Perivascular Progenitor Cells Derived From Human Embryonic Stem Cells Exhibit Functional Characteristics of Pericytes and Improve the Retinal Vasculature in a Rodent Model of Diabetic Retinopathy

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ABSTRACT
Diabetic retinopathy (DR) is the leading cause of blindness in working-age people. Pericyte loss is one of the pathologic cellular events in DR, which weakens the retinal microvessels. Damage to the microvascular networks is irreversible and permanent; thus further progression of DR is inevitable. In this study, we hypothesize that multipotent perivascular progenitor cells derived from human embryonic stem cells (hESC-PVPCs) improve the damaged retinal vasculature in the streptozotocin-induced diabetic rodent models. We describe a highly efficient and feasible protocol to derive such cells with a natural selection method without cell-sorting processes. As a cellular model of pericytes, hESC-PVPCs exhibited marker expressions such as CD140B, CD146, NG2, and functional characteristics of pericytes. Following a single intravitreal injection into diabetic Brown Norway rats, we demonstrate that the cells localized alongside typical perivascular regions of the retinal vasculature and stabilized the blood-retinal barrier breakdown. Findings in this study highlight a therapeutic potential of hESC-PVPCs in DR by mimicking the role of pericytes in vascular stabilization.

SIGNIFICANCE
This study provides a simple and feasible method to generate perivascular progenitor cells from human embryonic stem cells. These cells share functional characteristics with pericytes, which are irreversibly lost at the onset of diabetic retinopathy. Animal studies demonstrated that replenishing the damaged pericytes with perivascular progenitor cells could restore retinal vascular integrity and prevent fluid leakage. This provides promising and compelling evidence that perivascular progenitor cells can be used as a novel therapeutic agent to treat diabetic retinopathy patients.

INTRODUCTION
Diabetic retinopathy (DR) is a microvascular complication of diabetes in which the retinal blood vessels become weakened and rupture [1]. It is one of the highly prevalent ocular diseases worldwide, but currently no effective treatments are available. One of the earliest hallmarks of DR is the loss of pericytes. The retina is known to have the highest density of pericytes in the body [2], which are part of the neurovascular unit, and suggested to have active functions in brain-retinal barrier formation for microvascular homeostasis [3, 4]. Pericyte loss in DR threatens vision by causing exudative leakage of fluid into the macula, resulting in macular edema, or by formation of new vessels, resulting in proliferative DR. It has been suggested that chronic cellular changes of DR, especially pericyte loss, are virtually irreversible [5]. Current DR therapeutic strategies may include laser photocoagulation vitreoretinal surgery, and intravitreal injection of vascular endothelial growth factor (VEGF) neutralizing agents (e.g., ranibizumab) or corticosteroids, but these therapies have achieved only limited success [6, 7].

In recent years, stem cell-based approaches have intended to address pericyte loss in DR [8]. It has been reported that transplanted mesenchyme stem cells (MSCs) can serve as a protector against vascular regression by adapting both pericyte morphology and marker expressions in multiple murine models of vasculopathy [9].
Despite repopulating the damaged pericytes, it remains unclear whether they had acquired functional pericyte potentials [10, 11]. Furthermore, most of these studies have been performed in nondiabetic models (e.g., oxygen retinal ischemia-reperfusion injury) or genetic models of retinal degeneration (e.g., Akimba mouse lines) that inadequately represent long-term hyperglycemia-induced DR with in vivo relevance [12, 13]. Thus, needs are increasing for pericyte-equivalent cellular models to test their therapeutic potentials in animal models with sustained retinal vascular hyper-permeability for developing cell-based treatments.

Herein, we describe a highly efficient and feasible protocol to derive multipotent perivascular progenitor cells from human embryonic stem cells (hESC-PVPCs) as a cellular model for pericytes using a collagen mediated natural selection method. Upon transplantation into a rat model of streptozotocin (STZ)-induced diabetes, these cells improved damaged retinal vasculature to indicate promising therapeutic outcomes for DR. The present data demonstrate (a) an important insight into powerful cellular model systems to study the role of the pericytes as a regulator of vascular stability and (b) a source of preliminary data to design a future clinical investigation into cell-based therapy for DR.

**MATERIALS AND METHODS**

### hESC-Derived PVPCs

The undifferentiated H9-hESCs were cultured as previously described [14]. For embryoid body (EB) formation, hESCs were detached from feeder cells by dispase (Invitrogen, Carlsbad, CA, USA, https://www.thermoshifer.com/us/en/home.html) and were suspended in Dulbecco’s modified Eagle Medium (DMEM/F12), supplemented with 10% serum replacement (SR), 1 mM L-glutamine, 1% penicillin-streptomycin, 1% nonessential amino acids, 0.1 mM mercaptoethanol, and 40 nM bone morphogenetic protein 4 (BMP4; R&D Systems, Minneapolis, MN, USA, https://www.rndsystems.com/). On day 6, human EBs were dissociated with Tryp-LE (Invitrogen) and were plated onto plates coated with rat tail collagen type I (BM-MSCs) were obtained from PromoCell (Heidelberg, Germany, www.lonza.com/) for 1 day. The medium was changed the next day to discard nonadherent cells, and the adherent cells were passaged upon reaching confluence.

#### Cell Proliferation Analysis

Cell proliferation analysis was conducted with a CCK-8 kit (Dojindo, Kumamoto, Japan, http://www.dojindo.com/) according to the manufacturer’s instructions. Briefly, cells were seeded with serum-free medium at a density of 4 × 10^3 cells per well in 96-well plates (n = 6) and were washed with phosphate-buffered saline (PBS) the next day before incubating the plate for an appropriate length of time in the growth medium. Both human placenta-derived pericytes (hP-PCs) and human bone marrow-derived MSCs (BM-MSCs) were obtained from PromoCell (Heidelberg, Germany, http://www.promocell.com/).

### Microarray Analysis

Microarray experiments and statistical analysis were performed by using Illumina HumanHT-12 v4 Expression BeadChip, and they presented the data from the HumanHT-12 v4 array (Macrogen Inc., Seoul, Korea, http://www.macrogen.com/eng/). Total RNA from three independent cell cultures for each cell line was subjected to preparation of cDNA probes and microarray experiments. Array data processing and analysis were performed with the BeadStudio software (Illumina Inc., San Diego, CA, USA, http://www.illumina.com/). Genes were filtered out by using the detection p-value threshold (p < .05) in at least three samples. The differentially expressed genes were analyzed on the basis of their fold-change difference (>|2.0-fold change). Consequently, 20,127 probes were used in the final analysis. Hierarchical clustering was performed with PermutMatrix (http://www.lirmm.fr/~caraux/PermutMatrix/Download.htm), with the normalized significant genes [15]. Functional annotation was assigned using the Panther database (http://www.pantherdb.org).

#### Differentiation Potential of hESCs-PVPCs

For adipogenic differentiation, the cultured cells at 70% confluence were switched to low-glucose DMEM, 10% fetal bovine serum (FBS), 5 μg/ml of insulin, 1 μM of dexamethasone, 0.5 mM of isobutylylmethyloxanthine, and 60 μM of indomethacin (all from Sigma-Aldrich, St. Louis, MO, USA, http://www.sigmaaldrich.com/). After 14 days, differentiation into adipocytes was assessed by Oil Red O staining. For osteogenic differentiation, cells at 70% confluence were cultured in low-glucose DMEM, 10% calf serum, 1 μM of dexamethasone, 10 mM of β-glycerophosphate, and 60 μM of ascorbic acid-2-phosphate. After 21 days, differentiation into osteocytes was assessed by alkaline phosphatase staining (all reagents from Sigma-Aldrich). For smooth muscle cell (SMC) differentiation, the cells were cultured in a smooth muscle differentiation medium (SMDM) consisting of DMEM high glucose (Invitrogen), 5% FBS (Invitrogen), 1% minimum essential medium nonessential amino acids (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.1 mM β-mercaptoethanol (Invitrogen). For induction of initial smooth muscle-like cells (SMLCs), the cells were seeded on the dish coated with 0.1% gelatin with basal medium for 6 days before switching to the basal medium supplemented with platelet-derived growth factor (PDGF; 10 nM) and insulin (10 μM) for 3 days.

#### Dye Transfer Assay

The hESC-PVPCs were labeled with the lipophilic fluorescent dye DiI (1.5 μM; Invitrogen), and human umbilical vein endothelial cells (HUVECs) and umbilical artery SMCs (UASMCs) (Lonza) were loaded with 5 μM calcein-acetoxymethyl ester (Calcein; Invitrogen) for 30 minutes at 37°C. The cells were then washed in Ca2+/Mg2+-containing Hanks’ balanced salt solution, and DiI-labeled cells were cocultured overnight with Calcein-labeled HUVECs or UASMCs. The presence of green fluorescence in DiI-positive hESC-PVPCs was considered indicative of the transfer of Calcein through gap junctions from HUVECs or UASMCs. Dye transfer between the cells was detected with flow cytometer and fluorescence microscopy (10× objective lens; Nikon, Tokyo, Japan, http://www.nikon.com/).

### In Vitro Tube Assembly Assay Using Three-Dimensional Fibrin Gel

The in vitro angiogenic tube assembly model using three-dimensional (3D) fibrin gel was described previously [16]. In brief, HUVECs and hESC-PVPCs were labeled with 2.5 μM DIO and 1.5 μM DiI (Invitrogen), respectively. DIO-labeled HUVECs and Dil-labeled hESC-PVPCs in a ratio of 10:1 were suspended with Cytodex 3 microcarriers (GE Healthcare Life Sciences, Pittsburgh, PA, USA, http://www.ge lifesciences.com/) at a concentration of 400–450
mixed cells per bead in 1 ml of EGM-2 medium (Lonza). The bead-cell mixtures were shaken gently every 20 minutes for 4 hours at 37°C and 5% CO₂. After incubating, the beads were transferred to a 10-cm low-attachment surface culture plate and were incubated for 12–16 hours in 5 ml of EGM-2 at 37°C and 5% CO₂. On the following day, the beads were resuspended in 2.5 mg/ml of fibrinogen (Sigma-Aldrich). One milliliter of fibrinogen/bead solution was added to 0.625 units/ml thrombin (Sigma-Aldrich) in a glass-bottom 35-mm culture plate (ibidi, Martinsried, Germany, http://ibidi.com/). Fibrinogen/bead solution was allowed to clot for 5 minutes at room temperature and at 37°C and 5% CO₂ for 20 minutes. One milliliter of EGM-2 was added and equilibrated with the fibrin clot for 30 minutes at 37°C and 5% CO₂. Medium was removed from the well and replaced with 1 ml of fresh medium. Medium was changed every other day thereafter, and the bead assays were monitored for 7 days. Lumen formation and hESC-PVPCs’ localization were detected by z-stack confocal imaging the sprouts that had formed lumens.

**Brown Norway Rat Model of Diabetic Retinopathy**

All animal experiments were performed in accordance with the CHA University Institutional Animal Care and Use Committee and the National Research Council’s Guidelines for the Care and Use of Laboratory Animals. Normal Brown Norway (BN) rats were purchased from Central Laboratory Animal, Inc. (Seoul, Korea, http://www.hoovers.com/). Diabetes was induced with STZ administration, and the animals were maintained in a diabetic state for 8 weeks. Six-week-old BN rats weighing 150–180 g received a single 55 mg/kg intraperitoneal injection of STZ (Sigma-Aldrich) in 0.05 M citrate buffer (pH 4.5). Control nondiabetic mice received citrate buffer alone. One week after the STZ injection, BN rats with blood glucose levels above 250 mg/dL were deemed diabetic. In one group of animals, 50,000 to 100,000 hESC-PVPCs in 2 µl vehicle (Dulbecco’s PBS containing 0.5% bovine serum albumin [BSA] and 2 mM of EDTA) were injected into the vitreous using a Hamilton syringe fitted with a customized 31-gauge needle (Sigma-Aldrich). In the contralateral eye, vehicle alone was injected; in some cases no injection was performed to observe the natural course of the model.

**Retinal Vascular Image Analysis**

Retinas were harvested at various times for imaging of the vasculature and the localization of the transplanted and endogenous cells. In some cases, animals were anesthetized, and intravenous fluorescein-labeled high-molecular-weight dextran (fluorescein isothiocyanate [FITC]-Dextran; Sigma-Aldrich) was injected prior to dissection of the retinas to visualize the retinal vessels. Retinas were fixed in 4% paraformaldehyde for 1 hour at room temperature, then laid flat with radial-relaxing incisions to obtain whole-mount preparations. For phenotypic analysis, the following human-specific primary antibodies were used: NG2 (ab20156; Abcam, Cambridge, MA, USA, www.abcam.com) and major histocompatibility complex (MHC) class 1 (ab7855; Abcam). Quantification of diabetic retinal leakage was performed as previously described [17].

**Statistical Analysis**

All experiments were performed at least three times to assess the reproducibility of the results. Quantitative data are expressed as the mean ± SD or SEM. Student’s paired t test was performed to analyze the statistical significance in each response variable. Pre-specified comparisons between groups were made where appropriate with post hoc testing using Tukey’s method in SPSS, version 17. A p value of <.05 was considered statistically significant. Data histograms were fit with the GraphPad Prism program (version 5; GraphPad, San Diego, CA, USA, http://www.graphpad.com/).

Detailed methods for quantitative real-time polymerization chain reaction (qPCR), flow cytometry, and immunofluorescence analysis can be found in the supplemental online data.

**RESULTS**

**Derivation of CD140B⁺CD44⁺ Cells Using Collagen-Dependent Natural Selection**

Because CD140B (PDGF receptor β [PDGFRB]) has been shown to represent one of the earliest markers of mesoderm lineage [18, 19], we evaluated its expression in BMP4-induced EBs. As shown in Figure 1B, CD140B expression increased continuously to reach ~70% on day 6, and exhibited two distinct CD140B⁺ or “populations (Fig. 1B, case #1) or a CD140B⁺ population (Fig. 1B, case #2), depending on the starting hESC lines. EBs with the peak CD140B expression at this point (Fig. 1B) were dissociated into single cells and seeded onto the collagen-coated dishes. The unattached cells were removed by a fresh medium change (Fig. 1A, Day 7), and the remaining adherent cells coexpressed ~95% CD140B and CD44 (hyaluronic acid receptor) (Fig. 1B, Day 7). An additional analysis demonstrated that BMP4-induced mesodermal specification of EBs was also important to obtain a homogeneous population, and only the CD140B⁺ population was attached in a matrix-dependent manner (supplemental online Figs. 1, 2).

To better define the naturally selected CD140B⁺CD44⁺ population, we performed a microchip array among hESCs, BMP4-EBs, and CD140B⁺CD44⁺ populations isolated at Day 7 using 20,127 probes (respectively marked as hESCs, EBs, and PVPCs in Fig. 1C, 1D). There were 4,240 upregulated probes (fold change ≥ 2) in CD140B⁺CD44⁺ cells in comparison with hESCs. The heatmap and hierarchical clustering analyses showed that the global gene expression patterns of CD140B⁺CD44⁺ cells were distinct from those of hESCs and EBs (Fig. 1C, 1D). The expressions of pluripotency-, neuroectoderm-, and endoderm-related genes in CD140B⁺CD44⁺ cells were clustered differently from hESCs and EBs (supplemental online Fig. 3A–3C). In addition, the expression levels of meso-endoderm and epithelial-to-mesenchymal transition (EMT)-related genes were upregulated in PVPCs in comparison with hESCs and EBs (supplemental online Fig. 3C, 3D). Gene ontology (GO) analysis was conducted to categorize the functions of the differentially expressed genes in CD140B⁺CD44⁺ cells and hESCs. We isolated differentially expressed genes (fold change ≥ 3) in CD140B⁺CD44⁺ cells in comparison with hESCs and EBs. We observed 677 upregulated probes and 609 downregulated probes in CD140B⁺CD44⁺ cells in comparison with hESCs. We performed GO term annotation by using DAVID (http://david.abcc.ncifcrf.gov/) and found that the 10 most enriched categories of upregulated genes, which had a ≤ .01 p value and Benjamini score with the classification stringency set to medium, include “blood vessel development,” “response to wounding,” and “vasculature development” (supplemental online Table 1). In contrast, the 10 most enriched categories of downregulated genes in CD140B⁺CD44⁺ cells (i.e., upregulated genes in hESCs) were “DNA replication,” “M phase,” and “cell cycle phase” (supplemental online Table 2). These gene-profiling differences suggested that the naturally selected CD140B⁺CD44⁺ cells had acquired pericyte-like characteristics.

On the basis of these observations, it is suggested that adherence to collagen-coated surface has a critical role in inducing CD140B⁺CD44⁺ population of cells. As shown in Figure 1E, the
**Figure 1.** CD140B⁺CD44⁺ cells are derived from human embryonic stem cells (hESCs) in a matrix-dependent manner. (A): Schematic representation of matrix-dependent differentiation process for CD140B⁺CD44⁺ cells. (B): Two-color flow cytometry was used to check mesodermal specification following the differentiation schedule. All single cells were labeled with antibodies against CD140B and CD44. Obtained cells are distinguished from CD140B⁻ and CD140B⁺ by the horizontal lines in each plot. Cases #1 and #2 are typical representative aspects, depending on a different starting H9-hESC line. After collagen-dependent natural selection through a simple medium change, the attached CD140B⁺ population showed a strong CD44 expression at Day 7 (A). Values in each quadrant plot represent percentage of population, mean ± SD (n = 6). (C, D): The change of gene context by collagen-dependent attachment step showed a big difference. Differential gene expression profiling was performed among H9-hESCs, embryoid bodies (EBs) induced by bone morphogenetic protein 4 (BMP4) (EBs) and naturally selected CD140B⁺CD44⁺ population at Day 7 (perivascular progenitor cells, PVPCs, CD140B⁺CD44⁺ population) from three independent batches. (C): Heatmap of each group. Each column represents a single microarray analysis. (D): Hierarchical clustering analysis of the global gene expression profiles using the average linkage and the Pearson distance. (E): Expanded CD140B⁺CD44⁺ population exhibited a unique cell morphology. Scale bar = 20 μm. (F): The cell proliferation was monitored with the CCK-8 assay at different time points. Data are means of three separated experiments ± SD. Abbreviations: BM-MSC, human bone marrow-derived mesenchyme stem cells; h, hour; hPL-PC, human placenta-derived pericyte; MEF, mouse embryonic fibroblast; OD, optical density.
naturally selected CD140B+CD44+ cells were morphologically unique; the shortened spindle-shaped cells exhibited an end-to-end polarity, which remained consistent throughout passaging. Furthermore, they demonstrated a superior proliferative capacity in comparison with that observed with BM-MSCs and human placenta-derived pericytes (Fig. 1F).

**CD140B+CD44+ Cells Have the Characteristics of Multipotent Perivascular Progenitor Cells**

For characterization of the naturally selected CD140B+CD44+ cells, we analyzed phenotypic marker expressions using flow cytometry (Fig. 2A). The majority of cells expressed MSC and pericyte markers such as CD140A+, CD140B+, CD44+, CD105+, CD90+, CD106+, CD117+, and CD146+. Furthermore, CD140B+CD44+ cells also expressed perivascular marker such as NG2 (Fig. 2B). The skeletogenic differentiation of CD140B+CD44+ cells was shown in the differentiation condition of adipogenesis (left, Oil red staining) and osteogenesis (right, Alizarin red staining) (Fig. 2C). The smooth muscle cell (SMC) differentiation potential of CD140B+CD44+ cells was shown in SMC differentiation media (SMDM). Quantitative polymerase chain reaction values represent mean (n = 3) ± SD (***, p < .0001). SMC-specific markers, α-SMA (green), and CNN (red) were detected in CD140B+CD44+ cells after 6 days in SMDM differentiation (Fig. 2D). Dye transfer (circle in the plot, yellow-green) increased in a coculture of DiI-labeled CD140B+CD44+ cells and Calcein-labeled other vascular lineages (upper, human umbilical vein endothelial cell, HUVEC-Calcein; lower, umbilical artery smooth muscle cell, UASMC-Calcein, green). Values in each plot represent percentage of population, mean ± SD (n = 3). Dye-transferred CD140B+CD44+ cells were also detected on culture well (white dots) (Fig. 2E). Perivascular localization of CD140B+CD44+ cells was confirmed in three-dimensional fibrin gel bead assay with HUVEC (Fig. 2F, G). Magnified view (G) of rectangular region (F), and orthogonal projection images (white dotted lines 1 and 2). All scale bars = 100 μm. Abbreviations: α-SMA, α-smooth muscle actin; CNN, Calponin 1; d, days.
CD73\(^+\), NG2\(^+\), and CD146\(^+\), but the expressions of endothelial (CD144\(^-\) and CD31\(^-\)), hematopoietic (CD34\(^-\) and CD45\(^-\)) lineage, and progenitor (c-Kit\(^-\) and CD133\(^-\)) were absent. These surface marker expressions were stable through a long-term subculture (P18; data not shown). In addition, the naturally selected cells exhibited similar phenotypic characteristics with a previously reported perivascular progenitor cell identity [20]; thus we designated these cells as hESC-derived perivascular progenitor cells (hESC-PVPCs). These cells do not express immunologically relevant cell surface markers, including human leukocyte antigen DR (HLA-DR; Fig. 2A and supplemental online Fig. 3E), similarly to MSCs [21].

To further evaluate their potential as a cellular model for pericytes, we characterized the cells in accordance with previous reports [20, 22]. Similar to that of hPL-PCs, a spindle-like morphology with NG2 expression was observed (Fig. 2B), but not \(\alpha\)-smooth muscle actin (SMA) [supplemental online Fig. 4]. Moreover, under appropriate differentiation culture conditions, these cells exhibited multipotent potential akin to MSCs by differentiating into adipocytes and osteocytes (left and right sides, respectively, of Fig. 2C), as well as SMCs. The qPCR analysis revealed that the expression level of SMC-specific marker \(\alpha\)-SMA had remained low but increased threefold in a time-dependent manner during differentiation in SMDM (Fig. 2D, left). The differentiated SMCs expressed \(\alpha\)-SMA and Calponin (CNN, as a contractile SMC marker) with a fiber-lined shape pattern (Fig. 2D, right). In addition, we performed a dye transfer assay to detect the formation of functional junctions between hESC-PVPCs and other blood vessel component ECs and SMCs. Flow cytometry analysis revealed that the dye transfer between Dil-labeled hESC-PVPCs (red population) and Calcein-labeled HUVECs or UASMCs (green population) increased approximately 25% (yellow-green population) within 60 minutes (Fig. 2E). As is also shown in fluorescence images of coculture, there was a consistent appearance of green fluorescence adjacent to HUVECs or UASMCs (Fig. 2E). We then tested whether the cells had a more generalized characteristic of pericytes, referred to as perivascular localization in vascularization.

As is shown in Figure 2F, the image analysis of fibrin gel bead assay for 6 days revealed that vascular guidance lumen spaces were generated by HUVECs and served as matrix conduits whereby hESC-PVPCs were recruited during EC-pericyte sprouting tube coassembly. The aligned cells did not form any portion of the vessel lumen, as shown by the orthogonal projection images (Fig. 2G). Taken together, these analyses further confirmed that the naturally selected CD140B\(^+\)CD44\(^+\) population has similar functional characteristics to those of pericytes.

**hESC-PVPCs Improve the Retinal Microvasculature Integrity After Transplantation in Rodent DR Model**

We induced diabetes in BN rats using STZ administration to address whether hESC-PVPCs can ameliorate the early vascular damage inflicted by pericyte loss. In accordance with the experimental plan (Fig. 3A), BN rats (6 weeks old) were treated with 55 mg/kg of STZ to induce sustained hyperglycemia. The blood glucose level in nondiabetic animals was 157.4 \(\pm\) 18 mg/dL \((n = 20)\), whereas this was significantly elevated in diabetic animals (559.2 \(\pm\) 32 mg/dL, \(n = 60, p < .0001\)) as early as 1 week after STZ administration (Fig. 3B) and had remained \(>350\) mg/dL until the end of the study. Prior to injection, we excluded BN rats \((n = 13)\) that had lost more than 10% of their original body weight.

The use of paired eyes in the same animal (e.g., right, hESC-PVPC; left, sham) provided a biologically and statistically meaningful comparison that effectively equalized most environmental variables. BN rats (2 weeks after STZ-diabetes induction) were treated with a single intravitreal injection of PBS or hESC-PVPCs. Within the course of a week, an increased vascular leakage was shown significantly in the retina of STZ-diabetic BN rats that had received a sham injection in comparison with the nondiabetic animals (FITC-Dextran, sham in Fig. 3C and supplemental online Fig. 5). The quantification analysis revealed that diabetic vascular leakage of the sham group was much more in evidence than in the nontreated (NT) normal retina (3.27 \(\pm\) 0.7-fold at 1 week, 3.83 \(\pm\) 1.2-fold at 2 weeks, and 4.28 \(\pm\) 1.7-fold at 4 weeks, in relation to NT, \(n = 8\), in supplemental online Fig. 6), whereas the vascular leakage of hESC-PVPCs transplanted group were reduced in comparison with the sham group (2.71 \(\pm\) 0.6-fold at 1 week, 2.62 \(\pm\) 0.5-fold at 2 weeks, and 2.25 \(\pm\) 0.3-fold at 4 weeks, in relation to NT, \(n = 8\), in supplemental online Fig. 6; \(p < .05\) at 2 weeks, \(p < .0001\) at 4 weeks).
In addition, the single intravitreal injection of hESC-PVPCs significantly reduced the FITC-Dextran leakage in comparison with the retina from the sham group (PVPC in Fig. 3C and supplemental online Fig. 5). The transplanted DiI-labeled hESC-PVPCs acquired typical perivascular localization and remained viable under the hyperglycemic microenvironment up to 4 weeks after the intravitreal injection. This localization occurred more apparently alongside bigger vessels than around the plexus in the transplanted retina. A number of DiI-labeled hESC-PVPCs were found in close proximity to the host retina vasculature within 7 days after intravitreal injection (Fig. 4A and supplemental online Fig. 5). As shown in the magnified images of retina whole mount, the transplanted cells localized alongside the perivascular region of capillaries stained with FITC-Dextran (Fig. 4B). This localization occurred more frequently in diabetic BN rats when assessed 2 weeks posttransplantation (supplemental online Fig. 5). They did not form any portion of the vessel lumen nor invade the deeper layer of the host retina vascular plexus, as shown by the y-axis orthogonal images (Fig. 4B, 1-3) and 3D imaging (supplemental online Movie 1). Furthermore, DiI-labeled hESC-PVPCs coexpressed a marker for pericytes (NG2, Fig. 4C) and human-specific MHC class I (hMHC I, cyan) (D). All scale bars = 50 μm. Abbreviations: hNG2, human neural/glial antigen 2; MHC, major histocompatibility complex; NT, nontreated group; STZ, streptozotocin.

DISCUSSION

Pericytes play critical roles in providing vascular stability, and they control endothelial cell proliferation [23, 24]. Pericyte loss leads to conditions such as edema, DR, and even embryonic lethality during early development, because the blood vessels become hemorrhagic and hyperdilated [25]. Recently, pericytes have been recognized as functional and critical contributors to angiogenesis; hence their potential therapeutic applications for treating vascular diseases are highly anticipated [24]. Despite the growing interest in clinical applications, cells exhibiting...
robust pericyte characteristics or functionality have yet to be demonstrated.

This report describes an efficient protocol to generate highly purified CD140B+ “CD44” cell populations, designated as hESC-PVPCs, which share similar phenotypic and functional characteristics of pericytes. CD140B+ “CD44” cell population was naturally selected through a combination of BMP4 treatment and adherence to a collagen-coated surface. Our analysis demonstrates that the process of cell attachment on collagen was more efficient in generating these cells than was BMP4 treatment alone. One explanation would be that the cell-matrix interaction between CD140B+ cells derived from EBs and collagen matrix provides favorable microenvironments for EMT-like processes to take place. This system provides an efficient protocol for generating highly enriched PVPCs free of contaminating populations such as immature hESCs or other lineage cells. The derived cells demonstrated pericyte characteristics such as MSC and pericyte marker expressions, the absence of endothelial, hematopoietic lineage and progenitor marker expressions, and the multilineage differentiation potentials into SMCs, adipocytes, and osteocytes. In addition, a functional assay revealed that they are capable of incorporating into transplanted tissues by forming functional junctions between hESC-PVPCs and other blood vessel components such as ECs and SMCs.

Clinical application of hESC-PVPCs was analyzed in a rodent model of DR, a common blindness caused by retinal blood vessel damage in diabetic patients that is primarily induced by pericyte loss. Some recent MSC-based approaches have demonstrated functional and phenotypic overlap with pericytes in diabetic vascular abnormality [26, 27], but it is not clear whether the reported beneficial effects are a direct effect of donor MSCs in the damaged tissue. Previously, it has demonstrated that pericytes derived from hESCs can promote recovery in an ischemic animal model [22], but this study was performed in nondiabetic models, which was not sufficient to address the potential of pericytes with in vivo relevance. Because of their low sensitivity against STZ and autoregulation of diabetic symptom [28], the athymic nude rodents are not suitable for this study. Previous studies have shown that BN rats have higher retinal VEGF levels and more severe retinal vascular leakage than do Sprague Dawley rats in response to diabetes [29]. We have developed a type I diabetes model in these inbred rats to better study the in vivo function of hESC-PVPCs. Posttransplantation analysis at 4 weeks revealed (a) a long-term in vivo survival of hESC-PVPCs in STZ-induced diabetic rats, (b) functional integration at the typical perivascular region of the retinal vascular plexus, and (c) a decreased vascular leakage in comparison with the sham-injected group. The transplanted hESCs-PVPCs remained viable and functional during the course of the in vivo experiment without severe rejection. In contrast, hBM-MSCs failed to survive more than 2 weeks after transplantation when transplanted in the same model (data not shown). It has recently been reported that hESCs and their differentiated derivatives are less susceptible to immune rejection than are adult cells [30, 31]. Because hESC-PVPCs do not express immunologically relevant cell surface markers, including HLA-DR and costimulatory molecules (CD40, CD40L, B7-1, and B7-2), we carefully speculate that hESC-PVPCs may have evaded immune response, although further investigations are required to fully elucidate this possibility. The ability to provide such pericyte-like cells in the early stages of DR would represent a significant advancement in developing potential hESC-based treatments. This study describes a comprehensive proof of concept suggesting the potential of the hESC-PVPCs as a therapeutic cell source that can palliate vascular abnormality in the DR.

**CONCLUSION**

In this study, we established a highly efficient and feasible protocol to derive hESC-PVPCs using a combination of BMP4 treatment and collagen-dependent natural selection without cell-sorting processes. So far, there are no proper cellular models for pericytes and perivascular progenitors. We would expect hESC-PVPCs to play the role of a powerful cellular model for pericytes. Furthermore, we reported therapeutic potentials of hESC-PVPCs in the rodent DR, in which a single intravitreal injection of hESC-PVPCs improved damaged retinal vasculature by localizing alongside the perivascular region of the host-damaged retina vascular plexus. We believe that the findings we report here are significant and will be of great interest not only to investigators developing stem cell therapies but also to a broader audience interested in clinical implications of diabetic retinopathy.

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**AUTHOR CONTRIBUTIONS**

J.M.K.: conception and design, collection and/or assembly of data, manuscript writing; K.-S.H.: collection and/or assembly of data, data analysis and interpretation, manuscript writing and review; I.-K.H. and J.S.K.: collection and/or assembly of data; H.-M.C.: conception and design, manuscript writing and editing, project supervision.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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