Functional characterization of human pluripotent stem cell-derived arterial endothelial cells

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Here, we report the derivation of arterial endothelial cells from human pluripotent stem cells that exhibit arterial-specific functions in vitro and in vivo. We combine single-cell RNA sequencing of embryonic mouse endothelial cells with an *EFNB2-tdTomato/EPHB4-EGFP* dual reporter human embryonic stem cell line to identify factors that regulate arterial endothelial cell specification. The resulting xeno-free protocol produces cells with gene expression profiles, oxygen consumption rates, nitric oxide production levels, shear stress responses, and TNF α -induced leukocyte adhesion rates characteristic of arterial endothelial cells. Arterial endothelial cells were robustly generated from multiple human embryonic and induced pluripotent stem cell lines and have potential applications for both disease modeling and regenerative medicine.

arterial endothelial cells | arterial-specific functions | myocardial infarction | single-cell RNA-seq | human pluripotent stem cell differentiation

Bypass surgery is a common treatment for cardiovascular disease, which is the leading cause of death in the United States (1). From 2001 to 2006, at least 430,000 coronary artery bypass surgeries and 367,000 lower extremity bypass surgeries were performed annually in the United States (2, 3). Although venous grafts are most widely used for bypass surgery, some patients lack suitable veins for transplantation as a result of age or disease, and venous grafts are still prone to thrombosis, occlusion, and aneurysm (4). Primary arterial endothelial cells (AECs) have limited expansion potential and undergo de-differentiation in culture (5), making tissue engineering of human blood vessels for clinical use challenging. The ability to generate functional AECs from human pluripotent stem cells will provide a scalable, defined source of material for modeling vascular disease in vitro and for generating tissue-engineered blood vessels for transplantation.

In this study, we hypothesize that, when transplanted, properly specified AECs will improve arteriogenesis and collateral formation in ischemic tissues more robustly than more generic endothelial cells and that pluripotent stem cell-derived vascular progenitors will provide a superior, scalable, genetically defined cell source for completely tissue-engineered arteries. Previous studies have shown progress in AEC differentiation (6–14), but the arterial-specific functions in vitro and the protection of ischemic tissue in vivo have not been well demonstrated in the resulting cells. Here, we use single-cell RNA sequencing (RNA-seq) of early mouse AECs and a CRISPR/Cas9-generated *EFNB2-tdTomato/EPHB4-EGFP* dual reporter human embryonic stem cell line to develop a protocol for differentiating human pluripotent stem cells into AECs using fully defined culture conditions. The resulting cells demonstrate arterial-specific function in vitro and improve survival in a myocardial infarction model in vivo.

Results

Single-Cell RNA-Seq of Embryonic Mouse Endothelial Cells. Previously, we developed an efficient endothelial cell differentiation protocol (15), but the resulting cells lacked strong expression of arterial markers (*SI Appendix, Fig. S1 A and B*). To enhance AEC differentiation in vitro, we performed single-cell RNA-seq analysis for the endothelial cells isolated from the aorta–gonad–mesonephros region of E11.5 mouse embryos (*SI Appendix, Fig. S2A*). To distinguish AEC and venous endothelial cell (VEC) populations, we performed hierarchical clustering and principal component analysis with a set of arterial and venous markers (16). Among the five cell populations classified (*SI Appendix, Fig. S2B*), population 1 (P1) and population 2 (P2) were identified as AECs and VECs, respectively (*SI Appendix, Fig. S2 B–D*) (see *SI Appendix, Online Methods* for the detailed analysis). To identify the arterial-enriched genes, we compared the gene expression between P1 and P2 and identified 42 growth factor-related genes that can be further classified into 28 pathways (*SI Appendix, Fig. S2E* and *Datasets S1* and *S2*). Well-known arteriovenous regulators,

Significance

Generating fully functional arterial endothelial cells is a critical problem for vascular development and disease research. Currently, the arterial endothelial cells derived from human pluripotent stem cells lack the range of arterial-specific functions in vitro and the protective function for ischemic tissues in vivo. Here, we combine single-cell RNA sequencing and CRISPR-Cas9 technology to identify pathways for regulating arterial endothelial cell differentiation. We then manipulate these pathways and generate arterial endothelial cells that demonstrate unprecedented arterial-specific functions as well as improve survival of myocardial infarction. These findings facilitate the understanding of vascular development and disease and provide a source of cells that have broad applications for vascular disease modeling and regenerative medicine.

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including VEGF, WNT signaling (FZD4, FZD7, FZD10), and NOTCH signaling (DII4 and NOTCH4), were present on the list (Datasets S1 and S2). Therefore, we hypothesized that the other pathways may also play a key role in our AEC differentiation. To test this, we applied growth factor or small molecules to modulate these pathways (SI Appendix, Fig. S2F and Dataset S2).

CRISPR-Cas9 Generation of an *EFNB2*-tdTomato/*EPHB4*-EGFP Dual Reporter Cell Line to Monitor Arteriovenous Specification. To facilitate evaluating the function of these candidate factors (growth factors or small molecules) in human AEC differentiation, we developed a human embryonic stem cell reporter line using CRISPR-Cas9 technology to target *EFNB2* with tdTomato and *EPHB4* with EGFP (SI Appendix, Fig. S3A and B). *EFNB2* and *EPHB4* were the first identified and most widely used markers for AECs and VECs, respectively (6–13, 17). Specific targeting of the *EFNB2* and *EPHB4* locus was confirmed by junction PCR and Southern blot analysis (SI Appendix, Fig. S3C–F). Only single copies of each reporter were integrated into the genome (SI Appendix, Fig. S3G and H); the expression of *EFNB2* and *EPHB4*

was enriched in *EFNB2*-tdTomato⁺ and *EPHB4*-EGFP⁺ cells, respectively; and their expression in the reporter cell line was similar to that in wild-type cells (SI Appendix, Fig. S3I and J). Karyotypes were normal after dual targeting (SI Appendix, Fig. S3K), and DNA sequencing revealed no CRISPR-induced insertions or deletions in the wild-type alleles (SI Appendix, Fig. S4).

Evaluation of Candidate Factors for Arteriovenous Specification. The *EFNB2*-tdTomato/*EPHB4*-EGFP reporter cells were first differentiated to mesoderm, and then candidate factors identified by single-cell RNA-seq were added into or removed from the media (SI Appendix, Fig. S5A). Consistent with their previously described roles (14, 18, 19), we found that VEGFA, WNT3A, and resveratrol [RESV, a NOTCH agonist (20)] promoted arterial specification (SI Appendix, Fig. S5B–F).

Among the other candidate factors (Fig. 1 and SI Appendix, Fig. S6), insulin is particularly interesting. Insulin is one of the key components present in bovine serum or serum replacement widely used in previous studies (6–13), but our results showed that removing it did not affect the pan endothelial cell (CD31⁺CD144⁺) differentiation efficiency (Fig. 1A and B). Instead, insulin-free

Fig. 1. Identification of candidate factors critical to arteriovenous specification. (A and C) Flow cytometric analysis of CD31, CD144, EFNB2-TdTomato (EFNB2-Tom), and EPHB4-EGFP expression. The gate setting is based on undifferentiated embryonic stem cells for all of the flow cytometric data in this study. *EFNB2*-tdTomato/*EPHB4*-EGFP dual reporter cells were first differentiated to mesoderm cells using E8BAC media [E8 media (45) supplemented with 5 ng/mL BMP4, 25 ng/mL Activin A, and 1 μ M CHIR99021]. E5 (E8 media minus FGF2, TGF β 1, and insulin) media supplemented with 100 ng/mL FGF2, 50 ng/mL VEGFA, and 50 ng/mL BMP4 was used to induce mesoderm cells to differentiate into endothelial cells from day 2 to day 6. Insulin (20 μ g/mL) was added to or removed from the media from day 2 to day 6 as indicated. (B and D) Statistics of CD31⁺CD144⁺ and EFNB2-TdTomato^{high}/EPHB4-EGFP^{low} cells. Data are represented as mean $\pm$ SD. Student's *t* test; **P* < 0.05, *n* = 3. (E) Flow cytometric analysis of EFNB2-TdTomato and EPHB4-EGFP expression on CD31 and CD144 gated endothelial cells. E5 media supplemented with 50 ng/mL VEGFA and 10 μ M SB431542 was used to induce mesoderm cells differentiated into endothelial cells from day 2 to day 6. BMP4 (50 ng/mL) was added as indicated. Experiments were repeated three times. (F and H) Flow cytometric analysis of CD31⁺CD144⁺ and EFNB2-TdTomato^{high}/EPHB4-EGFP^{low} cells. Embryonic stem cells were first differentiated into mesoderm cells as mentioned above. E5 media supplemented with 50 ng/mL VEGFA and 10 μ M SB431542 was used as the base media to induce mesoderm cells to differentiate into endothelial cells from day 2 to day 6. Other factors were added to the base media as indicated. (G and I) Statistics of CD31⁺CD144⁺ and EFNB2-TdTomato^{high}/EPHB4-EGFP^{low} cells. Data are represented as mean $\pm$ SD. Student's *t* test; **P* < 0.05; *n* = 3. The following were used: 5 μ M L690, 5 μ g/mL LDL, and 100 ng/mL PDGF-BB.

media triggered EFN2-tdTomato^{high}/EPHB4-EGFP^{low} AEC differentiation (Fig. 1 C and D). We also tested the function of BMP4 as it is widely used for AEC differentiation (6, 9, 12, 13). The result showed that BMP4 suppressed the arterial marker (EFNB2) expression (Fig. 1E and SI Appendix, Fig. S7A). Therefore, the BMP4- and insulin-free medium was used to test the other candidates. Adding FGF2, L690 (an IMPase inhibitor), and native low-density lipoprotein (LDL) increased EFN2-tdTomato^{high}/EPHB4-EGFP^{low} AEC differentiation (Fig. 1 F-I), whereas removing SB431542 (a TGF- β 1 pathway inhibitor) or adding PDGF-BB inhibited AEC differentiation (Fig. 1 F-I). In summary, we demonstrated that VEGFA, WNT3A, RESV, FGF2, LDL, L690, and SB431542 promote AEC differentiation, whereas insulin, BMP4, and PDGF-BB inhibit AEC differentiation.

Cell fate is determined by the temporal exposure to combinatorial developmental cues (21). Therefore, we examined the combinatorial effect of these factors to further improve AEC differentiation. The combination of FGF2, VEGFA, SB431542, RESV, and L690 ("five factor") in the absence of insulin greatly

improved AEC differentiation (Fig. 2 A and B) compared with the individual influence of each of these factors (Fig. 1). The individual subtraction of FGF2, VEGFA, SB431542, RESV, or L690 or adding insulin significantly reduced EFN2-tdTomato^{high}/EPHB4-EGFP^{low} AECs (Fig. 2B). To better understand their functions, we further analyzed the expression of other key AEC markers, CXCR4 and DLL4. We found that CXCR4⁺CD144⁺ and DLL4⁺CD144⁺ AECs were also decreased after withdrawing FGF2, VEGFA, or SB431542 or after adding insulin (Fig. 2 C and D). Removing RESV or L690 did not affect the percentage of CXCR4⁺CD144⁺ and DLL4⁺CD144⁺ but significantly decreased EFN2-tdTomato^{high}/EPHB4-EGFP^{low} AECs (Fig. 2 B-D). The result demonstrates that FGF2, VEGFA, SB431542, RESV, L690, and insulin-free are the core factors of the five factor protocol, which generated $1.7\text{--}2.1 \times 10^6$ AECs from 1.0×10^6 starting cells or $1.4\text{--}1.7 \times 10^7$ AECs from one 10-cm dish in 6 d (Fig. 2 E and F).

The robustness of this protocol was tested in six human pluripotent stem cell lines, including H1 (male) and H9 (female)

Fig. 2. Combinatorial effects of the key factors. (A–D) Flow cytometric analysis of CD31⁺CD144⁺, EFN2-tdTomato^{high}/EPHB4-EGFP^{low}, CD144⁺CXCR4⁺, and CD144⁺DLL4⁺ cells. Five factor media (E5 media supplemented with 100 ng/mL FGF2, 50 ng/mL VEGFA, 10 μ M SB431542, 5 μ M RESV, and 5 μ M L690) was used as the base media. As indicated, single factors were either withdrawn from or added into the five factor media. Insulin, 20 μ g/mL. Statistical data of CD31⁺CD144⁺, EFN2-tdTomato^{high}/EPHB4-EGFP^{low}, CD144⁺CXCR4⁺, and CD144⁺DLL4⁺ cells are represented as mean $\pm$ SD. Student's *t* test; **P* < 0.05; *n* = 3. (E) Statistics of AEC number generated from 1.0×10^6 starting embryonic stem (ES) cells or one 10-cm dish with 8.3×10^6 starting ES cells after 6 d of differentiation (five factor media). *n* = 3. (F) A schematic of five factor protocol. EFN2-Tom, EFN2-tdTomato.

embryonic stem cell lines and DF19.11 (derived from foreskin fibroblast), 005B23.1 (derived from skin punch fibroblast), CD-3-1 (derived from bone marrow-CD34⁺ cells), and PBMC- (derived from peripheral blood mononuclear cell) induced pluripotent stem cell lines. After 6 d, $1.0\text{--}2.2 \times 10^6$ (44–90% purity) AECs were generated from a starting population of 1.0×10^6 cells, and the purity further increased when the cells were passaged (*SI Appendix, Fig. S8 A–D*). qRT-PCR was performed to characterize more arterial and venous genes of AECs generated from CD-3-1 and PBMCs. The results demonstrated that these cells expressed higher arterial markers but lower venous markers compared with human umbilical venous endothelial cells (HUVECs) (*SI Appendix, Fig. S8E*).

Molecular and Functional Characterization of Endothelial Cells Produced by the Five Factor Protocol. We next performed molecular and functional characterization of the five factor arterial-specific endothelial cells (AECs). For comparison, we generated EFN2-tdTomato^{low}/EPHB4-EGFP^{high} VECs as the control, which also expressed lower DLL4 and CXCR4 compared with AECs (Fig. 3*A* and *B*). qRT-PCR revealed that, compared with VECs, AECs expressed several arterial genes, including *CXCR4*, *DLL4*, *EFNB2*, *HEY1*, *NOTCH4*, and *NR1P1* at levels 10x or higher, whereas the venous master regulator, *NR2F2* (also known as *COUP-TFII*), was greatly diminished (Fig. 3*C*, logarithmic scale). Similar to more generic endothelial cells, AECs took up acetylated LDL, formed patent vascular networks in vitro that recruited pericytes, and formed perfused vascular networks in mice (Fig. 3*D–I*). However, unlike more generic endothelial cells, the AECs also expressed activated NOTCH intracellular domain (NICD1) and maintained expression of EFN2 in vascular networks (Fig. 3*E* and *F*).

Arterial-specific functions, including higher NO oxide production and oxygen consumption, lower leukocyte adhesion, and a more sensitive response to shear stress, are critical for modeling arterial disease in vitro and preventing vascular disease in vivo (22–27). Hence, we performed analyses of these functions by comparing our AECs with VECs and primary human endothelial cells. The results demonstrated that AECs produced NO and consumed oxygen at rates higher than VECs and HUVECs and at rates similar to primary human coronary arterial endothelial cells (HCAECs) (Fig. 4*A* and *B* and *SI Appendix, Fig. S7B*). In response to shear stress, AECs and HCAECs elongated to a greater degree than VECs and HUVECs, demonstrating a physiological response used to protect the vessel from this environmental strain (Fig. 4*C* and *D*). Finally, AECs and HCAECs exhibited a significantly lower TNF α -induced leukocyte adhesion (28, 29) than VECs and HUVECs (Fig. 4*E* and *F*). This result is consistent with the increased levels of NO production (Fig. 4*A*) as NO production by AECs limits leukocyte adhesion (30, 31). These arterial-specific functions could be maintained in AECs at passage 4 (Fig. 4). In summary, the functional properties of AECs differentiated from the five factor protocol characterize an arterial phenotype that is distinct from HUVECs and VECs.

The Function of AECs in a Model of Myocardial Infarction. Finally, we tested the function of the human pluripotent stem cell-derived AECs in a mouse model of myocardial infarction (32, 33). To induce myocardial infarction, the left coronary artery was permanently ligated. Two days postinfarction, the border zone of infarcts was injected with AECs, VECs, or PBS. Four weeks postinjection, the mice receiving AECs demonstrated a significantly higher survival rate (83%) compared with that of the control group receiving PBS (33%) (Fig. 5*A* and *B*). However, the survival rate of the AEC group was not significantly higher than the VEC group (Fig. 5*B*). Interestingly, although VECs also increased survival rate (Fig. 5*A*), the difference from the survival rate of the PBS control group was not statistically significant (Fig. 5*B*). Immunocytochemistry revealed that only limited transplanted

Fig. 3. Characterization of AECs. (*A* and *B*) Flow cytometric analysis of the expression of EFN2-tdTomato, EPHB4-EGFP, DLL4, and CXCR4. The experiment was performed three times; one of the typical results is shown. AECs were derived using the five factor protocol. VECs were derived using E6 media (E8 media minus FGF2 and TGF- β 1, containing insulin) supplemented with 50 ng/mL VEGFA and 50 ng/mL BMP4 to differentiate mesoderm cells into endothelial cells from day 2–6. (*C*) qRT-PCR of arterial and venous markers. AECs and VECs were purified by flow cytometry. Data are represented as mean $\pm$ SD. Student's *t* test; **P* < 0.05; *n* = 3. (*D*) Acetylated LDL (Ac-LDL) uptake. (Scale bar, 100 μ m.) AECs (passage 2) derived from wild-type H1 cells were used. The purity was ~93% after being passaged, so cells used in *C–H* were not purified. (*E*) Matrigel encapsulation assay. AECs (passage 3) derived from the reporter cell line were used. (Scale bar, 100 μ m.) (*F*) Immunostaining of NICD1 (activated). (Scale bar, 50 μ m.) AECs derived from wild-type H1 cells were used for *F–H*. (*G*) Vascular formation in fibrin gel. (Scale bar, 100 μ m.) (*H*) Lumen formation of endothelial cell and pericyte cocultured in fibrin gel. To visualize the lumen, cells were stained with CMFDA (green). Y-z and x-z projection was shown. (Scale bar, 100 μ m.) (*I*) Endothelial cells formed functional vessels in vivo. Wild-type H1-derived AECs (passage 2, purified by CD144 microbeads) were mixed with Matrigel and injected into SCID mice. After 4 wk, rhodamine-dextran was retro-orbital injected to highlight perfused vessels. (Scale bar, 100 μ m.) CD31, the antibody recognizes both human and mouse CD31. hCD144, anti-human CD144 antibody.

cells were detected 4 wk postinjection, however the results demonstrated that AECs contributed to the host arterial structures whereas VECs contributed to the venous structure (Fig. 5*C*).

Discussion

In this study, we used single-cell RNA-seq to identify pathways for improving the generation of functional AECs from human pluripotent stem cells. To evaluate the identified pathways, we used completely defined, serum-free media, which allowed us to evaluate all of the individual components and factors of the media, such as insulin. Insulin is present in serum, serum replacements, and xeno-free differentiation media used in previous endothelial cell protocols (6–13, 15, 34–38). Our results

Fig. 4. Arterial-specific functional characterization of endothelial cells. (A) NO production was revealed by the intensity of DAF-FM. AECs were derived from wild-type H1 cells using the five factor protocol and used for experiments at passage 2 or 4 as indicated. VECs were derived using E6 media (E8 media minus FGF2 and TGF- β 1) supplemented with 50 ng/mL VEGFA and 50 ng/mL BMP4 to induce mesoderm cells differentiated into endothelial cells from day 2–6. CD31⁺ CD144⁺ cells were purified for the experiments. DAF-FM is nonfluorescent until it reacts with NO to form a fluorescent benzotriazole. The fluorescent intensity was measured by flow cytometry. The experiment was performed three times; one of the typical results is shown. (B) Oxygen consumption rate was measured on XF24 analyzers (Seahorse Bioscience). Oligomycin was used to abolish the oxygen consumption. FCCP was used to uncouple the electron transport chain from the oxidative phosphorylation, thus measuring the maximal respiration capacity. Antimycin A and Rotenone were applied simultaneously to completely block the electron transport chain. Student's *t* test; **P* < 0.05; *n* = 4. The *P* value was calculated by comparing to HUVECs. (C) Shear stress response was performed on ibidi Pump System (Perfusion Set RED, μ -Slide VI 0.4). (D) Statistical data of shear stress response. Ratio of cell length to width was used to demonstrate the elongation of cells in response to shear stress. For each cell type, 100 cells were measured to do the statistics. Data are represented as mean $\pm$ SD. Student's *t* test; **P* < 0.05; *n* = 100 cells from three independent experiments. (E) Leukocyte (round cells) adhesion assay. (Scale bar, 200 μ m.) (F) Statistics of leukocyte adhesion assay. Leukocyte number was counted for each image. Data are represented as mean $\pm$ SD. Student's *t* test; **P* < 0.05; *n* = 3 images from three independent experiments. The *P* value was calculated by comparing to HUVECs with TNF α treatment. –, nontreated; +, TNF α treated.

demonstrate that insulin withdrawal just after mesoderm formation dramatically increases the percentage of AECs. Thus, the widespread use of insulin (6–13, 15, 34–38) in previous endothelial protocols may have inhibited arterial specification. Another unexpected set of findings is that inhibition of the TGF- β 1 pathway (SB431542) increased AEC differentiation and that BMP4, a commonly used growth factor in endothelial differentiation protocols, suppressed AEC differentiation. Although the function of TGF- β 1 and BMP4 signaling in deriving endothelial cells has been widely studied (6, 9–13, 35, 38, 39), the inhibitory effects of TGF- β 1 and BMP4 signaling on AEC differentiation was not previously described. Lastly, the function of NOTCH in promoting AEC differentiation has been previously demonstrated (18), and our study shows that RESV (a NOTCH agonist) promotes AEC differentiation of human pluripotent stem cells. This study provides insights of human arterial devel-

opment and differentiation and will facilitate the understanding of the underlying mechanisms of vascular disease.

The human pluripotent stem cell-derived AECs in the present study express markers and exhibit functional characteristics that define the arterial phenotype. A recent study, for example, demonstrated that AECs were confined to a CD184⁺ subfraction of CD34⁺ cells isolated from differentiating human embryonic stem cell-derived embryoid bodies (9). The human embryonic stem cell-derived AECs described here also expressed high levels of CXCR4 (CD184), consistent with those previous results. However, in addition to appropriate marker expression, the AECs derived here demonstrate arterial-specific functions that have not been fully assessed before, including characteristic oxygen consumption rates, NO production levels, shear stress responses, and TNF α -induced leukocyte adhesion rates. Because these functions are related to the formation of aneurysms, atherosclerosis, hypertension, intimal

Fig. 5. AECs improve cardiovascular function. (A) Survival rate of the mice. AECs group: $n = 12$, 2.5×10^6 AECs were injected per mouse. VECs group: $n = 8$, 2.5×10^6 (30) VECs were injected per mouse. PBS group: $n = 12$. (B) Kaplan–Meier survival curve. $*P < 0.05$. The P value between the AECs and PBS group or the VECs and PBS group was calculated by Mantel–Cox test. AECs group, $n = 12$. VECs group, $n = 8$. PBS group, $n = 12$. (C) Immunostaining. AECs and VECs were stained with human-specific CD31 antibody. Alexa Fluor 647 Hydrasize (1 $\mu\text{g}/\text{mL}$) was used to label the elastin produced by arteries (46). hCD31, anti-human CD31 antibody. (Scale bar, 50 μm .)

hyperplasia, and thrombosis (22, 23, 27), these AECs will be useful for modeling human vascular disease.

Finally, the AECs generated here were able to significantly enhance survival rates in a mouse myocardial infarction model. The mechanism underlying this improvement is unclear. In addition to the direct contribution of arteriogenesis, other mechanisms maybe also involved. For example, AECs expressed high levels of DLL4, which can activate the Notch pathway and promote cardiac regeneration (40–45). Another mechanism may be high NO production as NO plays a pivotal role in ischemic protection (46). Results suggest that these AECs could have therapeutic value by themselves or in more complex tissue constructs.

Materials and Methods

All research involving human pluripotent stem cells and mice are approved by the University of Wisconsin–Madison Institutional Review Board (IRB). Informed consent was obtained. Detailed protocols for single-cell RNA-seq, gene targeting, arterial and VEC differentiation, endothelial cell functional characterization, and myocardial infarction mouse model are provided in *SI Appendix, SI Materials and Methods*.

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