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## Quality-Quantity Control Culture Enhances Vasculogenesis and Wound Healing Efficacy of Human Diabetic Peripheral Blood CD34+ Cells

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### ABSTRACT

Autologous endothelial progenitor cell (EPC) therapy is commonly used to stimulate angiogenesis in ischemic repair and wound healing. However, low total numbers and functional deficits of EPCs make autologous EPC therapy ineffective in diabetes. Currently, no known ex vivo culture techniques can expand and/or ameliorate the functional deficits of EPCs for clinical usage. Recently, we showed that a quality-quantity culture (QQc) system restores the vasculogenic and wound-healing efficacy of murine diabetic EPCs. To validate these results and elucidate the mechanism in a translational study, we evaluated the efficacy of this QQc system to restore the vasculogenic potential of diabetic human peripheral blood (PB) CD34+ cells. CD34+ cells purified from PB of diabetic and healthy patients were subjected to QQc. Gene expression, vascular regeneration, and expression of cytokines and paracrine mediators were analyzed. Pre- or post-QQc diabetic human PB-CD34+ cells were transplanted into wounded BALB/c nude mice and streptozotocin-induced diabetic mice to assess functional efficacy. Post-QQc diabetic human PB-CD34+ cell therapy significantly accelerated wound closure, re-epithelialization, and angiogenesis. The higher therapeutic efficacy of post-QQc diabetic human PB-CD34+ cells was attributed to increased differentiation ability of diabetic CD34+ cells, direct vasculogenesis, and enhanced expression of angiogenic factors and wound-healing genes. Thus, QQc can significantly enhance the therapeutic efficacy of human PB-CD34+ cells in diabetic wounds, overcoming the inherent limitation of autologous cell therapy in diabetic patients, and could be useful for treatment of not only wounds but also other ischemic diseases.

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### SIGNIFICANCE STATEMENT

Quality-quantity culture (QQc) is the only ex vivo culture system that can expand and/or ameliorate the functional deficits of endothelial progenitor cells for clinical usage. Due to disease oriented cell dysfunction, CD34+ cell therapy has a limited role in vasculogenesis and tissue regeneration for diabetic patients. After QQc, diabetic CD34+ cells have improved vasculogenic and tissue regenerative potential and can function much better than pre-QQc diabetic CD34+ cells and at comparable levels as pre-QQc healthy CD34+ cells. Therefore, QQc CD34+ cell therapy could be the most useful treatment for diabetic wounds and other ischemic diseases.

### INTRODUCTION

Refractory wounds in diabetes patients are a major cause of nontraumatic amputation and death, leading to serious social and economic burdens worldwide [1–3]. Endothelial progenitor cells (EPCs) are novel targets to induce therapeutic angiogenesis in ischemic repair and wound healing. However, in diabetic patients, both the number and functional capacity of EPCs are severely impaired, resulting in a lack of clinical value for autologous EPC therapy. Ameliorating functional

deficits and increasing cell counts to a sufficient level are therefore central to successful autologous EPC therapy in diabetes patients [4].

CD34+ cells comprise the endothelial and hematopoietic progenitor-enriched fraction of EPCs and possess high functionality and superior regenerative efficacy [5]. Although CD34+ cell transplantation is clinically helpful for ischemic patients, it is not effective for diabetic patients. In a recent clinical trial, we demonstrated that G-CSF-mobilized PB-CD34+ cell therapy is safe and feasible for patients with non-healing diabetic

foot ulcer, and that a higher total number of transplanted CD34/KDR double-positive cells accelerates wound healing [6]. However, the therapeutic success of autologous EPC therapy in diabetes remains limited because of low counts and functional deficits of EPCs in diabetic patients. Despite considerable attempts to increase the number and ameliorate the functional impairment of diabetic EPCs via ex vivo culture systems [7–9], CD34+ cells do not proliferate and tend to differentiate, effectively losing their stem cell capacities.

To overcome these deficits, we recently identified suitable culture conditions that could restore the functionality of bone marrow (BM)-derived murine diabetic c-kit + Sca-1 + lin- (KSL) cells [10]. Most importantly, after treatment with this serum-free quality-quantity control culture (QQc), adoptive transfer of murine BM diabetic KSL cells resulted in significantly increased euglycemic wound closure, matching the therapeutic efficacy of non-diabetic healthy KSL cells.

In this preclinical study, we further evaluated the efficacy of QQc in ex vivo culturing of peripheral blood (PB)-CD34+ cells from diabetic patients for effective wound healing. We analyzed the effect of QQc-treated diabetic PB-CD34+ cells on vasculogenesis and tissue regeneration in diabetic wounds and identified potential mechanisms underlying the functional and therapeutic enhancements after QQc, including KDR positivity, differentiation capacity, vasculogenic potential, expression of cytokines at the wound site, PGC-1 $\alpha$  expression, and Notch signaling. This is the first ex vivo suspension culture system that could be clinically applied to expand the number of PB CD34+ cells and enhance their vasculogenic potential in diabetic patients.

## MATERIALS AND METHODS

### Patients

Diabetes mellitus patients ( $n = 19$ ) were recruited from the type 2 diabetic outpatients of Juntendo University Hospital, Tokyo, Japan. Non-smoking, 20- to 80-year-old men ( $\text{HbA}_{1\text{C}} < 8.0\%$ , 64 mmol/mol), excluding those who had severe heart failure, hemodialysis, peritoneal dialysis, infectious disease, hematologic disease, inflammatory disease, or malignant tumors were included in the study. Age-matched non-diabetic healthy volunteers ( $n = 17$ ) were selected as controls using the same criteria (Supporting Information Table S1). All participants provided informed consent. The study was conducted in full accordance with the Declaration of Helsinki and was approved by Juntendo University ethics committee and review board.

Blood (50 ml) was withdrawn from each donor for isolation of CD34+ cells. Initial in vitro assays (cell count, pre- and post-QQc culture assays, and colony forming assay) were performed using samples from all patients. However, because CD34+ cells are rare in PB (less than 0.1%), it was not feasible to perform all experiments using CD34+ cells from 50 ml of blood, and collecting more than 50 ml of blood from all patients was not approved by the clinical ethical committee of Juntendo University. To overcome this limitation, donors whose blood samples had cell count and pre- and post-QQc values within the median range were chosen randomly and requested to undergo a second blood withdrawal to perform additional experiments. The chosen subset was thus representative of the entire group of donors.

### Preparation of CD34+ Cells and QQc

CD34+ cells were prepared as previously described [10–12]. The CD34+ cell fraction had a purity of 80%–90% as determined by fluorescence-activated cell sorting (FACS) analysis. Freshly isolated CD34+ (pre-QQc) cells from healthy and diabetic patients were placed in an ex vivo serum-free expansion culture QQc as previously described [13]. In brief, the cells were seeded at a density of  $1 \times 10^4$  cells per well in 24-well Primaria plates (BD Falcon, Franklin Lakes, NJ), with 0.5 ml/well StemSpan SFEM medium (Stem Cell Tech., Vancouver, BC, Canada) supplemented with recombinant human (rh) VEGF (50 ng/ml), rhIL-6 (20 ng/ml), rhFlt3-ligand (100 ng/ml), rh thrombopoietin (20 ng/ml), rhSCF (100 ng/ml) (all from PeproTech, Rocky Hill, NJ), and antibiotic cocktail (Invitrogen, Carlsbad, CA), and cultured for 7 days at 37°C in 5% CO<sub>2</sub>.

### Wound Model and Human CD34+ Cell Therapy

Eight to 10-week-old male BALB/c nude mice (Crea Japan, Kawasaki, Japan;  $n = 45$ , four per experimental group) and 50 mg/kg streptozotocin-induced diabetic mice (Sigma-Aldrich, St. Louis, MO) were used ( $n = 28$ ). This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This research was approved by Juntendo Animal Care and Ethical Committee. Mice were considered diabetic if they maintained glucose levels above 300 mg/dl for at least 4 weeks before wounding. The animals were anesthetized and depilated. Two wound sites were made by biopsy punches (6 mm, Kai Industries, Gifu, Japan) on the dorsum; full-thickness excisions included the hypodermis and panniculus carnosus. The wound edges were marked with India ink. Silicon stents with an 8-mm internal diameter were sutured with 5-0 nylon around each wound to minimize skin contracture and ensure secondary intention healing [10, 14]. On the third post-operative day, each wound was injected with either phosphate-buffered saline (PBS) or CD34+ cells ( $1 \times 10^4$  cells per wound) from one of the following groups: pre-QQc healthy control, pre-QQc DM, post-QQc healthy control, and post-QQc DM. Wounds were photographed and digitally measured (BZ II Analyzer application software, BZ-H2A, Osaka, Japan). The percent wound closure was measured photogrammetrically on days 0, 3, 7, 10, and 14, using the formula  $(1 - [(wound area on examination day)/(wound area at day 0)]) \times 100$ .

### Fluorescence-Activated Cell Sorting

Cells in EDTA-PBS containing 2% fetal bovine serum (FBS) (FACS buffer) were treated with an FcR blocking reagent (Miltenyi Biotech, Auburn, CA) at 4°C for 30 minutes, and then stained with specific anti-human antibodies, namely, phycoerythrin-Cy7-labeled anti-CD34 (clone: 581), and allophycocyanin-labeled anti-CD133 (clone: AC133, Miltenyi Biotec, Bergisch Gladbach, Germany) plus phycoerythrin-labeled anti-hVEGFR2/KDR (R&D Systems FAB357P, Minneapolis, MN) (or isotype controls for each color), at 4°C for 30 minutes. The cells were detected on an LSR Fortessa cell analyzer (BD Biosciences, San Jose, CA), and the data were processed using FlowJo software (Tree Star, Ashland, OR).

### Colony-Forming Assay

Pre- and post-QQc CD34+ cells were subjected to EPC colony-forming assays using a semi-solid culture medium as described previously [10, 11]. Colonies were counted 21 days later using an inverted microscope (Nikon ECLIPSE, Tokyo, Japan) at  $\times 4$

magnification; EPC stages were determined as primitive EPC (pEPC)-, definitive EPC (dEPC)-, and total EPC (tEPC) colony-forming units (CFUs) based on morphology by two investigators as previously described [10, 11, 13].

### Tube Formation Assay

The tube formation assay was performed per Tsukada et al. [15]. Briefly, CD34+ cells were labeled with low-density lipoprotein from human plasma, acetylated, Dil complex (Dil-Ac-LDL) (Biomedical Technologies Inc. #BT-902, Lancashire, UK) at 37°C for 2 hours. 96-well plates were pre-coated with 50 µl/well of Biocoat Matrigel (Corning #356234, Corning, Corning, NY). The gels were then overlaid with  $1 \times 10^3$  pre- and post-QQc CD34+ cells and co-cultured with  $1.5 \times 10^4$  human umbilical vein endothelial cells (HUVECs, #2519A; Lonza, Basel, Switzerland) in 50 µl of EBM-2 containing 2% FBS (Lonza #CC-4147) and incubated at 37°C and 5% CO<sub>2</sub> for 15 hours. HUVEC-only wells were used as controls. The wells were photographed with a phase-contrast microscope. Total Dil-Ac-LDL-labeled CD34+ cells incorporated into the tubes were analyzed by fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan).

### Real-Time PCR Analysis of Cells and Wound Tissues

Pre- and post-QQc CD34+ cells were lysed in RLT solution (Qiagen, Hilden, Germany) and stored at -80°C. On Day 14, the wounded skin was collected in Micro Smash tubes (MS-100 TOM) with TRIzol (Thermo Fisher Cat # 10296010). Total RNA was prepared following the manufacturer's protocol and used to synthesize cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed using cDNA as a template. The data were analyzed as delta ( $\Delta$ )CT (target CT-18S-rRNA CT) and reported as 2- $\Delta$ CT to indicate fold differences in expression. Primers for the human genes are provided in Supporting Information Table S2. The mouse SYBR Green primer sequences are shown in Supporting Information Table S3. TaqMan probes were obtained from Applied Biosystems (Foster City, CA) and are listed in Supporting Information Table S4.

### Histology and Immunofluorescence

On Day 14, the wounded skin was harvested from euthanized mice with a full-thickness excision that extended 3 mm laterally past the original edge demarcated with India ink. Each sample was dissected for paraffin or frozen sectioning. Sections were cut from the central region of the wound at a thickness of 5 µm. Sections were deparaffinized and rehydrated before staining with hematoxylin and eosin to measure epithelial and granulation thicknesses via fluorescence microscopy (BZ-9000). Epithelial thickness included the distance from the surface to the basic membrane of the epithelium. Granulation thickness was measured by the distance from the base of the epithelium to the surface of the panniculus carnosus layer.

For detection of vascular regeneration, paraffin sections were stained with rat anti-mouse CD31 antibody (clone: MEC13.3, BD) as an endothelial marker and developed with 3, 3-diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA). The CD31 positive vessels per field were counted at  $\times 200$ .

Wound maturity was quantified by the van Gieson staining protocol, which simultaneously stains mature collagen deep red and immature collagen pink. Paraffin sections were processed with staining solution as described previously [16]. Images were digitalized (BZ-9000) and were analyzed using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Six-micron-thick frozen tissue sections were stained with TGF-β antibody (1:100, ab66043; Abcam, Cambridge, MA) per the manufacturer's protocol. Stained sections were imaged and digitized in their entirety at  $\times 200$  resolution with a BZ-9000 microscope. TGF-β levels within the wound were calculated by measuring total DAB pixelation intensity.

To follow cell trafficking of the adoptively transferred CD34+ cells, the frozen tissues were stained with mouse anti-human mitochondrial antibody using a rabbit anti-mouse kit (Discovery MoMap kit, 760-137, Ventana Medical Systems, Oro Valley, AZ) and anti-mouse CD31 antibody (clone: MEC13.3, BD). Tissue sections were mounted using a mounting medium with DAPI (VECTASHIELD H-1200; Vector Laboratories). Slides were digitally captured, and the double-positive vessels were counted at  $\times 40$  and  $\times 20$  using a confocal microscope (Olympus FV1000D IX81, SPD; Olympus, Tokyo, Japan). Human mitochondria antigen (HMA)-positive cell counting was performed blindly by two independent researchers. Each section of the entire wound was cut equivalently into four areas such that each slide represented 1/4 of the wound, which ensured that entire wound was evaluated for HMA-positive cells.

### Statistics

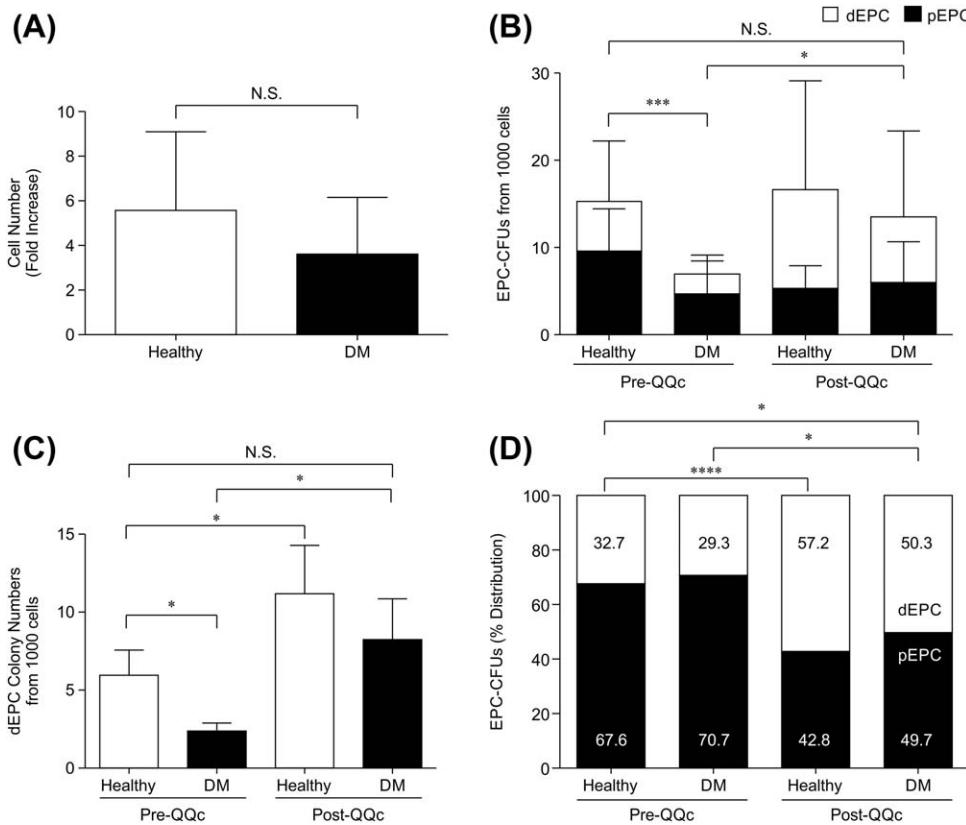
Wilcoxon signed-rank test (paired non-parametric test) was performed for the *in vitro* assays between pre- and post-QQc groups. The Mann-Whitney test (non-paired non-parametric test) was performed for comparisons between healthy and diabetic groups as well as for *in vivo* data comparisons for which we compared data from different donors. All analyses were performed using GraphPad Prism 5. Statistical significance was defined as  $p < .05$ . Data are presented as the mean  $\pm$  SD.

## RESULTS

### QQc Increases the Number and Restores the Differentiation Ability of Diabetic CD34+ Cells

PB-CD34+ cell counts were significantly lower in diabetic patients than in healthy volunteers ( $3.21 \pm 1.74 \times 10^4$  cells/50 ml vs.  $5.53 \pm 3.55 \times 10^4$  cells/50 ml, respectively,  $p < .05$ ). Although CD34+ counts significantly increased in both diabetic and healthy groups ( $8.45 \pm 8.71 \times 10^4$  cells/50 ml vs.  $25.07 \pm 17.92 \times 10^4$  cells/50 ml, respectively;  $p < .01$ ) post-QQc (Supporting Information Fig. S1), there was no significant difference in the fold cell number increase in the two groups (Fig. 1A). KDR positivity significantly increased in diabetic and in healthy CD34+ cells post-QQc ( $p < .05$ ) (Supporting Information Fig. S2).

pEPC can be morphologically identified as small round cells, whereas dEPC form larger spindle-like cells that indicate differentiated cells. PB-CD34+ from diabetic patients demonstrated significantly lower pEPC-CFUs ( $4.47 \pm 3.97$  vs.  $9.73 \pm 4.94$ ;  $p < .01$ ), dEPC-CFUs ( $2.38 \pm 2.18$  vs.  $5.95 \pm 7.04$ ;  $p < .05$ ), and tEPC-CFUs ( $6.97 \pm 5.62$  vs.  $15.28 \pm 8.27$ ;  $p < .001$ ) than CD34+ cells isolated from healthy volunteers (Fig. 1B, 1C). QQc increased the numbers of pEPC-CFU ( $6.18 \pm 4.80$  vs.  $5.42 \pm 2.63$ ; NS), dEPC-CFU ( $7.67 \pm 10.24$  vs.  $12.53 \pm 12.78$ ; NS), and tEPC-CFUs ( $14.14 \pm 11.32$  vs.  $16.63 \pm 12.94$ ; NS) in diabetic CD34+ cells to the levels of healthy CD34+ cells (Fig. 1B). Importantly, the increase of dEPC-CFUs was remarkable compared to that of pEPC-CFUs (Fig. 1C, 1D).



**Figure 1.** QQc increases cell counts and differentiation capacity of diabetic (DM) CD34+ peripheral blood (PB) cells. **(A)**: Post-QQc fold increase in the cell counts of healthy ( $n = 17$ ) and diabetic CD34+ cells ( $n = 12$ ). **(B)**: The number of EPC CFUs from pre- and post-QQc healthy ( $n = 19$  each) and DM PB-CD34+ cells (pre-QQc  $n = 17$ ; post-QQc  $n = 13$ ). **(C)**: The frequency of dEPC-CFU production from pre-QQc and post-QQc healthy and PB-CD34+ cells.  $n$  is the same as for (B). **(D)**: The percent distribution of pEPC-CFUs and dEPC-CFUs among total EPC-CFUs. \*,  $p < .05$ ; \*\*\*,  $p < .001$ ; \*\*\*\*,  $p < .0001$  samples evaluated in triplicate. Abbreviations: CFUs, colony-forming units; dEPC, definitive endothelial progenitor cell; DM, Diabetic; EPC, endothelial progenitor cell; NS, not significant; pEPC, primitive endothelial progenitor cell; QQc, quality-quantity culture.

### QQc Enhances Incorporation of Diabetic CD34+ Cells and Tubule Formation

Diabetic CD34+ cells elicited significantly fewer tubules per high-powered field than HUVECs alone. Post-QQc, the number of tubes formed increased compared with pre-QQc (pre-QQc vs. post-QQc:  $0.95 \pm 0.07$  vs.  $1.12 \pm 0.06$ ;  $p < .01$ , and  $1.07 \pm 0.07$  vs.  $1.16 \pm 0.05$ ;  $p < .01$ , diabetic and healthy, respectively). The pre-QQc diabetic CD34+ cell group showed significantly lower incorporated cell numbers than the pre-QQc healthy CD34+ group ( $12.15 \pm 3.93$  vs.  $25.85 \pm 6.24$ , respectively;  $p < .01$ ). The incorporated cell number significantly increased post-QQc in both groups (pre-QQc vs. post-QQc:  $12.15 \pm 3.93$  vs.  $45.15 \pm 9.89$ ;  $p < .01$ , and  $25.85 \pm 6.24$  vs.  $57.15 \pm 21.32$ ;  $p < .01$ ; diabetic and healthy, respectively) with no significant difference between post-QQc diabetic and healthy groups ( $45.15 \pm 9.89$  vs.  $57.15 \pm 21.32$ , respectively) (Fig. 2A–2C). Furthermore, the number of tubes formed and cells incorporated significantly increased in post-QQc diabetic versus pre-QQc healthy cells ( $p < .1$  and  $p < .0001$ , respectively).

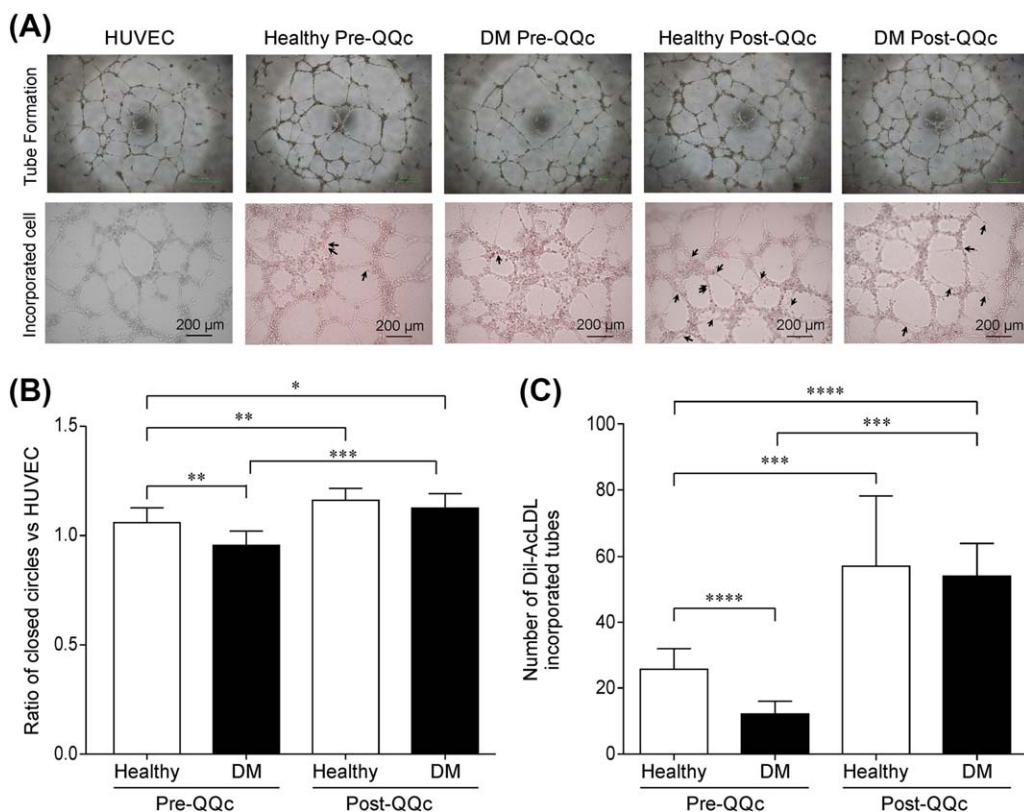
### QQc Enhances Expression of Vasculogenic and Wound Healing Factors in CD34+ Cells

Diabetic PB-CD34+ cells, compared to healthy PB-CD34+ cells, showed significantly lower expression levels of the angiogenesis-related genes Ang-1 and HGF. Although not significant, we

observed a trend for lower expression levels of Ang 2, VEGF-A, VEGF-B, and pro-angiogenic cytokine IL-1 $\beta$  as well as wound healing-related genes TGF- $\beta$  and MMP-2. Post-QQc, diabetic CD34+ cells showed significantly increased expression of Ang-1, Ang-2, VEGF-B, and HGF in both groups. IL-10 expression was not detectable in pre-QQc CD34+ cells but was present in post-QQc healthy and diabetic CD34+ cells. MMP-2 expression also increased post-QQc in the diabetic group (5.9-fold;  $p < .05$ ). VEGFR-1 and VEGFR-2 expression was, however, significantly decreased post-QQc in both healthy and diabetic CD34+ cells. Both Leptin and MMP-9 expression was higher in the post-QQc diabetic cells, although not statistically significant (Supporting Information Fig. S3).

### QQc Affects the Expression of PGC-1 $\alpha$ and Notch Signaling in Both Healthy and Diabetic PB-CD34+ Cells

Diabetic PB-CD34+ cells showed a high abundance of PGC-1 $\alpha$  mRNA and mRNA of Notch-related genes such as Hey, Hes, and RBPJ compared to healthy CD34+ cells, although statistically significant only for HEY and RBPJ. Notably, QQc led to a decrease in PGC-1 $\alpha$  expression in CD34+ cells from both healthy subjects ( $5.03 \pm 3.42$  vs.  $34.19 \pm 28.70$ ;  $p < .01$ ) and patients with diabetes ( $3.53 \pm 2.83$  vs.  $57.60 \pm 36.28$ ,  $p < .01$ ). Similarly, expression of Notch and its related genes also significantly decreased in both healthy subjects and diabetic patients post-QQc (Fig. 3). Notably,



**Figure 2.** In vitro tube formation assay. CD34+ peripheral blood (PB) cells labeled with Dil-ac-LDL were co-cultured with HUVEC. **(A)**: Representative microphotographs demonstrating tube formation and incorporation of PB CD34+ cells in the newly formed vessels. The ratio of HUVEC:CD34+ cells is 15:1. **(B)**: Number of tubules formed in each group, \*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ . **(C)**: Dil-ac-LDL incorporation into HUVEC-formed tubes in each group. The data are shown as the mean  $\pm$  SD;  $n = 13$  wells/group from five healthy individuals and five DM patients. \*\*\*,  $p < .001$ ; \*\*\*\*,  $p < .0001$ . Abbreviations: Dil-ac-LDL, low-density human plasma lipoprotein-acetylated Dil complex; DM, Diabetic; HUVEC, human umbilical vein endothelial cells; QQc, quality-quantity culture.

there was either no difference or a significant decrease in Notch1, PGC-1 $\alpha$ , and HES mRNA expression in post-QQc diabetic versus pre-QQc healthy cells.

#### QQc Diabetic PB-CD34+ Cell Therapy Accelerates Wound Closure, Maturation, and Vascularization

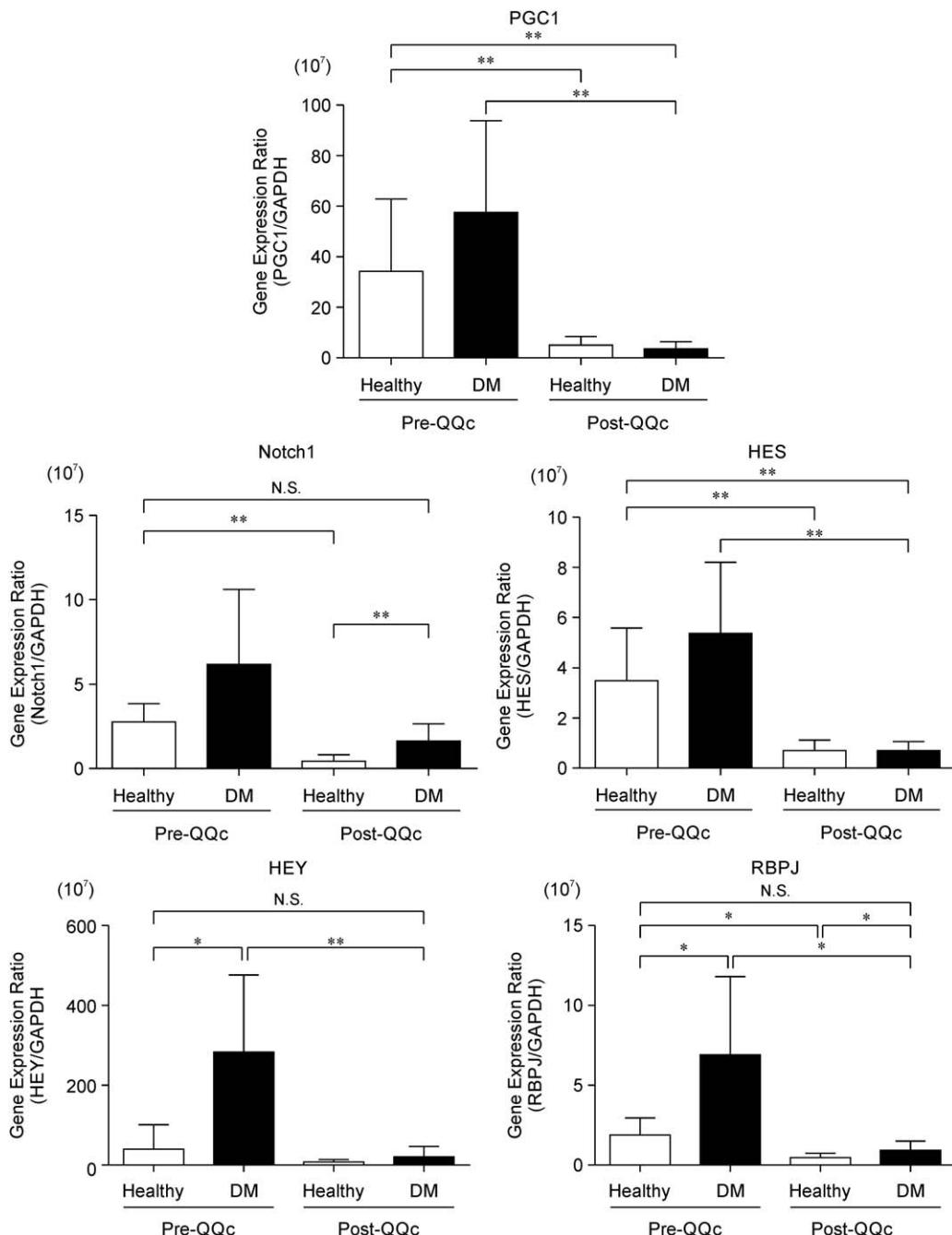
To determine the effect of adoptive cell therapy on wound healing, wounds produced in euglycemic mice were injected with CD34+ cells from diabetic and healthy volunteers before and after QQc. Transplantation of pre-QQc diabetic CD34+ cells had no effect on wound closure examined on day 14 compared to the PBS control ( $68.0\% \pm 9.8\%$  vs.  $66.3\% \pm 11.8\%$  for pre-QQc diabetic CD34+ cells vs. PBS, respectively), and resulted in significantly lower wound closure on day 14 compared to pre-QQc healthy CD34+ cell therapy ( $68.0\% \pm 9.8\%$  vs.  $76.9\% \pm 6.2\%$  for pre-QQc diabetic CD34+ cells vs. pre-QQc healthy CD34+ cells, respectively;  $p < .01$ ). Conversely, post-QQc diabetic CD34+ cells elicited significantly faster wound closure than pre-QQc healthy CD34+ cells ( $92.6\% \pm 5.5\%$  vs.  $76.8\% \pm 6.2\%$ , respectively;  $p < .001$ ) and even post-QQc healthy CD34+ cells ( $92.6\% \pm 5.5\%$  vs.  $88.4\% \pm 7.0\%$ , respectively;  $p < .05$ ). Furthermore, no significant differences were seen between any of the groups prior to day 14 (Fig. 4A, 4C and Supporting Information Fig. S4).

Pre-QQc diabetic PB-CD34+ cell-treated wounds had a significantly lower epithelial thickness ( $24.2 \pm 5.3$  vs.  $34.1 \pm 8.4 \mu\text{m}$ ;  $p < .05$ ) and granulation thickness ( $158.7 \pm 76.8$  vs.  $215.1 \pm 38.7 \mu\text{m}$ ;  $p < .01$ ) than healthy PB-CD34+ cell-treated wounds after 14 days (Fig. 4C). Post-QQc diabetic CD34+ cell-treated wounds had significantly increased the epithelial thickness ( $43.8 \pm 11.1$  vs.  $24.2 \pm 5.3$ ;  $p < .05$ ) and granulation thickness ( $497.9 \pm 258.3$  vs.  $158.7 \pm 76.8$ ;  $p < .01$ ) compared to the pre-QQc diabetic CD34+ cell treated group. The proportion of mature collagen deposition mirrored the findings for epithelial and granulation thickness (Fig. 4B, 4C).

The vascular density of both healthy and diabetic post-QQc cell-injected wounds was significantly higher than that of control PBS-injected wounds. Wounds injected with post-QQc diabetic CD34+ cells showed significantly higher vascular density than wounds injected with pre-QQc diabetic CD34+ cells ( $76.9 \pm 27.7$  vs.  $44.4 \pm 33.25$ , respectively;  $p < .05$ ). There was no difference in vascular density between pre-QQc healthy and post-QQc diabetic CD34+ cell-injected wounds (Fig. 4C).

**QQc Enhances Expression of Wound Healing Factors in the Cell-Transplanted Wound**

The angiogenesis-related genes expressed in the cell-transplanted and PBS-treated wounds after 14 days are shown in Supporting Information Figure S5. Expression of anti-inflammatory IL-10 and MMP-9 was higher in post-QQc diabetic CD34+ cell-transplanted wounds compared to all other groups. Conversely, the level of inflammatory cytokine IL-12 was significantly higher in PBS-treated wounds than post cell therapy. Additionally, expression of



**Figure 3.** QQc attenuates diabetes (DM)-induced PGC-1 $\alpha$  and Notch signaling. The expression of each gene normalized to that of GAPDH is shown for each group. Data are reported as the mean  $\pm$  SD;  $n = 7$ /group. \*,  $p < .05$ ; \*\*,  $p < .01$ . Abbreviations: DM, Diabetic; NS, not significant; QQc, quality-quantity culture.

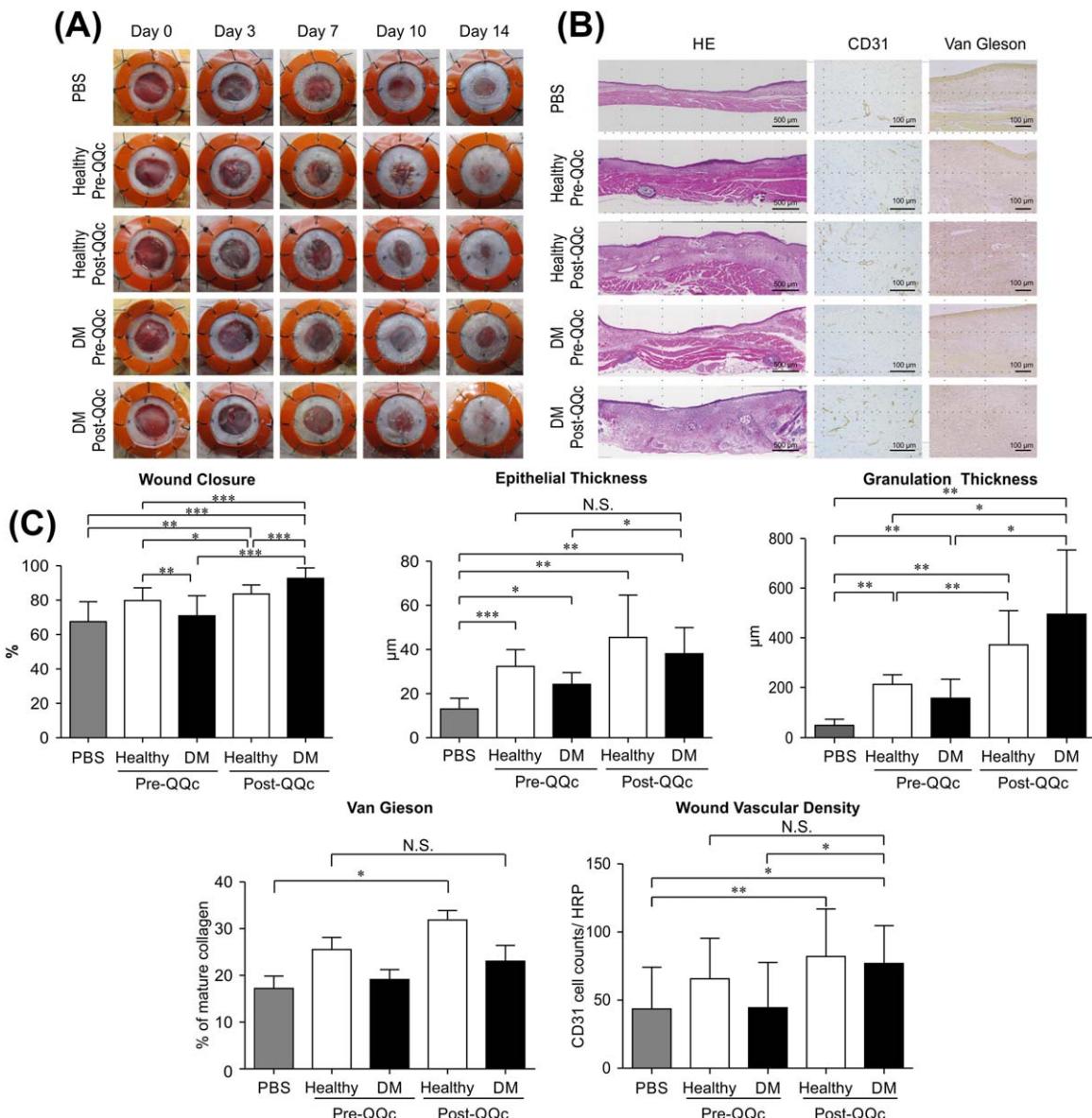
VEGFR-1 and VEGFR-2 was significantly higher in wounds treated with post-QQc diabetic CD34+ cells compared to pre-QQc healthy CD34+ cells. Expression of VEGFR-1 and VEGFR-2 was also higher in post-QQc diabetic CD34+ cells than in pre-diabetic QQc and post-QQc healthy CD34+ cells; however, the differences were statistically significant only for VEGFR-1.

As shown by immunofluorescence, TGF- $\beta$  expression was also significantly lower in wounds transplanted with diabetic PB-CD34+ cells than in wounds transplanted with healthy PB-CD34+ cells ( $74,599 \pm 20,139$  vs.  $192,254 \pm 75,594 \mu\text{m}^2$ , respectively;

$p < .05$ ). The levels of TGF- $\beta$  increased significantly post-QQc (pre-QQc vs. post-QQc diabetic CD34+ cell therapy:  $74,599 \pm 20,139$  vs.  $219,124 \pm 56,349 \mu\text{m}^2$ , respectively;  $p < .05$ ) (Supporting Information Fig. S6).

#### QQc Diabetic PB-CD34+ Cell Therapy Has High Potential for Direct Vasculogenesis

The number of vessels with HMA-positive cells co-stained with CD31 were significantly lower in the wounds transplanted with pre-QQc diabetic CD34+ cells than in those transplanted

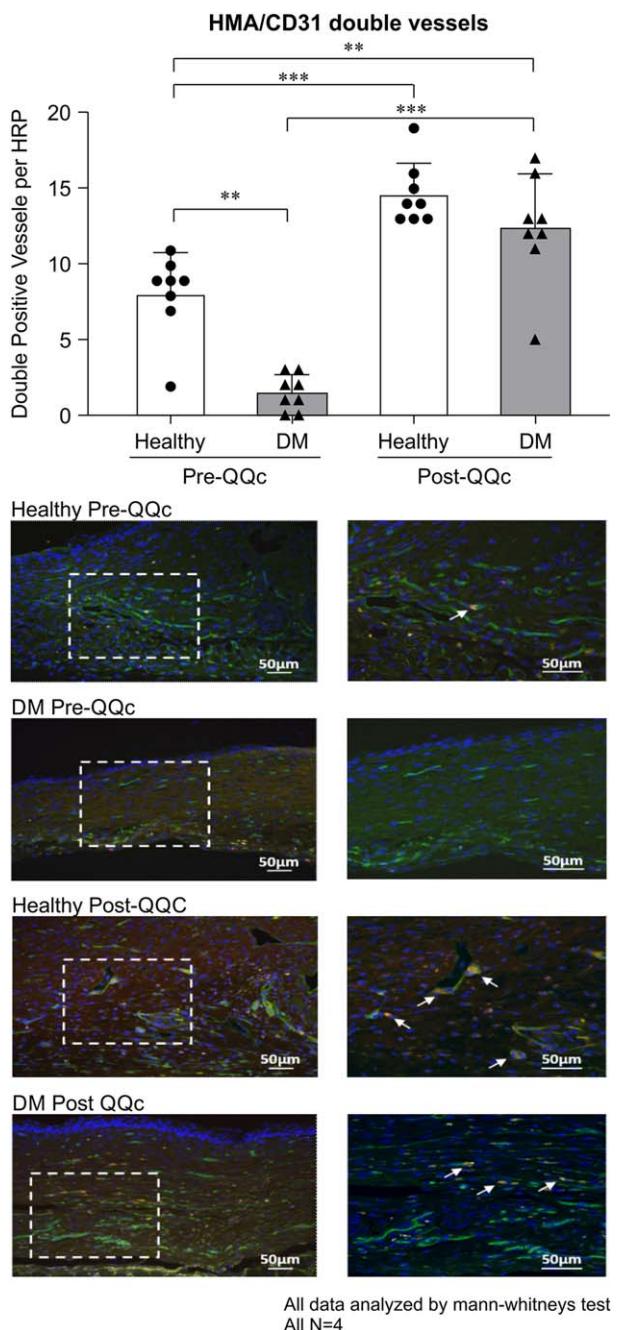


**Figure 4.** Therapy with diabetic (DM) peripheral blood CD34+ cells subjected to QQc accelerates wound closure and maturation in euglycemic mice. Full-thickness wounds in mice were treated with PBS or with pre-QQc or post-QQc healthy and DM CD34+ cells, and evaluated on day 14. **(A)**: Representative photographs of wounds taken every 3–4 days up to day 14. **(B)**: Representative photographs of wound sections stained with HE (scale bar: 500 µm, ×20) and Van Gieson (scale bar: 100 µm, ×20), and immunostained with the anti-CD31 antibody (scale bar: 100 µm, ×40). **(C)**: Quantification of histological examination and immunostaining. The following number of wounds were counted. Wound closure: PBS  $n = 8$ ; pre-QQc healthy cells  $n = 9$ ; pre-QQc DM cells  $n = 9$ ; post-QQc healthy cells  $n = 9$ ; post-QQc DM cells  $n = 7$ . Epithelial thickness: PBS  $n = 7$ ; pre-QQc healthy  $n = 7$ , pre-QQc DM  $n = 5$ ; post-QQc healthy  $n = 6$ ; post-QQc DM  $n = 5$ . Granulation thickness: PBS  $n = 5$ ; pre-QQc healthy  $n = 7$ ; pre-QQc DM  $n = 5$ ; post-QQc healthy  $n = 7$ ; post-QQc DM  $n = 5$ . Van Gieson staining (maturity): PBS  $n = 3$ ; pre-QQc healthy  $n = 7$ ; pre-QQc DM  $n = 3$ ; post-QQc healthy  $n = 6$ ; post-QQc DM  $n = 6$ . Wound vascular density: PBS  $n = 7$ ; pre-QQc healthy  $n = 8$ ; pre-QQc DM  $n = 7$ ; post-QQc healthy  $n = 7$ ; post-QQc DM  $n = 5$ . Each bar represents the mean ± SD; \*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ . Abbreviations: DM, Diabetic; HE, hematoxylin and eosin; NS, not significant; PBS, phosphate-buffered saline; QQc, quality-quantity culture.

with pre-QQc CD34+ healthy cells at day 14 ( $1.5 \pm 1.2$  vs.  $8.1 \pm 2.7$ , respectively;  $p < .01$ ). The number of co-stained vessels in both diabetic and healthy groups significantly increased post-QQc ( $14.6 \pm 2.1$  and  $12.4 \pm 3.6$ , respectively;  $p < .001$ ), and the values were significantly higher in wounds transplanted with post-QQc diabetic CD34+ cells than in wounds transplanted with pre-QQc healthy CD34+ cells ( $12.4 \pm 3.6$  vs.  $8.1 \pm 2.7$ , respectively;  $p < .01$ ) (Fig. 5).

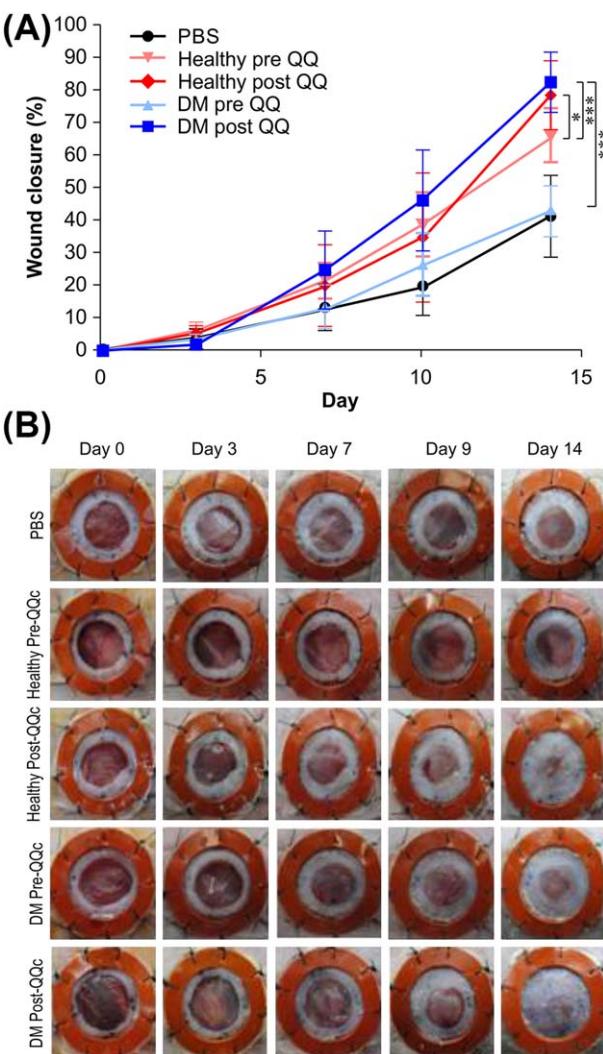
#### QQc Diabetic PB-CD34+ Cell Therapy Is Effective for Wound Healing in Diabetic Animals

In addition to being effective in normal mice, post-QQc diabetic CD34+ cell therapy also resulted in significantly faster wound healing in diabetic mice compared to pre-QQc diabetic CD34+ cells ( $82.1 \pm 9.6$  vs.  $42.3 \pm 7.8$ , respectively;  $p < .001$ ) and even pre-QQc healthy CD34+ cells ( $82.1 \pm 9.6$  vs.  $65.53 \pm 8.27$ , respectively;  $p < .001$  respectively;  $p < .05$ ); its effect was similar to the



**Figure 5.** Post-QQc diabetic (DM) peripheral blood CD34+ cells induce direct vasculogenesis as indicated by HMA/CD31 double-positive staining. Frozen tissues were stained with anti-human mitochondrial antibody and co-stained with anti-mouse CD31. Representative results of confocal microscopy are shown. The highlighted areas on the left panels are magnified on the right. Quantitation of double-stained vessels is shown as bar graphs (mean  $\pm$  SD  $n = 4$  group) and also as dot plots for individual experiments. \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ . Abbreviations: DM, Diabetic; HMA, human mitochondria antigen; QQc, quality-quantity culture.

function of post-QQc healthy CD34+ cells ( $82.1 \pm 9.6$  vs.  $78.22 \pm 11.21$ , respectively;  $p > .05$ ). Cell therapy with pre-QQc diabetic CD34+ cells had little impact on diabetic wound healing and exhibited effects similar to those of PBS-treated controls ( $42.3 \pm 7.8$  vs.  $41.1 \pm 12.6$ ;  $p > .05$ ) (Fig. 6).



**Figure 6.** Therapy with diabetic (DM) peripheral blood (PB)-CD34+ cells subjected to post-QQc is effective for wound healing in diabetic animals. (A): Time course of wound closure in streptozotocin (STZ)-induced DM mice transplanted with PB-CD34+ cells; two wounds were made in each animal. The number of animals in each experimental group was as follows: PBS  $n = 4$ ; pre-QQc healthy PB-CD34+ cells  $n = 5$ ; pre-QQc DM PB-CD34+ cells  $n = 4$ ; post-QQc healthy PB-CD34+ cells  $n = 6$ ; post-QQc DM PB-CD34+ cells  $n = 5$ . \*,  $p < .05$ ; \*\*\*,  $p < .001$ . (B): Representative photographs of wounds in STZ-induced DM mice treated with PBS, or pre-QQc or post-QQc healthy and diabetic cells. The photographs were taken every 3–4 days up to day 14. Abbreviations: DM, Diabetic; PBS, phosphate-buffered saline; QQc, quality-quantity culture.

## DISCUSSION

Emerging evidence suggests that EPC dysfunction in diabetes leads to poor vasculogenesis and impaired wound regeneration [9, 17–20]. CD34+ cells are highly functional EPCs [21, 22], and have been clinically used for effective autologous therapy in non-diabetic individuals [9, 23–25]. However, autologous, freshly isolated CD34+ cells are not effective in patients with diabetes because they are both low in number and dysfunctional. Therefore, there is a need to pre-treat these cells before therapy. We recently filled this gap by developing a serum-free QQc culture system that significantly increased the number of total and differentiated

colony-forming EPCs [13], restored their functionality, and showed promising wound-healing characteristics for diabetic wounds in mice [10]. Similar to our results for murine cells, we have now identified a method to expand human diabetic CD34+ cells ex vivo and to restore their impaired functioning such that post-QQc, they exhibit a markedly higher potential for wound healing and vasculogenesis than pre-QQc healthy or pre-QQc diabetic CD34+ cells. Interestingly, post-QQc human diabetic CD34+ cells showed significantly improved wound closure even when compared to post-QQc human healthy CD34+ cells in the euglycemic model. Although this was an unexpected observation, we have previously shown that prior to QQc, diabetic cells are stressed and therefore unable to function appropriately. After QQc, this stress is removed [26]. It is likely that the removal of stress results in a more robust wound healing process. Further studies would be needed to determine how this robust effect comes into play. Most importantly, post-QQc human diabetic PB-CD34+ cell therapy resulted in significantly accelerated wound closure in diabetic mice.

In contrast to our previous results for murine BM diabetic KSL cells [10], human diabetic PB-CD34+ cell therapy exhibited therapeutic feasibility even in diabetic animals, after the cells were subjected to QQc. We attribute the poor therapeutic efficacy of BM diabetic KSL cells to metabolic memory [27]. The difference between our previous study and this study can be traced to intrinsic differences in the regenerative potential and functionalities of CD34+ cells and KSL cells and/or to differences in the cell origin, that is, human PB versus murine BM [10, 28, 29]. The reasons for such differences could be multifactorial, and further studies are needed to characterize murine EPC subtypes for wound healing. This study, however, is not just a validation of the efficacy of QQc using PB from diabetic patients to effectively use CD34+ cells as a cell therapy for diabetic wounds; it also provides evidence of high translational impact.

In an attempt to identify underlying mechanisms involved in restoring diabetic CD34+ cell functionality, we tested several hypotheses including modulation of EPCs, alteration of inflammatory milieu at the wound site, and the PGC-1 $\alpha$ -Notch signaling pathway. We found a significant increase in the proportion of dEPC-CFUs post-QQc. It is known that the numbers of differentiated EPCs (dEPC-CFUs) are low among diabetic CD34+ cells [10, 22, 29]. Because these EPCs have high potential to adhere to the interior surface of the vascular lumen and to migrate and differentiate within extravascular spaces, they likely contribute to vasculogenesis [30]. In our post-QQc study, human diabetic PB-CD34+ cells also exhibited increased potential for direct vasculogenesis *in vivo*. Wounds transplanted with pre-QQc diabetic cells showed only a few vessels double-stained for HMA and CD31; conversely, a significantly higher number of double-stained vessels was observed in wounds transplanted with post-QQc cells. Our data do not allow estimation of the proportion of transplanted human cells incorporated into mouse vessels because diabetic PB-CD34+ cells also enhanced mouse vasculogenesis in paracrine fashion as shown by the increased number of CD31-positive vessels. It remains unclear whether the relationship between increased human and mouse endothelial cells is linear. However, our results clearly demonstrate that treatment in QQc enhanced the vasculogenic ability of diabetic cells in addition to their paracrine activity.

The paracrine effect of the post-QQc diabetic PB-CD34+ cells on the wound environment resulted in altered gene expression of inflammatory markers, growth factors, and cytokines at the wound site. The elevated expression of IL-10 and reduced IL-12 in the wound tissues after transplantation of the post-QQc diabetic

PB-CD34+ cells suggests a shift in the wound environment from an inflammatory to an anti-inflammatory state. Protein expression of VEGFR-1 and 2, implicated in angiogenesis, was lower in diabetic PB-CD34+ cells, while gene expression was higher when compared to healthy control cells. Similar to our protein KDR expression data, it has been previously reported that the number of CD34+/KDR+ cells is significantly lower in diabetic patients [8, 31]. Diabetic EPCs are reported to have a VEGF-VEGFR signaling defect, and PCR data for murine EPCs show high VEGFR expression similar to our RT-qPCR data [32]. It is likely that decreased VEGF expression leads to an increase in expression of VEGFR-2 to compensate for the deficiency of VEGF signaling. We expect that future studies will shed light on the proposed mechanism.

TGF- $\beta$  was found to be released in significantly higher amounts at the wound site after post-QQc human diabetic PB-CD34+ cell therapy. Higher TGF- $\beta$  has been known to play a key role in stimulation of keratinocyte and fibroblast migration and proliferation [33, 34] as well as in granulation formation and wound closure [18, 35].

PGC-1 $\alpha$  is a transcriptional co-activator involved in mitochondrial biogenesis and function; it can block endothelial migration and vasculogenesis, causing endothelial cells to become unresponsive to angiogenic factors [36]. PGC-1 $\alpha$  also induces Notch signaling and can therefore affect Notch-associated pathways that are critical in endothelial migration and sprouting angiogenesis [36–38]. We previously observed that murine diabetic BM and PB EPC have high Notch activity, leading to inhibition of migration and differentiation [34]. In this study, pre-QQc diabetic CD34+ cells also demonstrated significantly higher expression of PGC-1 $\alpha$  and Notch pathway signature genes than healthy CD34+ cells; expression of these genes was significantly decreased post-QQc, suggesting that the attenuation of PGC-1 $\alpha$  and Notch may play a role in restoring the vasculogenic potential of diabetic PB-CD34+ cells. Further inhibitor studies are required to confirm the role of PGC-1 $\alpha$  and Notch pathways in diabetic human PB-CD34+ cells and whether QQc alters these pathways.

## CONCLUSION

In conclusion, we have devised a novel therapeutic modality for improving angiogenesis and vasculogenesis in refractory diabetic wounds. Our approach overcomes the inherent limitation of autologous cell therapy in diabetic patients, as QQc not only increases cell counts but also markedly enhances their functionality. To our knowledge, this is the first study to investigate tubule formation, EPC colony formation, and the *in vivo* wound healing potential of human diabetic PB-CD34+ cells. Because QQc is a serum-free suspension culture requiring only 1 week of incubation, it can be used effectively without the burden of passaging the cells into clinically applicable pools. Rapidly expanded post-QQc cells can be aliquoted and cryopreserved for future use in case of wound recurrence or other diabetic complications. Furthermore, because post-QQc CD34+ cells are highly vasculogenic, they could be applied not only for wounds but also for other ischemic conditions such as myocardial ischemia, cerebral infarction, and peripheral arterial diseases. Additional experiments on the underlying mechanisms could facilitate the design of effective therapeutic modalities for diabetes and ischemic conditions.

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## AUTHOR CONTRIBUTIONS

R.T.: conception and design, financial support, manuscript writing, provision of the study material, collection of data, data analysis, and interpretation; H. Masuda: conception and design; S.F. and N.S.: collection and assembly of data, data analysis and interpretation, and manuscript writing; K.A., R.H.-I., Y.K., H.H., M.K., and A.H.: collection and assembly of data and data analysis and interpretation; T.M., T.O., and H.W.: collection and assembly of data; H. Mizuno and T.A.: administrative support and financial support; R.T., H. Masuda, S.F., R.H.-I., K.A., Y.K., H.H., M.K., A.H., T.M., T.O., H.W., H. Mizuno, N.S., and T.A.: final approval of the manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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