Functional Arterial and Venous Fate Is Determined by Graded VEGF Signaling and Notch Status During Embryonic Stem Cell Differentiation

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Objective—The aim of this work was to develop a mouse embryonic stem (ES) cell system addressing the early specification of the developing vasculature into functional arteries and veins.

Methods and Results—ES cells were differentiated 4 days on collagen-type IV coated dishes to obtain Flk1+ endothelial precursors. Sub-culture of these precursors for additional 4 days robustly generated, in a VEGF dose-dependent manner, mature endothelial cells. Arterial marker genes were specifically expressed in cultures differentiated with high VEGF concentration whereas the venous marker gene COUP-TFII was upregulated in endothelial cells induced through low and intermediate VEGF concentrations. This VEGF-dependent arteriolarization could be blocked by inhibition of Notch resulting in an arterial to venous fate switch. Functional and morphological studies, ie, measurement of sprout length, pericyte recruitment, and interleukin-I–induced leukocyte adhesion, further confirmed their arterial and venous identity.

Conclusions—We conclude that endothelial cells with distinct molecular, morphological, and functional characteristics of arteries and veins can be derived through in vitro differentiation of ES cells in a VEGF dose-dependent and Notch-regulated manner. (Arterioscler Thromb Vasc Biol. 2007;27:487-493.)

Key Words: angiogenesis ■ embryonic stem cells ■ arteries and veins ■ VEGF ■ Notch

During early embryogenesis, blood vessels are formed de novo as a primary capillary plexus by endothelial cell precursors derived from nascent mesodermal cells. This process is known as vasculogenesis (1, for review). The capillary plexus is eventually remodeled into organized vessels, composed of endothelial and smooth muscle cells. Vascular endothelial growth factor (VEGF) signaling plays a critical role in this process as mice lacking VEGF or its receptor (VEGFR2, Flk1) fail to develop any vasculature at all.2–4 Other signaling pathways like the angiopoietin/Tie, receptor (VEGFR2, Flk1) fail to develop any vasculature at all.2–4 Other signaling pathways like the angiopoietin/Tie, receptor (VEGFR2, Flk1) fail to develop any vasculature at all.

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Materials and Methods

Cell Culture
CCE ES cells (a gift from Dr M. J. Evans, Welcome/CRC Institute, Cambridge, UK) were cultured on gelatin-coated tissue culture dishes in Glasgow MEM (Sigma) supplemented with 15% FBS (Hyclone), 1000 IU/mL LIF, 2-mercaptoethanol, and Non-Essential Amino Acids (Gibco). For differentiation, 6 × 10^4 ES cells were cultured 4 days on type-IV collagen-coated dishes (BD Labware) in IMDM medium, 15% FBS (Gibco), 1.5 × 10^{-5} M MTG, and Ascorbic acid (Sigma). Cells were dissociated with collagenase (Sigma) 20 minutes and passed through 20G needle. Flk1^+ cells were subcultured 4 days on collagen type-IV with hrVEGF 50 ng/mL (R&D Systems). To block Notch signaling γ-secretase inhibitor (L685.485; 4 μmol/L; BachemAG) was added. The monocyte-like cell line U937, cultured in RPMI medium with 5% FBS, was kindly provided by Georg Klein (Karolinska Institute, MTC).

RNA Extraction and Quantitative RT-PCR
RNA extracted with Qiagen RNeasy Mini Kit and transcribed using Invitrogen’s SuperScriptIII First-Strand kit. RT-PCR was performed using Applied Biosystem 7000. Primer sequences are available on request. Equal amount of cDNA were loaded and normalized to B-actin. Results were evaluated using Student t test.

Flow Cytometry
Staining was performed in PBS with 4% FBS 30 minutes on ice before analysis/sorting using a FACSaria (Becton Dickinson). Antibodies used: Flk1-PE (1:200, Avas12α1), CD31-PE/APC (1:200; MEC13.3), E-selectin-PE (1:500; 10E9.6) (BD Pharmingen).

Immunocytochemistry
Cells were fixed in 4% paraformaldehyde, blocked with 4% FCS and incubated with primary antibody overnight. Antibodies used: PE-CD31 (MEC13.3, BD Pharmingen), Smooth-Muscle-Actin (mouse-IgG, 1A4, Sigma), ephrin-B1, a reported pan-EphrinB antibody (1:200C-18, rabbit-IgG, Santa Cruz Biotechnology), SMA-antibody was detected by goat-anti-mouse-alexa488 antibody (Molecular Probes). The EphrinB staining was detected using Vectastain-ABC-kit (Vector Laboratories). Pictures were taken with Axiovert200N 40x, and LSM 510 Meta microscopes, (Zeiss).

EB-Sprouting Assay
Embryonic bodies (EBs) differentiated 4 days were further subcultured an additional 4 days in low attachment plates with 10 or 50 ng/mL VEGF with or without γ-secretase inhibitor, as indicated. EBs were then harvested, washed, and embedded in collagen type-I gel containing VEGF to form sprouts during 5 days. The collagen gels were fixed in PFA 4% and stained with PE-conjugated anti-CD31 antibody (1:200), SMA-antibody (1:500) with Alexa-488 conjugated anti-mouse IgG antibody as secondary (1:500). Pictures were taken with (Axiovert200M, Zeiss) and confocal microscope (Eclipse TE300, Nikon). The longest sprout of each EB was measured. 20 EBs in each group were counted. Coverage by αSMA^+ pericytes were counted along 10 sprouts per treatment and normalized by sprout-length. Significance was assessed using Student t test.

Results

VEGF Is Required for Endothelial Differentiation and Controls Arterial Venous Fate by Graded Signaling
Differentiation of ES cells generated up to 80% CD31^+ and acetyl-LDL incorporating endothelial cells in large sheets (Figure 1A and 1B). As has been shown previously, VEGF is required for, but also regulates in a dose-dependent manner, the endothelial differentiation efficiency (Figure 1C).^2,3\] Cultures without VEGF generated primarily mural cells expressing α-smooth muscle actin (SMA) and no endothelial cells. In addition to being essential in endothelial differentiation, there is evidence from studies of embryonic cells that VEGF can induce arterialization in primitive venous endothelial cells. Based on this observation, one would expect ES cell–derived endothelial cells to be predominantly arterial. In accordance with this EphrinB2 expression could be detected using an
pan-EphrinB antibody (Figure 1D), as RT-PCR showed EphrinB2 to be the predominant transcript in these endothelial cells (supplemental Figure I, available online at http://atvb.ahajournals.org).

To further investigate the potential arterialization in our cultures we examined the expression levels of genes reported to be arterial- or venous-specific throughout the progression of differentiation. Multiple sets of ES (Day 0), Flk1\(^{+}\) (day 4), and VEGF-induced endothelial cells (Day 8) were isolated and gene expression analyzed by quantitative PCR. This revealed that initially both EphrinB2 and EphB4 were upregulated in the Flk1\(^{+}\) angioblast precursor population compared with ES cells. EphrinB2 transcripts increased further when the angioblasts were differentiated into endothelial cells whereas EphB4 level decreased. Turning to the Notch signaling pathway Dll4, Notch4, and Hey1 are all upregulated with VEGF induced transition from angioblast to endothelium (Figure 2A). Notch1, Jag1, and Jag2 transcripts were detected throughout differentiation but the levels remained unchanged. However, in the nonendothelial cells, ie, SMA\(^{+}\) cells, Notch1 transcripts were decreased in contrast to Notch3 being expressed at higher levels (data not shown). The VEGF coreceptor Neurupilin1 (Nrp1),\(^{18}\) reported to be arterial specific is strongly upregulated in the initial step from ES cells to angioblasts. The venous-associated Neurupilin2 (Nrp2)\(^{18}\) is also upregulated but to a much lesser degree. During endothelial specification Nrp1 expression was further increased, whereas Nrp2 level remained low. Finally Alk1 and the gap junction gene Connexin40, both with reported arterial expression,\(^{19,20}\) were strongly up regulated during endothelial differentiation (Figure 2A).

Yurugi-Kobayashi et al\(^{17}\) did not report upregulation of arterial genes without cAMP activation, keeping in mind that they examined gene expression one day earlier compared with this study. FACS analysis show that CD31\(^{+}\) cells at 6, 7, and 8 days of differentiation incorporate acetylated-LDL with high specificity indicative of functional endothelial population, allowing us to dissect the temporal lineage commitment. Gene expression analysis showed progressive arterialization with increased transcripts of Dll4, EphrinB2, and Connexin40 over time (Figure 2B).

Having confirmed that VEGF induces endothelial differentiation we wanted to further investigate whether VEGF dosage also influence arterial and venous specification. ES cells were differentiated with decreasing levels of VEGF; 50, 10, and 2 ng/mL. CD31\(^{+}\) endothelial cells were then FACS sorted to pure populations and their gene expression profiles were analyzed. This revealed strong expression of the arterial genes Dll4, Notch4, EphrinB2, and Nrp1 exclusively in cultures with 50 ng/mL VEGF. In contrast, the venous gene COUP-TFII was upregulated in cultures differentiated with lower concentrations of VEGF (Figure 3). These findings suggest a bipotent fate for the primitive endothelial cells, where arterial specification is dependent on VEGF dosage.

Inhibition of Notch Signaling Overrides VEGF-Driven Arterial Induction Resulting in Venous Identity

Recent literature has postulated a close connection between VEGF-induced Notch activation and endothelial arterialization.\(^{7–11}\) To evaluate this, FACS sorted Flk1\(^{+}\) cells were differentiated with or without \(\gamma\)-secretase inhibitor (GSI) to

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Expression of arterial and venous marker genes during differentiation. A, Gene expression analyses by RT-PCR were performed on ES (day 0), Flk1\(^{+}\) (day 4), and VEGF-differentiated cells (day 8). B, FACS analysis of acetylated-LDL incorporation and expression analysis on 6, 7, and 8 days of differentiation. The data represent the mean of 4 biological samples with error bars showing SEM. *\(P<0.05\), **\(P<0.01\), ***\(P<0.005\) by Student t test.

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Gene expression in endothelial cells differentiated with 50, 10, or 2 ng/mL VEGF. Endothelial cells were FACS sorted to purity before cDNA preparation. The data represent the mean of 4 biological samples with error bars showing SEM. *\(P<0.05\), **\(P<0.01\), ***\(P<0.005\) by Student t test.
phenotype switch

Evidence of Arterial-Venous Specification

Monocyte Adhesion Assay Shows Functional Evidence of Arterial-Venous Specification

Venous endothelium has higher monocyte adhesiveness than arterial endothelium in response to inflammatory stimuli. Taking advantage of this we performed an IL-1 triggered monocyte (U937-cells) adhesion assay to test functionality of the putative arterial and venous cells derived. Endothelial cultures were differentiated as previously described and then incubated with IL-1 for 4 hours. IL-1 incubation increased the monocyte adhesiveness of the cultures with GSI 4-fold over baseline. In contrast to the arterial like cultures, stimulation with IL-1 gave no significant increase in adhesiveness (Figure 6A and 6B). Culture with 10 ng/mL VEGF could not be scored accurately as this protocol generated fewer and significantly smaller endothelial sheets. One of the initial and key events in the endothelium’s response to inflammatory stimuli is the expression of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and E-selectin, which mediate monocyte adhesion. In cultures with low VEGF or blocked notch signaling, IL-1 induced an 11% and 25% upregulation of E-selectin. No significant regulation was observed in the arterial like cells (Figure 6C, positive and negative control in supplemental Figure II). These data show

Morphological Data Supporting a Phenotype Switch

In a recent study, explants of rat aorta or vena cava embedded in collagen generated endothelial sprouts, decorated with pericytes. Venous sprouts were longer and had less pericytes than arterial sprouts, resulting in a quantifiable morphological phenotype that potentially could be recapitulated in our system. This was addressed using EB differentiation followed by sprouting assay in collagen type I gel. EBs were formed in hanging drop cultures for 4 days and were then further cultured under arterial or venous promoting conditions during 4 days, ie, 50 ng/mL VEGF only for arterial and either 10 ng/mL VEGF alone or 50 ng/mL VEGF with GSI added for venous. These EBs were then embedded in a collagen type I gel and sprouts were quantified on day 5. The longest protruding sprout was measured for each EB. Venous conditions generated significantly longer sprouts (Figure 5A and 5B). Thicker smooth muscle wall is also a well-documented, characteristic of arterial vessels. Thus, pericyte recruitment was scored using CD31 and α-SMA staining. Compared with arterial cultures, both venous conditions reduced the number of pericytes attached along the sprouts by 50% (Figure 5C).

block Notch signaling. Compared with control cultures, GSI treatment did neither alter CD31 and αSMA expression levels (Figure 4A) nor the numbers of CD31+ cells analyzed by FACS. This is in accordance with previous in vivo data from Notch mutants, where endothelial cells do form but fail to remodel properly.

As was expected, inhibition of Notch signaling resulted in lower transcript levels of direct downstream genes Hey1 and Hey2 (Figure 4B). Expression of the Notch ligand and arterial marker Dil4 decreased as did EphrinB2 levels, whereas expression of its receptor EphB4 remained unaltered at mRNA level. Connexin40 was strongly downregulated, and EphrinB2 immuno-staining showed more than 50% reduction of the number of positive cells (Figure 4C). Blocking Notch signaling did not inhibit VEGF-induced upregulation of Nrp1 and had no effect on Nrp2 levels (data not shown). This supports the idea that Nrp1 is upstream of and potentially activates Notch signaling. Interestingly, blocking Notch in our differentiating endothelial cultures resulted in upregulation of COUP-TFII (Figure 4B) implying either that COUP-TFII may be downstream of Notch, or the presence of an autoregulatory loop between the two.
that it is possible to induce functional specific arterial or venous cell phenotype in vitro.

Discussion

Endothelial differentiation of ES cells is described in the literature mainly in the context of studies on hematopoietic development. However, unlike in the hematopoietic system, less progress has been made in characterizing the steps inducing lineage commitment among endothelial cells. In the present study, we describe a protocol to derive endothelial cells with arterial and venous like properties from early mesodermal precursors. These cells not only show lineage specific gene expression patterns but also display corresponding morphological as well as functional phenotypes.

As VEGF is an absolute requirement for endothelial development in vivo and targeted haploid disruption reveals a strong gene dosage effect, it is not surprising that our in vitro differentiation system robustly displays a strong VEGF dose-dependency. Without VEGF the sorted angioblast developed into SMA-expressing cells instead of endothelial cells. Comparing gene expression in ES cells, angioblasts and endothelial cells show an initial upregulation of the arterial genes EphrinB2 and Nrp1, as well as the venous genes EphB4 and Nrp2. Further studies have provided evidence of VEGF promoting arterial fate in addition to endothelial induction in vertebrates. In the zebrafish VEGF is placed downstream of Sonic hedgehog but upstream of Notch signaling driving arterialization. Overexpression of VEGF-A in mouse heart results in increasing numbers of cardiac arterial vessels, and

![Figure 5](http://example.com/figure5.png)

**Figure 5.** VEGF level and Notch status effect endothelial sprouting and pericyte recruitment. A, EBs were differentiated with 50 and 10 ng/mL VEGF only or 50 ng/mL VEGF with γ-Secretase inhibitor (GSI) before being embedded in collagen type-I to form sprouts during 5 days. B, Quantification of sprout length and relative length distribution <300 μm, 300 to 400 μm, and >400 μm (n=20). C and D, Pericyte coverage of endothelial sprout, CD31 in red and SM-actin in green (n=10). Error bars show SEM. *P<0.05, **P<0.01 by Student t test Scale bars: A, 200 μm; C, 50 μm.

![Figure 6](http://example.com/figure6.png)

**Figure 6.** Monocyte adhesion assay. A, Monocyte adhesion to endothelial sheets (CD31, red) differentiated in VEGF (50 ng/mL) with or without γ-Secretase inhibitor (GSI). Endothelial cultures were IL-1 stimulated for 4 hours before DiO-labeled monocytes were allowed to adhere. B, The data represent the mean of leukocytes attached per area in each treatment. Error bars show SEM. *P<0.05 by Mann–Whitney U test (n=16 without and n=21 with IL-1 stimulation). C, FACS analysis showing E-selectin upregulation by IL-1 stimulation.
mouse embryonic endothelial cells can be driven to express EphrinB2 in response to VEGF in vitro. In agreement with these studies we show that VEGF-driven endothelial differentiation acquires an arterial phenotype, and several of the tested arterial markers show a tight dose-dependent response to VEGF during in vitro differentiation. Interestingly, decreasing levels of VEGF result in a loss of arterial markers and a strong upregulation of the venous transcription factor COUP-TFII indicating a spatial patterning of the vasculature dependent on graded VEGF signaling, not only to promote endothelial induction but also inducing arterial specification. This effect may be ameliorated through Nrp1 acting as a VEGF coreceptor at high VEGF concentration. It has been shown that Nrp1 enhances the binding of VEGF_{64} to its receptor Flk1 increasing mitogenic activity, even though Nrp1 itself does not show such function. Mutation of Nrp1 in mouse produced a variety of vascular defects. Stalmans et al showed that mice lacking the VEGF_{64} isoform also displayed a variety of vascular defects. Notably, the arterial vessels of the eye were affected, whereas the veins appeared normal. Because Nrp1 selectively binds the VEGF_{64} isoform, this defect could be attributed to loss of Nrp1 potentiated VEGF signaling.

There is a strong link between VEGF, Notch, and endothelial development. Angioblast differentiation into endothelial or smooth muscle cells suggests a specific function for different Notch members, because SMA cells only express Notch3, whereas endothelial cells express Notch1, 4, and DI4. However, inhibition of Notch signaling still allowed establishment of both lineages. VEGF induction of arterial genes can to a great extent be blocked through inhibition of Notch signaling, identifying both Notch-dependent and -independent response to VEGF. GSI blocked the upregulation of Hey1 and 2, but also DI4, EphrinB2, and Cx40. However, Nrp1 was unchanged indicating a function upstream of Notch. The fact that VEGF-induced DI4 expression could be reduced with GSI points to a positive feedback loop in Notch signaling where receptor activation increases ligand expression. The other Notch ligands and receptors did not show such regulated expression. Furthermore, inhibition of Notch signaling also increased expression of COUP-TFII. You et al have previously shown that overexpression of COUP-TFII gave defective vessels indicating an arterial to venous fate shift and their study placed COUP-TFII upstream of Notch. Our data indicate a regulation of COUP-TFII by Notch signaling possibly through a feedback loop triggered by strong VEGF signaling.

Yurugi-Kobayashi et al report that AM-mediated cAMP enhances endothelial induction and is required for arterial differentiation. In their system, 50 ng/mL VEGF alone induces 30% endothelial cells and EphrinB2, DI4, Notch4, Nrp1, or Alk1 expression was not detected, whereas addition of cAMP together with VEGF induces 70% endothelial cells giving detectable arterial transcripts. In our hands, the same concentration of VEGF can induce up to 80% endothelial cells and give strong upregulation of multiple arterial markers during the progression from ES cells to mature endothelial cells. However, if we decrease VEGF concentration, the endothelial induction is reduced as well as the arterial transcripts. We show that these seemingly contrasting findings partly could be attributed to temporal progression, because Yurugi-Kobayashi et al evaluate arterial/venous status at day 7 as compared with the present study at day 8.

In addition to the molecular characterization, 3 in vitro quantifiable readouts confirmed the arterial and venous identity of our endothelial cells. Morphologically, arteries extend shorter sprouts and recruit more pericytes then veins when cultured ex vivo in collagen type-I gel and in vivo they recruit thicker smooth muscle walls. Both of these characteristics could be confirmed in our system. A critical and specific hallmark of venous cells in vivo is the ability to respond to inflammatory stimuli by leukocyte adhesion. This differential response has also been documented in arterial and venous cell lines and held true for our in vitro derived cells, further supporting a mature venous cell commitment and not merely undifferentiated venous-like endothelial precursor.

Summary

We developed an ES cell in vitro differentiation system for endothelial arterial and venous lineage specification and show that not only is VEGF required for endothelial differentiation but the subsequent lineage fate is also dose-dependent. High VEGF concentration is needed for the expression of arterial marker genes, whereas lower levels of VEGF are permissive for venous differentiation. This VEGF-induced arterialization can be blocked by inhibition of Notch signaling, which results in an arterial to venous fate shift. Morphological and functional studies further confirmed their identity as arterial and venous endothelial cells.

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Disclosures

None.

References

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**Supplementary Figure I.** RT-PCR showing individual transcript levels of EphrinB1, 2, and 3. Each lane was loaded with 5µl from corresponding RT-PCR reaction.

**Supplementary Figure II.** FACS characterization of the mouse specific E-selectin antibody. The antibody only reacts to mouse SVR endothelial cells but not human HUVEC cells after 4h IL-1 stimulation, confirming its specificity. SVR cells obtained from ATCC (CRL-2280) and HUVECs were a kind gift from Lena Claesson-Welsh (Rudbeck Laboratory, Uppsala).