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Heterogeneity and reciprocity of FVIII and VWF Expression, and the Response to Shear Stress in Cultured Human Endothelial Cells

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Abstract

Background.—Substantial phenotypic heterogeneity exists in endothelial cells and while much of this heterogeneity results from local microenvironments, epigenetic modifications also contribute.

Methods.—Cultured HUVECs, hPMECs, hHSECs, hLECs and 2 different isolations of ECFCs were assessed for levels of FVIII and VWF RNA and protein. The intracellular location and co-localization of both proteins was evaluated with immunofluorescence microscopy and stimulated release to FVIII and VWF from Weibel-Palade bodies was evaluated. Changes in expression of FVIII and VWF RNA after hLECs and ECFCs were exposed to 2 or 15 dynes/cm^2 of laminar shear stress were also assessed.

Results.—We observed considerable heterogeneity in FVIII and VWF expression among the endothelial cells. With the exception of hLECs, FVIII RNA and protein were barely detectable in any of the endothelial cells and a reciprocal relationship between levels of FVIII and VWF appears to exist. When FVIII and VWF are co-expressed, they do not consistently colocalize in the cytoplasm. However, in hLECs where significantly higher levels of FVIII are expressed, FVIII and VWF colocalize in Weibel-Palade bodies and are released together when stimulated. Expression of both FVIII and VWF is markedly reduced when hLECs are exposed to higher or lower levels of laminar shear stress, while in ECFCs there is a minimal response for both proteins.

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Author Contribution

C. Hough designed experiments, interpreted data, performed statistics, and wrote the manuscript. C. Notley, and A. Mo, performed experiments, and helped write the manuscript. B. Videl provided technical assistance. D. Lillicrap designed experiments and edited the manuscript.

Conflicts of Interest

CH, CN, AM, BV, and DL have no conflicts to declare.

Conclusions.—Variable levels of FVIII and VWF RNA and protein exist in a subset of cultured human endothelial cells. Higher levels of FVIII present in hLECs colocalize with VWF and are released together when exposed to a secretagogue.

Keywords

Endothelial cells; Factor VIII; von Willebrand factor; gene expression; shear stress

Introduction

The two proteins, von Willebrand Factor (VWF) and factor VIII (FVIII) have vital functions in hemostasis. In primary hemostasis VWF mediates adhesion and activation of platelets, while in secondary hemostasis FVIII acts as a cofactor for factor IXa to activate factor X in the intrinsic pathway of blood coagulation. FVIII binds to VWF and circulates in plasma with a stoichiometry of one FVIII molecule to fifty VWF monomers.[1] This high affinity interaction imparts reciprocal outcomes for the two proteins. VWF stabilizes the heterodimeric structure of FVIII and protects it from premature proteolytic inactivation[2,3] while FVIII facilitates proteolytic cleavage of membrane-bound ultra-large VWF multimers.[4] Although it has long been recognized that VWF is synthesized in megakaryocytes[5,6] and vascular endothelial cells,[7,8] the cellular source of FVIII has been controversial, with reports showing evidence of FVIII synthesis in both hepatocytes and endothelial cells. It is now well-established that FVIII synthesis is restricted to a subset of endothelial cells including liver sinusoidal,[9–13] microvascular[14,15] and lymphatic endothelial cells.[16] While both glycoproteins are synthesized in endothelial cells, it is unclear if they are co-expressed in the same subsets of endothelium, and stored together in Weibel-Palade bodies (WPBs). Although this has been demonstrated artificially *in vitro*,[17–19] i*n vivo* FVIII and VWF expression is poorly correlated,[16,20] and no studies have looked for evidence of colocalization in WPBs in native endothelial cells.

There is considerable heterogeneity in the gene expression profiles in endothelial cells throughout the vasculature and although much of this can be attributed to the specific microenvironments,[21] other factors such as permanent epigenetic changes established during embryogenesis also contribute.[22] Vascular heterogeneity in VWF expression is well documented, with venous endothelium being more productive than arterial vascular cells, and mechanisms underlying this heterogeneity are beginning to be understood.[23–25] However, this is not the case for FVIII where the current understanding of its expression throughout the vasculature and mechanisms regulating this expression are very limited. The challenge in identifying the cellular source of FVIII hindered such studies and until recently,[26] *in vitro* studies identifying cis-regulatory elements in the FVIII promoter were carried out in human hepatocyte-derived cell lines.[27–31] Likewise, sequences within the coding region that repress *F8* gene expression have not been evaluated in endothelial cells.[32–35] Importantly, no studies have examined levels of coincident expression of VWF and FVIII in different endothelial subtypes.

Most gene expression studies have been carried out *in vitro* under static conditions, a state that does not recapitulate their natural environment where endothelial cells are exposed

to varying levels of biomechanical forces that can influence levels of gene expression. While studies have shown an increase in VWF gene expression when human umbilical vein endothelial cells (HUVECs) are exposed to arterial levels of laminar shear stress,[36,37] similar studies have not been carried out in different subsets of endothelial cells, or using different levels of shear stress. Furthermore, no studies have been carried out to determine if endothelial levels of FVIII expression are affected by shear stress.

The objective of this study was to evaluate FVIII and VWF expression and possible colocalization in several subsets of human endothelial cells regularly used in *in vitro* studies (HUVECs and endothelial colony forming cells [ECFCs]) or those specifically shown to express human FVIII *in vivo* – human pulmonary microvascular endothelial cells, human hepatic sinusoidal endothelial cells and human lymphatic endothelial cells (hPMECs, hHSECs and hLECs respectively). In addition, we have investigated whether levels of expression of these two proteins change when endothelial cells are exposed to physiologically relevant levels of laminar shear stress.

Materials and Methods

Human Endothelial Cell Culture

Human ECFCs were isolated from 60 mL of venous blood obtained from healthy human volunteers as previously described.[38] HUVECs were obtained from Lonza (Mississauga, Canada). The hPMECs, hHSECs and hLECs were purchased from Sciencell Research (Carlsbad, CA USA). Baby hamster kidney (BHK) cells containing a *gfp*-inserted *F8* gene were generously provided by Dr Robert Hebbel.[39] Cell surface expression of CD31, CD144, CD146, CD14 and CD34 was carried by FAC analysis as previously outlined[40] and cells were seeded onto rat tail type-I collagen-coated (BD Biosciences, Mississauga, Canada) plates and cultured in endothelial cell growth media (Promocell). Cultured endothelial cells were detached from the collagen using 1X TRYPle (Life Technologies, Burlington, Canada). All endothelial cells were evaluated between passages 4 and 6. For all experiments, BHK cells were used as a negative control and BHK-hF8 are used as a positive control for FVIII expression. Cultured human microvascular endothelial cells (HMVEC-L) obtained from Lonza were used as a positive control for endothelial expression of the vasopressin V2 receptor and DDAVP stimulated release of VWF.[41]

Absolute Gene Copy Number Determination

Cells were cultured in 6-well plates and total RNA was isolated using the RNAEasy Mini Kit (Qiagen, Montreal, Canada). cDNA was generated from 200 ng of total RNA using SuperScript III (Invitrogen, Grand Island NY, USA). RNA copy numbers were determined with 1 or 2 μL of FVIII or VWF cDNA respectively using the Applied Biosystems® ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific). Pre-designed probe and primer sets for TaqMan® Gene Expression Assays are listed in Supplementary Table 1. FVIII and VWF RNA copy numbers were determined using a standard curve that was generated using *F8* and *VWF* gene-containing plasmids, diluted in ten-fold increments from 10^8 to 10 copies/μL. All reactions were carried out in triplicate.

FVIII and VWF ELISAs

Endothelial cells, seeded at 500,000 cells were grown for 72 hours and cell lysates were obtained using CelLytic M Lysis Buffer (Sigma Aldrich, Oakville, Canada) supplemented with Protease Inhibitor tablet (Roche, Mississauga, Canada). When required, media was concentrated using Amicon filters (Millipore, Etobicoke, Canada). Lysates and media were stored frozen at −80°C. Quantification of FVIII and VWF antigen was assessed using sandwich ELISAs. For the FVIII ELISA, the coating antibody used was a sheep anti-human FVIII, (Catalogue # SAF8C-AP, Affinity Biologicals, Ancaster, ON, Canada). To enhance the sensitivity of the assay, a biotin-conjugated sheep anti-human FVIII detection antibody (Catalogue # SAF8C-APBIO, Affinity Biologicals) was used together with an HRP-conjugated streptavidin (Pierce/Thermo Scientific, Rockford, IL, USA) and developed with o-Phenylenediamine (Sigma Aldrich). The lower limit of detection for this FVIII ELISA is 1.4 mU/mL, however only levels above 3.0 mU/mL are on the linear portion of the standard curve and levels below this were not considered accurate. The capture (Catalogue A0082) and detection (Catalogue P0226) antibodies used to quantify VWF antigen were obtained from Agilent (Santa Clara, CA, USA) and the lower limit of detection for this assay is 2.5 mU/mL. the standard curve was generated using Normal Human Reference Plasma (Precision Biologic, Dartmouth, NS, Canada).

FVIII and VWF Antibody Binding Assays

Maxisorb plates were coated overnight at 4°C with 1 IU/mL of either full-length FVIII (Kogenate), plasma-derived FVIII (VWF-free) or VWF (FVIII-free) (Biotest, Dreieich, Germany). After removal of unbound protein, 1 μg/mL of the various anti-FVIII or anti-VWF antibodies were incubated for 1 hour followed by a 1-hour incubation with streptavidin poly-HRP as outlined above.

Confocal Immunofluorescence Microscopy

Endothelial cells were grown on collagen-coated coverslips and fixed 48 hours post-confluency with Cytofix/Cytoperm (Becton Dickenson, Mississauga, ON, Canada). Cells were permeabilized with 0.1% Triton X-100 for 10 minutes and blocked for nonspecific binding using Protein Block (Agilent, Santa Clara, CA). Rabbit anti-VWF (Agilent) and Sheep anti-FVIII biotinylated antibody (Affinity Biological, Ancaster, On) were incubated overnight at 4ºC followed by a 1-hour incubation with the appropriate secondary antibodies. Nuclei were counterstained with 0.4% 4’,6-diamidino-2-phenylindole (DAPI, Sigma) and mounted using fluorescent mounting media (Agilent). All images were taken by a Widefield Super-Resolution system Leica SR GSD microscope. The amount of detected FVIII protein was quantified using ImageJ software. Fluorescent intensity from 5 separate images was obtained after subtracting the average of the corresponding isotype.

Stimulated Release of FVIII and VWF

Endothelial cells (500,000 per well of a 6-well plate) were cultured for 72 hours before stimulating with 160 mM PMA, 100μM histamine or 1μM DDAVP with or without 100 μM IBMX for 1 hour. Culture media and cell lysates were harvested to assess FVIII and VWF antigen as above.

Exposure of Endothelial Cells to Laminar Shear Stress

ECFCs and hLECs were grown in EMB2 media (Lonza, Mississauga, ON, Canada) supplemented with EGM Endothelial SingleQuots Kit (Lonza) and 10% BSA and 5% L-glutamine, on collagen coated plates. At 95% confluency, cells were harvested and 1.5 X 10^5 cells in 100 μL of media were seeded onto an ibidi μ-Slide, then returned to the incubator for 2 hours to adhere. Laminar shear stress experiments used the ibidi Pump and Perfusion System. Shear stress experiments at 2 or 15 dynes/cm^2 were carried out at 37°C for 48 hours. Initial 30-minute exposures of 2 and 15 dynes cm^2 were employed for the 15 dynes/cm^2 studies to enable cells to adhere to the slides. For static conditions, cells on ibidi μ-Slide were maintained at 37°C for 48 hours.

Relative Quantitative Real-time PCR

To assess relative changes in FVIII and VWF RNA expression after exposure to laminar shear stress, cells were lysed on the ibidi μ-Slide and total RNA was isolated using the RNAqueous-micro kit (Invitrogen). RNA (200 ng) was reverse transcribed using SuperScriptIII Reverse Transcriptase (Invitrogen) and 10 (VWF) or 20 (FVIII) ng of the cDNA was used for each real-time PCR reaction. qRT-PCR was used to measure FVIII, VWF, KLF2, THBD and VCAM1 mRNA expression from cells exposed to laminar shear stress relative to static conditions. GAPDH was used as the internal control. Each sample was run in triplicate and the fold change in gene expression was calculated using the comparative C_T method.[42]

Statistics

Statistical analysis was performed with GraphPad prism 4.03 for Windows (GraphPad Prism, San Diego, CA, USA). Data are presented as the mean ± SD and statistical comparisons were calculated using a two-tailed Student’s t test.

Results

We confirmed the endothelial phenotype of all endothelial cell subtypes using flow cytometric analysis for positive expression of CD31, CD144, CD146 and CD54 and absence of hematopoietic (CD14) and progenitor (CD34) markers. The results in Supplementary Table 2 show a characteristic endothelial molecular signature for hPMECs, hHSECs, HUVECs and ECFCs (two isolates M1 and M7). Lower levels of CD31 and CD146 expression were observed on hLECs, however, these cells are positive for podoplanin, a characteristic of lymphatic endothelial cells.

Levels of FVIII and VWF Gene Expression Vary Among Endothelial Subsets

To assess FVIII and VWF gene expression levels, qRT-PCR was carried out on total RNA and levels are presented in Figures 1A and 1B respectively. Overall, absolute FVIII RNA expression in this subset of endothelial cells is very low and only barely detectable in HUVECs (135 copies/per 10 ng total RNA) and the two individual isolations of ECFCs (mean 189 copies/per 10 ng total RNA). While hLECs express approximately 12-fold higher levels of FVIII than the other endothelial cells, levels of expression are 100- to 2,000-fold

less than that observed for absolute levels of VWF mRNA, where levels ranged between 134,571 (hHSECs) and 391,602 (ECFCs-M7) copies/per 10 ng total RNA.

In general, endothelial cell subtypes that express lower levels of FVIII, for example ECFCs, were associated with higher VWF expression and subtypes of endothelial cells with higher levels of FVIII expression, such as hLECs, were associated with lower VWF expression. A summary of mean FVIII and VWF mRNA expression in the various endothelial cells is presented in Supplementary Table 3.

During this study, frozen stocks of hPMECs and hLECs were depleted and additional cells were acquired from the supplier. We observed some variability between the original and newly acquired cells in FVIII expression for hLECs and VWF expression for hPMECs. We wanted to investigate if levels of expression also varied between different ECFC isolations and therefore isolated ECFCs from 2 additional healthy volunteers, M10 and M30. Additionally, to assess differences in individual cells within a specific isolation, single cells were identified and expanded (clones 1–3) for both M10 and M30. FVIII and VWF RNA expression was assessed and the results are presented in Supplementary Figure 1. As observed with the original ECFC isolations, levels of FVIII expression in all clones from both individuals were barely above the limits of detection (1.4 mU/mL) and no significant differences in expression were observed between the individual isolated ECFC clones for M10 ($p=0.331$) or for M30 ($p=0.271$). VWF RNA expression in all the isolated ECFC clones was approximately 100-fold higher than FVIII levels and significant differences in levels of VWF were observed between the different clones isolated from M10 ($p=0.0021$) and M30 ($p=0.0042$). A similar reciprocal relationship between FVIII and VWF expression exists in these individual ECFC clones as with all subtypes of endothelial cells analyzed in this study.

Colocalization of FVIII and VWF in Weibel-Palade Bodies is Limited to hLECs

Since levels of RNA are not necessarily predictive of protein expression, we evaluated FVIII and VWF protein levels in our subsets of endothelial cells. Despite enhancing the sensitivity of our ELISA with a biotinylated FVIII capture antibody in conjunction with Streptavidin Poly-HRP, a 23-fold concentration step of the culture media was necessary to detect FVIII protein production from any of these endothelial cells. Despite these efforts, FVIII could only be detected in hHSECs, hPMECs and hLECs (Figure 2A). While a concentration step was not necessary to detect FVIII in cell lysates, Levels of FVIII antigen were barely above detection levels (Figure 2B). Similar to results for FVIII RNA expression, levels of FVIII antigen were considerably higher in hLECs compared to the other endothelial cells. Interestingly, while levels of FVIII mRNA expression (339.2 ± 240.4 copies/10 ng total RNA) were detected in HEK293 cells, no FVIII antigen could be detected in the lysate of these cells indicating that FVIII RNA is not always translated. This may suggest a role for post-transcriptional regulation in endothelial-specific expression of FVIII.

Levels of VWF antigen were readily detected in all endothelial cells in both the culture media and cell lysates, and as the results in Figures 2C and 2D illustrate, VWF protein levels varied between the different endothelial subsets with lower levels of expression observed in

hHSECs, hPMECs and hLECs. A summary of mean protein levels for both FVIII and VWF is presented in Supplementary Table 3.

To confirm FVIII protein expression, assess intracellular location and possible co-localization of FVIII with VWF, we carried out immunofluorescence microscopy. To begin this analysis, we verified the specificity of various anti-FVIII and anti-VWF antibodies by assessing binding of each antibody to human recombinant FVIII or VWF. The results presented in Supplementary Figure 2, clearly show that anti-FVIII antibodies MA1–10589 and B3124 bind to VWF and not to FVIII. However, anti-FVIII antibodies F8C-E1A-D, GMA 8015 and GMA 8018 bind only to FVIII while the anti-VWF antibody PO226, binds specifically to VWF. Representative images showing FVIII expression for each of the endothelial subsets are presented in Figure 3 and for all but hLECs, FVIII is barely detectable. When compared to hLECs, the mean fluorescent intensity is at least 2-fold lower in the other endothelial subsets. In contrast, VWF is readily detectable in the cytoplasm and Weibel-Palade bodies of all the different types of endothelial cells (Figure 4). However, there is considerable variability in levels of fluorescent intensity between the endothelial subsets with the lowest and highest levels observed in hHSECs and HUVECs, respectively.

Colocalization of FVIII with VWF was only detected in hLECs and this colocalization was limited to cytoplasmic granules showing some features of WPBs with no evidence of co-localization in the cytoplasm (Supplementary Figure 3). Co-localization of P-selectin and Ang-2 with VWF in these granules (data not shown) confirmed that the granules are WPBs. None of the other endothelial cell subtypes, demonstrated FVIII-VWF colocalization.

Regulated secretion of VWF and FVIII

Regulated release of VWF from Weibel-Palade bodies using secretagogues such as PMA and histamine is well documented, and since this is the first reported observation of FVIII colocalization with VWF in WPBs in unmodified endothelial cells (hLECs), we sought to determine if it can be released in a similar manner. While we were unable to detect FVIII in culture media after stimulation, levels of FVIII in the cell lysate were within the limits of sensitivity of the FVIII ELISA and therefore we used the relative reduction in intracellular levels as a surrogate for exocytosis of these two proteins. As the results presented in Supplementary Figure 4 clearly show, relative to unstimulated hLECs, there is a highly significant ($p<0.0001$) reduction of both FVIII and VWF in the cell lysates when these cells are stimulated with PMA. A similar relative reduction was not observed after histamine stimulation, presumably because it is a much weaker agonist.

When humans are treated with DDAVP there is an increase in plasma VWF that is presumed to originate from endothelial cells. However, similar findings have not been documented in cultured endothelial cells. DDAVP acts specifically on the vasopressin V2 receptor and to investigate DDAVP-stimulated release in our subset of endothelial cells, we first assessed levels of *AVPR2* gene expression in these cells. Using GAPDH to normalize data to cell numbers, the ΔCT was determined and are shown in Supplementary Figure 5 along with levels of expression relative to HEK293 cells ($2^{-\Delta\Delta CT}$). In general, levels of expression of *AVPR2* are very low but are substantially higher in hLECs and hPMECs compared to the other endothelial subsets. We therefore examined DDAVP-simulated release of VWF

from these two endothelial cell types along with HUVECs since they have been extensively studied in the past. In addition to DDAVP, we also tested PMA and histamine as well-known stimulants of VWF secretion.

Stimulated release of VWF was assessed by calculating the percentage of the total VWF produced that was secreted into the media after stimulation with each of the agonists. As the results presented in Supplementary Figure 6 show, of the total VWF produced by hLECs, hPMECs and HUVECs, substantially more VWF is secreted (27.1% versus 18.1% and 15.6% respectively) by hLECs after PMA stimulation. Similarly, more VWF was released from hLECs (7.13%) compared to hPMECs (4.15%) and HUVECs (5.4%) when the cells were stimulated with histamine.

These results are in contrast to that observed when cells were stimulated with DDAVP as no significant release of VWF relative to unstimulated cells was detected. DDAVP is a weak agonist whose mechanism of action is dependent on cAMP, and cAMP has a very short half-life. To preserve intracellular levels, IBMX was included with DDAVP and regulated secretion was reassessed. While this combination causes a significant release of VWF from hLECs and HUVECs relative to unstimulated cells, there is no significant difference in release of VWF between the DDAVP/IBMX combination and IBMX alone. Taken together, the low expression of *AVPR2* along with the lack of a significant release of VWF after DDAVP stimulation suggests that these endothelial cell subsets may not account for the increase of VWF seen in patients treated with DDAVP.

Laminar Shear Stress Modifies FVIII and VWF Gene Expressions

Evaluating gene expression in endothelial cells under static conditions does not recapitulate conditions *in vivo* since endothelial cells are exposed to shear stresses associated with flowing blood and levels of these forces vary throughout the vasculature. To assess effects of laminar flow on FVIII and VWF expression, we exposed ECFCs and hLECs to arterial (15 dynes/cm^2) or venous/lymphatic (2 dynes/cm^2) levels of shear stress for 48 hours and compared relative levels of RNA expression between static and flow conditions. We used increased expression of KLF2 and THBD along with decreased expression of VCAM1 as controls for laminar shear stress exposure. The results presented in Figure 5 show when ECFCs are exposed to higher levels of laminar shear stress there is a modest 1.33-fold increase in FVIII RNA expression along with a 1.42-fold decrease in VWF expression (Figure 5B). The effect of higher levels of shear stress on FVIII and VWF RNA expression is much more significant in hLEC with a 6.10-fold and 2.44-fold reduction respectively (Figure 5D). When ECFCs are exposed to lower levels of laminar shear stress (Figure 6B) there is a 2.07-fold and 1.22-fold increase in levels of FVIII and VWF RNA expression, respectively. In contrast, levels of FVIII and VWF expression are decreased 4.13-fold and 1.29-fold respectively in hLECs. A summary of the fold-change in FVIII and VWF expression after exposure to 2 or 15 dynes/cm^2 in hLECs and ECFCs is presented in Supplementary Figure 7.

Discussion

Much of the phenotypic heterogeneity of vascular endothelial cells results from their sensing and responding to specific tissue microenvironmental cues, and thus when these cells are removed from the body and grown in static culture, they become uncoupled and undergo phenotypic drift.[43,44] While this presents limitations for using cultured endothelial cells in assessing transcriptomes associated with site-specific heterogeneity, permanent epigenetic modifications to genes also contribute to vascular bed-specific heterogeneity and these epigenetic marks are resistant to the cellular microenvironment and are mitotically stable.[45] Prior studies have shown heterogeneity in levels of FVIII[14,20,46] and VWF[23,47–49] expression across different vascular beds, and our study has evaluated whether this heterogeneity is preserved in cultured human endothelial cells. The results we present here clearly show heterogeneity in expression of both FVIII and VWF within subsets of endothelial cells, and while it is important to bear in mind that this heterogeneity may be influenced by phenotypic drift, the results highlight that these cells could provide an opportunity to investigate epigenetic mechanisms that contribute to the heterogeneity..

Overall, levels of FVIII expression in our subset of cultured endothelial cell are extremely low, reflecting levels observed *in vivo* and highlighting why its site of production has been controversial. There is a very significant difference between levels of expression of FVIII and VWF, and within different endothelial cell subtypes, VWF RNA expression is between 100-fold to 1,000-fold higher than that observed for FVIII. Similar to other reports,[16,20] we have shown consistent evidence of reciprocity for FVIII and VWF expression. While the explanation for this phenomenon is unknown, we do know that high level FVIII expression can induce cellular stress[50] and thus one could speculate that most cells would avoid expressing both these large and complex proteins to minimize the biosynthetic stress that would likely result.

FVIII protein is barely detectable in any of the endothelial cells other than hLECs and these very low levels present significant technical challenges, not only in quantifying protein levels but also in assessing its subcellular location and stimulated release. FVIII protein levels were among the lowest in HUVECs and were barely visible in confocal analysis of immunostained FVIII. Our results are in contrast to those of Pan et al[16] who detected significant amounts of intracellular FVIII, and Turner and Moake[9] who present images showing high levels of FVIII in HUVECs. In these studies however, the anti-human FVIII antibody clones F8 2.2.9 (LSBioies; LS-B3124) and F8–5.5.72 (Thermo Fisher Scientific; MA1–10589) were used. In our hands, both these antibodies bind to VWF and not FVIII, thus emphasizing the critical requirement of appropriate controls for determining antibody specificities.

With the exception of hLECs, visualizing FVIII by confocal analysis or detecting it in an ELISA required considerable optimization and even then, FVIII protein could not be detected in unconcentrated culture media, was at the limit of detection in all cell lysates and was barely detectable in immunocytochemistry images. However, levels of FVIII RNA expression are 4.6-fold, 7.9-fold and 15.61-fold higher in hLECs than hPMECs, hHSECs and HUVECs, respectively. The higher levels of FVIII in hLECs presented an opportunity

to gain insights into the storage and stimulated release of FVIII from Weibel-Palade bodies. Confocal analysis of these cells shows that FVIII can naturally be stored together with VWF in WPBs. While very low amounts of FVIII can be seen in the cytoplasm of the other endothelial cell subsets, very little, if any is detected in WPBs. Given that WPBs are present in all the endothelial subtypes, it is possible that a minimum threshold of expression much exist before FVIII can be trafficked into these storage organelles. Our studies of the stimulated release of FVIII from Weibel-Palade bodies was inconclusive as the FVIII ELISA was not sensitive enough to detect the very low levels of FVIII released into media. However, after hLECs were stimulated with the potent secretogogue PMA, a significant reduction in the level of intracellular FVIII was observed, suggesting it was released into the media. Collectively, these results indicate for the first time that in primary unmodified endothelial cells, FVIII can be stored with VWF in WPBs and that regulated release of this protein occurs after stimulation with a strong agonist.

Liver sinusoidal endothelial cells regarded as an important source for plasma FVIII and yet when cultured *in vitro,* hHSECs express very little FVIII. It is possible that the microenvironment plays an important role in regulating FVIII expression in the liver and accounts for these low levels. However, given the large size of the liver (25 billion cells in an average adult liver)[51] and that 15–20% of the total number of cells are sinusoidal endothelial cells,[52] very low expression levels of FVIII may be sufficient to produce the low levels of circulating plasma FVIII. Interestingly, we observed a 7.9-fold reduction in FVIII RNA expression between hLECs and hHSECs, whereas the BioGPS database reports a 2.3-fold reduction. This would suggest that epigenetic mechanisms have a significant influence on endothelial heterogeneity but that these influences can be overridden by the microenvironment.

Cultured endothelial cells are not exposed to the biomechanical forces they normally experience within the vasculature, and it is well established that variations in levels of shear stress are associated with endothelial heterogeneity. Our results demonstrate that laminar shear stress effects levels of FVIII expression and this effect is remarkably different between the two subtypes of endothelial cells studied. While there is a large reduction in FVIII expression when hLECs are exposed to either higher or lower levels of laminar shear stress, a marked increase in FVIII expression occurs in ECFCs under identical conditions. Furthermore, the level of shear stress affects the level of FVIII expression, with higher levels associated with lower levels of FVIII expression. This suggests that shear stress may contribute to the observed vascular heterogeneity in FVIII expression and may play a role in down-regulating its expression. In stark contrast to FVIII, laminar shear stress does not influence VWF expression to the same extent. While there are reductions in VWF expression at both levels of shear stress in hLECs, the reduction is substantially less that that observed with FVIII. Furthermore, changes in VWF expression are not as pronounced in ECFCs as that observed for FVIII under these conditions. Laminar flow is associated with a healthy, non-dysfunctional endothelial phenotype and our results illustrate its role in contributing to the anti-coagulant component of this phenotype by reducing expression levels of both FVIII and VWF.

Most *in vitro* studies to assess various physiological functions of endothelial cells have been carried out using cells such as HUVECs and ECFCs, and results are proposed as representative of endothelial cells *in vivo.* However, the response of cultured endothelial cells to DDAVP is an example where an obvious correlation with *in vivo* effects does not exist. In patients treated with DDAVP, an increase in high molecular weight VWF in plasma results from its exocytosis from WPBs. In contrast, this phenomenon is not observed in cultured HUVECs[41] or ECFCs.[53] DDAVP acts via the vasopressin V2 receptor[41,54] and while evidence of V2 receptor presence on various endothelial cell subtypes has been assessed,[9,41] DDAVP induced VWF release has only been demonstrated in cultured human lung microvascular endothelial cells (HMVEC-L).[41] We assessed AVPR2 expression in the subset of endothelial cells examined in this project, and while levels of expression are generally very low, they are significantly higher in hLECs and hPMECs. However, we were unable to detect DDAVP-stimulated release of VWF from any of these endothelial cell subtypes. This is in contrast to results presented by Kaufmann et al[41] where they reported a significant release of VWF into culture media from human lung microvascular endothelial cells. However, it has been reported that FVIII is expressed in both kidney[20] and bladder[55] epithelial cells and the bladder may be the extrarenal source of the DDAVP induced release of FVIII. Alternatively, there is evidence that DDAVP induces release of platelet-activating factor from monocytes and it is this factor that facilitates WPB exocytosis.[56] Clearly additional studies will be required to address the disparity between *in vivo* and *in vitro* responses of endothelial cells to DDAVP.

At first glance it is difficult to rationalize why FVIII is expressed at such high levels in lymphatic endothelial cells (LECs). Release of this coagulation co-factor into the lymph is not a timely or direct route to circulating blood where it would be needed for hemostasis. The only blood cells present in lymph are lymphocytes. With these facts in mind, it is possible that the FVIII expressed in LECs does not play an initial role in hemostasis but rather has an important function in inducing immunological tolerance. While the thymus is the primary site for induction of central tolerance, not all self-reactive T cells are eliminated here and for a growing number of antigens, central tolerance is not established in the thymus at all.[57] Lymph nodes are increasingly recognized as important secondary compartments for T cell deletion, anergy and regulatory T cell differentiation and are essential for induction of peripheral tolerance to self-antigens. LECs play an important part in this process by expressing peripheral tissue antigens in an Aire-independent manner, and serve as an antigen reservoir in lymph nodes.[58] These peripheral antigens are transferred to dendritic cells for subsequent presentation to $CD4^+$ T cells and the induction of anergic and regulatory T cells associated with peripheral tolerance.[59] Mechanisms associated with immunological tolerance to FVIII are poorly understood and therefore cultured hLECs present an opportunity to gain novel insights into mechanisms contributing to immunological tolerance to FVIII.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Essentials

- Endothelial cells in different vascular beds exhibit significant phenotypic heterogeneity.
- There is heterogeneity and reciprocity in FVIII and VWF expression in a subset of cultured human endothelial cells.
- Elevated levels of FVIII colocalize with VWF in Weibel-Palade bodies and are released together when endothelial cells are stimulated.
- Arterial, venous and lymphatic levels of laminar shear stress influence FVIII and VWF expression.

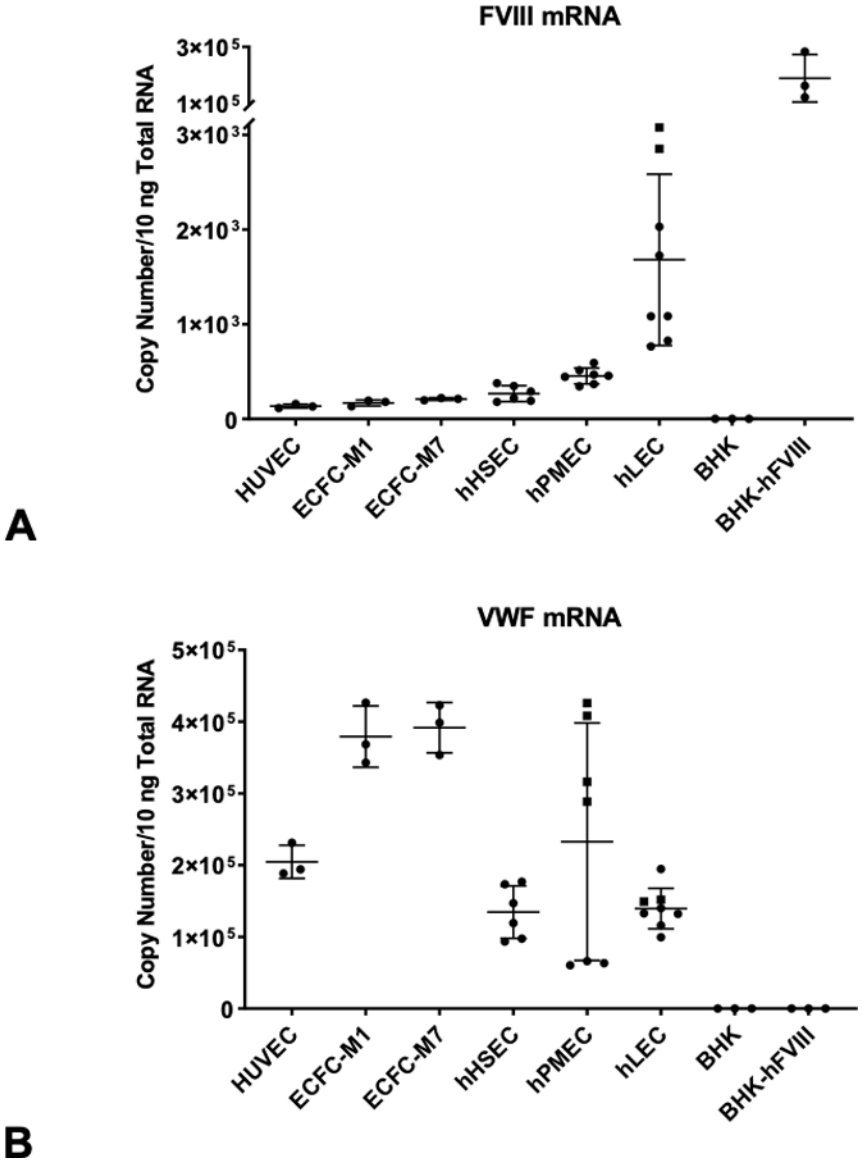

Figure 1.

Levels of FVIII and VWF RNA expression in cultured endothelial cells. Total RNA was isolated from cultured endothelial cells and copy numbers for FVIII (A) and VWF (B) were determined based on 10 ng (VWF) and 20 ng (FVIII) of total RNA using quantitative real-time PCR. Standard curves of FVIII and VWF mRNA copy numbers were generated by quantifying the concentration of *F8* and *VWF* cDNA-containing plasmids. All reactions were carried out in triplicate and copy numbers were assessed for both FVIII and VWF from a minimum of 3 independent experiments. Circles and squares represent mean values obtained from different purchased endothelial cell lots. Mean values (horizontal lines) and standard deviations are shown. Cultured BHK cells were used as a negative control for FVIII and VWF expression. BHK cells containing a hFVIII plasmid (BHK-hFVIII) were used as a positive control for FVIII mRNA and also served as a negative control for VWF mRNA.

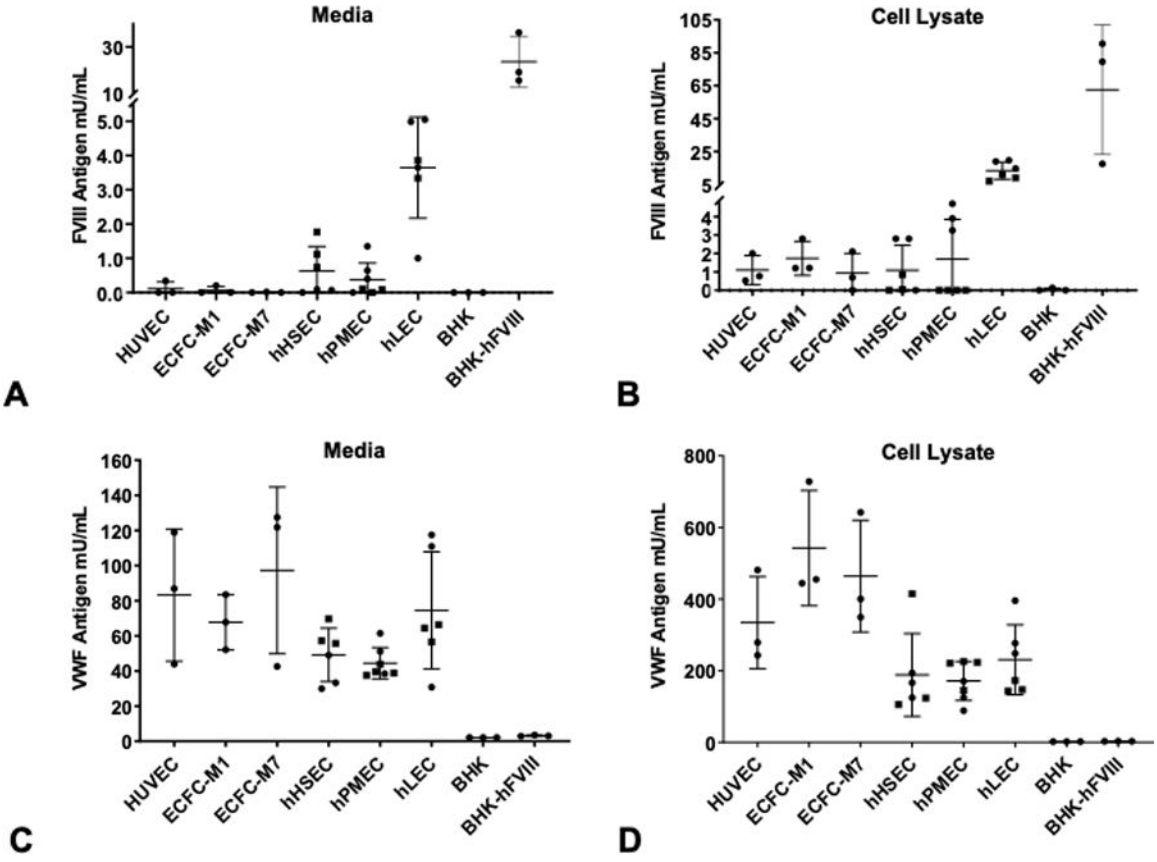

Figure 2.

Quantification of FVIII and VWF protein in cultured endothelial cells. Endothelial cells seeded at 500,000 cells in a 6-well plate were cultured for an additional 72 hours after reaching confluence. Levels of FVIII (A and B) and VWF (C and D) protein in media and cell lysates were determined using sandwich ELISAs. To measure FVIII in the media a 23-fold concentration step was required, and levels presented (A) represent converted values for unconcentrated media. All assays were carried out in duplicate and individual experiments are depicted and represent the average values. Closed circles and squares represent levels of FVIII or VWF obtained from different endothelial cell lots. Mean values (horizontal lines) and standard deviations are shown. BHK cells were used as a negative control for FVIII and VWF and BHK-hFVIII was used as a positive control for synthesis of FVIII and additional negative control for VWF.

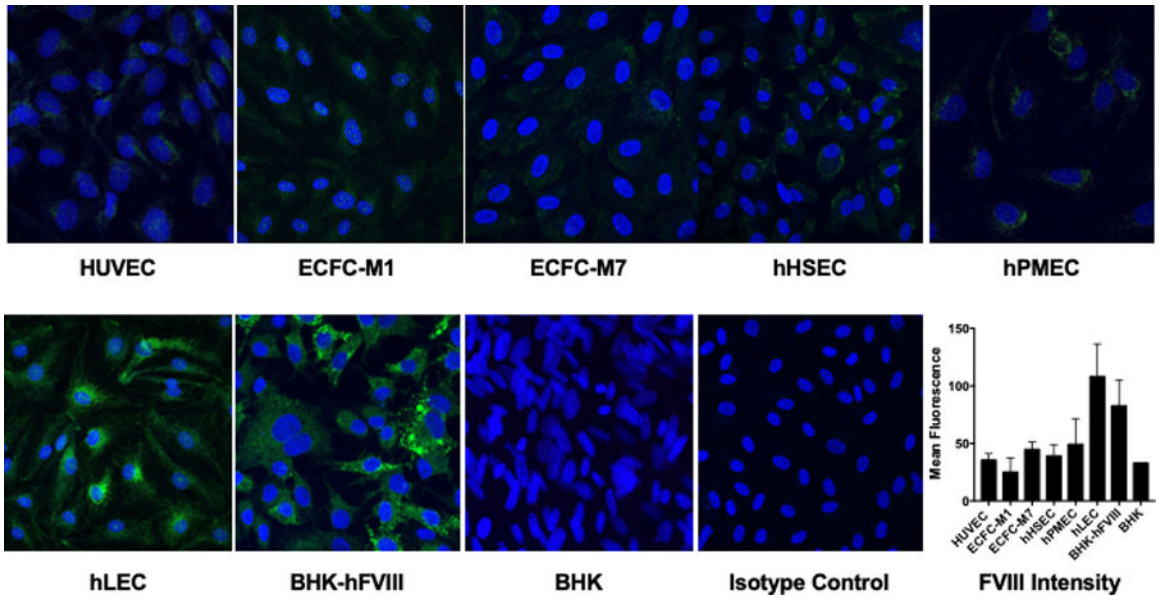

Figure 3.

Immunolocalization of FVIII in cultured endothelial cells. Endothelial cells were cultured on collagen-coated coverslips and permeabilized cells were stained for FVIII (green) with the polyclonal biotinylated anti-FVIII antibody F8C-E1A-D (Affinity Biologicals). Representative images show that FVIII is barely detectable in any of the subset of endothelial cells other than hLECs. BHK-hFVIII and BHK were used as a positive and negative controls respectively for FVIII expression. Isotype controls were used for each endothelial cell and representative images show virtually no background fluorescence of FVIII. FVIII protein was quantified using ImageJ software and the mean fluorescent intensity from 5 separate images is shown in the graph at the bottom right. The images are magnified 63X

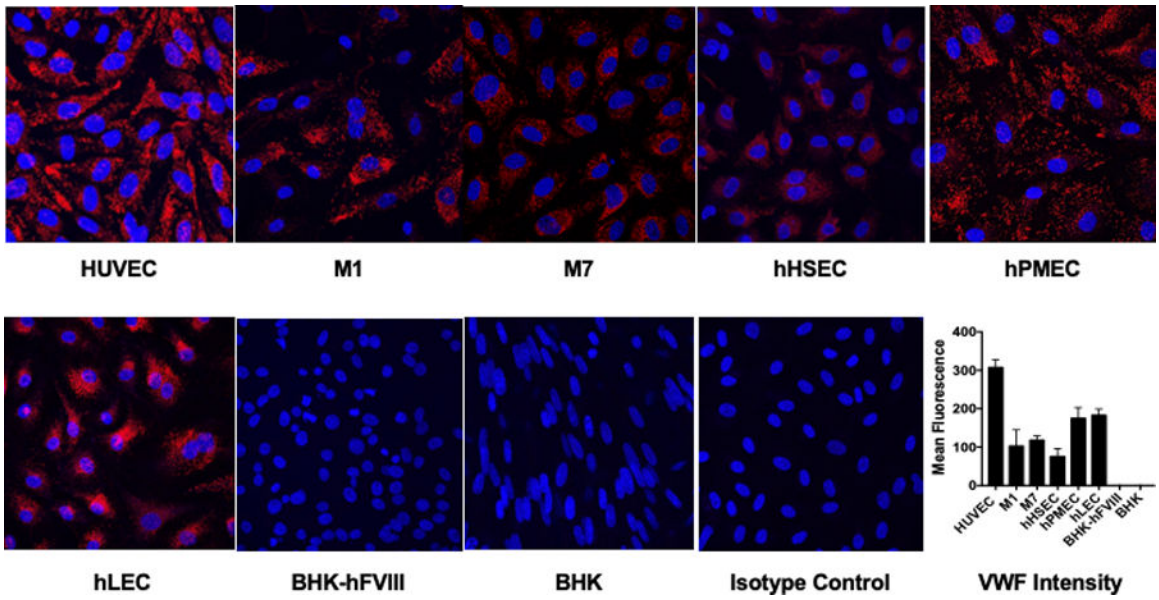

Figure 4.

Subcellular localization of VWF in cultured endothelial cells. The subgroups of endothelial cells were cultured for 48 hours post-confluency and stained for VWF (red) with the anti-VWF antibody P0226 (Dako). BHK-hFVIII and BHK which were used as a negative controls for VWF expression and along with the isotype control show virtually no background fluorescence of VWF. VWF protein was quantified using ImageJ software and the mean fluorescent intensity from 5 separate images is shown in the graph at the bottom right. The magnification of the images is 63X.

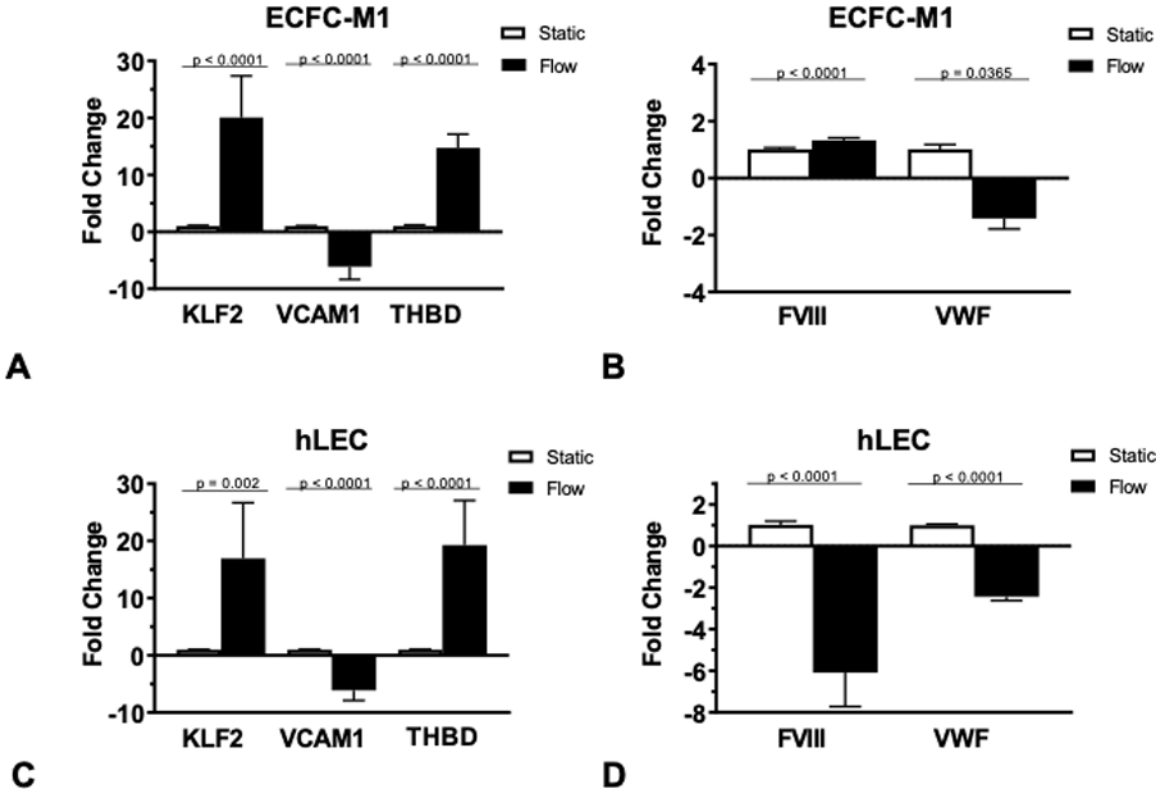

Figure 5.

Changes in FVIII and VWF RNA expression when endothelial cells are exposed to arterial levels of laminar shear stress. ECFCs isolated from individual M1 and hLECs were exposed to arterial levels (15 dynes/cm^2) of laminar shear stress for 48 hours. Levels of expression of KLF2, VCAM1, THBD, FVIII and VWF RNA were determined using real-time quantitative PCR. GAPDH was used to control for cell numbers. The fold-change in FVIII and VWF expression relative to cells exposed to static conditions is shown in panels B and D respectively. KLF2, VCAM1 and THBD are used as controls for exposure to laminar shear stress. KLF2 and THBD expression should increase while VCAM1 levels should decrease. Samples were run in triplicate and experiments were repeated a minimum of 3 times. Mean fold-change in levels of RNA expression are shown with error bars representing standard deviation. An unpaired t test was used to compare the fold-change in expression between static (white) and flow (black) conditions and p values displayed above the bars for each gene.

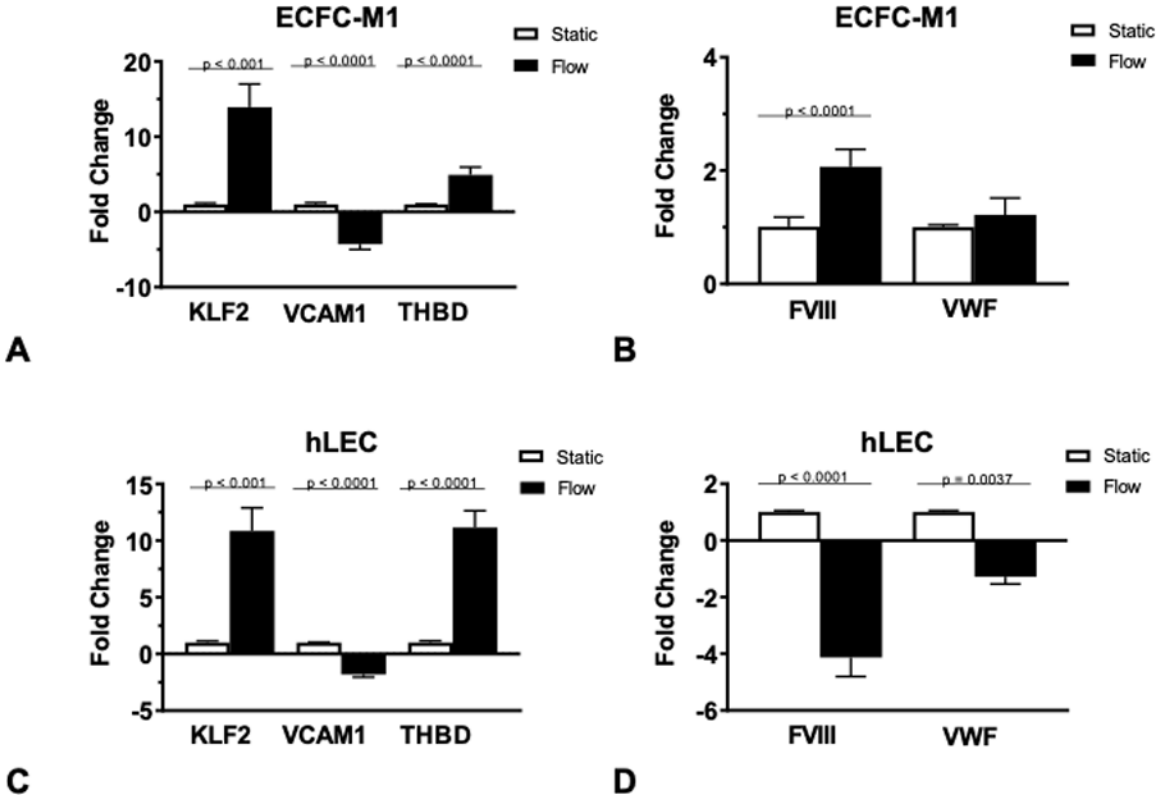

Figure 6.

Changes in FVIII and VWF RNA expression when endothelial cells are exposed to low levels of laminar shear stress. ECFCs (panels A and B) and hLECs (panels B and D) were exposed to venous and lymphatic levels (2 dynes/cm^2) of laminar shear stress for 48 hours. Levels of expression of KLF2, VCAM1, THBD, FVIII and VWF RNA were determined using real-time quantitative PCR. GAPDH was used to control for cell numbers. The fold-change in FVIII and VWF expression relative to cells exposed to static conditions for ECFCs and hLECs is shown in panels B and D respectively. KLF2, VCAM1 and THBD are used as controls for exposure to laminar shear stress (panels A and C). KLF2 and THBD expression should increase while VCAM1 levels should decrease. Samples were run in triplicate and experiments were repeated a minimum of 3 times. Mean fold-change in levels of RNA expression are shown with error bars representing standard deviation. An unpaired t test was used to compare the fold-change in expression between static (white) and flow (black) conditions and p values displayed above the bars for each gene.