Gene expression profile of human endothelial cells exposed to sustained fluid shear stress

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Wasserman, Scott M., Fuad Mehraban, Laszlo G. Komuves, Ruey-Bing Yang, James E. Tomlinson, Ying Zhang, Frank Spriggs, and James N. Topper. Gene expression profile of human endothelial cells exposed to sustained fluid shear stress. Physiol Genomics 12: 13–23, 2002. First published November 5, 2002; 10.1152/physiolgenomics.00102.2002.—Biomechanical forces can modulate endothelial phenotype through changes in gene expression. We hypothesized that physiological laminar shear stresses (LSS) act as differentiative stimuli on endothelial cells (EC) to alter gene expression, creating an antioxidant, anti-apoptotic and anti-proliferative environment. The transcriptional profile of cultured human umbilical vein endothelial cells (HUVEC) exposed to LSS was evaluated by GeneCalling; 107 genes demonstrated at least a twofold change in expression at 24 h (LSS vs. static). These flow-responsive genes represent a limited number of functional clusters that include transcription factors, antioxidants, signaling molecules, cell cycle regulators, and genes involved in cellular differentiation. Immunohistochemistry and in situ hybridization confirmed that many of these flow-responsive genes, including the novel basic helix-loop-helix transcription factor Hath6, are expressed in EC in vivo. Thus these data identify a limited set of flow-responsive genes expressed in the endothelium that may be responsible for the establishment and maintenance of the flow-adapted endothelial phenotype in vivo.

transcriptional profile; laminar shear stress; endothelium; biomechanical force; vascular biology

PULSATILE BLOOD FLOW generates an array of biomechanical forces that modulate the structural and functional phenotype of the endothelial monolayer that lines the vascular tree. These complex stimuli play a critical role in the maintenance of vascular homeostasis and the development of diseases such as atherosclerosis, thrombosis, and restenosis. For example, early atherosclerotic lesions occur primarily in regions of nonlaminar flow, such as vessel branch points and curvatures, whereas areas of time-averaged uniform laminar flow appear to be protected (14, 23). Previous in vitro studies have demonstrated that prolonged, physiological levels of laminar shear stress (LSS) can induce the expression of individual endothelial genes with anti-inflammatory, antioxidant, anti-apoptotic, and anti-proliferative properties, whereas nonuniform or low-shear flow patterns can initiate the transcription of species that mediate inflammation, oxidative stress, proliferation, and apoptosis (2, 6, 12, 17, 19, 21, 38, 42, 47, 59, 61). In addition, several studies have suggested that genes selectively upregulated by LSS in vitro are enriched for species present in the vascular endothelium in vivo (38, 60). These observations have provided a putative molecular basis for the strong correlation between flow and vascular disease; however, this hypothesis has not been tested comprehensively at the level of the endothelial genome.

We hypothesized that physiological levels of sustained LSS act as differentiative stimuli on cultured endothelial cells (EC) to induce a “protective,” antiatherosclerotic phenotype and that genes upregulated by LSS in vitro would be expressed in normal endothelium in vivo. To test this hypothesis directly, we employed a comprehensive, unbiased gene discovery technique, called GeneCalling, to characterize the transcriptional profile of cultured human umbilical vein endothelial cells (HUVEC) exposed to sustained physiological levels of LSS. GeneCalling is a high-throughput expression profiling technique that combines PCR-based linear amplification of gene fragments and in silico database queries to generate a genome-wide assessment of differences in gene expression (30, 55). Our study identified 107 genes in cultured HUVEC that were significantly and reproducibly regulated by 24 h of LSS at 10 dyn/cm² (an arterial magnitude of shear stress) vs. no flow. Interestingly, the LSS-regulated genes fell into a limited number of functional clusters that include oxidative metabolism, inflammation, apoptosis, cellular growth/proliferation, and cell differentiation. Taken as a whole, this shear-induced transcriptional profile is highly consistent with a “protective,” quiescent EC phenotype. Immunohistochemistry and in situ hybridization demonstrated in vivo endothelial expression for many of the LSS-upregulated genes including several novel genes, sug-
ggesting that flow is an important biomechanical regulator of endothelial gene expression in vivo and that these LSS-regulated genes likely play a role in the maintenance of endothelial homeostasis in vivo.

METHODS

EC culture. HUVEC from pooled donors (Clonetics) were cultured in Medium 199 (Mediatech Cellgro) supplemented with EC growth supplement (50 μg/ml), Biomedical Technologies, Stoughton, MA), penicillin-G/streptomycin (100 U/ml and 100 μg/ml, respectively; Mediatech Cellgro), l-glutamine (2 mM, Mediatech Cellgro), porcine intestinal heparin (50 μg/ml, Sigma), and 20% fetal bovine serum (HyClone Laboratories). Cells at passage level 2–6 were grown on 0.1% gelatin-coated (Sigma) 150 mm × 25-mm treated cell culture dishes (Corning) or specially designed 178-mm diameter maxi-plates made from the same tissue culture plastic (Corning).

Shear stress apparatus. Confluent HUVEC monolayers grown on 0.1% gelatin-coated (Sigma) maxi-plates were exposed to 6 and 24 h of LSS in a cone-plate viscometer at 37°C, 5% CO₂-95% air atmosphere and the described protocol (21). The calculations and equations entailing the shear stresses produced in the cone-plate flow cuvette have been detailed (10, 54). For control maxi-plates, culture media was changed, and the cells were maintained under static conditions at 37°C, 5% CO₂-95% air atmosphere for 6 and 24 h. Independent sets of shear/static experiments were performed for GeneCalling (n = 3), microarray (n = 2), and SYBR green real-time quantitative PCR (RT-qPCR) (n = 3) (67).

Cytokine exposure. Confluent monolayers of HUVEC grown in 0.1% gelatin-coated 150 × 55-mm treated cell culture dishes (Corning) were exposed to 24 h of 10 U/ml recombinant human interleukin-1β (IL-1β, Biogen), 200 U/ml recombinant human TNFα (Genzyme), 5 ng/ml human TGFβ1 (R&D Systems), and no cytokine control by replacing the culture medium with standard medium or medium supplemented with cytokine. Cells were maintained at 37°C in 5% CO₂-95% air atmosphere. All cytokine and control exposures were performed 3–7 times.

RNA isolation and cDNA synthesis. Total RNA was isolated utilizing TRizol LS reagent (GIBCO BRL). Poly(A)⁺ RNA isolation, cDNA synthesis, and second-strand synthesis for GeneCalling were performed as described previously (30, 55). For RT-qPCR, total RNA was DNase treated with the RNase-free DNase kit (Qiagen) per the manufacturer’s protocol and purified on RNaseasy mini columns (Qiagen). cDNA synthesis was performed using random or oligo-dT primers and the Superscript First-Strand Synthesis System for RT-PCR (GIBCO BRL) on RNA with an A260/A280 ratio >1.9 and a 28S/18S ratio of >1.4 (Agilent 2100 Bioanalyzer RNA 6000 LabChip kit).

GeneCalling. GeneCalling was performed as described (30, 55). For quantitative expression analysis, double-stranded cDNA digested with 93 pairs of restriction enzymes (RE) underwent linear PCR amplification (20 cycles) followed by high-resolution electrophoretic separation (Fig. 1A). This process was repeated in triplicate for each experiment (9 times for the 3 LSS experiments and 9 times for the 3 controls). Statistical algorithms identified bands whose expression was at least twofold different between LSS and control samples. Probable gene identities for the bands (i.e., GeneCalls) were determined by querying sequence databases based on band length and the unique RE pair. Bands not found in the database were isolated, sequenced, and analyzed (43). GeneCalls in the database were confirmed by “poisoning,” which involved repeating the PCR amplification with excess unlabeled gene-specific oligonucleotides designed for the putative gene fragment (Fig. 1B); repeat PCR with the oligonucleotides for the correct gene ablated the corresponding band. Gene function was determined with internet-based tools that included National Center for Biotechnology Information (NCBI) LocusLink, NCBI PubMed, Proteome Public HumanPSD database, GeneCards, Gene Ontology Consortium, and Online Mendelian Inheritance in Man (OMIM). GeneCalls were sorted into nine functional categories based on their demonstrated role in EC and/or vascular biology (Table 1).

Real-time quantitative PCR. SYBR green RT-qPCR was performed with 1 μl of cDNA reverse transcribed from 50 ng per μl of experimental RNA, 100–200 nM of gene-specific primers, and SYBR green master mix (Applied Biosystems) in an Applied Biosystems ABI Prism 7700 sequence detector according to the manufacturer’s protocol. Each experimental sample was run in duplicate. Gene-specific primers were designed using PrimerExpress 1.0 (Applied Biosystems) and spanned an intron when possible (Supplemental Table 1; Supplemental Data for this paper is available from the Physiological Genomics web site). All primer sets were analyzed with RT ± cDNA, RT – cDNA and no template control prior to experimental use. Amplification of a single PCR product of the expected size was confirmed by polyacrylamide gel electrophoresis on 10% TBE gels (Bio-Rad Laboratories). Standard curves were generated for each gene by serial dilution of HUVEC cDNA. The average of duplicate RT-qPCR reactions for an experiment was used to quantify the amount of mRNA relative to the standard curve. All experimental samples were normalized to human GAPDH ( Biosource International) (18). Normalized fold-inductions were calculated for each experiment by dividing the normalized-experimental mRNA quantity by the normalized-control mRNA quantity. The average fold-induction and standard error for the distinct genes were calculated for each condition (Fig. 2). An additional RT-qPCR analysis for Hath6 was performed on human cDNA derived from a variety of tissues (human MTC panel I and II, Clontech). Normalization for this study was based on equivalent amounts of input cDNA (50 ng).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were used following heat-induced antigen retrieval using Target Retrieval Solution, pH 6.1 (DAKO, Carpinteria, CA), in a pressure cooker (BioCare Medical, Walnut Creek, CA). Antibodies for Id2 (1:100, rabbit polyclonal), Id3 (1:100, rabbit polyclonal), BMP6 (1:100, goat polyclonal), Jagged2 (1:50, rabbit polyclonal), Smad6 (1:100, rabbit polyclonal), TGFβ1 (1:100, rabbit polyclonal), and GADD34 (1:50, rabbit polyclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas vascular endothelial cadherin (VE-cadherin) antibody (1:50, polyclonal goat) was from Research Diagnostics (Flanders, NJ). Secondary antibody labeling was performed with biotinylated anti-rabbit IgG or anti-goat IgG (1:500; Vector Laboratories, Burlingame, CA) and detected with avidin-biotin complex-horseradish peroxidase (Vector Laboratories). Signal detection was performed with DAB substrate (Vector Laboratories).

In situ hybridization. Antisense and sense digoxigenin-labeled riboprobes for human Smad6 and human Hath6 were employed for in situ hybridization on formalin-fixed, paraf-
fin-embedded sections of human umbilical vessels and various cynomolgus monkey (Macaca fascicularis) tissues (Supplemental Table 2). Tyramide-mediated signal amplification was used to detect probe hybridization. The in situ hybridization procedure was described earlier (35, 56).

Microscopy. Digitized images (1,280/H11003 1,024 pixel resolution) were acquired with an Olympus BX50 microscope (Olympus US, Melville, NY) equipped with a Nikon DXM1200 digital camera (Technical Instruments San Francisco, Burlingame, CA) using differential-interference contrast illumination and ACT-1 software (Nikon, Melville, NY).

Northern blot analyses. A human multiple tissue Northern (MTN) blot (BD Biosciences Clontech) was probed with a [32P]dCTP-radiolabeled probe generated from the Hath6 in situ probe. The blot was washed with 2× SSC, 0.05% SDS at room temperature and 0.1× SSC, 0.1% SDS at 55°C. After stripping, the blot was reprobed with human β-actin to normalize for loading.

RESULTS

Identification of LSS-regulated genes in cultured human EC. Using GeneCalling to compare the gene expression profile of cultured HUVEC exposed to 24 h of
LSS at 10 dyn/cm² and static conditions, we assayed a total of 35,985 bands for at least twofold differential regulation by LSS in three independent experiments (Fig. 1C). This magnitude of shear-regulation was observed in 484 (1.3%) bands at 24 h. Assuming that the ratio of bands to genes was constant, we estimate that 1–2% of the expressed genes in cultured HUVEC were responsive to prolonged LSS. The 484 bands represented at least 107 distinct genes that were differentially regulated by LSS (Fig. 1C, Supplemental Table 3, A–C); 60 transcripts were upregulated and 47 were downregulated (Table 1). These included many genes known to be regulated by biomechanical forces in EC such as ICAM-1, cyclooxygenase 2, endothelial constitutive nitric oxide synthase (NOS3), Smad6, TGF-β1, Cu/Zn superoxide dismutase, and thrombomodulin (46–48, 51, 58–60). Of the 107 shear-regulated genes, 89 (83%) represent species that have been characterized to some extent. Only 33% (35 genes) of the LSS-responsive genes have been described in vascular endothelium, and only 20% (21 genes) have been demonstrated to be responsive to biomechanical stimuli (asterisks in Supplemental Table 3, A–C). Comparison of the 21 endothelial shear-responsive genes that overlap between our study and previously published work corroborate the regulatory effect (up or down) of LSS for 20 transcripts (asterisks in Supplemental Table 3, A–C). The only gene whose expression differed markedly was the IL-1 receptor-like 1 transcript. We observed a shear-mediated 3.2-fold increase in mRNA level by GeneCalling which was confirmed by microarray analysis and RT-qPCR. Garcia-Cardena et al. (21) observed a 1.79-fold decrease. Plausible explanations for this observation range from differences in cell derivation (i.e., origin, harvesting technique) to technical issues (i.e., commercial array annotation errors).

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GeneCalling was also performed on cDNA generated from HUVEC subjected to 6 h of LSS at 10 dyn/cm² and static controls. A total of 33,232 bands were assayed at 6 h, and 872 bands (2.6%) were regulated at least twofold by LSS, indicating an apparent 50% reduction in the number of LSS-regulated genes from the 24 h time points. Thus the 6 h data suggest that the expression of a significant number of genes was only transiently altered in response to the acute transition from no flow to sustained laminar flow.

Microarray analyses and RT-qPCR were employed to independently corroborate the GeneCalling data. A series of microarray experiments evaluating the transcriptional profile of HUVEC exposed to 24 h of LSS at 10 dyn/cm² and static conditions were performed (67). These data independently confirmed the shear-regulation of 95 genes (88.8%) identified by GeneCalling (Supplemental Table 3, A–C). Six transcripts (5.6%) could not be mapped to our arrays. A subset of 24 transcripts identified by GeneCalling as being regulated by 24 h of LSS was chosen for independent confirmation by SYBR
green RT-qPCR (Fig. 2). With a separate set of experimental samples, RT-qPCR confirmed the shear-regulation of all 24 species in cultured HUVEC. A SYBR green RT-qPCR analysis capable of differentiating two unique splice variants of Smad8, a receptor-activated Smad protein that mediates bone morphogenic protein (BMP) signaling, demonstrated that both transcripts were upregulated by prolonged LSS in EC in vitro. The magnitude of shear-regulation determined by RT-qPCR and GeneCalling differed for a number of these genes, but in all cases the direction of regulation (up or down) was confirmed (Fig. 2).

The majority of the upregulated genes could be assigned to a limited set of functional clusters that included metabolism, signal transduction, cell cycle/growth, and transcription (Table 1). A closer evaluation of the upregulated species revealed that the majority of the genes in these clusters have antioxidant, anti-inflammatory, anti-proliferative, and anti-apoptotic, and differentiative properties. For example, thioredoxin reductase, cyclooxygenase 2, aldehyde dehydrogenase 6, and heme oxygenase-1 are all oxidoreductases whereas pregnancy-induced growth inhibitor OKL38 and N-myc downstream regulated gene-1/Cap43 are tumor suppressors. Many of the oxidoreductases, such as heme oxygenase-1 and Cu/Zn superoxide dismutase, also have anti-apoptotic functions. The transcription factors Id1, Id2, Id3, HEY2, interferon regulatory factor 6, Snail, and GATA2 have all been implicated in cellular differentiation. Excluding the uncharacterized genes, the most represented functional category among the downregulated species was cell cycle/growth. This set of downregulated genes included a variety of species that are necessary for cell cycle progression and cellular proliferation. For example, cyclins A and B and cell cycle controller CDC2 are required for cell cycle progression, whereas placenta growth factor, chemo kinase HCC-1, deoxythymidylate kinase, and polo-like kinase are growth factors or proteins involved in cellular proliferation.

The response of LSS-regulated genes to humoral stimuli in cultured human EC. In an effort to examine the cytokine-responsiveness of the 24 RT-qPCR-confirmed shear-responsive genes, further RT-qPCR was performed on cDNA derived from cultured HUVEC exposed to 24 h of 10 U/ml IL-1β, 200 U/ml TNFα, 5 ng/ml TGFβ1, or no cytokine control (Fig. 2). ICAM-1 and PAI1, genes known to be regulated by IL-1β, TNFα, and TGFβ in EC, were used as positive controls (39, 49, 57, 65). Interestingly, prolonged exposure to TGFβ and the inflammatory cytokines IL-1β and TNFα did not result in a significant change in mRNA expression for the majority of transcripts in this subset of LSS-regulated genes. Excluding the positive controls, the majority of cytokine-responsive genes demonstrated a less than twofold change in response to 24 h of IL-1β, TNFα, and TGFβ.

Genes upregulated by LSS in vitro are expressed in the endothelium in vivo. To evaluate our hypothesis that genes upregulated by sustained LSS in cultured EC will be present in the vascular endothelium in vivo, immunohistochemistry or in situ hybridization were used to examine the expression of eight upregulated genes. Human umbilical vessels stained with antibodies for Smad6, TGFβ1, Id2, Id3, Jagged2, and GADD34 demonstrated clear endothelial expression in vivo (Fig. 3, A–F). Some adventitial and smooth muscle cell staining was noted for all of the proteins except TGFβ1, indicating that the expression of these proteins was not restricted to the endothelium. In situ hybridization with antisense probes for Smad8 and the novel basic helix-loop-helix (bHLH) transcription factor Hath6 revealed endothelial-selective expression for both of these genes (Fig. 3, I–P). Evaluation of a panel of tissues by in situ hybridization revealed Smad8 expression in the endothelium of human umbilical vessels and cynomolgus monkey femoral artery, kidney, and lung (Fig. 3, I–L). In particular, there was pronounced expression of Smad8 in the endothelium lining the glomerulus, afferent and efferent arterioles, and vasa recta of the kidney, as well as the endothelium of the entire pulmonary vasculature (Fig. 3, K and L). Smad8 sense control probe did not reveal any signal (data not shown). Endothelial-specific expression of Hath6 was seen in human umbilical vessels and cynomolgus monkey femoral artery (Fig. 3, M and N) as well as highly vascular tissues such as the cynomolgus monkey kidney and lung (Fig. 3, O and P). A survey of Hath6 expression in 46 different cynomolgus monkey tissues by in situ hybridization demonstrated endothelial-selective expression in all tissues (data not shown). Sense Hath6 probe did not reveal signal in any tissues (data not shown).

The human homolog of murine Math6 (Hath6) is a novel shear-responsive bHLH transcription factor. As noted above, the GeneCalling study identified a novel bHLH transcription factor that was similar to human NeuroD1/neurogenic differentiation factor 1. BLAST analyses revealed that this gene was identical to FLJ14708 and that it was 93% identical to the murine Math6 gene. Comparison of hypothetical protein sequence FLJ14708 demonstrated 98% identity to Math6, indicating that this novel LSS-responsive bHLH was the human homolog of Math6, termed Hath6 (Fig. 4A). Northern analysis of Hath6 in human tissues demonstrated a single transcript of the predicted length of ~3 kb (Fig. 4C). Its expression was most pronounced in the highly vascularized tissues lung, liver, kidney, and heart. RT-qPCR for Hath6 expression in human tissues confirmed the Northern analysis with the highest levels of expression being in the lung, liver, kidney, heart, and pancreas (Fig. 4D). Multiple sequence alignment of Hath6 with a selection of bHLH transcription factors by PileUp algorithm in SeqLab (Genetics Computer Group) demonstrated that it was a member of the atonal-related protein family and was most similar to the NeuroD group, the neurogenin group, and Hath1. This family of bHLH proteins is part of a tissue-restricted class of bHLH transcription factors that includes MyoD, NeuroD, Hand1, and Hand2 (41).
DISCUSSION

The transcriptional profile of flow-adapted endothelium. We present here a comprehensive, genome-wide analysis of the transcriptional profile of HUVEC exposed to sustained physiological levels of LSS. GeneCalling, which does not require a priori sequence information and provides ~95% coverage of the transcriptome, identified 484 bands (~1.3% of the EC transcriptome) whose expression in cultured HUVEC was significantly altered following 24 h of an arterial magnitude of LSS (10 dyn/cm²) compared with no flow (24). These bands represent at least 107 unique human genes. Two independent techniques, microarray and RT-qPCR analyses, corroborated these results in separate in vitro experiments. By utilizing this approach, we have established a reproducible transcriptional fingerprint of cultured EC in the setting of sustained uniform LSS. Prior studies have employed differential display PCR, candidate gene screening, and microarray analyses in an effort to characterize this LSS-mediated endothelial transcriptional profile (1, 6, 12, 21, 42, 59). Based on these reports and the literature, only 20% of the genes identified in this study have been reported to be LSS-responsive in EC. Furthermore, the expression of the majority of these species (72/107) has not been described in EC in vitro or in vivo.

Interestingly, our functional analyses demonstrated that these 107 LSS-responsive genes have a limited set of common functional properties. In particular, endothelial genes upregulated by LSS included a significant number of proteins with antioxidant, anti-apoptotic, anti-proliferative, and cellular differentiative charac-

Fig. 3. Genes upregulated by LSS are expressed in endothelium in vivo. A–H: immunohistochemistry. Formalin-fixed, paraffin-embedded sections of human umbilical vessels were stained with polyclonal antibodies against Smad6 (A), TGFβ1 (B), Id2 (C), Id3 (D), Jagged2 (E), and GADD34 (F). G: VE cadherin is a known endothelial-selective protein that was used as a positive control. H: a representative negative control stained with a nonspecific polyclonal antibody. I–P: in situ hybridization of two LSS-inducible genes identified by GeneCalling. Anti sense digoxigenin-labeled riboprobes for human Smad8 (I–L) and Hath6 (M–P) were employed for in situ hybridization on formalin-fixed, paraffin-embedded sections of human umbilical vessels (I and M) and cynomolgus monkey femoral artery (J and N), kidney (K and O), and lung (L and P). Tyramide-mediated signal amplification was used to detect probe hybridization. Sense control probes did not yield signal (data not shown).
Characteristics, whereas the major downregulated class was genes involved in cell cycle progression or cellular growth/proliferation (Table 1). Taken together, these data indicate that only a small proportion of the endothelial transcriptome is flow-responsive and that these genes may act coordinately to maintain endothelial phenotype and preserve vascular health.

Uniform shear stress induces an antioxidant, anti-apoptotic molecular phenotype in EC in vitro. Hemodynamic forces have been demonstrated to contribute to the redox state of the endothelium in vivo and in vitro (13, 17, 36). In this study, species with antioxidant and anti-inflammatory properties, such as NOS3, cyclooxygenase 2, aldehyde dehydrogenase 6, thioredoxin reductase, glucose-6-phosphate dehydrogenase, NAD-(P)H:menadione oxidoreductase, heme oxygenase-1, Cu/Zn superoxide dismutase, and ferritin (heavy and light chain), were upregulated by LSS. The coordinated regulation of a diverse set of antioxidant effectors strongly suggests that LSS can promote an in vivo endothelial phenotype that is antioxidant. This concept is supported by the observation that the level of intracellular reactive oxygen species increases after 15–30 min of LSS, but that with continued LSS these radicals decrease significantly over the course of hours (13, 17). Interestingly, sustained oscillatory laminar shear up-

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**Fig. 4. Hath6.** A: the novel basic helix-loop-helix (bHLH) is the human homolog of murine Math6. BlastP of hypothetical protein FLJ14708 demonstrates 95% homology to murine Math6. This novel bHLH transcription factor is the human homolog of Math6, Hath6 (accession no. AF529205). B: multiple sequence alignment of Hath6 and its neighbors. Hath6 falls into the atonal family of bHLH which includes Hath1, the NeuroDs, and the neurogenins. This family of bHLH transcription factors is a tissue-restricted class of bHLH and has been implicated in neural and pancreatic development. C: expression of Hath6 in human tissues. Northern analysis was performed on a human MTN multiple tissue blot (Clontech). The blot was reprobed with β-actin to control for loading. A single major band of the expected size of ~3 kb was detected in highly vascularized tissues, such as heart, lung, liver, and kidney. D: RT-qPCR analysis of Hath6 expression in variety of human tissues. SYBR green RT-qPCR was performed with cDNA from a variety of human tissues (Clontech) in an Applied Biosystems ABI Prism 7700 sequence detector according to the manufacturer’s protocol. Each experimental sample was run in duplicate and normalized to input cDNA. A standard curve was made by serial dilution of HUVEC cDNA to calculate relative transcript expression. Hath6 expression was most pronounced in the lung, liver, heart, kidney, and pancreas. PBL, peripheral blood leukocyte.
regulated heme oxygenase-1, but did not augment Cu/Zn superoxide dismutase expression in cultured EC, suggesting that the type of biomechanical stimulus was crucial to the generation of a complete antioxidant profile (17). Antioxidant genes, such as NOS3, Cu/Zn superoxide dismutase, and heme oxygenase-1, are also known to serve anti-apoptotic roles in the endothelium (8, 19, 32). Cu/Zn superoxide dismutase and NOS3 have been shown to inhibit endothelial apoptosis through abrogation of caspase activity, whereas the production of carbon monoxide by heme oxygenase-1 suppressed apoptosis in EC. Iron and its metabolites have also been implicated in apoptosis (16). Thus the observed downregulation of transferrin receptor and the upregulation of the ferritin heavy and light chains by LSS could also be predicted to be anti-apoptotic (45). The LSS-induced coordinate regulation of a set of genes with antioxidant and anti-apoptotic properties provides a plausible molecular mechanism for the protective endothelial phenotype observed in areas of the vasculature exposed to time-averaged uniform LSS (14, 23, 62).

Regulation of EC cell cycle and cellular proliferation by LSS. Fluid mechanical forces also appear to play an important role in regulating cell cycle and proliferation. As noted, LSS inhibits cell cycle progression and does not promote cell turnover in EC in vitro (15, 38). Thus it was not surprising that genes encoding proteins necessary for the cell cycle comprise a large fraction of the downregulated species. Essential components of the cell cycle, such as cyclin A, cyclin B, polo-like kinase, and CDC2, and oncogenes, such as E1A enhancer-binding protein, pituitary tumor transforming gene protein 1, and erythroblastosis virus oncoprotein homolog 2 were downregulated by LSS (20, 25, 28, 50, 52). Some of the other downregulated genes implicated in cellular proliferation included protein regulating cytokinesis 1, RNA helicase p68, ribosomal protein L23a, cavelin-1, deoxythymidylate kinase, and placenta growth factor. Conversely, growth inhibitory genes including N-myc downstream regulated gene, pregnancy-induced growth inhibitor OKL38, and XAPI-1 were upregulated (26, 31, 69). The overall cellular effect of this transcriptional profile suggests that LSS-induced endothelial quiescence is mediated, in part, through changes in gene expression.

The soluble factors, chemokine HCC-1, TGFβ1, and BMP6, also play roles in cell proliferation. HCC-1, which was downregulated by LSS, is a constitutively expressed chemokine that causes CD34+ myeloid progenitor cells to proliferate, whereas TGFβ1 and BMP6, both of which were upregulated by LSS, can inhibit cell cycle progression in many cell types including EC (5, 53, 66). Interestingly, we detected Smad8, an intracellular protein involved in BMP signaling and the inhibition of apoptosis, as a LSS-induced gene (33, 44). This gene has been reported to be expressed in two distinct isoforms, both of which were upregulated by sustained, uniform LSS (Fig. 2). Previous work has demonstrated the upregulation of two other Smads, Smad6 and Smad7, in EC by LSS (60). Unlike the receptor-activated Smad8, these Smad proteins are involved in the inhibition of TGFβ/BMP signaling. Unexpectedly, Smad8 also demonstrates EC-selective expression in many tissues (Fig. 3, M–P). These data implicate BMP signaling as an important modulator of endothelial phenotype, and suggest that like TGFβ, BMP signaling in EC may be modulated by biomechanical forces.

LSS acts as a differentiative stimulus. Surprisingly, many of the genes identified in this study play critical roles in development and cellular differentiation. In particular, there was a group of LSS-regulated genes including Id1, Id2, HEY2, EphB4, GATA2, Jagged2, and CXCRI4 that is known to modulate endothelial phenotype during development. The Ids are a family of four helix-loop-helix transcription factors that serve as transcriptional repressors by heterodimerizing with other bHLH proteins to prevent their DNA binding. They have been implicated in vascular development, angiogenesis, cellular growth, and differentiation (3, 7, 29, 40, 68). GeneCalling demonstrated that confluent cultured EC in a no-flow environment express very little Id1, Id2, and Id3 mRNA (data not shown). This was consistent with previous observations (11, 40). Sustained LSS activated the transcription of these rare transcripts (Supplemental Table 3, A–C). This observation was independently confirmed by RT-qPCR and microarray analyses (Fig. 2; Supplemental Table 3, A–C). Furthermore, Id2 and Id3 protein expression in the endothelium of human umbilical vessels and cynomolgus monkey tissues suggests that these transcriptional repressors are playing an as yet undefined role in the vessel wall in vivo (Fig. 3, C–D). GATA2 and Jagged2 have been implicated in the differentiation of endothelial and hematopoietic progenitor cells, whereas the human homolog of the Xenopus bHLH transcription factor gridlock known as HES-related repressor protein 1 (HEY2) and the receptor tyrosine kinase EphB4 play critical roles in arterial and venous development, respectively (9, 22, 63, 64, 70, 71). All of these transcripts with defined endothelial differentiative properties were upregulated by flow compared with static conditions, and many were found to be expressed in the endothelium in vivo. These data raise the intriguing hypothesis that LSS modulates endothelial phenotype, in part through the upregulation of several endothelial fate-determining genes, and that these genes modulate the expression of downstream effectors necessary to impart a functional endothelial phenotype.

Hath6 is a novel human shear-responsive bHLH transcription factor that is selectively expressed in the endothelium in vivo. We identified Hath6, a novel member of the tissue-restricted class of bHLH transcription factors, as a shear-responsive, endothelial-selective gene. This group of bHLH proteins that includes Hath1, the NeuroD family, and the neurogenin family are homologs of the Drosophila proneural atonal gene (ato). Atonal encodes a bHLH protein required for the production of Drosophila chordotonal organs which are stretch and/or vibration receptors. Mice lacking Math1 or NeuroD1 are deaf due to the absence of inner ear hair cells or the developmental failure of inner ear
sensory neurons, respectively (4, 34). The necessity of atonal-related proteins for mechanotransduction (i.e., development of mechanoreceptors) and the endothelial selectivity of Hath6 expression raise the interesting possibility that the genetic program downstream of Hath6 allows the EC to sense its biomechanical environment. Math6, the murine homolog of Hath6, has been demonstrated to be instrumental in determining cell fate in the developing mouse nervous system; however, its role in the vasculature, embryonic or adult, has not been appreciated (27). To date, a number of endothelial-selective bHLH proteins, such as EPAS-1, bHLH-EC2, Tal1, and CLIF, have been described. However, unlike Hath6, the expression of these genes does not appear to be restricted to the mature endothelium in vivo. Thus Hath6 appears to an endothelial-selective bHLH protein that may mediate the transcriptional events necessary for EC differentiation, phenotypic modulation, and possibly the genetic program that permits mechanotransduction in EC.

**Study limitations.** There are several limitations to this study. The first is the use of a single type of EC, HUVEC, for these analyses. We chose to utilize these cells because they are arguably the most well-characterized human primary EC type. In our hands, these cells yield reproducible biologic responses and demonstrate many of the same patterns of gene expression as those of other EC types, such as those derived from the coronary arteries and the aorta. However, whether cells derived from other areas of the vascular tree will behave similarly across all of the genes studied is currently unknown.

A second limitation of this study is the use of EC cultured in a no flow environment as a control. We chose to utilize a static control for several reasons. First, it was our primary hypothesis that a defined, uniform fluid mechanical stress (i.e., LSS) would induce an in vivo-like “protective” phenotype in these cultured cells. To address this question, the use of a standard set of control culture conditions was required, and thus the absence of flow stimulus was used as the control. In addition, other investigators have demonstrated that EC cultured under static conditions harbor a number of phenotypic characteristics of cells found at branch points and bifurcations in vivo, suggesting that the static phenotype may provide insights into the initiation and progression of vascular disease (37, 61). Due to the complexity of flow environments in vivo, there is no ideal in vitro system to mimic the entire spectrum of flow regimes. Our model evaluated the effect of a physiological magnitude of uniform laminar flow vs. no flow on endothelial gene expression. It will be very interesting in the future to examine the response of these cells to distinct, nonlaminar flow regimes (i.e., turbulent or disturbed flows) in vitro that reproduce intricate aspects of in vivo flow patterns utilizing whole-genome approaches.

Finally, there are some limitations of the GeneCalling technique as utilized here. As reported previously, the use of 72 unique RE pairs will produce at least one detectable fragment for at least 90% of the expressed genes (55). At 88–96 RE pairs, ~95% of the transcriptome is covered. Theoretically, the technique could be modified to encompass more of the transcriptome by increasing the number of RE pairs; however, there appear to be diminishing returns for these efforts (55). Therefore, we chose to perform this study with 93 distinct RE pairs as described. To our knowledge, this technology has no bias in terms of gene class or expression levels. Thus it appears to be truly genome-wide and unbiased. Despite these strengths, it is likely that some regulated transcripts were not identified.

**Conclusion.** Through the use of multiple gene expression profiling techniques, we have obtained a reproducible, genome-wide snapshot of the molecular phenotype of EC maintained under a physiological level of LSS in vitro. The shear-modulated expression profile, which comprises only a small fraction of the endothelial transcriptome, is enriched for species with molecular properties that create an antioxidant, anti-inflammatory, anti-apoptotic, anti-proliferative, and differentiated environment. These observations are consistent with the known cellular characteristics of the endothelium under flow. Furthermore, the demonstrated expression of many of the upregulated species in the vascular endothelium in vivo suggests that these genes do indeed contribute to the establishment of endothelial phenotype in vivo. These data provide a putative mechanistic link between flow, endothelial phenotype, and the balance between vascular health and disease.

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