Ciliary Regulation of Endothelial Response to Shear Stress

Consequences for Tgfβ Signaling and Endothelial-to-Mesenchymal Transition

Anastasia Dmitrievna Egorova

Colofon

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This thesis was prepared at the Department of Anatomy & Embryology and Department of Molecular Cell Biology of the Leiden University Medical Center, Leiden, The Netherlands.

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Ciliary Regulation of Endothelial Response to Shear Stress

Consequences for Tgfβ Signaling and Endothelial-to-Mesenchymal Transition

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Science is like a well-crafted matreshka - the more insight you seek, the more questions you discover.

Dedicated to my mother and grandparents

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KLF2
* * *
0
0.5
2
shear stress (Pa)

Chapter 3

Endothelial Colony-Forming Cells Show a Mature Transcriptional Response to Shear Stress

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In Vitro Cellular & Developmental Biology-Animal, 48(1): 21-9 (2012)

Abstract

Endothelial progenitor cells (EPCs) play a central role in endothelial maintenance and repair. Endothelial colony-forming cells (ECFCs) form a subpopulation of EPCs. ECFCs are readily attainable, can be easily isolated, possess a high proliferation potential, and are therefore a promising source of endothelial cells (ECs) for future cardiovascular therapeutic applications. The extent to which these cells respond to shear stress as adult vascular ECs remains to be elucidated. Here, we study the transcriptional response of ECFCs induced by shear stress and compare it with the response of mature arterial and venous cells. ECFCs, as well as human umbilical vein ECs (HUVECs) and human umbilical artery ECs (HUAECs), were subjected to low (0.5 Pa) and high (2.5 Pa) shear stress. The endothelial differentiation phenotype and transcriptional responses were analyzed using immunocytochemistry and quantitative polymerase chain reaction (Q-PCR). Performing absolute quantification of copy numbers by Q-PCR allows comparing the responses of cell types relative to each other. Our data show that isolated ECFCs resemble mature ECs in cobblestone morphology and endothelial marker expression. Absolute Q-PCR quantification revealed that although being truly endothelial, ECFCs do not fully resemble HUVECs or HUAECs in the expression of specific differentiation markers. When subjected to shear stress, ECFCs show a mature response to fluid flow, comparable to that of HUVECs and HUAECs. The capacity of endothelial progenitors to respond to fluid flow in a similar manner to HUVECs and HUAECs highlights the universal response of ECs to fluid shear stress, independently of their endothelial differentiation status. This property supports the use of these cells as an EC source for tissue engineering applications.

Introduction

Chronic exposure to cardiovascular risk factors leads to elevated endothelial cell (EC) turnover and ultimately to EC damage and death. Mature endothelial cells lining the vessels possess insufficient proliferative capacity to allow for substitution at lesion sites[1]. Circulating endothelial progenitor cells (EPCs) have been described to play a role in vascular endothelial repair and maintenance[2-6]. Endothelial injury is associated with activation of the coagulation system and recruitment of platelets. These platelet-rich sites facilitate the recruitment and homing of EPCs that further integrate into sites of vessel formation *in vivo*. Under the control of local conditions of platelet-derived matrix proteins and growth factors, these cells further differentiate into a more mature endothelial phenotype[7-9]. In addition to the exposure of the progenitor cells to a pro-angiogenic environment, the arrest of EPCs at the site of vascular injury exposes the progenitor cells to wall shear stress. Several studies have suggested that this friction force may well be an important factor to the capacity of homed EPCs to undergo further differentiation into a mature endothelial phenotype characterized by expression of e.g. platelet endothelial cell adhesion molecule1 (PECAM1/CD31) and von Willebrand factor (vWF)[1,10,11].

ECs lining the vessel wall are constantly influenced by biomechanical forces inherent to the blood flow. Physiological stresses manifested as wall shear stress and cyclic strain modulate vascular remodeling, development, stability, and pathogenesis. Elucidation of the functional and morphological responses of ECs to blood flow, which translates into shear stress, is instrumental for the understanding of vascular function and pathology. Shear stress regulates gene expression in ECs *in vitro*[12-14] and *in vivo* in both the adult[15-17] and embryonic vasculature[18,19] underlining the potential of differentiating ECs in remodeling (damaged) vasculature. High and pulsatile vascular shear stress is correlated in ECs with increased levels of the transcription factor Krüppel-like factor 2 (KLF2) and of endothelial nitric oxide synthase, the enzyme responsible for the production of the vasodilator nitric oxide[20]. It elicits an anti-inflammatory, antithrombotic, and anti-proliferative phenotype of the endothelium[12,16,21]. Expression of endothelin-1 (ET1) and vascular cell adhesion molecule 1 (VCAM1) is negatively regulated by KLF2[22-24]. ECs activation and modulation of their mechanosensing capacity focally occur in areas of low and oscillatory shear[25], leading e.g. to atherosclerosis[2,26].

EPCs have first been defined in 1997 by Asahara et al.[11], based on their surface antigen expression, morphology, and ability to incorporate into vessels. Since then, numerous groups have focused on cell morphology and surface antigen expression, resulting in different cell populations demonstrating a mixed potential in blood vessel formation[27-29]. The three currently used methods for isolation and identification of putative EPCs from human mononuclear cells have recently been reviewed by Critser and Yoder (2010)[30]. Where all EPC populations have been shown to contribute to angiogenesis, only

endothelial colony-forming cells (ECFCs)[31], also termed blood outgrowth endothelial cells[29,32], have been demonstrated to possess the characteristics of a true endothelial progenitor and have the ability to form *de novo* blood vessels *in vivo*. ECFCs can be readily isolated from human cord blood, have a high proliferative potential, and can achieve over 100 population doublings, replicate into secondary and tertiary colonies and retain high levels of telomerase activity[31]. The capacity of these cells to respond to shear stress remains of interest.

In the current study, we characterize the response of ECFCs derived from umbilical cord blood circulating mononuclear cells[32,33] to shear stress and compare this to that of two mature cell types—human umbilical venous ECs (HUVECs) and human umbilical arterial ECs (HUAECs). The cells were subjected to shear stress of 0, 0.5, and 2.5 Pa corresponding to conditions of static, low, and high flow rates, respectively[34]. The effect of fluid shear stress on the expression levels of a specific set of candidate genes was then analyzed using real-time PCR followed by absolute quantification of copy numbers, allowing to compare the responses of the cells relative to each other. The findings provide further insight into the relation between differentiation status and functional shear response of endothelial progenitors with respect to mature arterial and venous ECs.

Methods

Isolation, generation, and culture of cells

ECFCs were isolated from human umbilical cord blood as previously described[35]. Briefly, the mononuclear cell fraction was isolated from whole blood using Ficoll density gradient centrifugation. Cells were plated on fibronectin-covered six-well culture plates (Costar, Corning Incorporated, Corning) in a final concentration of 1×10^7 per well in M199 medium (Invitrogen) supplemented with 20% fetal calf serum (Invitrogen), 0.05 mg/ml bovine pituitary extract (Invitrogen), 1× antibiotic/antimyotic solution (Invitrogen), and 10 units/ml heparin (Leo Pharma BV). ECFCs were cultured on collagen type I (50 µg/ml; BD Biosciences), medium was refreshed twice a week, and the cells were used after the fifth passage.

HUVECs and HUAECs were isolated from umbilical cords, collected with informed consent, according to the method of Jaffe et al. (1973)[36]. Cells were cultured on 1% (w/v) gelatin (Merck) in M199 medium supplemented with 20% (v/v) heat-inactivated fetal calf serum, 1× antibiotic/antimyotic solution, 2 mmol/L L-glutamine (Invitrogen), 10 units/ml heparin, and 15 µg/ml endothelial cell growth supplement (Sigma-Aldrich Chemie). Cells were passed once a week and were used after the third–fourth passage. The investigation conforms with the principles outlined in the Declaration of Helsinki for use of human tissue.

Immunocytochemistry

We analyze the phenotype of ECFCs and compare it to that of HUVECs. Cells were cultured to confluency on coverslips, and their cobblestone morphology was determined before fixation. After fixation and permeabilization of the cells (10 min in ice-cold methanol), immunocytochemical reactions for endothelial markers were performed. Staining for PECAM1/CD31 (Southern Biotechnology Associates, Inc.), vWF (DakoCytomation), Endoglin/CD105 (BD Pharmingen), and vascular endothelial (VE)- cadherin/CD144 (BD Pharmingen) was used to compare the endothelial phenotype of ECFCs with HUVECs. Isotype controls for mouse IgG (BD Biosciences) and rabbit IgG (DakoCytomation) were performed. Primary antibodies were detected with secondary antibodies against mouse IgG (Alexa 488 conjugated) or rabbit IgG (Alexa 568 conjugated), both from Molecular Probes (Eugene). Fluorescence was detected with a Leica SP5 confocal scanning laser microscope, and ImageJ imaging software was used for processing the data.

Shear stress exposure

ECFCs, HUVECs, and HUAECs were seeded on fixed 1% (w/v) gelatin-coated coverslips and grown to confluency. Cells were plated in a parallel plate flow chamber and exposed to laminar shear stress in a recirculation flow system for 5 hours at 37°C and 5% CO_2 to create a steady, laminar flow, essentially as described before[37,38]. Shear stress levels of 0.5 (5 dyn/cm^2) and 2.5 Pa (25 dyn/cm^2)

were used to compare the response of ECFCs (n=4), HUVECs (n= 4), and HUAECs (n=4) to low and high flow rates, typically found in the venous and arterial parts of the vasculature, respectively.

Q-PCR

Total RNA from each sample was isolated using RNeasy (Qiagen) and treated with DNAse-I (Qiagen) according to the manufacturer's protocol. M-MuLV Reverse Transcriptase (Amersham) was used to reverse transcribe 500 ng of RNA into cDNA. Real-time Q-PCR was performed on equal amounts of cDNA using the Brilliant SYBR green Master Mix kit (Stratagene) in a Mx3000 real-time thermocycler (Stratagene), essentially as described before[39]. In short, the reaction mixture consisted of the following: 1× PCR Master Mix, 1 µl cDNA template, and 10 pmol of each specific primer. Genespecific primers that were used are listed in Table 3.1. The PCR program consisted of a hot start activation step, followed by 50 cycles of 30 seconds at 95°C, 60 seconds annealing at 58°C (62°C for *HEY2*), and 30 seconds extension at 72°C. Dissociation analysis was performed in all reactions to exclude the presence of primer–dimer complexes and confirm the amplification of unique targets. No-template controls were used as negative controls. In order to perform absolute quantification of the expression levels of genes of interest, plasmids with the insertions of genes of interest were constructed using One Shot TOP10 kit (Invitrogen), according to the manufacturer's protocol. Absolute quantification by standard curve analysis of serial dilutions of cloned plasmid products was performed allowing for the analysis of the response of cells to shear stress and for comparison of expression levels between primary cells (ECFCs, HUVECs, and HUAECs).

Statistical analysis

For comparison of the means, independent experiments were performed and analyzed using SPSS 14.0 (SPSS Inc.). Results are expressed as means of four independent experiments±standard error of the mean (SEM). Independent t tests, including Levene's analyses for equality of variances, were used to analyze differences between groups. Values of $p<0.05$ and a power ≥ 0.80 were considered to be statistically significant.

Gene of interest	Gene ID	Oligonucleotide sequence
ET1	1906	F: 5'-TCCTCTGCTGGTTCCTGAC-3'
		R: 5'-AGCCAGTGAAGATGGTTGG-3'
CD34	947	F: 5'-GGTGGCTGATACCGAATTG-3'
		R: 5'-CACGTGGTCAGATGCAGAG-3'
CD44	960	F: 5'-TGTGGGCAGAAGAAAAAGC-3'
		R: 5'-TGAGACTTGCTGGCCTCTC-3'
DLL4	54567	F: 5'-TATGTGTGCCAGCCAGATG-3'
		R: 5'-ATGACAGCCCGAAAGACAG-3'
EphB4	2050	F: 5'-GAAAAGGAAGTGCCCAACA-3'
		R: 5'-CTGGCAAGGGAGTCACACT-3'
EphrinB2	1948	F: 5'-GAAGTACGAGCCCCACAGA-3'
		R: 5'-CCCAACGCAGAAATAAACG-3'
HEY2	23493	F: 5'-GATTCAGCCCTCCGAATG-3'
		R: 5'-TGGCAGAGAGGGACAAGAG-3'
KLF2	10365	F: 5'-CTACACCAAGAGTTCGCATCTG-3'
		R: 5'-CCGTGTGCTTTCGGTAGTG-3'
NOTCH4	4855	F: 5'-GTGGTCATGGGTGTGGATT-3'
		R: 5'-CAGCAAGGAAGCGGAGTAG-3'
VCAM1	7412	F: 5'-GCTGCTCAGATTGGAGACTCA-3'
		R: 5'-CGCTCAGAGGGCTGTCTATC-3'
vWF	7450	F: 5'-ATTCAAGCTGGAGTGCAGG-3'
		R: 5'-GTTGTGGGAGATGTTTGCC-3'

Table 3.1. Sequences of oligonucleotide primers of genes of interest used for Q-PCR. F, forward primer; R, reverse primer.

Results

Endothelial phenotype of ECFCs

To confirm the endothelial phenotype of ECFCs, the morphological and immunocytochemical characteristics of these cells were compared with cultured HUVECs. Phase contrast microscopical evaluation showed comparable monolayer morphology for both ECFCs and HUVECs in culture (compare Figure 3.1A and B). Reactivity of both cell types for antibodies directed against endothelial markers PECAM1/CD31, vWF, Endoglin/CD105, and VE-cadherin/CD144 was analogous (Figure 3.1C–J). Isotype controls for mouse IgG and rabbit IgG were negative (Figure 3.1K, L).

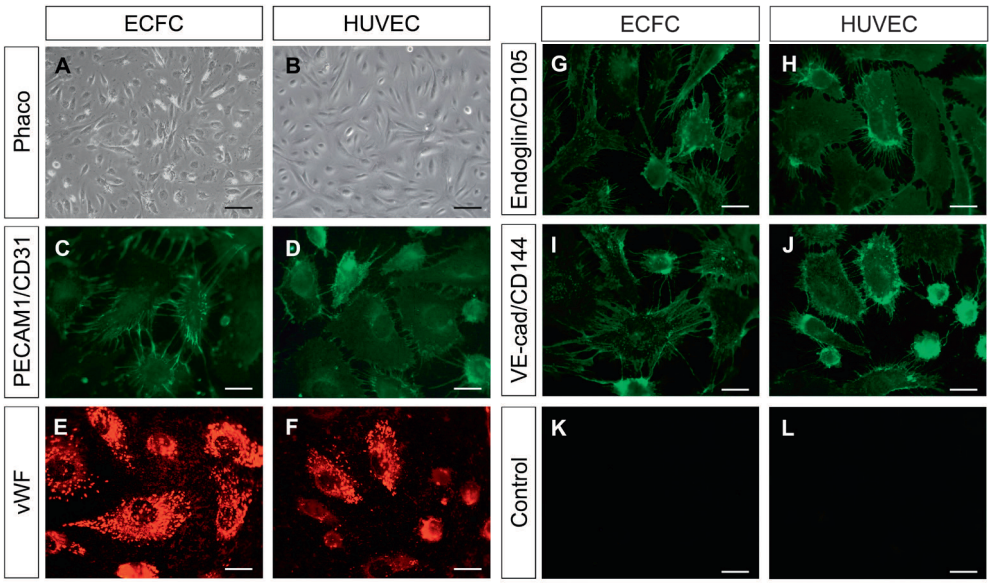


Figure 3.1. **Immunocytochemistry of ECFCs and HUVECs.** A comparison of the morphological and immunocytochemical characteristics of ECFCs and HUVECs. Phase contrast microscopy showing monolayer morphology of ECFCs (A) and HUVECs (B). Reactivity for antibodies directed against PECAM1/CD31 (C, D), vWF (E, F), Endoglin/CD105 (G, H), and VE-cadherin/CD144 (I, J) was comparable in ECFCs and HUVECs. Isotype controls (K, L). Note that the phase contrast images (A, B) were made prior to methanol fixation, a treatment that causes shrinkage of the cells and reduced cell contacts (C–J). Scale bars: 35 µm (A and B), 10 µm (C through L).

Activation of shear responsive genes under flow

The response of ECFCs to various shear stress levels was compared to that of HUVECs and HUAECs. Activation of flow-regulated gene expression was quantified by measuring absolute mRNA levels of *KLF2*, *ET1*, and *VCAM1* (Figure 3.2). Basic expression levels of *KLF2* did not differ among the cell types. *KLF2* expression was induced in ECFCs, HUVECs, and HUAECs to reach comparable copy numbers under 0.5 and 2.5 Pa shear stress. The basic expression of *ET1* was comparable in ECFCs and HUAECs, but was significantly lower in HUVECs. *ET1* was significantly downregulated under high shear compared to static conditions in all three cell types to reach comparable levels. *VCAM1* levels were significantly higher in HUAECs than in the ECFCs and HUVECs under static and shear conditions. ECFCs and HUAECs showed a significant decrease in *VCAM1* expression under high flow. A similar trend was seen in the HUVECs.

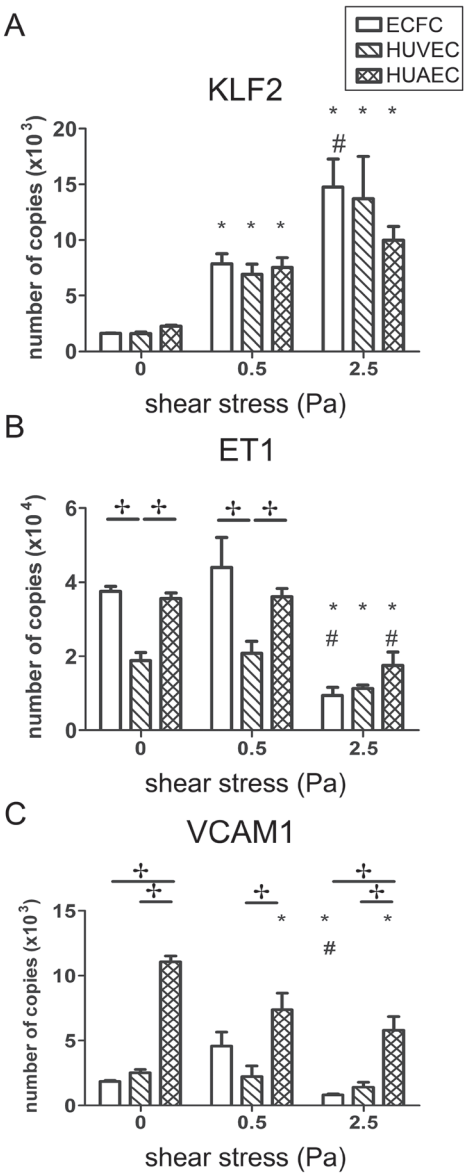
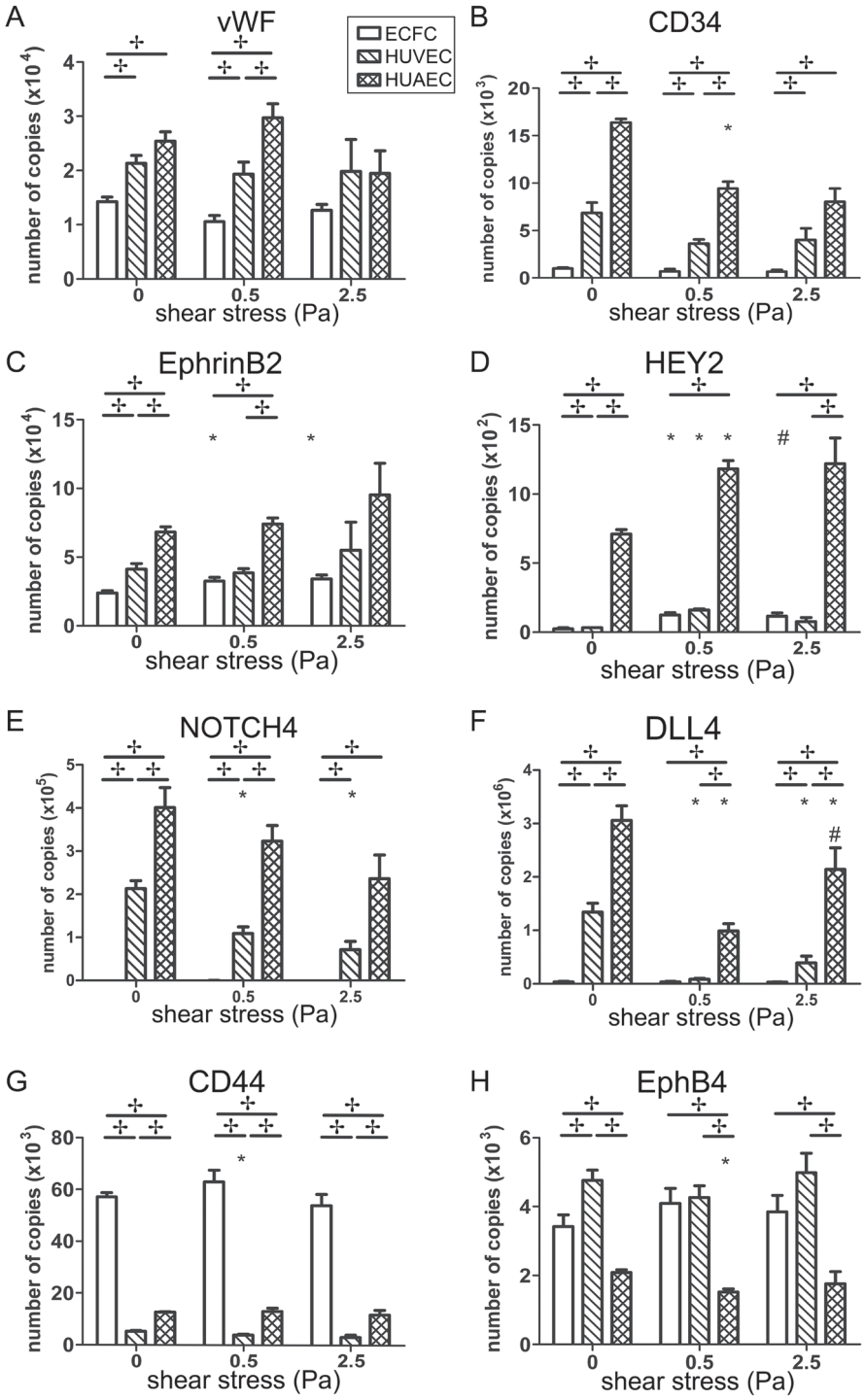


Figure 3.2. Shear stress markers. mRNA expression of flow-regulated genes *KLF2* (A), *ET1* (B), and *VCAM1* (C) in ECFCs, HUVECs, and HUAECs after exposure to 0, 0.5, and 2.5 Pa shear stress (n=4). *p< 0.05 compared to 0 Pa; #p<0.05 compared to 0.5 Pa; +p<0.05. Bars represent SEM values. Note that the scale of the y-axis differs between the genes of interest.

Endothelial differentiation markers under shear stress

Expression levels of *vWF*, *CD34*, *EphrinB2*, *HEY2*, *NOTCH4*, *DLL4*, *CD44*, and *EphB4* were studied to analyze the response of endothelial phenotype markers to shear stress in ECFCs in comparison to that of differentiated venous and arterial ECs (Figure 3.3A–H). *vWF* expression did not change under low or high shear stress in any of the cell types. Under static conditions, the expression levels of *CD34*, *EphrinB2*, *HEY2*, *NOTCH4*, and *DLL4* were significantly lower in ECFCs than in HUVECs and HUAECs. This difference in expression levels largely persisted under exposure to shear stress. Expression of *CD34* was the highest in the arterial cells, intermediate in venous cells, and the lowest in ECFCs. Despite the fact that *CD34* was downregulated under shear in the HUAECs, the levels in the flow, the levels remained substantially lower in these cells than in the HUAECs. Expression of arterial markers *NOTCH4* and *DLL4* was higher in the HUAECs than in the HUVECs and was very low in the ECFCs. Expression of these markers was slightly downregulated under shear in the venous and arterial ECs. Expression of *CD44* in the HUAECs was higher than in the HUVECs, yet about four times as low as *CD44* levels in the ECFCs. Essentially no response of *CD44* expression to shear stress was seen in any of the primary cell types. The expression levels of the venous marker *EphB4* were high in the HUVECs and ECFCs and low in the HUAECs. The relative expression levels remained effectively unaltered in the cell types independent of the magnitude of shear stress.

Figure 3.3. **Endothelial phenotype markers.** mRNA expression of endothelial markers *vWF* (A), *CD34* (B), *EphrinB2* (C), *HEY2* (D), *NOTCH4* (E), *DLL4* (F), *CD44* (G), and *EphB4* (H) in ECFCs, HUVECs, and HUAECs after exposure to 0, 0.5, and 2.5 Pa shear stress (n=4). *p< 0.05 compared to 0 Pa; #p<0.05 compared to 0.5 Pa; +p<0.05. Bars represent SEM values. Note that the scale of the y-axis differs between the genes of interest.


A
vWF
ECFC
HUVEC
HUAEC
number of copies (x10^4)
shear stress (Pa)
B
CD34
number of copies (x10^3)
shear stress (Pa)
C
EphrinB2
number of copies (x10^4)
shear stress (Pa)
D
HEY2
number of copies (x10^2)
shear stress (Pa)
E
NOTCH4
number of copies (x10^5)
shear stress (Pa)
F
DLL4
number of copies (x10^6)
shear stress (Pa)
G
CD44
number of copies (x10^3)
shear stress (Pa)
H
EphB4
number of copies (x10^3)
shear stress (Pa)

Discussion

Endothelial mechanobiology is currently receiving vast interest in the field of cardiovascular research as emerging evidence supports its role as a major contributor in cardiovascular pathology and regeneration. Different shear conditions result in distinct responses of ECs, which can be assessed by their cellular and monolayer morphology, biological function, and gene expression profiles[40-43]. ECs lining the mature vessel wall react to low and disturbed flow by activation of pro-inflammatory and pro-apoptotic pathways leading to recruitment of EPCs with regeneration potentials[16,23,44]. Umbilical cord blood-derived EPCs are readily attainable, can be easily isolated, possess a high proliferation potential, and are therefore a promising source of ECs for future cardiovascular therapeutic applications. Endothelial colonyforming cells (ECFCs) form a subpopulation of EPCs and have previously been described in detail by several groups[31,45]. These cells are easily attainable, express endothelial markers, and do not exhibit monocyte macrophage properties, making them a favorable source for endothelial reconstitution. However, the extent to which these cells respond to shear stress as adult vascular ECs remains to be defined.

This study aims at elucidating the influence of shear stress on gene expression regulation of ECFCs compared to that of differentiated venous and arterial ECs. Our results show that ECFCs isolated from human umbilical cord blood[33,35] resemble HUVECs in cobblestone morphology, expression of endothelial markers PECAM1/CD31, vWF, Endoglin/CD105, and VE-cadherin/CD144. These results are consistent with previous descriptions of ECFCs[28,45,46]. ECFCs were subjected to fluid shear stress *in vitro*, and their response was compared to that of HUVECs and HUAECs. Just like HUVECs and HUAECs, these cells preserved their endothelial phenotype after exposure to low (0.5 Pa) and high (2.5 Pa) shear stress. This indicates that responses of these cell types in our model system can be fully attributed to the effects of shear stress and are not secondary to the loss of endothelial phenotype under experimental conditions.

The response of ECFCs to fluid shear stress was further analyzed by measuring and comparing the expression levels of a selection of shear stress markers, particularly *KLF2*, which encodes a zinc finger protein belonging to a multigene family of Krüppel-like transcription factors[22]. KLF2 expression in the embryonic and adult cardiovascular system has previously been described to be confined to areas of high and pulsatile shear stress[17,19]. In the present study, *KLF2* expression levels were induced under 0.5 and 2.5 Pa shear stress in HUVECs, HUAECs, and ECFCs. The relative induction of *KLF2* in HUVECs under 0.5 Pa shear stress was approximately factor 5, comparable to the previously reported values in these cells and adult human aortic EC[38]. The absolute *KLF2* mRNA levels under low and high flow did not differ among the three cell types, indicating their universal capacity to upregulate *KLF2* in response to shear stress. ECFCs, therefore, show a mature dose-dependent response to flow, not distinct from differentiated venous and arterial cells. *ET1* and

VCAM1, which are negatively regulated by KLF2[22-24], show a trend towards downregulation in all cell types, compared to their static controls. The absolute expression levels of *ET1* and *VCAM1* in the three cell types, however, are distinct and not universal as shown for *KLF2*. This suggests that additional factors, possibly nonshear responsive, or in any case not KLF2 mediated, co-regulate *ET1* and *VCAM1* expression.

Expression analysis of a set of dedicated arterial (*EphrinB2*, *HEY2*, *NOTCH4*, and *DLL4*) markers under static conditions showed that all genes were expressed at a significantly higher level in the arterial ECs compared to the venous cells. An inverse relation was observed for the venous marker *EphB4*[47]. Although the progenitor cells show an expression profile that resembles the venous profile to some extent under static conditions (e.g., high *EphB4* and low *HEY2*), their responses to shear stress were clearly distinct from both venous and arterial cells. This confirms observations on the plasticity of ECs and the potential role of signaling cascades such as VEGF and NOTCH in regulating and fine-tuning their differentiation[48]. Interestingly, the marker *NOTCH4* was not detectable in ECFCs, and its receptor *DLL4* was expressed at very low levels, independent of the level of shear stress. *NOTCH4* and its receptor *DLL4* are downregulated by flow in mature cells. A differential expression and regulation under flow between mature and immature ECs supports the potential role of NOTCH4 and DLL4 in regulating the flow (in-) dependent differentiation in e.g. angiogenesis[49]. The remarkably high expression levels of *CD44* in ECFCs compared with differentiated ECs reflect their stem cell origin. CD44 has been shown to be involved in homing to the endothelial monolayer by binding to the hyaluronic acid component of the glycocalyx[50,51]. CD44, therefore, plays a role in the repopulation of the endothelial monolayer, in line with the functional role of EPCs in endothelial damage repair[52].

Here we show that absolute quantification allows for the analysis of the response of cells to shear stress, and also for comparison of expression levels between primary cells (ECFCs, HUVECs, and HUAECs). This permits the comparison of the responses of the cells relative to each other. The benefit of this application can best be illustratedwith an example from the data. *CD34* expression showed no response to flow in all the three cell types, with the exception of a decrease in HUAECs at 0.5 Pa relative to the static conditions. These data could also be obtained from a relative PCR analysis. However, only the absolute quantification allows the conclusion that ECFCs have fairly low levels of *CD34* mRNA, the levels found in the HUVECs are about four to six times as high as those in the ECFCs, and the levels found in the HUAECs are an additional factor 2 higher than those in the HUVECs under all tested conditions. The low expression levels of *CD34* found in the cultured ECFCs reflect the fact that these cells are not entirely non-differentiated progenitors (they have already committed to an ECs phenotype), an observation that would have been missed if absolute Q-PCR analysis by standard curve analysis of serial dilutions of cloned plasmid products was not performed.

In conclusion, we studied the flow-induced transcriptional response of ECFCs and compared it with the response of mature arterial and venous cells. ECFCs do not fully resemble HUVECs or HUAECs in the expression of specific differentiation markers; however, they do show a mature response to shear stress, comparable to that of adult arterial and venous cells. The functional property of ECFCs provides further support for the use of these cells as a possible EC source for tissue engineering applications. Further studies will need to address the effects of differential flow conditions (e.g., oscillatory profile) in conjunction with specific cytokines, as well as the use of differential coatings, to better represent the local environment *in vivo*.

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