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Research Article

Proteomic Analysis Reveals GLUT1 to be a Novel Discriminating Marker of Human Arterial Endothelium *In vivo* and loss of Venous Identity in Cell Culture

Abstract

Despite greatly improved understanding of endothelial heterogeneity, the number of molecules discriminating human arterial and venous endothelium remains limited. Indeed, there have been few reports validating markers proposed in animal model studies in freshly isolated human tissues. We report here the global characterization of freshly isolated human umbilical arterial and venous endothelial cell (HUAECs and HUVECs) plasma membrane proteins using an experimentally validated label-free quantitative LC-MS/MS platform. ECs were harvested by enzymatic digestion and purified by flow cytometry (CD31⁺, CD45⁻) prior to quantitative analyses. Following plasma membrane fractionation, we identified 4,300 proteins with high confidence using LC-MS/MS. GLUT1, an important regulator of endothelial function, was found to be up regulated in HUAECs 2.6 fold at the protein level and confirmed at the mRNA level using qRT-PCR. Using tissue immunohistochemistry, we discovered that GLUT1 expression was restricted to the cell surface in human arterial endothelium using umbilical cord and adult peripheral vascular sections. Importantly, GLUT1 mRNA levels decreased 20 fold in cultured arterial ECs within 48hrs of culture and continued to decline for 12 days *in vitro*. Principal Component Analyses demonstrated a profound effect of cell culture on protein expression signatures with cultured HUVECs, fresh HUVECS, and fresh HUAECs equally distinct. GLUT1 expression serves as a robust discriminator of arterial versus venous ECs *in vivo* and marks a loss of venous EC identity *in vitro*.

novel regulating factors beyond the standard transcriptional networks. The majority of EC-related proteomic publications have relied upon studies of cultured human umbilical venous endothelial cells (HUVECs), and those instances where freshly isolated vascular tissues were studied have been restricted to non-human samples [5].

Importantly, environmental differences between vessels (e.g. hemodynamic forces, direction of blood flow, sub-endothelial matrices, and interaction with smooth muscle cells) have been validated as important drivers of EC identity [6-8]. Recent work has revealed that gene expression signatures of freshly isolated arterial and venous endothelium are lost within 48 hours of cell culture [9]. Accordingly, we sought to use a proteomic approach to identify accurate and reliable protein signatures of human arterial and venous ECs *in vivo*, thus accounting for environmental differences in phenotypes by incorporating freshly isolated tissues, and, at the same time, characterizing the effect of standard cell culture conditions. Because plasma membrane-associated protein profiles are likely to include a repertoire of relevant cell surface proteins that can be probed readily by antibody, we fractionated the samples into plasma membrane and non-plasma membrane fractions using a recently developed membrane protein enrichment method [10]. Accordingly, we have established a novel method for characterizing protein signatures of endothelial heterogeneity.

Introduction

HUVECs were first isolated and cultured successfully in 1973 [1] and have been used thereafter as a model of human endothelial function and behavior predominantly via cell culture [2]. It is known that endothelial cell (EC) culture conditions do not mimic *in vivo* conditions in several important ways, including oxygen concentration, shear stress, growth media, and artificial contact surfaces. Although HUVECs are the most commonly used model of human endothelium, few studies have addressed the effects of cell culture and even fewer focused solely on the effects of cell culture on endothelium.

Through use of zebra fish, mouse, chick, and other animal models, numerous markers discriminating arterial and venous endothelial cells (ECs) have been proposed [3], with few validated in freshly isolated human tissues [4]. Despite efforts to find unique vascular endothelial markers that have ranged from peptide phage display to global transcript expression profiling, the number of useful markers that can discriminate arterial from venous EC states in a binary fashion remain limited. Moreover, few studies have investigated

Materials and Methods

Media and reagents

Endothelial Growth Medium-2 (EGM-2), which is Endothelial Basal Medium-2 (EBM-2) (Lonza) with additives (Bullet Kit) provided by the manufacturer supplemented with 10% fetal-bovine serum (FBS; Hyclone), 2% penicillin/streptomycin (Invitrogen), and 0.25 µg/mL amphotericin B (Invitrogen).

HUVEC and HUAEC isolation

Human umbilical cords were obtained from healthy newborns (38 – 40 weeks gestation). In each cord, either an umbilical artery or umbilical vein was canalized, rinsed twice with DPBS supplemented with 4.8 mM sodium pyruvate (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and then infused with 100 µg/mL Liberase Blendzyme2 (Roche Applied Science, Indianapolis, IN, USA) and supplemented DPBS. After 14 minutes of incubation at 37°C, detached ECs were eluted into a 50mL tube (Falcon) and centrifuged at 400 x g for 5 minutes. The cell pellet was resuspended and cells plated as described below for cell culture or directly purified for freshly isolated cell comparisons via flow cytometric cell sorting prior to lysis for either RNA or protein isolation. Briefly, cells were stained with anti-human CD31 and CD45 antibodies (BD Biosciences Pharmingen, San Diego, CA) and were sorted in a BD FACSAria cell sorter (BD Biosciences) to isolate a CD31⁺CD45⁺ population as purified ECs. Samples of freshly isolated HUVECs (n=10) or HUAECs (n=7) were pooled totaling 1.02 x 10⁷ and 1.1 x 10⁷ cells/group, respectively.

Cell culture

Cells were seeded onto tissue culture plates pre-coated with type-1 rat-tail collagen (BD Biosciences) in EGM-2 at 37°C, 5% CO₂, in a humidified incubator. HUVECs and HUAECs were released from the primary culture dish by TrypLE™ Express (Gibco) and re-plated onto tissue culture flasks pre-coated with Type I rat-tail collagen for expansion. Three HUVEC clones were propagated to the third passaged and pooled to gain 1.02 x 10⁷ cells (3.4 x 10⁶ from each clone). To minimize the effect of proliferation that occurs in culture, cells were collected on the third day of the third passage when they had become 100% confluent.

Proteomics

Materials: Urea, DL-Dithiothreitol (DTT), triethylphosphine (TEP), iodoethanol, and ammonium bicarbonate (NH₄HCO₃) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade acetonitrile (ACN) with 0.1% formic acid (v/v) and water (H₂O) with 0.1% formic acid (v/v) were purchased from Burdick & Jackson (Muskegon, MI, USA). Modified sequencing grade porcine trypsin was obtained from Princeton Separations (Freehold, NJ, USA).

Membrane Protein Enrichment: The membrane protein enrichment was performed according to a published procedure which includes a cell disruption step and multiple wash steps [10]. Briefly, after 500 µL of H₂O were added to a sample, the sample was pipetted up and down 10 times, incubated for 10 min on ice, frozen for 1 min in liquid N₂, thawed at room temperature, and centrifuged for 10 min at 10,000 × g, 4 °C. The supernatant was transferred to

a new tube and collected as the cytoplasm (non-plasma membrane, non-PM) fraction. After 500 µL of H₂O were added to the pellets, the sample was pipetted up and down 20 times, incubated for 10 min on ice, centrifuged for 20 min at 100,000 × g, 4 °C. After two more wash steps, 200 µL of lysis buffer (8 M urea, 10 mM DTT solution freshly prepared) were added to the pellets. Fully solubilized samples and the non-PM fraction samples were then stored at -80°C until LC-MS/MS analysis. Protein concentration was determined by the Bradford Protein Assay using Bio-Rad protein assay dye reagent concentrate [11].

Protein Reduction, Alkylation, and Proteolysis for LC-MS/MS:

A 100 µg aliquot of each sample was placed in a new tube. The volume and concentration of each sample were adjusted to 200 µL with 4 M urea and then reduced and alkylated by triethylphosphine and iodoethanol as previously described [12]. The sample was incubated at 35°C for 60 min, dried by a Vacuum Concentrator Centrifugal System (RC 10.10, JOUAN), and reconstituted with 100 µL of 100 mM NH₄HCO₃ at pH 8.0. A 150 µL aliquot of a 20 µg/mL trypsin solution was added to the sample and incubated at 35°C for 3 h, after which another 150 µL of trypsin was added, and the solution incubated at 35°C for 3 h.

LC-MS/MS: The tryptic peptides were analyzed using a Thermo-Finnigan linear ion-trap (LTQ) mass spectrometer coupled with a Surveyor autosampler and MS HPLC system (Thermo-Finnigan). Peptides were injected onto the C18 RP column (TSKgel ODS-100V, 1.0 mm x 150 mm) at a flow rate of 50 µL/min. The mobile phases A and B were 0.1% formic acid in water and 50% ACN with 0.1% formic acid in water, respectively. The gradient elution profile was as follows: 10.0% B (90.0% A) for 7 min, 10.0-20.6% B (90.0-79.4% A) for 5 min, 20.6-65.6% B (79.4-34.4% A) for 148 min, 65.6-100.0% B (34.4-0.0% A) for 10 min, 100.0% B for 10 min. The data were collected in the “Data dependent MS/MS” mode with the ESI interface using normalized collision energy of 35%. Dynamic exclusion settings were set to repeat count 1, repeat duration 30 s, exclusion duration 120 s, and exclusion mass width 0.6 m/z (low) and 1.6 m/z (high).

Protein Identification and Quantification: The acquired data were searched against the International Protein Index (IPI) HUMAN database (ipi.HUMAN.v3.83.fasta) using SEQUEST (v. 28 rev. 12) algorithms in Bioworks (v. 3.3). General parameters were set to: peptide tolerance 2.0 amu, fragment ion tolerance 1.0 amu, enzyme limits set as “fully enzymatic - cleaves at both ends”, and missed cleavage sites set at 2. The searched peptides and proteins were validated by PeptideProphet [13] and ProteinProphet [14], in the Trans-Proteomic Pipeline (TPP, v. 3.3.0) ([http:// tools.proteomecenter.org/software.php](http://tools.proteomecenter.org/software.php)). Only proteins with probability ≥ 0.9000 and peptides with probability ≥ 0.8000 were reported. Protein quantification was performed using a label-free quantification platform, IdentiQuantXL™ [15].

Computational analyses: All dendrograms were generated by performing unsupervised hierarchical clustering with 1-Pearson correlation coefficient as the distance metric, and the ‘average’ linkage method. Differential expression analysis was performed using Student’s t-Test and false discovery rates [16], were computed using R’s p.adjust function. Unless otherwise noted, differentially

expressed proteins were defined as those with FDRs <5% and artery/vein ratios equal to or greater than +1.5 or equal to or less than -1.5. Gene (protein) enrichment analysis was determined by Chi-square test, comparing the proportion of differentially expressed genes to all genes overall to those genes in each gene set from Gene Ontology Biological Process [17-19]. Unless otherwise described, we defined enriched categories as those with Holm-multiple corrected p-values < 0.10 [20]. We used a previously described human endothelial cell gene regulatory network (GRN) to find the predicted transcriptional regulators of genes differentially expressed between vein and arterial samples [21].

Quantitative real time RT-PCR

HUVECs and HUAECs were released from the primary culture dish (2D) by TrypLE™ Express (Gibco) and centrifuged at 400xg for 5 min before homogenization in RLT buffer (QIAGEN). RNA was isolated using the RNeasy MicroKit (QIAGEN) according to the manufacturer's instructions using DNaseI on column genomic DNA digestion. RNA was quantified using a Nanodrop 1000 (Thermo Scientific) and quality was assessed by the A260/A280 and A260/A230 ratios. Reverse transcription was carried out using the Omniscript RT Kit (Qiagen) incorporating Oligo (dT) 15 primer (Promega). Quantitative Real-Time Reverse Transcript PCR (qRT-PCR) was performed using the FastStart Universal SYBR Green Master (ROX) (Roche) and 25 ng cDNA from three different patients' ECFCs per reaction. Amplification was performed in an ABI7500 Real-Time PCR system (Applied Biosystems). Cycling conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. 7500 Software (Applied Biosystems) was used to determine the cycle threshold (Ct) values. Data were analyzed using the 2- Δ Ct method using the housekeeping gene ATP5B for normalization. This method reports expression levels of transcripts relative to ATP5B. Each sample was measured in triplicate in three separate experiments, and a maximum standard deviation between Ct values of 0.3 was considered acceptable.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and immersed in a retrieval solution (Dako, Carpinteria, CA) for 20 minutes at 95°C to 99°C. Slides were incubated at room temperature with anti-human CD31 (clone JC70A, Dako) or anti-human SLC2A1/GLUT1 (clone SPM498, LSBio) for 30 minutes followed by 10-minute incubations with LSAB2 link-biotin and streptavidin-HRP (Dako), then developed with DAB solution (Dako) for 5 min.

Results and Discussion

Integrative analysis identifies SLC2A1 as a robust marker of *in vivo* AEC

Messenger RNA levels have been reported to explain about half of protein variation [22], implying that explanations of functional differences between AEC and VEC based on genomic profiling alone are incomplete. To address this inadequacy by characterizing novel protein markers of endothelial diversity, we performed label-

free quantitative liquid chromatography-tandem mass spectrometry (LFQMS) on freshly isolated and pooled HUVEC and HUAEC. We detected 2,796 and 2,227 proteins in plasma membrane-associated (PM) and non-plasma membrane-associated (non-PM) fractions, respectively, 1,276 of which were detected in both fractions (Supplemental Tables 1,2).

Differential protein expression analysis ($p \leq 0.05$; FDR $\leq 5\%$; Fold change ≥ 1.5 or ≤ -1.5) of fresh tissue arterial and venous signature proteins revealed 181 PM and 100 non-PM venous, and 218 PM and 78 non-PM arterial differentially expressed proteins (Supplemental Tables 3,4). To assess the differences between the proteome profiles of freshly isolated HUVECs and HUAECs within each fraction, we performed unsupervised hierarchical clustering, finding that cell types clustered with each other and were distinct from the other cell type, as expected (Figure 1A). From the clustered PM data, plasma membrane – cell surface protein signatures were derived (Figure 1B).

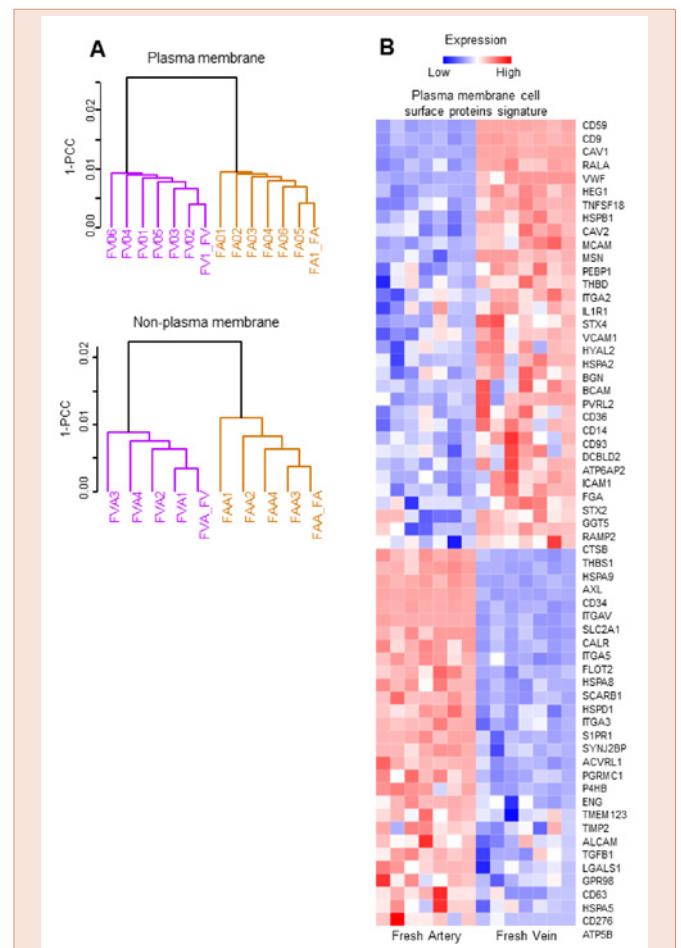


Figure 1: Freshly isolated human umbilical venous and arterial endothelium (HUVEC and HUAEC) plasma membrane protein heterogeneity. (A) Unsupervised hierarchical clustering of protein expression of freshly isolated HUVEC and HUAEC in PM and non-PM proteome data sets (1-PCC, Pearson correlation coefficient). FA = Fresh Artery (HUAEC); FV = Fresh Vein (HUVEC); numbers refer to individual samples. (B) Plasma membrane – cell surface protein signatures (highly differentially expressed proteins) in HUVEC and HUAEC. PM = plasma membrane.

To compare and contrast the functional pathways that characterize AEC and VEC, based on their respective protein expression profiles, we performed gene (protein) enrichment analysis. Notably, in both the PM and non-PM fractions, only gene annotations dominated by histone members (e.g. 'Packaging of telomere ends' and Nucleosome assembly') were highly enriched in the vein signatures (Figure 2).

The most differentially expressed cell surface proteins in the PM artery were associated with Gap junctions (e.g. connexins). Intercellular current spread in the vessel wall is essential to propagation and coordination of vasomotor signaling, a function easily carried out by endothelial cells because they are intensively coupled by means of gap junctions, including HUVEC and HUAEC [23], and these cells selectively regulate both metabolic and electrical coupling by changing the ratio of Cxn-43, Cxn-40, and Cxn-37 by differential expression of these gap functional proteins. We identified Gap junction alpha-1 protein (Cxn-43; GJA1) and Gap junction alpha-5 protein (Cxn-40; GJA5) in the PM fractions of fresh and cultured EC, though neither was differentially expressed. Nevertheless, consistent with previous observations [23-26], Cxn-40 was far more abundant in all cells than Cxn-43, especially in fresh arterial cells. The other prominent EC connexin, Gap junction alpha-4 protein (Cxn-37; GJA4), was not detected.

Because we observed little overlap between our protein signatures and mRNA expression datasets from previous studies comparing either freshly isolated HUVECs and HUAECs [4], or cultured bovine aortic ECs [27], we speculated that genes whose expression was consistently different at both mRNA and protein levels would be robust markers of endothelial identity. We further specified that suitable PM protein candidate markers should include the repertoire of cell surface proteins and thus be readily probed by antibody. SLC2A1, a glucose transporter also known as GLUT1, was a key candidate for a novel discriminating marker and one of the most highly differentially expressed PM proteins in our study (Figure 1B).

Using tissue immunohistochemistry, we validated that expression of SLC2A1 (GLUT1) was restricted to the extracellular plasma membrane of arterial endothelium, thus providing a discernable biomarker of HUAECs (Figure 3A). It is possible that this marker is tissue specific. To address this, we were able collect human peripheral artery and vein tissues from a senescent human male. We found that SLC2A1 was indeed restricted to arterial endothelium cell surface in this tissue as well (Figure 3B).

AEC and VEC identity is lost in cell culture

Cell culture conditions differ from normal, *in vivo* conditions in several key ways, including the substrate, static flow, and gas concentrations, with oxygen being much higher than physiologic states. Additionally, EC culture medium contains growth factors to ensure adequate cellular proliferation *in vitro*. To avoid obtaining proteome profiles characteristic of an *in vitro* proliferative state, we collected the cells after they reached 100% confluence. Global effects of cell culture on EC identity were assessed by comparing the protein expression profile of freshly isolated HUVECs and HUAECs to cultured HUVECs at passage 3. Using Principal Component Analysis (PCA), we found that these three cell types were equally distinct, indicating that cultured HUVECs were as different from fresh

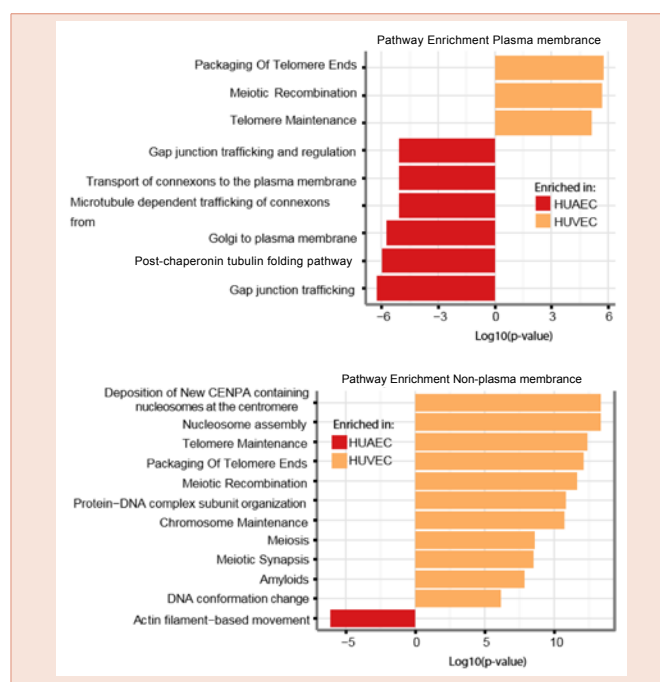


Figure 2: Biological process enrichment analyses in freshly isolated HUVEC and HUAEC. Biological pathways enriched in HUVEC and HUAEC PM (top) and non-PM (bottom) data sets. Enrichment was determined by Chi-square test, comparing the proportion of differentially expressed proteins to all proteins overall to those from the Gene Ontology Biological Process aspect. PM = plasma membrane.

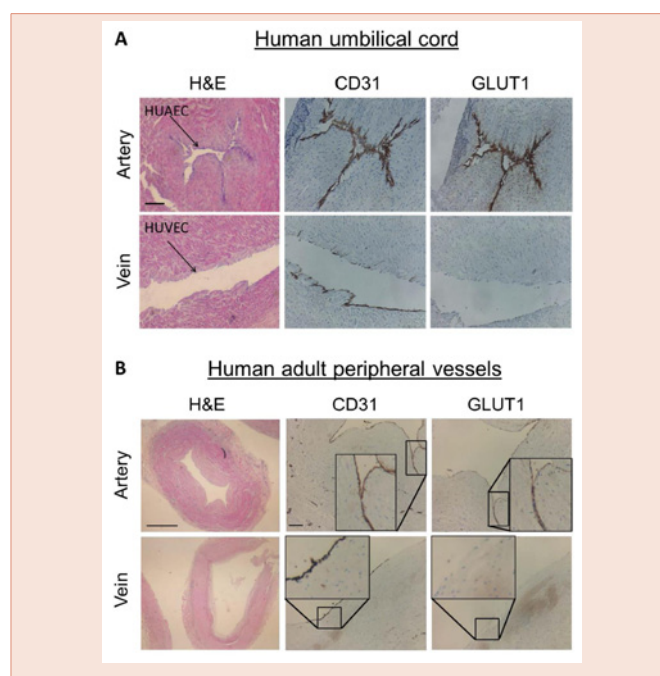


Figure 3: GLUT1, a novel marker to distinguish human venous and arterial endothelium. (A) Immunohistochemical validation of restricted expression of GLUT1 to arterial EC PM in human umbilical cord tissue and (B) adult human peripheral vessels. H&E (hematoxylin and eosin stain) is used to identify high level tissue structure. CD31, or PECAM1 (platelet endothelial cell adhesion molecule) is used as standard marker of EC.

HUVECs as fresh HUAECs, and the cultured cells appeared to have a phenotype between that of the freshly isolated HUVECs and HUAECs (Figure 4A). Interestingly, we found that 371 PM and 279 non-PM vein signature proteins were reduced in expression in the passage 3 cultured VEC (Figure 4B, top). In contrast, 347 PM and 214 non-PM artery signature proteins had increased expression in cultured VEC (Figure 4B, bottom), suggesting that cell culture conditions enact a shift toward an arterial protein profile [4]. Moreover, the histone-dominating gene sets that were enriched in the fresh vein signature (relative to artery) were also enriched in fresh vein versus cultured vein, suggesting that reduction of histone protein levels contributes in some way (or at least corresponds to) this shift (Figure 5A). On the other hand, pathways dominated by ribosomal proteins were highly enriched in cultured versus fresh vein non-PM protein signatures (Figure 5B). These gene sets were not enriched in fresh artery versus vein, suggesting that their up-regulation is an artifact of the *in vitro* culture that pushes the cells away from resembling either vein or artery.

While it is possible that the ECs simply underwent a shift in cell state, the loss and normalization of SLC2A1 (GLUT1) expression in culture (Figure 6), suggest that their arterial and venous subtype identities were lost, while they maintained their identities as ECs, as suggested by morphology and EC marker expression (PECAM-1, VECAD, etc) maintained over time.

To determine whether SLC2A1 (GLUT1) would remain a faithful arterial marker in culture, we also assessed SLC2A1 mRNA expression from freshly isolated and cultured (passage 0-5) HUVECs and HUAECs by qRT-PCR. We found that SLC2A1 expression decreased approximately 20 fold within 48 hours of culturing freshly isolated HUAECs but remained higher than in cultured HUVECs. SLC2A1 mRNA expression continued to decline over passaging *in vitro* and remained differentially expressed between HUVECs and HUAECs until passage 3 ($p \leq 0.05$) (Figure 6).

Our observations are consistent with Aranguren et al. [4], regarding the effect of cell culture: they discovered a set of transcription factors

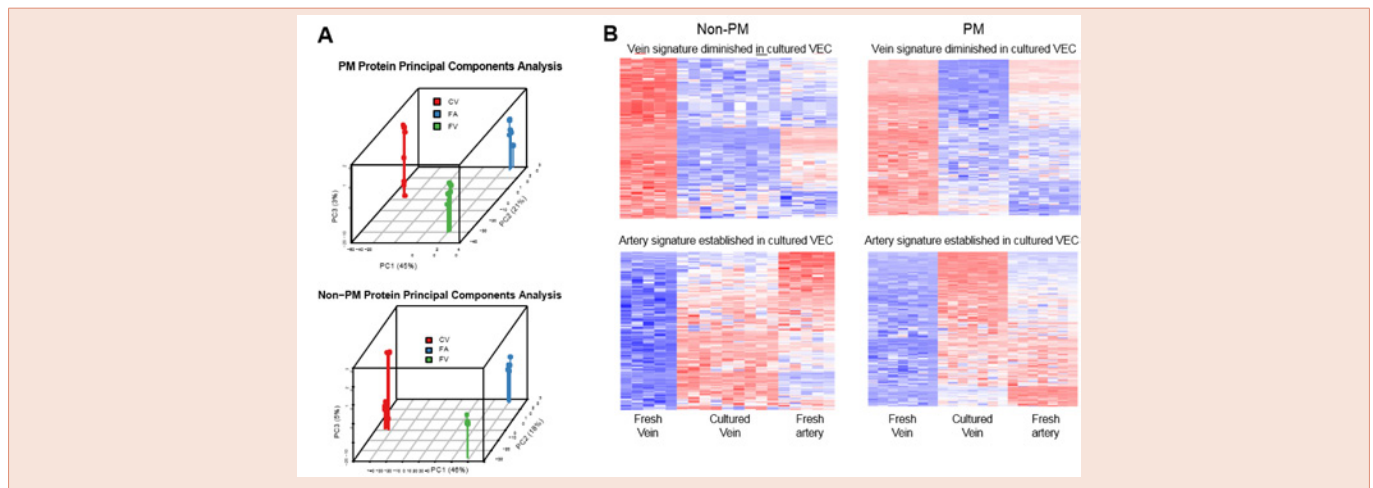


Figure 4: Profound effect of cell culture on HUVEC identity. (A) PCA (Principal Component Analysis) of cultured HUVEC and freshly isolated HUAEC and HUAEC proteome demonstrates a shift of cultured HUVEC protein expression toward HUAEC but remains distinct from both freshly isolated cell types. (B) heat map of fresh artery and vein protein expression signatures across three conditions: fresh HUVEC, cultured HUVEC, and fresh HUAEC (from left to right). Clearly the fresh venous protein expression signature is lost in culture while the arterial expression signature is trending upward in the cultured cells. CV = cultured HUVEC; FA = Fresh HUAEC; FV = Fresh HUVEC; PM = plasma membrane.

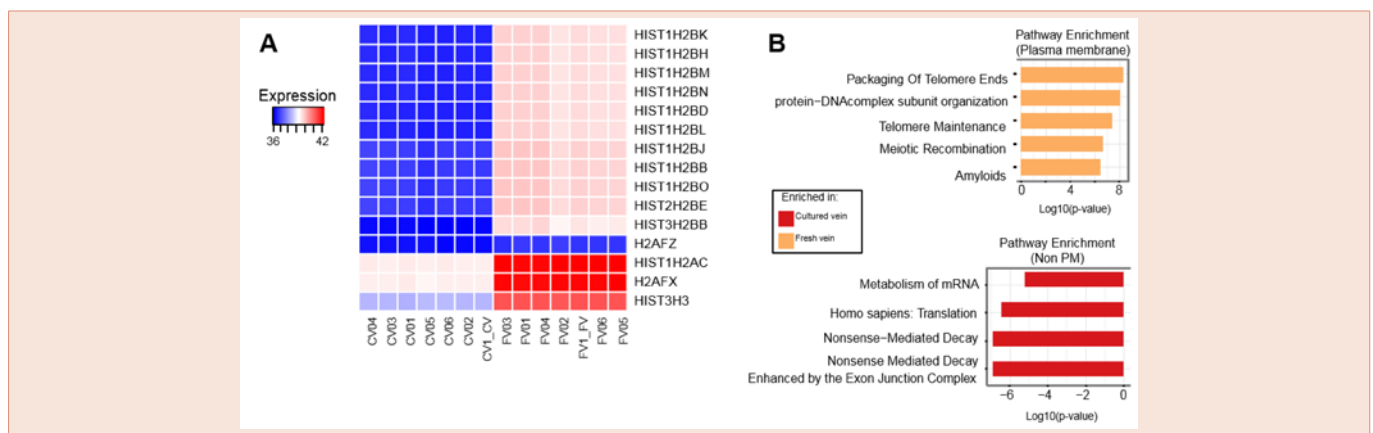


Figure 5: Histone attenuation defines cell culture effect at the protein level. (A) Heat map of the most highly differentially expressed proteins in cultured HUVEC demonstrates a striking enrichment in histone proteins. (B) Biological pathways enriched in HUVEC and HUAEC PM (top) and non-PM (bottom) data sets. PM = plasma membrane; CV = cultured HUVEC; FV = fresh HUVEC.

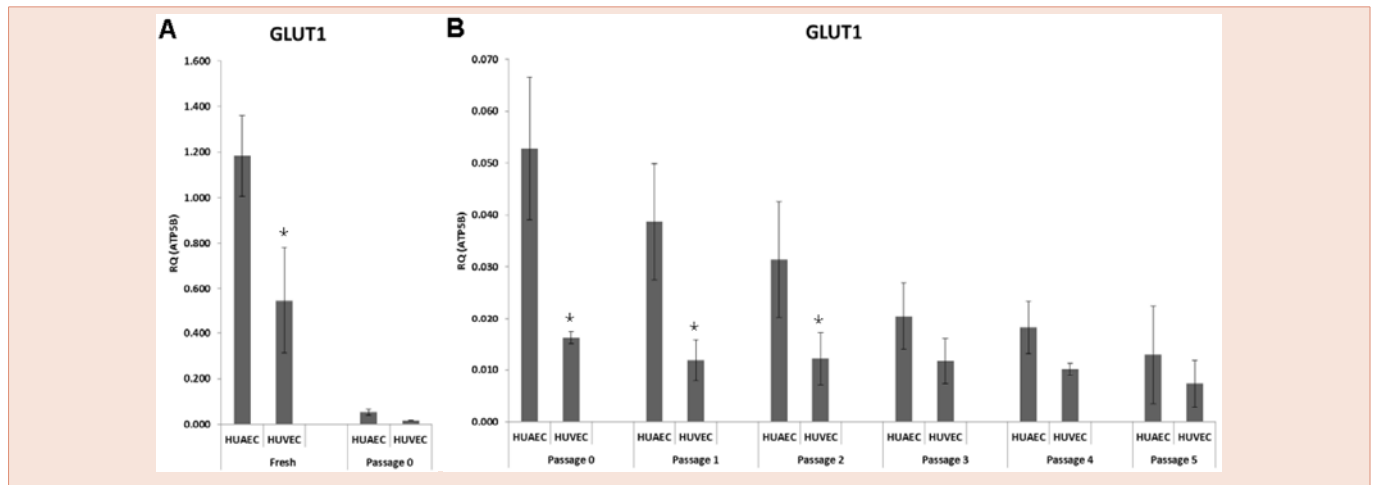


Figure 6: Profound and immediate effect of cell culture on venous identity. GLUT1 mRNA is also differentially expressed between freshly isolated HUVEC and HUAEC and remains so through passage 2, although the overall expression of GLUT1 declines greatly within 48 hrs of culture and continues to decline over time. RQ = relative quantity; * $p \leq 0.05$ vs. HUAEC at the same time point.

(TFs) responsible for generating an arterial phenotype and that within 48 hrs. of cell culture, the TFs no longer were differentially expressed. We observed GLUT1 arterial marker expression to be diminished, dramatically and consistently, during that time. Importantly, our studies are not the first to indicate a loss of EC identity *in vitro* [28], but provide novel insight through global protein profiling and PCA. Although currently unclear, it will be interesting to see if future work sheds light on the observation of histone expression being distinctly altered by cell culture. Further work remains to determine how each variable of cell culture contributes to the overall effect.

Concluding Remarks

We report novel protein signatures of human umbilical arterial and venous ECs. Many of these include cell surface proteins that can be probed readily by antibodies and thus may serve as accessible biomarkers. We validated one such potential antigen, GLUT1 (SLC2A1) that functions as a binary marker of arterial ECs. Many potential protein markers may be expressed to varying extent by many cell types. GLUT1 serves as an ideal marker in that it is highly expressed on the cell surface of arterial ECs, but it is not detected on venous ECs. This provides a clear distinction between these two cell types. Future work is necessary to determine at what stage in development GLUT1 begins to function as a marker of arterial endothelium and if it is indeed universal, distinguishing the cell types throughout the vascular tree. It is important to note that vascular tone is regulated by arterial endothelium, and it is already known that GLUT1 is an important regulator of endothelial function as its expression is required for normal endothelium-dependent vascular relaxation [29].

Acknowledgments

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