Low Oxygen Tension Enhances Endothelial Fate of Human Pluripotent Stem Cells

Sravanti Kusuma, Elizabeth Peijnenburg, Parth Patel, Sharon Gerecht

Objective—A critical regulator of the developing or regenerating vasculature is low oxygen tension. Precise elucidation of the role of low oxygen environments on endothelial commitment from human pluripotent stem cells necessitates controlled in vitro differentiation environments.

Approach and Results—We used a feeder-free, 2-dimensional differentiation system in which we could monitor accurately dissolved oxygen levels during human pluripotent stem cell differentiation toward early vascular cells (EVCs). We found that oxygen uptake rate of differentiating human pluripotent stem cells is lower in 5% O₂ compared with atmospheric conditions. EVCs differentiated in 5% O₂ had an increased vascular endothelial cadherin expression with clusters of vascular endothelial cadherin+ cells surrounded by platelet-derived growth factor β+ cells. When we assessed the temporal effects of low oxygen differentiation environments, we determined that low oxygen environments during the early stages of EVC differentiation enhance endothelial lineage commitment. EVCs differentiated in 5% O₂ exhibited an increased expression of vascular endothelial cadherin and CD31 along with their localization to the membrane, enhanced lectin binding and acetylated low-density lipoprotein uptake, rapid cord-like structure formation, and increased expression of arterial endothelial cell markers. Inhibition of reactive oxygen species generation during the early stages of differentiation abrogated the endothelial inductive effects of the low oxygen environments.

Conclusions—Low oxygen tension during early stages of EVC derivation induces endothelial commitment and maturation through the accumulation of reactive oxygen species, highlighting the importance of regulating oxygen tensions during human pluripotent stem cell-vascular differentiation. (Arterioscler Thromb Vasc Biol. 2014;34:913-920.)

Key Words: endothelial cells ■ markers, differentiation ■ oxygen ■ pluripotent stem cells

Low oxygen environments drive blood vessel growth in both the embryo and the adult. In the developing human embryo, the earliest tissue to form is the vascular system because of the necessity of oxygen and nutrients for tissue growth and survival. In the adult, ischemia, characterized by a deficit in nutrients and oxygen, stimulates blood vessel recruitment. In stark contrast to typical cell culture environments that consist of ≈20% O₂, tissues in the body experience 1% to 5% O₂ and blood vessels experience 5% to 7% O₂ based on vessel type.1

Human stem cells provide the opportunity to study the effects of low oxygen environments on vascular differentiation in controlled in vitro conditions. Toward these ends, human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have been studied widely because of their abilities to self-renew and differentiate into any cell type of the body. Recapitulation of low O₂ tensions during hPSC differentiation offers insights into embryonic development and, in turn, may yield the generation of cells from a renewable source for therapeutic use.

Endothelial cells (ECs), which make up the inner lining of blood vessels, are in direct contact with blood, thus positioning them as the first responders to changes in O₂ levels. Indeed, previous studies have revealed that O₂ availability plays a vital role in EC differentiation from a variety of stem cell sources, including human embryonic stem cells,2-3 mouse ESCs,4 human endothelial progenitor cells,5 human bone marrow CD133+ cells,6 and amniotic mesenchymal side population cells.7 Previous studies examining the influence of low O₂ tensions on EC differentiation from PSCs have relied on 3-dimensional embryoid body differentiation—in which the local oxygen environment of each cell varies slightly according to the cell’s position in the sphere as result of an oxygen gradient—or spontaneous differentiation from pluripotent cultures on feeder layers that may contribute to oxygen consumption.2 In our previous study, we found that hPSCs can be induced to codifferentiate into early vascular cells (EVCs) using a step-wise differentiation protocol that uses a feeder-free monolayer culture and avoids an EB intermediate and sorting, thus enabling the study of the role of low O₂ in a controlled system. The EVCs were composed of ECs and pericytes, determined by their expression of vascular endothelial cadherin (VEcad) and platelet-derived growth factor β (PDGFRβ), respectively.9

Received on: December 8, 2013; final version accepted on: January 27, 2014.
From the Department of Chemical and Biomolecular Engineering, Johns Hopkins Physical Sciences–Oncology Center, Institute for NanoBioTechnology (S.K., P.P., S.G.), Department of Biomedical Engineering (S.K.), Cellular and Molecular Biology (E.P.), and Department of Materials Science and Engineering (S.G.), Johns Hopkins University, Baltimore, MD.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.303274/-/DC1. Correspondence to Sharon Gerecht, PhD, Johns Hopkins University, 3400 N Charles St, Baltimore, MD 21218. E-mail Gerecht@jhu.edu

© 2014 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.114.303274
Low oxygen environments have been shown to accelerate the generation of vascular precursors, characterized by the expression of Brachyury, vascular endothelial growth factor (VEGF) receptor-2, and Bone morphogenetic protein 4, from mouse ESCs. Common markers of vascular precursors applicable to hPSCs include kinase domain receptor, CD34, CD36, and CD65. Furthermore, in response to changing oxygen (DO) levels, cells increase production of reactive oxygen species (ROS). With respect to ECs, ROS levels increase in response to hypoxia and reoxygenation. The role of ROS has also been implicated in cardiovascular differentiation of mouse ESCs to transduce mechanical signals. In ROS-induced conditions, hESCs were observed to differentiate into mesodermal and endodermal lineages. Here, we investigated whether exposure to 5% O2 affected expression of early vascular markers as well as yielded ROS expression to transduce oxygen signals.

The role of low oxygen tension in vascular growth is further complicated by the fact that arterial and venous ECs are exposed to disparate oxygen tensions. Macroscopically, arterial ECs are subject to oxygenated blood flow, in contrast to venous ECs, which experience deoxygenated blood. Previous studies examining the role of low oxygen tension in EC fate specification has yielded confounding results. One study demonstrated that newborn mice exposed to moderate hypoxia (10% O2) failed to express arterial markers but maintained vein-specific marker expression. However, endothelial progenitor cells acquired an arterial fate on exposure to hypoxic conditions.

In the present study, we hypothesized that physiological oxygen conditions direct endothelial fate of hPSCs. We speculated that by differentiating hPSCs in controlled conditions, we could precisely elucidate the role of low O2 tension as a means to augment EC differentiation capacity and to direct EC fate specification. Furthermore, we sought to understand whether the influence of low O2 was maintained in hiPSC differentiation, which has not been investigated previously. Toward this end, we used a fully genetically sequenced hiPSC line, BC1, which is also derived nonvirally, making it a clinically relevant model with translational importance. We measured the local O2 microenvironment of the cells under 5% O2 and atmospheric differentiation conditions and assessed the expression of early vascular markers under each condition. Next, we evaluated the phenotypes of EVCs under continuous 5% O2 and control (atmospheric) conditions. Finally, we determined the role of ROS in the differentiation phenotypes.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

DO Measurements and Oxygen Uptake Rate

Three classes of hypoxia have been described in correlation with the oxygen concentration in the blood: moderate hypoxia (≈5% O2), severe hypoxia (≈1% O2), and anoxia (no O2). Moderate hypoxia corresponds to physiological oxygen tension, or the concentration of oxygen typically found in blood vessels. Alternatively, severe hypoxia is found within certain tissues, the developing embryo, and in tumors; anoxia arises from complete lack of blood flow and has been suggested to be present in the bone marrow niche. Previous studies have reported that prolonged differentiation in 1% O2 conditions yielded cell death and low-quality RNA; thus, we focused on a 5% O2 environment as a representative of physiologically relevant conditions.

In this study, hPSCs were differentiated in feeder-free monolayer cultures following our established protocol. We began by measuring DO levels during the first 6 days of differentiation to determine the oxygen uptake rate (OUR). Toward this end, hPSCs were dissociated into a single-cell suspension and seeded on a collagen IV substrate with oxygen sensor patches affixed to the bottom. Using this system, it is possible to measure DO levels precisely at the cells’ microenvironment. We measured the DO levels every 30 minutes for 6 days and changed media on the third day. Under atmospheric conditions (≈20% O2), DO levels decreased to ≈15% O2 for both hESC-H9 and hiPSC-BC1 differentiating cells (Figure 1A; for ease of visualization, measurements every 60 minutes are presented on the graph).

To obtain 5% O2 conditions, differentiation occurred in a hermetically sealed chamber that was flushed with a nitrogen/carbon dioxide/oxygen mixture to obtain 5% O2 conditions before DO measurements. Under these conditions, we detected oscillations in DO levels initially (data not shown). We attribute these fluctuations to the low temperature of the gas mixture used to flush the chamber to obtain a 5% O2 environment. Thus, our measurements begin once the temperature within the chamber is constant (ie, after 2.5 hours). We found that DO levels of differentiating cultures decreased to ≈1% O2 during the first 6 days of 5% O2 differentiation (Figure 1B). DO levels did not seem to reach equilibrium after 3 days, as evidenced by the downward sloping trend during the first 3 days. During the next 3 days, DO levels decreased in a gradual fashion and reached a steady state on the sixth day. After 6 days, cell density was not significantly different between the 2 hPSC lines or variable oxygen tensions (Figure 1C).

The difference in the oxygen partial pressure in the chamber and the DO level measured by the sensors allows us to calculate the oxygen gradient. Using this gradient and taking into account the cell growth after 6 days, we can also calculate the OUR per cell (see Materials and Methods in the online-only Data Supplement). The difference in OURs between hPSC
Assessment of Early Vascular Marker Expression

After 6 days of differentiation in either atmospheric or 5% O₂ conditions, we assessed the expression of early vascular markers by the differentiated cells. Flow cytometry revealed no difference in protein expression of kinase domain receptor (also known as human VEGF receptor-2), CD34, or CD56 between the differentiation conditions (Figure 1D). However, reverse transcription polymerase chain reaction (PCR) analysis revealed that CD34, kinase domain receptor, and CD56 expression is significantly increased in 5% O₂-differentiated cells compared with that in lower oxygen conditions. ²⁹

Effect of 5% O₂ Environment on Endothelial Differentiation

Next, we assessed the effect of variable oxygen conditions on our bicellular EVCs. We compared the EVCs derived in continuous 5% O₂ tension with control conditions (Figure 2A). Our control conditions were differentiating cells exposed to atmospheric conditions for all 12 days (control EVCs). ⁹ In continuous 5% O₂ conditions, cells were exposed to a 5% O₂ environment for all 12 days of differentiation. Light microscopy images revealed drastic morphological differences in the cell derivatives (Figure 2B). EVCs differentiated in control conditions appeared spread with no visible organization. Remarkably, EVCs differentiated in continuous 5% O₂ conditions adopted an organized structure and exhibited 2 distinct morphologies: elongated cells bundles (arrows) and cobblestone area–forming cells (arrowheads). Flow cytometry analysis of these derivatives revealed that 5% O₂-differentiated EVCs exhibited greater VECad expression compared with control conditions (Figure 2C; P<0.05).

Immunofluorescent staining revealed further differences between the EVCs. Some VECad expression in control EVCs was membrane localized, but most was intracellular (Figure 2D and 2E). Control EVCs exhibited PDGFRβ expression localized to the nucleus, most likely as a result of nonspecific binding. Contrastingly, immunofluorescent images uncovered a unique organization of 2 distinct populations of VECad+ and PDGFRβ+ cells in 5% O₂ EVCs (Figure 2D). VECad+ clusters were observed to be surrounded by PDGFRβ+ pericytes. Moreover, VECad expression was localized appropriately to the cellular membrane in EVCs derived via continuous 5% O₂ environments (Figure 2E). PDGFRβ expression in 5% O₂ EVCs was largely expressed in cells’ cytoplasm.

Effects of Temporal Low Oxygen Differentiation Environments

To determine whether low oxygen environments play a role in a temporal manner, we compared the effect of 5% O₂ environments during the first half of differentiation with the effect of 5% O₂ environments during the second half of differentiation (Figure 3A). In the secondary 5% O₂ conditions, cells were exposed to control (ie, atmospheric) conditions for the first 6 days of differentiation followed by 6 days in 5% O₂ conditions. Conversely, in the 5% O₂ primed condition, cells were exposed to a 5% O₂ environment for the first 6 days of

---

Table. Calculation of OUR

<table>
<thead>
<tr>
<th>Condition</th>
<th>P₅ₒ₂ (%)</th>
<th>xO₂</th>
<th>C*, μmol/L</th>
<th>H9</th>
<th>Cᵣ, μmol/L</th>
<th>Pₒ₂ (%)</th>
<th>OUR, pmol/s per 10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21</td>
<td>5</td>
<td>4x10⁻⁶</td>
<td>224</td>
<td>159.5±9.2</td>
<td>14.9</td>
<td>73.0±16.1</td>
</tr>
<tr>
<td>BC1</td>
<td>5% O₂</td>
<td>3.5±1.6</td>
<td>169.8±16.7</td>
<td>15.8</td>
<td>51.7±17.9</td>
<td>36.9±5.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21% O₂</td>
<td>3.5±1.6</td>
<td>169.8±16.7</td>
<td>15.8</td>
<td>51.7±17.9</td>
<td>36.9±5.3</td>
<td></td>
</tr>
</tbody>
</table>

xO₂ is molar fraction of oxygen in the liquid. OUR indicates oxygen uptake rate.
differentiation followed by 6 days in control conditions. We found that mRNA levels of VEcad and CD31 were significantly increased in continuous or primed 5% O\textsubscript{2} conditions compared with control conditions (Figure 3B). Interestingly, when cells were alternately cultured in secondary 5% O\textsubscript{2} conditions, VEcad and CD31 mRNA expression levels were similar to that of control conditions. Correspondingly, light microscopy revealed that secondary 5% O\textsubscript{2} EVCs lacked any organization (similar to control conditions), whereas primed 5% O\textsubscript{2} EVCs resembled continuous 5% O\textsubscript{2} EVCs with an organization composed of elongated cell bundles (arrows) and cobblestone area--forming cells (arrowheads; Figure 3C). Flow cytometry for VEcad expression confirmed that primed 5% O\textsubscript{2} EVCs had significantly greater VEcad protein expression compared with secondary 5% O\textsubscript{2} EVCs (Figure 3D). In fact, VEcad expression by primed 5% O\textsubscript{2} EVCs was similar to that by continuous 5% O\textsubscript{2} EVCs (from Figure 2C). Finally, we confirmed that primed 5% O\textsubscript{2} EVCs also displayed clusters of VEcad+ cells surrounded by PDGFRβ+ pericytes (Figure 3E, left), with appropriately localized VEcad and PDGFRβ (Figure 3E, right). Altogether, these data demonstrate that low oxygen conditions during the early stages of differentiation enhance endothelial lineage commitment.

**Effect of 5% O\textsubscript{2} on Endothelial Fate**

Because our data suggested augmented EC differentiation, we further investigated EC characteristics. For these studies, we compared control EVCs with primed and continuous 5% O\textsubscript{2} EVCs. Flow cytometry analysis revealed significantly augmented CD31 expression under either 5% O\textsubscript{2} condition (Figure 4A; P<0.05). Immunofluorescent assessment of cultures corroborated this result and revealed the expression of clusters of cells expressing CD31 appropriately localized to the cell membrane (Figure 4B). We also observed increased lectin binding in EVCs differentiated under either primed or continuous 5% O\textsubscript{2} conditions (Figure 4C). Lectin was not expressed in control EVCs; however, we could observe clusters of lectin+ cells under both 5% O\textsubscript{2} conditions (Figure 4C).

As part of the body’s cholesterol metabolism, ECs are able to incorporate acetylated low-density lipoprotein (acLDL). When we examined the ability for the 3 classes of EVCs to endocytose acLDL in vitro, we found that primed and continuous EVCs were able to uptake acLDL again following a clustered phenotype (Figure 4D). We could not detect acLDL uptake in control EVCs.

We next assessed the ability for EVCs derived by the 3 conditions to form cord-like structures on Matrigel. After 4 hours of culture, we could observe some spreading in control EVCs (Figure 4E). Intriguingly, primed or continuous EVCs were able to reorganize into cord-like structures after just 4 hours. Quantification of mean tube length revealed both types of 5% O\textsubscript{2} EVCs formed structures with significantly larger mean tube lengths compared with control EVCs after 4 hours (Figure 4F). During 24 hours, the mean tube length of structures formed by continuous or primed EVCs did not change. Contrastingly, the mean tube length of structures formed by control EVCs gradually increased during 24 hours, approaching that of primed and continuous EVCs. Examining the mean tube thickness, we found a similar trend (Figure 4F). After 4 hours of culture, primed or continuous EVCs reached their maximum thickness, whereas networks formed by control EVCs increased in mean tube thickness during 24 hours.

To further define the phenotype of derived ECs, we examined the expression of arterial EC markers, ephrinB2 and Nrp1, and venous markers, EphB4 and Nrp2, compared with control cell types, human umbilical artery ECs and human umbilical vein ECs. Expression of these markers was normalized to that by endothelial colony--forming cells. We took the natural log of the ratio of the relative expression of arterial markers ephrinB2 and Nrp1 to the relative expression of venous markers EphB4 and Nrp2. This form of analysis yields positive values for arterial cells (eg, human umbilical artery ECs) and negative values for venous cells (eg, human umbilical vein ECs; Figure 4G). When

---

**Figure 2.** Effect of 5% O\textsubscript{2} on endothelial differentiation. A, Schematic of manipulated oxygen environments. Comparison of control and continuous 5% O\textsubscript{2} conditions demonstrated by (B) light microscopy images (arrows, elongated cell bundles; arrowheads, cobblestone area--forming cells; scale bar, 100 μm), (C) flow cytometry for vascular endothelial cadherin (VEcad) expression, and (D) and (E) immunofluorescence images of VEcad (red) and platelet-derived growth factor β (PDGFRβ; green). Nuclei in blue. Scale bar, (D) 500 μm and (E) 100 μm.
we assessed these values for control, primed, and continuous EVCs, we found that both primed and continuous EVCs took on a phenotype more similar to that of human umbilical artery ECs (Figure 4G). Control EVCs did not demonstrate a clear fate identity toward either lineage. These data suggest that a 5% O₂ environment may promote EVCs toward the arterial lineage.

Role of ROS in 5% O₂ Differentiation
Low oxygen environments promote the generation of ROS, which allow the cells to adapt to lower O₂ tensions. The generation of ROS was analyzed by 2′,7′-dichlorodihydrofluorescein diacetate, a redox-sensitive fluorescent dye, during differentiation in 5% O₂ and control conditions (Figure 5A). After just 3 days, we observed ROS accumulation in the 5% O₂-differentiated cells to a greater extent than that in control cells (Figure 5B). To understand whether there was an association between ROS generation and the distinct phenotypes observed after 12 days, we treated the cells with diphenyleneiodonium (DPI), a potent inhibitor of ROS. Because we found that low oxygen conditions during the early stages of differentiation affect endothelial fate, we examined the effect of the addition of DPI to the first half of the differentiation. When DPI was added at day 0, cell attachment and proliferation were severely limited and we could not obtain enough cells for analysis. Alternatively, we added DPI after 3 days of differentiation to first allow the cells to attach and grow and then treated them with DPI for a total of 3 days (days 3–6 of differentiation). After 3 following days of differentiation under DPI-treated conditions (day 6 total), ROS production was reduced drastically (Figure 5C).

DPI-treated cells were maintained in 5% O₂ conditions for the second 6 days of differentiation but without DPI. After 12 days, DPI-treated cells exhibited similar expression of VEcad and CD31 compared with control EVCs without DPI (Figure 5D). Both VEcad and CD31 expression in DPI-treated
cells were significantly lower than EVCs differentiated in 5% O₂ conditions without DPI (from Figure 3B). Thus, we suggest that the presence of DPI abolished differentiating cells' downstream signaling in response to 5% O₂ and led to a phenotype more similar to that of atmospheric-derived (ie, control) cells, implicating ROS in the augmented vascular differentiation capacity under 5% O₂ conditions.

**Discussion**

Because of their ability to differentiate into every cell type of the body and to self-renew indefinitely in culture, hPSCs represent an important channel toward the progress of tissue regenerative therapies owing to their potential to generate clinically relevant numbers of any cell type of interest. Means to improve hPSC differentiation capacity will advance their clinical application toward repairing tissue in vivo or rebuilding tissue in vitro. Vital to these efforts is the regeneration of a functional blood supply to sustain tissue survival. Low oxygen tension is an important regulator in blood vessel growth. In the present work, we demonstrated that a low oxygen environment is especially critical during the early half of differentiation exhibited 2 distinct cell morphologies, characterized as VEcad⁺ colonies surrounded by PDGFβR⁺ pericytes. When alternately exposed to 5% O₂ only during the second half of differentiation, EVCs resembled control. The effect of early low oxygen tension is suggestive of embryonic differentiation in which the vascular system itself forms under hypoxic oxygen levels, which increase to more physiological levels after the onset of flow.

In the previous and current study, DO levels were monitored via a fluorescent quenching technique, in which a noninvasive sensor patch composed of a ruthenium-based metal complex is excited by an external fluorescence light source. In our present study, we confirmed that DO levels experienced by the cells are lower than that of the macroenvironment. Under atmospheric conditions, differentiating hPSCs experience DO levels closer to 15%. Contrastingly, hPSCs maintained in pluripotent culture under atmospheric conditions experience decreasing DO levels to ≈5% O₂ along a 3-day culture period, as we have previously reported. These differences could be a result of the lower cell seeding density in our differentiation system and perhaps a slower proliferation rate. In 5% O₂ conditions, differentiating hPSCs actually experience DO levels close to 1% after 3 days in culture. These differences play a vital role in cellular responses as different pathways are activated under 5% O₂ versus 1% O₂.

Despite similar cell growth rates after 6 days, cells differentiated in 5% O₂ conditions demonstrated a lower OUR compared with control cells, concordant with our previous findings that various cell types, including somatic and pluripotent cells, cultured under lower oxygen conditions exhibit lower OURs than cell cultured under atmospheric conditions. Six-day differentiated cells did not express vascular progenitor markers CD34, kinase domain receptor, or CD56 at the protein level. However, it is clear that the lower oxygen tension did affect mRNA expression of these vascular progenitor markers as reverse transcription PCR revealed significantly increased expression of all 3 markers after 6 days under 5% O₂ conditions compared with control conditions. We suspect that this discrepancy is because of the fact that although the low oxygen conditions are affecting the internal cellular machinery of the differentiating cells, 6 days may not be sufficient time to observe the differences at the protein level.

After 6 days of differentiation in either atmospheric or 5% O₂ conditions, cells were then subjected to a subsequent 6 days of differentiation in either condition. Indeed, continuous exposure to 5% O₂ for 12 days yielded EVCs with vastly different cell morphologies and cellular phenotypes compared with control EVCs. The low oxygen differentiation environment stimulated increased expression of EC markers VEcad and CD31 as well as their appropriate junctional localization. Low oxygen differentiation environments also yielded the generation of these derived ECs in discrete colonies throughout the culture, surrounded by PDGFβ cells. This EC colony phenotype is reminiscent of an adult stem cell population, endothelial colony–forming cells, known for their ability to differentiate to mature ECs.

Although we implicate the role of 5% O₂ in EC differentiation from hPSCs, an intriguing caveat to this generalization is that a low oxygen environment is especially critical during the early time points of differentiation. EVCs exposed to 5% O₂ only during the first half of differentiation exhibited 2 distinct cell morphologies, characterized as VEcad⁺ colonies surrounded by PDGFβR⁺ pericytes. When alternately exposed to 5% O₂ only during the second half of differentiation, EVCs resembled control. The effect of early low oxygen tension is suggestive of embryonic differentiation in which the vascular system itself forms under hypoxic oxygen levels, which increase to more physiological levels after the onset of flow.
Another important aspect of the low oxygen differentiation environment is its ability to accelerate EC maturation. EVCs differentiated in either continuous or primed 5% O₂ conditions exhibited appropriate membrane localization of CD31, in addition to VEcad. As we previously reported, such robust membrane localization of VEcad or CD31 was only observed on sorted, subcultured VEcad+ cells on day 18 (ie, mature EC derivatives).⁷ ECs in both primed and continuous EVCs also exhibited lectin binding and were able to uptake acLDL, a functionality associated with more mature EC phenotypes. Furthermore, continuous and primed EVCs demonstrated more rapid cord-like structure formation compared with control EVCs. Cord-like structures were observed as early as 4 hours after culture on Matrigel, whereas a comprehensive network was not observed by EVCs until 24 hours. Control EVCs reached similar network formation capability on Matrigel with respect to mean tube length and mean tube thickness after 24 hours. Taken together, these data demonstrate that low oxygen differentiation conditions enhance EC commitment as exemplified by the boost in EC marker expression and functionalities.

The derivation of ECs specified for a particular function may yield more effective repair of dysfunctional or injured vasculature. That oxygen tension differs in arteries and veins warrants continued study of the role of this instructive signal in EC specification. Our studies revealed that a 5% O₂ differentiation environment promoted the derivation of arterial-like ECs as demonstrated by increased expression of arterial markers ephrinB2 and Nrp1. Because our ECs are coderived with pericytes, we speculate the ECs may be more appropriately labeled as arteriole ECs. Previous studies have implicated a role for fluid shear stress³² or activation of cAMP pathway³³ in arterial specification from endothelial progenitor cells or mouse PSCs, respectively. Furthermore, it has been demonstrated that low oxygen conditions (≈1% O₂) activate the Notch signaling pathway, which in turn upregulates arterial marker expression and represses venous marker expression in murine embryonic endothelial progenitor cells. Inhibition of Notch signaling under hypoxic conditions led to markedly reduced levels of arterial markers. Contrastingly, other studies demonstrate that moderate hypoxia (10% O₂) promoted vein-specific marker expression but not artery marker expression. Our finding that low oxygen tension promotes arterial EC fate specification is in agreement with the former of these 2 studies, linking low oxygen tension to arterial fate. To our knowledge, ours is the first study to make this connection in hPSC differentiation. The platform established here could be further leveraged to investigate these signaling pathways that drive specification more extensively.

Another common byproduct of hypoxic culture conditions is increased expression of VEGF, which acts upstream of Notch.⁴⁴ Corroborating this finding, previous literature supports that arteriovenous specification may be regulated by distinct VEGF levels, indicating that higher VEGF concentrations yield arterial ECs, whereas lower VEGF concentrations yield venous ECs. Intriguingly, Lanner et al⁴⁷ found that arterial EC differentiation from mouse PSCs under hypoxic conditions was independent of increases in VEGF. Future work using our differentiation practice could help to understand this regulation more closely and expose whether the same is true during human PSC differentiation.

To better understand the differences between 5% O₂ and atmospheric differentiated cells, we examined the role of ROS on derivative phenotypes. We observed increased ROS expression under 5% O₂ conditions and confirmed that treatment with DPI diminished ROS generation. We found that when we added DPI at the beginning of differentiation, cell attachment and proliferation were diminished. This decreased cell viability may be a result of the necessity of a basal level of ROS for cell survival in hPSCs, a dependency that has been demonstrated in other cell types.³⁸,³⁹ To assess whether ROS generation under 5% O₂ conditions was critical to the observed phenotypes of primed and continuous EVCs, we treated the differentiating cells with DPI from days 3 to 6 of 5% O₂ differentiation. Our data indicated that VEcad and CD31 mRNA levels of DPI-treated EVCs were similar to or lower than that of control (ie, atmospheric) EVCs, demonstrating that treatment with DPI abrogated the endothelial inductive effect of the low oxygen differentiation environments.

Although our system presents a controlled method to study varying oxygen tensions on EC differentiation from hPSCs, there are certain notable limitations. For one, because we are only subjecting the system to 5% O₂ conditions every 3 days, the DO level drops in between flushes in a manner that we are not controlling. Future studies using constant 5% O₂ exposure would further help validate our findings. Second, the first half of our differentiation scheme includes media rich in serum (10% serum), which is known to contain a wide variety of undefined growth factors and cues. Reduction of this unknown variable could further help unveil the role low oxygen tension, unbridled by extraneous cues.

These studies demonstrate the importance of low oxygen tension in EC differentiation in a controlled environment and importantly for the generation of ECs with clinical translatability.

Acknowledgments
We thank Dr Hasan Erbil Abaci for his assistance and productive discussions and Dr Linzhao Cheng for providing human-induced pluripotent stem cell lines.

Sources of Funding
We gratefully acknowledge support for this work by predoctoral awards from the American Heart Association and National Institutes of Health (NIH) grant F31HL112644 (both to S. Kusuma), NIH grants R01HL107938 and U54CA143868, an American Heart Association Scientist Development grant, and National Science Foundation grant 1054415 (to S. Gerecht).

Disclosures
None.

References
Improving endothelial cell (EC) differentiation from human pluripotent stem cells is a key area of research for regenerative medicine. As a stimulant of vascular growth in vivo, low oxygen tension has been studied widely for its role in vascular biology, including EC differentiation from a variety of stem cell types. In this study, we implicate the role of low oxygen tension in the controlled differentiation of ECs from human pluripotent stem cells, primarily during the early stages of differentiation. Our differentiation scheme yields ECs with protein expression and functionalities similar to that of mature, arterial-like ECs. Using a clinically relevant human-induced pluripotent stem cell type as well as a controlled differentiation strategy, these derived ECs could be integral to regenerative medicine therapeutics.

**Significance**

**Arterioscler Thromb Vasc Biol** April 2014
Low Oxygen Tension Enhances Endothelial Fate of Human Pluripotent Stem Cells
Sravanti Kusuma, Elizabeth Peijnenburg, Parth Patel and Sharon Gerecht

Arterioscler Thromb Vasc Biol. 2014;34:913-920; originally published online February 13, 2014;
doi: 10.1161/ATVBAHA.114.303274
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/4/913

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/02/13/ATVBAHA.114.303274.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

**hPSC culture.** Human ESC line H9 (passages 15 to 40; WiCell Research Institute, Madison, WI) and hiPSC line BC1 were cultured as previously described. Cell lines were routinely examined for pluripotent markers using immunofluorescence staining and flow cytometry analysis for TRA-1-60, TRA-1-81, SSEA4, and Oct4.

**Cell culture.** ECFCs, HUAECs, and HUVECs were cultured as previously described. Briefly, ECFCs (Lonza, Walkersville, MD, USA), HUAECs and HUVECs (Promocell, Heidelberg, Germany) were cultured in endothelial growth media-2 (EGM2, Lonza) containing 10% fetal bovine serum (FBS) on type I collagen (BD Biosciences, Franklin Lakes, NJ, USA). Media was changed every other day. Cells were passaged every three to four days with 0.05 trypsin/0.1% ethylenediaminetetraacetic acid (EDTA; Invitrogen, Carlsbad, CA, USA) and maintained in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

**Differentiation protocol.** Human PSCs were collected through digestion with ethylenediaminetetraacetic acid (EDTA; Promega, Madison, WI), separated into an individual cell suspension using a 40-µm mesh strainer (BD Biosciences), and plated onto collagen IV (Trevigen) coated plates at a concentration of 5x10⁴ cells/cm². Cells were cultured in a differentiation medium composed of alpha-MEM (Invitrogen Carlsbad, CA), 10% FBS (HyClone) and 0.1 mM β-mercaptoethanol (β-ME) as previously described. For continuous and primed 5% O₂ conditions, we modified a previously published protocol. Cells were allowed to attach in normoxic (21% O₂) conditions for 4 hours, and then subjected to physiologic (5% O₂) conditions in a hermetically sealed chamber. 5% oxygen concentration was achieved by flushing the chamber with a 5% O₂-5%CO₂-N₂ balance for 3 min at 3 psi and three times every thirty minutes. Humidity was maintained in the chamber by inclusion of sterile water in a petri dish at the bottom of the chamber. The chamber was flushed and media was changed after 3 days to avoid slight changes in control oxygen concentrations due to oxygen consumption by the cells and to replenish nutrients to support cell growth. On day 6, differentiated cells were collected through digestion with TrypLE (Invitrogen), separated with a 40-µm mesh strainer, and seeded at a concentration of 1.25x10⁴ cells/cm² on collagen-type-IV-coated plates in endothelial cell growth media (ECGM) (PromoCell, Heidelberg, Germany) supplemented with 2% FBS, 50ng/ml VEGF, and 10µM SB431542 (Tocris) for an additional 6 days. For continuous 5% O₂ conditions, cells were allowed to attach for 4 hours in normoxic conditions, and then subjected to 5% O₂ conditions as described above. Media was changed and where appropriate, the chamber was flushed, every 3 days.

**DO measurements.** Dissolved oxygen (DO) levels were measured as previously described. Briefly, DO was measured noninvasively, using a commercially available sensor dish reader (SDR; PreSens GmbH, Regensburg, Germany) capable of reading DO levels from an immobilized fluorescent patch affixed to the bottom of culture plate (Oxo-Dish OD-6; PreSens). The plates are sterilized and calibrated by the manufacturer for consistency in measurements. The dishes were then coated with collagen IV in a manner identical to dishes without sensor patches; All measurements were performed in a controlled environment within an incubator at 37 °C, and were taken every five minutes. Collected data were exported for analysis into Excel (Microsoft, Inc., Redmond, WA) and GraphPad Prism (4.02, GraphPad Software, San Diego, CA).

**Calculation of OUR.** We calculated the OUR based on several key assumptions as previously: (1) equilibrium is achieved at the liquid-gas interface, (2) oxygen concentration remains steady, (3) oxygen diffusivity in medium is similar to that in water, and (4) population changes are slow. From these assumptions, the OUR is calculated by:
\[ OUR = D_{O2} \frac{(C^* - C_0)}{h \varphi} \]

where \( D_{O2} \) is the oxygen diffusivity in water (3.35 x 10^{-5} \text{ cm}^2/\text{s}), \( C^* \) is the oxygen at the bottom surface in \text{mol/cm}^3, \( C_0 \) is the concentration of oxygen in the gas phase in \text{mol/cm}^3, \( h \) is the height of the liquid in the well in cm, and \( \varphi \) is the number of cells per cm². (For the complete derivation, please refer to our previous publication.)

**Flow cytometry.** Flow cytometry was performed as previously described.\(^5\) Briefly, cells were incubated with FITC- or PE-conjugated antigen specific antibodies for markers outlined in the text. All analyses were done using corresponding isotype controls. Forward-side scatter plots were used to exclude dead cells. User guide instructions were followed to complete the flow cytometry analysis via Cyflogic v1.2.

**Real-time quantitative RT-PCR.** Two-step reverse transcription polymerase chain reaction (RT-PCR) was performed on differentiated and undifferentiated (day 0) hPSCs as previously described in accordance with Applied Biosystems manufacturer instructions.\(^5\) For each primer set (CD34, KDR, CD56, VEcad, CD31, ephrinB2, EphB4, Nrp1, Nrp2), we used the comparative computerized tomography method (Applied Biosystems, Foster City, CA) to calculate the amplification differences between different samples. The values for experiments were averaged and graphed with standard deviations.

**Immunofluorescence.** Cells were prepared for immunofluorescence as previously described.\(^5\) Briefly, fixed cells were blocked in 1% BSA, treated with 0.1% Triton-X (Sigma-Aldrich, St. Louis, MO), and incubated with the antigen specific antibodies for the markers outlined in the text, followed by an appropriate secondary, and DAPI (Roche Diagnostics). The immunolabeled cells were examined using a fluorescent microscope (Olympus BX60).

**Matrigel.** Cord formation on Matrigel was assessed as previously described.\(^3,6\) Briefly, Matrigel was cast into 16 well chamber slides (Lab-Tek). After polymerization, 20,000 cells were seeded per well in 50 ng/ml VEGF media. Cord formation was observed after 4, 12 and 24 h. Quantification was performed using the Angiogenesis Tube Formation application module in Metamorph.

**Arteriovenous fate determination.** To assess fate specification, RT-PCR analysis was performed on control ECFCs, HUAECs, and HUVECs, and derived control, primed, and continuous EVCs. All data were normalized to expression by ECFCs. The ratio of ephrinB2 to EphB4 and Nrp1 to Nrp2 was calculated. The natural log of the ratio was taken and graphed versus cell type.

**ROS detection and inhibition.** We followed previously published protocols to detect and inhibit ROS production.\(^10\) For ROS detection, we replaced the culture media with 10 \text{µM} 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Invitrogen) in PBS and incubated for 30 min at 37ºC. Samples were washed with PBS three times prior to imaging. For ROS inhibition, we treated cells with diphenyleneiodonium (DPI) (Sigma-Aldrich) at a concentration of 10 \text{µM}.

**Graphs and Statistics.** All analyses were performed in triplicate samples for \( n=3 \) at least. Real-time RT-PCR were also performed on triplicate samples (\( n=3 \)) with triplicate readings. One Way ANOVA with Bonferroni post-hoc test were performed to determine significance (GraphPad Prism 4.02).
References


