 (Table s1) and include, for

example, heat shock 70kDa protein 8 (HSPA8), heat shock 90kDa protein 1, alpha (HSPCA), DnaJ (Hsp40) homolog, subfamily B, member 1 (DNAJB1), DnaJ (Hsp40) homolog, subfamily C, member 8 (DNAJC8), heat shock 70kDa protein 5 (HSPA5), heat shock 70kDa protein 1A (HSPA1A), heat shock 90kDa protein 1, alpha (HSPCA) and glutathione S-transferase pi (GSTP1). Cluster 3 also contains basic helix-loop-helix domain containing, class B, 2 (BHLHB2) and it has previously been suggested that this transcription factor is a critical component of the cellular response to hypoxia (37). We also found a number of other stress response genes whose expressions were elevated by hypoxia that did not reach statistical significance but were previously shown to be responsive to hypoxia in HAECs (40). These include heat shock 60kD protein 1 (HSPD1) (~2-fold), heat shock 10kD protein 1 (HSPE1) (~3-fold), heat shock 105kD (HSPH1) (~6-fold) and chaperonin containing TCP1, subunit 6A (CCT6A) (~1.7-fold).

Short-Term Chronic Hypoxia Elicits Coordinated Changes in the Expressions of Apoptotic Genes in HPAECs

In keeping with our previous observations in HAECs, we found that hypoxia resulted in gene expression changes that are consistent with a pro-apoptotic molecular phenotype. For example, as shown in Table s1, lymphotoxin beta receptor (LTBR), reticulon 4 (RTN4), etoposide induced 2.4 mRNA (EI24), peptidylprolyl isomerase F (PPIF) and programmed cell death 4 (PDCD4) were all significantly increased by hypoxic exposure. Similarly, a number genes previously identified in HAECs were elevated although these did not reach statistical significance. These include, apoptosis-inducing factor (AMID) (~4-fold) and BCL2/adenovirus E1B 19kD-interacting protein 3-like (BNIP3L) (~3-fold). Notably, the antiapoptotic factor

BCL2/adenovirus E1B 19kDa interacting protein 2 (BNIP2) was significantly reduced in HPAECs within 8 hours following the onset of hypoxia and this reached significance in our HPAEC data (Table s1).

Exposure to Hypoxia Results in an Antiproliferative Phenotype in HPAECs

Exposure to hypoxia resulted in both an increase in the expressions of genes encoding antiproliferative factors and a reduction in genes encoding proteins involved in cell cycle progression in HPAECs (Table s1). For example, the antiproliferative genes sialomucin (CD164) and Insulin-like growth factor binding protein 7 (IGFBP7) were both found to be significantly elevated by hypoxia. Similarly, there were decreases in the expressions of a number of cell cycle-associated genes previously identified as hypoxia inducible in HAECs such as cyclin D1 (CCND1) (~2-fold reduction at 24h), minichromosome maintenance deficient 2 (MCM2) (~5 fold reduction at 24h), enhancer of rudimentary homolog (ERH) (~2-fold reduction at 8h) (Table s1). As previously demonstrated in HAECs (40) there was a concomitant increase in the negative cell cycle regulator retinoblastoma-binding protein 1 (RBBP1), (~3-fold reduction).

Hypoxia Causes Changes in the Expression of Genes Encoding Extracellular matrix Factors

Short-term chronic hypoxia also caused significant elevation in the expressions of genes encoding extracellular matrix factors (Table s1). These include procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), lysyl oxidase-like 2 (LOXL2), microfibrillar-associated protein 2 (MFAP2), connective tissue growth factor (CTGF), matrix metalloproteinase 2 (MMP2) and EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1). With the

exception of MFAP2 all these genes were also significantly upregulated by hypoxia in HAECs (40).

Other Notable Hypoxia-Responsive Genes in HPAECs

A number of genes of significant functional interest were also altered by exposure of HPAECs to hypoxia. These include angiopoietin-like 4 (ANGPTL4) in cluster 1 which we previously found to be similarly elevated in HAECs (40) and cysteine-rich motor neuron 1 (CRIM1) in cluster 6, both of which have been shown to be involved in angiogenesis (16, 27). Elevation of ANGPTL4 in response to hypoxia is consistent with previous reports (29) and may indicate an angiogenic response to hypoxia. Furthermore, we found that the transcription factor, signal transducer and activator of transcription 3 (STAT3) was undetectable at 0h but rapidly induced by 8h hypoxia in HPAECs whereas STAT5B was reduced between 0h and 8h hypoxia. STAT3 has been shown to be involved in the protective cellular response to hypoxic injury (47) and is involved in the HIF-1 alpha-dependent induction of vascular endothelial growth factor (VEGF) in renal (25) and prostate carcinoma cells (18). STAT5B has recently been shown to be involved in cell proliferation in vascular endothelial cells (13).

Confirmation of HPAEC Expression Changes by RTPCR

Real time TaqMan RTPCR was carried out on five genes (CAV1, MET, MMP2, SERPINE1 and CTGF) to confirm the transcriptional changes identified by SAGE. These were chosen for further analysis because they are all well characterized genes and representative of a broad range of functional classes. It can be seen from Figures 2A and 2B that the hypoxia-responsive differential expression identified by SAGE was quantitatively corroborated by RTPCR for these

five genes.

Direct Comparison of the Response to Short-Term Chronic Hypoxia in HPAECs Versus HAECs

We took advantage of the digital nature of SAGE data to formally compare our HPAEC data with previously published SAGE data derived from HAECs grown under identical conditions and using cells obtained from the same donor to reduce potential confounding results due to polymorphic genetic variation. Genes whose expressions were found to differ significantly between HPAECs and HAECs are shown in Supplementary Table s2.

In general we found marked similarity between the HPAEC and HAEC transcriptomes under both normoxic and hypoxic conditions. Despite this, however, there was limited overlap between the genes flagged as significant when SAGE data from HPAEC and HAECs were analyzed independently. This likely reflects the high stringency at which significant genes were flagged since overall trends between the two cell types are highly similar. Specifically, 25 of 354 (7%) significantly altered pulmonary endothelial genes were found to overlap with an identical analysis of aortic endothelial genes. Table 2 shows that the genes that did significantly overlap were strongly representative of a small number of functional groups including those involved in extracellular matrix structure and remodeling, metabolic energy production and the response to stress. Other important groups such as blood clotting and angiogenesis were also represented.

Table s2 shows that the genes that distinguish HPAEC from HAECs include representatives from a variety of functional classes. One interesting feature of the comparison between the HPAECs and HAECs under hypoxic conditions is the response of genes encoding factors involved in metabolic energy production. For example, ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1 (ATP5G1) and ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (ATP5O), which encode components of the F-type ATPase complex are differentially modulated in the two cell types. Specifically, we found a strong reduction in expression following 24h exposure to hypoxia in HPAECs versus either an induction (ATP5O) or only moderate reduction (ATP5G1) in HAECs. Similarly, we found that transcription of suppressor of S. cerevisiae gcr2 (HSGT1), which is a transcriptional regulator of glycolytic genes, was reduced by hypoxia in HPAECs and induced in HAECs and adenylate kinase 1 (AK1) was elevated in HPAECs and reduced in HAECs. Notably, glycerol-3-phosphate dehydrogenase 2 (GPD2), which is involved in lipid metabolism, was elevated by hypoxia in HPAECs and reduced in HAECs. Patatin-like phospholipase domain containing 2 (PNPLA2), which is also involved in lipid metabolism showed a similar pattern of expression to that of GPD2.

Significant transciptional changes between the two cell types in a number of genes involved in the response to oxidative stress were observed. For example, the thioredoxin reductase 1 (TXNRD1) and thioredoxin-like 1 (TXNL1) were elevated in HPAECs and reduced in HAECs. Ferridoxin reductase gene (FDXR) was reduced by hypoxia in HPAECs and induced in HAECs. Similarly, peroxiredoxin 6 (PRDX6) was induced between 0 and 8h in HPAECs and then dramatically reduced between 8h and 24h whereas it was relatively unchanged in HAECs.

Genes encoding cytoskeletal factors were also differentially responsive to short-term chronic hypoxia in HPAECs versus HAECs (Table s2). For example, LIM domain kinase 2 (LIMK2) is repressed in HPAECs and induced in HAECs by hypoxia, as are a number of other genes including emerin (EMD) and titin-cap (TCAP). One gene of particular interest in this context is paxillin (PXN). Although PXN differential expression in HPAECs (tag ATTTTCAAAA) did not reach statistical significance, we found its expression to be induced 6.5-fold between 0h and 8h (Table s1). We have previously shown that PXN is not altered at the level of transcription by hypoxia in HAECs (40). We further explored this apparent difference between HPAECs and HAECs at the level of transcription. The northern analysis data presented in Figure 3 shows that this cell type-specific induction is indeed confined to HPAECs.

Other significant differences between HPAECs and HAECs include the induction of inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta (IKBKB), which is reduced by 8h in HPAECs and induced in HAECs; latent transforming growth factor beta binding protein 2 (LTBP2), which is increased in HPAECs and reduced in HAECs; and the propapoptotic factor tumor necrosis factor (ligand) superfamily, member 14 (TNFSF14), which is unchanged in HPAECs and dramatically increased in HAECs. Of particular interest is the observation that corin, serine protease (CORIN) is dramatically induced by 8h hypoxia in HPAECs but unaffected in HAECs. The fact that this gene encodes a protein involved in blood pressure regulation, whose absence has been shown to lead to elevated blood pressure (6) is intriguing given that hypoxia is known to have a constrictive effect in pulmonary vessels. We also noted a dramatic reduction in the expression at 8h in HPAECs of CREBBP/EP300 inhibitor 1 (CRI1),

which is significant given the documented involvement of CREBBP/EP300 in gene transcription via the hypoxia-inducible factor (HIF) pathway (28) and the fact that we also found significant cell type-specific differences in the response of known HIF1-alpha-inducible genes to hypoxia Table 3).

We also found that the endothelin gene (EDN1) was differentially responsive in HPAECs compared to HAECs. Specifically, we found EDN1 to be elevated by ~2.5-fold in HPAECs between 8h and 24h hypoxia, whereas it was unresponsive in HAECs. This observation was confirmed by real time PCR (Figure 4), using RNA derived from a second female donor, in which the magnitude of fold change between HPAECs versus HAECs at 24h was 1.5-fold.

Finally, we also observed alterations in the expressions of genes involved in the pathobiology of Alzheimer's Disease (AD) including amyloid beta (A4) precursor protein (APP), amyloid beta (A4) precursor-like protein 2 (APLP2), beta-site APP-cleaving enzyme 2 (BACE2) and amyloid beta (A4) precursor protein-binding, family B, member 1 (APBB1). All of these are elevated in HPAECs and, with the exception of BACE2, are relatively unchanged (or slightly down-modulated) by hypoxia in HAECs. In contrast, anterior pharynx defective 1 homolog A (APH-1A) is significantly reduced by hypoxia in HPAECs and unchanged in HAECs. These changes were statistically significant in HPAECs except for APBB1 (2-fold increase at 24h).

Identification of Previously Described Hypoxia-Responsive Genes

A number of known hypoxia responsive genes are known to be regulated specifically by the activities of hypoxia inducible factor 1 (HIF-1) alpha. We therefore compared our data with

previous data in a search for genes that may be modulated by HIF1-alpha. Table 3 shows that our data are in general agreement with previously published data. For example, known hypoxia-responsive genes such as adrenomedullin, aldolase A, endothelin1, enolase 1, gluose transporter 1, glyceraldehyde phosphate dehydrogenase, hexokinase 2 and lactate dehydrogenase A, p21, phosphofructokinase, phosphoglycerate kinase 1 and plasminogen activator inhibitor 1 were all found to be increased by exposure to hypoxia. However, aldolase C, endothelin converting enzyme 1, hemeoxygenase1 and pyruvate kinase M were not and a number of other known HIF1-alpha inducible genes were expressed at levels too low to make any comparison possible.

Discussion

We present a comprehensive analysis of the temporal genomic response to short-term chronic hypoxia at the level of transcription in primary cultures of HPAECS. This global and unbiased analysis builds upon previous SAGE analysis of HAECs cultured under identical conditions and provides a resource for the characterization of the endothelial cell transcriptome under pathologically important stresses. SAGE is a valuable tool in this context since the resulting data is considered to be immortal and can be readily compared to other distinct SAGE data generated at different times and in different laboratories (22). Thus, in addition to identifying a number of genes representing distinct functional classes whose expressions are modulated by short-term chronic hypoxia in HPAECs, we have also identified genes whose expressions are altered in both a similar or disparate fashion in HAECs. We have also identified numerous uncharacterized hypoxia-responsive genes. Clearly these observations will require further confirmation at the level of protein expression and *in vivo.* Confirmation of these data is important given the complex mechanisms by which hypoxia is known to alter physiology *in vivo*, such as by altering

shear stress via polycythemia, that cannot be reproduced in a model cell-based system such as ours.

In general, we found that the response of HPAECs for a number of gene categories (e.g. heat shock, cell cycle, apoptosis, glycolysis/ATP, extracellular matrix, thrombosis) was markedly similar to that previously described for HAECs cultured under identical conditions (40). The significance of these gene families in the endothelial response to hypoxia has been discussed previously (40).

There were, however, notable differences between the response to hypoxia between HPAECs and HAECs. These include genes encoding proteins that are involved in cellular protection against oxidative stress such as PRDX6 (7) and FDXR, which has recently been shown to sensitize cells to oxidative stress-induced apoptosis (31). Also differentially responsive were genes involved in thioredoxin signaling including thioredoxin reductase 1 (TXNRD1) and thioredoxin-like 1 (TXNL1). Interestingly, thioredoxin activity is thought to lead to elevated hypoxia-inducible factor 1alpha (HIF1A) protein expression resulting in increased vascular endothelial growth factor (VEGF) expression and angiogenesis (52) and thioredoxin TXNDC5 has been shown to be involved in cytoprotective response to hypoxia (45).

Another significant outcome of our comparison of the cell type-specific response to hypoxia was the observation that cytoskeletal genes are differentially expressed in HPAECs versus HAECs including LIMK2 and PXN. The LIMK2 protein is phosphorylated and activated by ROCK, a downstream effector of Rho, and once activated in this fashion it phosphorylates cofilin,

inhibiting its actin-depolymerizing activity. It is thought that this pathway contributes to Rho-induced reorganization of the actin cytoskeleton (50) and, significantly, Rho signaling has been shown to be critically important for hypoxia-dependent alterations in endothelial cell structural alterations (2). Furthermore, it has been previously demonstrated that HAECs display significantly greater motility in response to hypoxia than do HPAECs (38). In keeping with this, Tian and Phillips (2003) (48) showed that paxillin expression is inversely correlated with motility. The fact that we observed elevated expression of paxillin (PXN) in HPAECs supports these findings.

The fact that EDN1 was only hypoxia-responsive in HPAECs is significant. Endothelin is a well characterized vasoconstrictor whose hypoxia-responsive mRNA induction has been previously described in umbilical vein endothelial cells (21). Furthermore, it is known that EDN1 is a major mediator of hypoxia-induced pulmonary vasoconstriction (8). This cell type-specific hypoxia responsive induction of EDN1 clearly deserves further investigation at the functional level.

Also significant with regard to the difference between the HPAEC- and HAEC-specific responses to hypoxia is the fact that four genes associated with Alzheimer's Disease (AD) pathobiology, APP, BACE2, APBB1 and APLP2 were coordinately elevated by exposure to hypoxia whilst APH-1H was reduced. These changes were statistically significant. In contrast, in our previous analysis of the hypoxia responsive transcriptome in HAECs we did not find these genes to be significantly altered although BACE2 was upregulated at 24h (40). Numerous reports have linked the expression of AD-associated genes with hypoxia and ischemia but these observations have almost exclusively been made in neuronal tissue (3, 39).

In conclusion, we have used SAGE to characterize the global temporal response of HAECs to short-term chronic hypoxia at the level of transcription. This identified numerous hypoxia-responsive genes representing a variety of functional classes. This information can be collated to build up a relatively detailed picture of the way in which HAEC molecular physiology is reprogrammed following exposure to hypoxia. In addition to providing comprehensive data regarding the hypoxia-responsive HCAEC transcriptome *in vitro,* it provides a foundation for further studies of the molecular mechanisms by which cells respond to hypoxic stress. Further experiments will require validation of our findings in experimental systems that more closely represent physiological conditions. Until then, the current data provide a reference point for biologists interested in the genomic response to hypoxia in an *in vitro* vascular model system.

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Figure 1. Normalized tag count plotted against the duration of exposure to hypoxia for representative genes from each of clusters 1-9 (A-I respectively).

Figure 2(A). The relative expression level of selected genes in HPAECs after 8 and 24 h exposure to hypoxia (1% O_2) confirmed by Taqman RTPCR. CAV1 = caveolin 1, MET = met proto-oncogene, MMP2 = matrix metalloproteinase 2, SERPINE1 = plasminogen activator inhibitor type 1, CTGF = connective tissue growth factor. Normalized tag counts are displayed on the vertical axis. **(B)**. Comparison between fold change in mRNA expression between samples determined by SAGE and TaqMan. Expression changes are shown relative to time = 0h.

Figure 3. Northern analysis of paxillin expression in HPAECs and HAECs at 0h, 8h and 24h exposure to hypoxia (1% O_2). Corresponding SAGE tag counts are indicated by *.

Figure 4. The relative expression level of endothelin 1 in HAECs and HPAECs after 8 and 24 h exposure to hypoxia (1% O_2) confirmed by Taqman RTPCR

Supplementary Table s1. Genes whose expressions were significantly modulated by hypoxia in HPAECs. Genes in bold did not reach statistical significance but are discussed in the text because they have functional significance and are altered by hypoxia. Tag counts are shown for each time point and are expressed as tags per 30,000.

Supplementary Table s2. Genes whose hypoxia-responsive expression patterns were significantly different between HPAECs versus HAECs. Tag counts are shown for each time point and are expressed as tags per 30,000.

Figure 1.

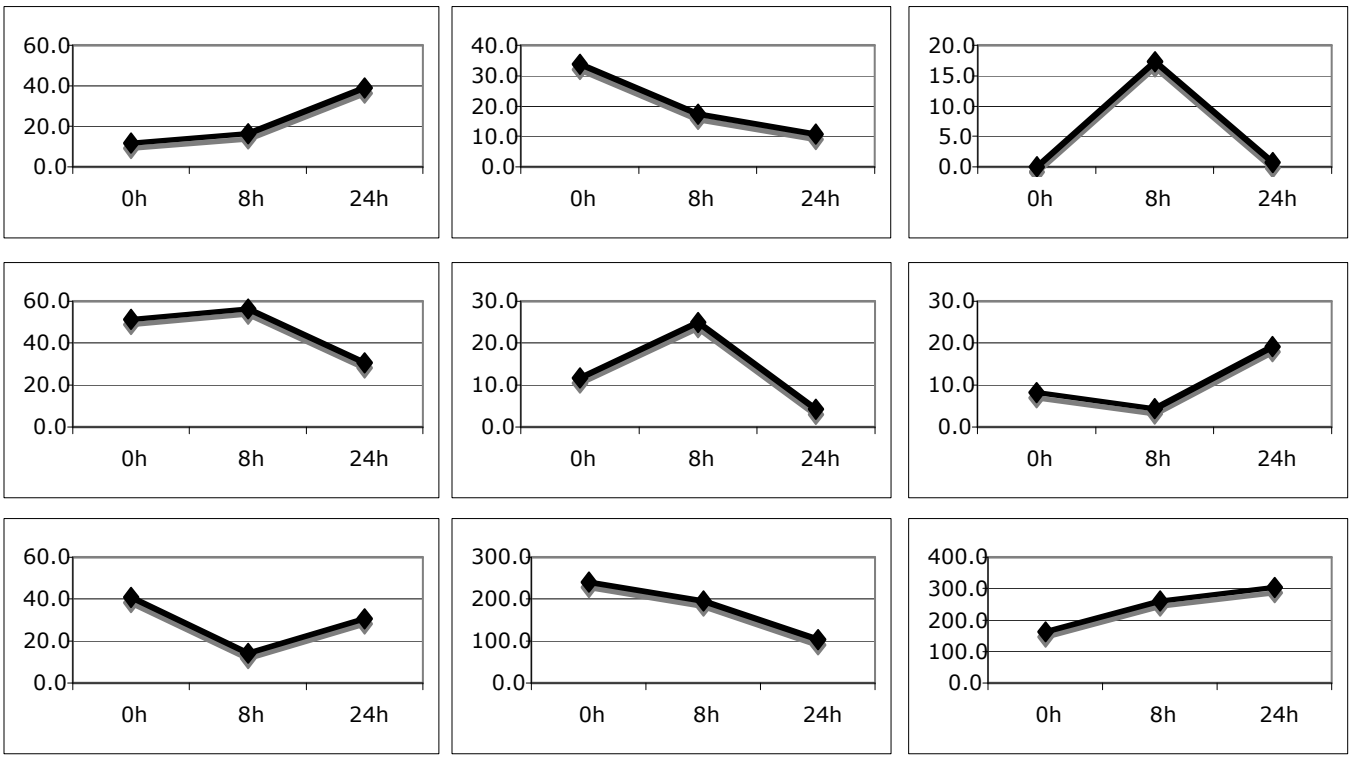
60.0
40.0
20.0
0.0
0h
8h
24h
40.0
30.0
20.0
10.0
0.0
0h
8h
24h
20.0
15.0
10.0
5.0
0.0
0h
8h
24h
60.0
40.0
20.0
0.0
0h
8h
24h
30.0
20.0
10.0
0.0
0h
8h
24h
30.0
20.0
10.0
0.0
0h
8h
24h
60.0
40.0
20.0
0.0
0h
8h
24h
300.0
200.0
100.0
0.0
0h
8h
24h
400.0
300.0
200.0
100.0
0.0
0h
8h
24h

Figure 2A

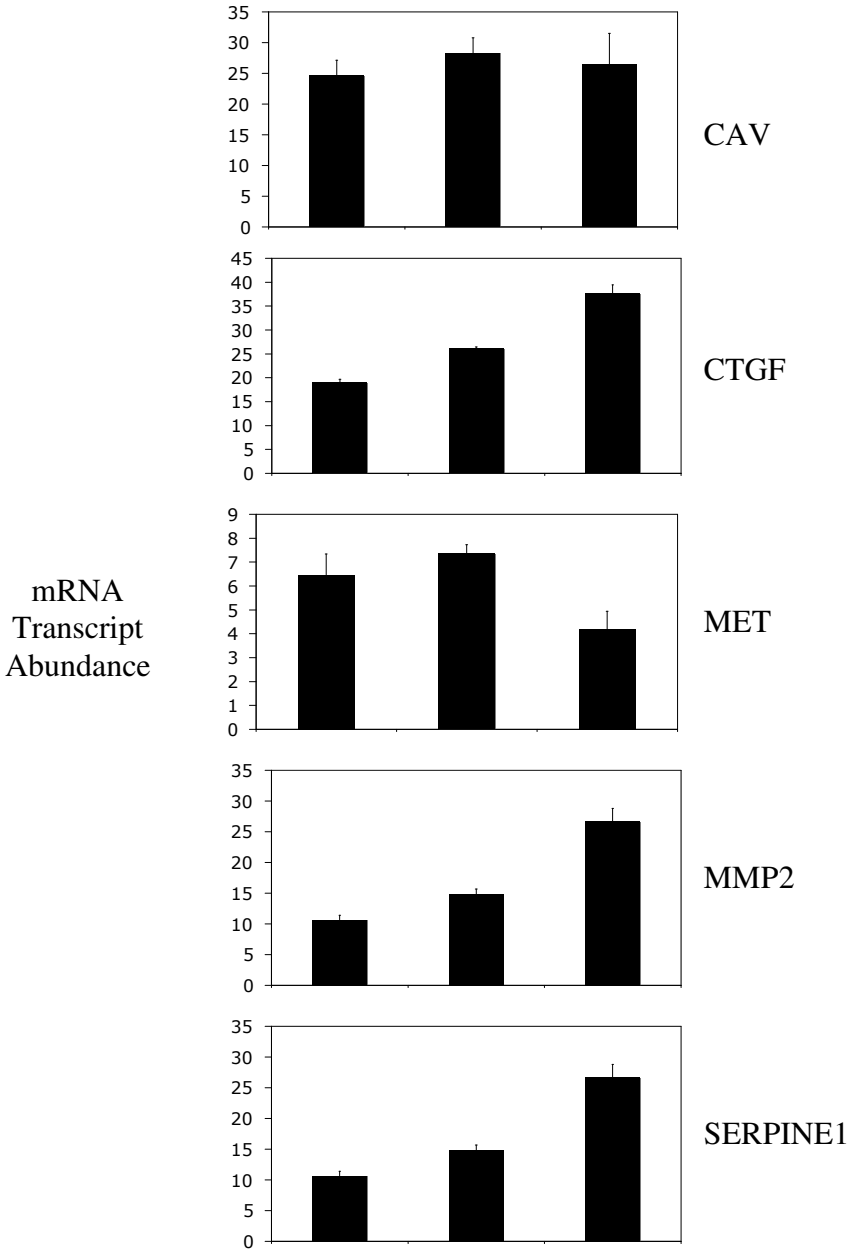

Figure 2B

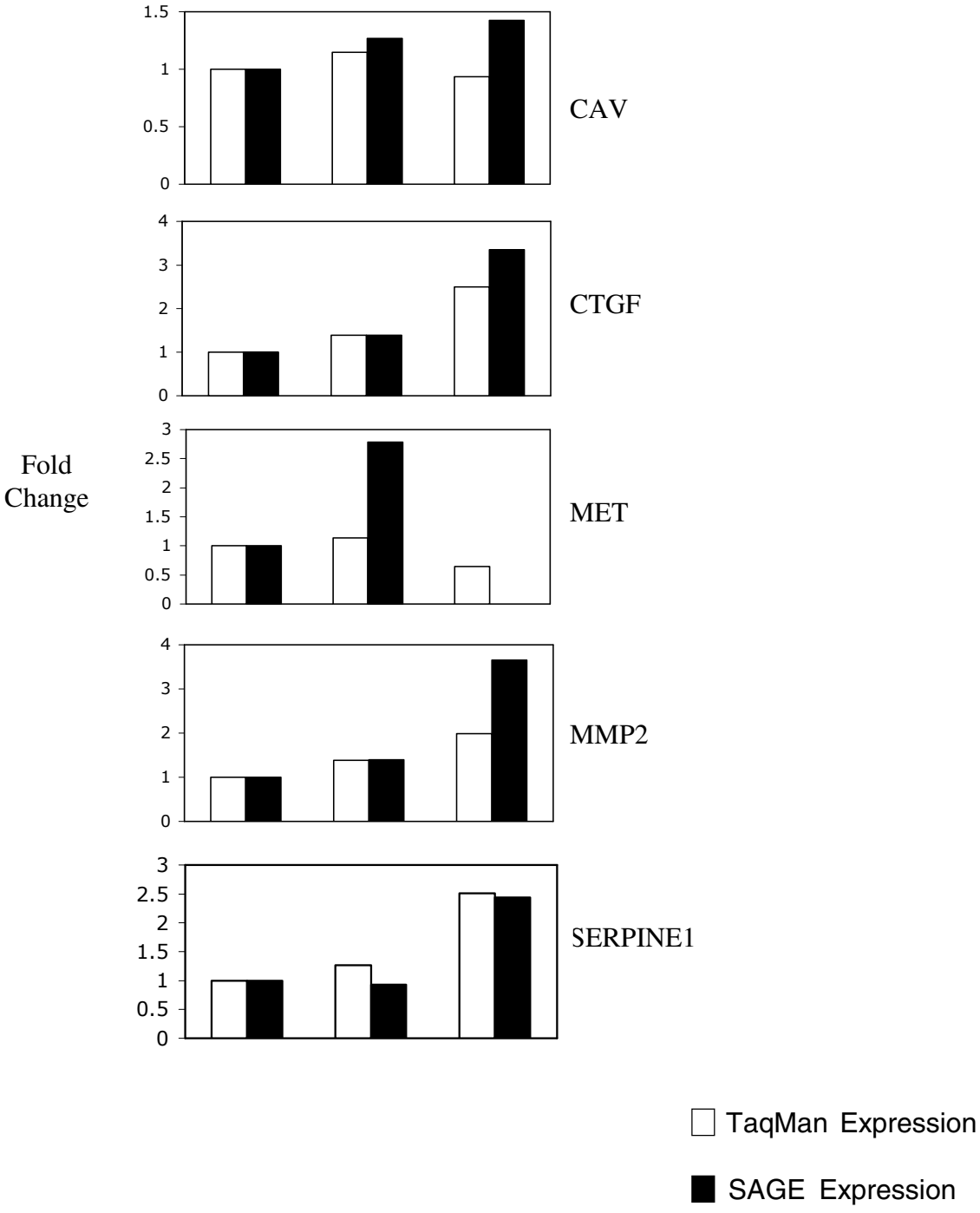
Fold Change
CAV
CTGF
MET
MMP2
SERPINE1
TaqMan Expression
SAGE Expression

Figure 3

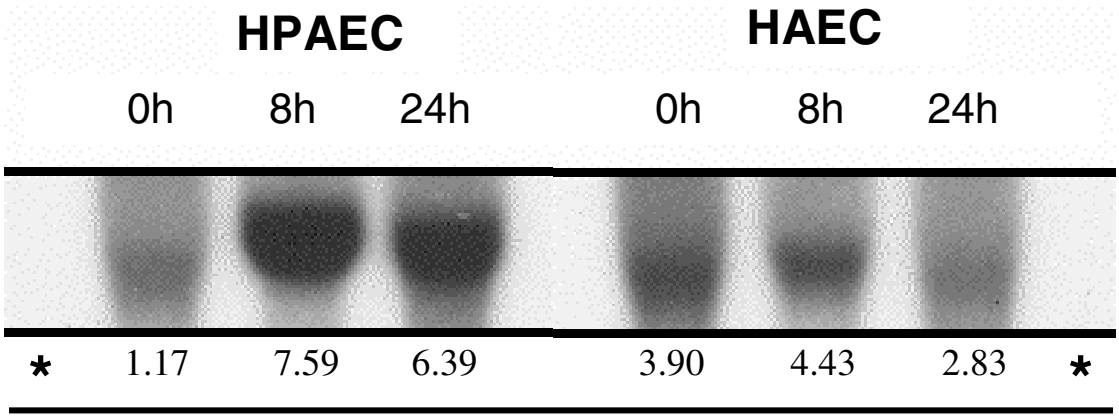

Figure 4

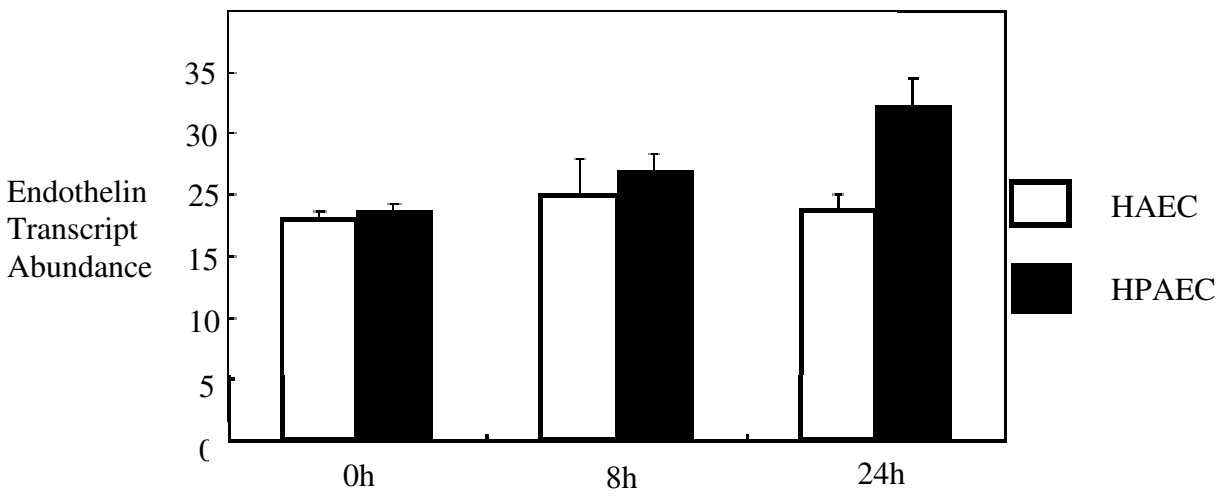
Endothelin
Transcript
Abundance
35
30
25
15
10
5
0h
8h
24h
HAEC
HPAEC

Table 1. EASE analysis. Matching differentially expressed genes to gene ontology (GO) terms using the Biological Process category. Unigene accession numbers are shown for members of statistically significant gene categories (p=<0.05).

Gene Category	EASE score	Unigene clusters	Cluster
cell communication	0.003	HS.1116; HS.15154; HS.26010; HS.280342; HS.410104; HS.421986; HS.440848; HS.447899	1
signal transduction	0.028	HS.1116; HS.26010; HS.280342; HS.410104; HS.421986; HS.447899	1
protein biosynthesis	0.002	HS.118183; HS.256184; HS.333823; HS.386384; HS.408018; HS.408073; HS.418233	2
RNA metabolism	0.002	HS.118183; HS.130098; HS.168799; HS.77897; HS.83753; HS.98541	2
macromolecule biosynthesis	0.004	HS.118183; HS.256184; HS.333823; HS.3439; HS.386384; HS.408018; HS.408073; HS.418233	2
metabolism	0.036	HS.106534; HS.118183; HS.130098; HS.168799; HS.178728; HS.181300; HS.250581; HS.256184; HS.268742; HS.333823; HS.3439; HS.374262; HS.386384; HS.408018; HS.408073; HS.418233; HS.436187; HS.436687; HS.5298; HS.77897; HS.83753; HS.89545; HS.98541	2
metabolism	0.030	HS.120; HS.16130; HS.178551; HS.180909; HS.374503; HS.381072; HS.409065; HS.422585; HS.433529; HS.446522; HS.5245; HS.78183	5
response to oxidative stress	0.043	HS.120; HS.180909	5
integrin-mediated signaling pathway	0.027	HS.158237; HS.410037	6
cell adhesion	0.048	HS.158237; HS.410037; HS.76206	6
cell growth and/or maintenance	0.039	HS.108371; HS.170622; HS.250758; HS.26516; HS.414565; HS.429; HS.7476; HS.789	8
proton transport	0.058	HS.429; HS.7476	8

Tag	Cluster	HAEC 0h	HAEC 8h	HAEC 24h	chi2 HAEC	HPAEC 0h	HPAEC 8h	HPAEC 24h	chi2 HPAEC	Unigene	Symbol	Name	Function
CAGAGATGAA	1	2	11	0	21.0	0	17	1	35.2	8997	HSPA1A	heat shock 70kDa protein 1A	stress response
GACCGAGGTG	1	0	4	0	10.0	0	3	0	7.4	129953	EWSR1	Ewing sarcoma breakpoint region 1	unknown
TGCATCTGGT	1	55	88	64	10.8	41	94	44	30.3	75410	HSPA5	heat shock 70kDa protein 5	stress response
CTGTACAGAC	2	66	52	33	15. 0	51	56	31	10.2	433615	TUBB2	Tubulin beta 2	cytoskeletal
GATCCCAACT	2	66	56	35	13.2	55	38	23	15.6	118786	MT2A	metallothionein 2A	cytoprotective
GGCTGGGGGC	2	112	103	69	14.5	118	92	56	25.8	75721	PFN1	profilin 1	cytoskeletal
TGCAGTCACT	2	400	340	221	72.1	240	195	104	66.3	83169	MMP1	matrix metalloproteinase 1	extracellular matrix
GAAAACAAAG	3	4	1	0	7.7	0	3	0	7.4	99936	KRT10	keratin 10	cell structure
GAAGGCATCC	3	10	4	2	9.2	8	7	1	6.1	250758	PSMC3	proteasome 26S subunit, ATPase, 3	protein catabolism
GGGGGTCACC	3	23	12	13	6.9	12	25	4	19.5	80986	ATP5G1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, isoform 1	metabolic/ energy
GTTTCAAACG	3	8	4	1	8.6	12	4	3	7.8	180535	URP2	UNC-112 related protein 2	cytoskeletal
CTATGGCTTC	4	9	1	6	8.4	0	2	6	6.9	75618	RAB11A	RAB11A, member RAS oncogene family	signal transduction
GCTGACGTCA	4	101	72	94	7.3	60	62	114	26.3				unknown
GTGCTGGTGC	5	1	2	7	8.6	0	4	12	12.8	9613	ANGPTL4	angiopoietin-like 4	angiogenesis
TGTTAGAAAA	5	5	11	16	7.1	8	4	19	11.7	41270	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	extracellular matrix
CAAGGGTAAG	5	54	71	96	16.1	105	115	171	21.7	76224	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	extracellular matrix
TACCATCAAT	5	181	219	266	22.2	163	260	304	42.1	169476	GAPD	glyceraldehyde-3-phosphate dehydrogenase	metabolic/ energy
TAGGTTGTCT	5	187	233	261	16.4	212	241	295	15.8	279860	TPT1	tumor protein, translationally-controlled 1	unknown
CTGCTAAGGT	6	4	0	8	10.6	4	0	0	8.2	49500	KIAA0746	KIAA0746 protein	unknown
GCCCCTGAAG	6	2	0	4	6.5	6	0	1	9.9	152663	PAK3	p21 (CDKN1A)-activated kinase 3	signal transduction
GGAAATGTCA	6	17	13	29	9.3	12	16	39	20.0	111301	MMP2	matrix metalloproteinase 2	extracellular matrix
TGTCATCACA	6	23	24	38	6.4	20	17	35	8.2	83354	LOXL2	lysyl oxidase-like 2	extracellular matrix
GGGTTGGCTT	6	17	18	45	25.4	29	16	37	8.3	73818	UQCRH	ubiquinol-cytochrome c reductase hinge protein	metabolic/ energy
TGAGGGAATA	6	46	44	80	19.6	49	75	78	7.1	83848	TPI1	triosephosphate isomerase 1	metabolic/ energy

Table 3. Comparison of SAGE-identified hypoxia-responsive genes to previously identified HIF-1 regulated hypoxia responsive genes. Abbreviations: SMC (smooth muscle cells), BPAEC (bovine pulmonary endothelial cells), BAEC (bovine aortic endothelial cells), BVEC (bovine venous endothelial cells), RPAEC (rat pulmonary endothelial cells), RVSMC (rat venous smooth muscle cells), HUVEC (human umbilical vein endothelial cells), ES (embryonic stem) cells. Rows shaded light grey contain SAGE-identified genes closely related to previously described hypoxia-responsive genes. Tag counts are shown for each time point and are expressed as tags per 30,000.

Gene	UniGene ID	Reference	Context	HPAEC 0h	HPAEC 8h	HPAEC 24h	HAEC 0h	HAEC 8h	HAEC 24h	SAGE tag
Adenylate kinase 3	Hs.274691	(54)	HT1080	-	-	-	-	-	-	-
Adenylate kinase 1	Hs.203	-	-	5.84	4.34	16.33	10.14	14.77	6.37	GACAGCTGAG
Adenylate kinase 2	Hs.204	-	-	2.33	3.25	2.84	0.78	2.95	4.96	GTGTAGTTGA
Beta-Adrenergic receptor 1	Hs.123055	(14)	SMC	-	-	-	-	-	-	-
Beta-Adrenergic Receptor Kinase 2	Hs.157	-	-	0	1.08	0	2.34	0	0	AAGCCTTTTT
Adrenomedullin	Hs.394	(11, 42)	Cardio-myocytes HUVEC	2.33	4.33	4.97	0.78	0	0.70	AAAGAGAAAG
Aldolase A	Hs.273415	(23)	ES cells	15.17	21.69	29.82	23.41	34.70	34.69	GCGACCGTCA
Aldolase C	Hs.155247	(23)	ES cells	4.67	4.34	2.13	5.46	5.91	4.25	-
Transaldolase 1	-	-		4.67	4.34	2.13	5.46	5.91	4.25	GGCGCCTCCT
Endothelin 1	Hs.2271	(21)	HUVEC	3.50	3.25	8.52	3.12	2.95	2.12	CAAGTAAAAA
Endothelin converting enzyme 1	Hs.1889	(46)	Venous tissue	8.17	5.42	5.68	7.02	5.91	5.66	GCTCAGGTCT
Enolase 1 (alpha)	Hs.254105	(1, 23)	ES cells BPAEC BAEC	9.34	7.59	14.20	12.49	9.60	20.53	TGAGCCTCGT
Erythropoietin	Hs.2303	(24)	Hep3B	1.17	1.08	0.00	0.00	0.74	0.00	AATTGAAACC
Beta Galactosidase 1	Hs.2730	-		5.84	3.25	3.55	4.68	2.95	3.54	TTACCTTTTT
Glucose transporter 1	Hs.169902	(34)	BAECs BVECs	1.17	2.17	4.26	0.78	2.22	1.42	GAGACTCCTG

Glyceraldehyde phosphate dehydrogenase	Hs.169476	(17)	BPAEC BAEC	163.39	260.25	303.90	181.03	218.56	266.22	TACCATCAAT
Heme oxygenase 1	Hs.202833	(19)	RPAECs	5.84	4.34	4.97	4.68	7.38	9.20	CGTGGGTGGG
Hexokinase 1	Hs.118625	(23)	ES cells	0.00	0.00	0.00	0.00	0.74	0.00	
Hexokinase 2	Hs.198427	(43)	A549	3.50	2.17	2.13	3.90	2.22	7.08	CCAGGCACGC
Lactate dehydrogenase A	Hs.2795	(35)	RVSMC	5.84	13.01	12.07	11.70	15.51	17.70	TCTTGTGCAT
p21 (WAF-1, Cip1)	Hs.228665	(5)	ES cells	3.50	7.59	5.68	5.46	8.12	4.25	TGTCCTGGTT
Phosphofructokinase L	Hs.155455	(23)	ES cells	5.84	13.01	22.01	11.70	22.15	18.41	CTTTCAGATG
Phosphoglycerate kinase 1	Hs.78771	(15)	HepG2	7.00	13.01	14.91	14.04	15.50	16.28	GAAACAAGAT
Plasminogen activator inhibitor-1	Hs.5054	(49)	BAEC	88.70	94.34	156.21	84.27	76.05	101.25	TACAGGATCC
Pyruvate kinase M	Hs.198281	(23)	ES cells	11.67	9.76	9.23	13.27	14.03	16.99	TGGCCCCACC
Vascular endothelial growth factor	Hs.73793	(32)	BPAEC	1.17	0.00	0.00	0.00	0.00	0.00	-
Vascular endothelial growth factor C	Hs.7424	-	-	4.67	4.34	8.52	3.90	2.22	7.08	CAAAATATGT
Vascular endothelial growth factor-related protein	Hs.252820	-	-	2.33	6.51	3.55	0.78	1.48	2.83	CAGCTCCTAA