Quality Control Culture System Restores Diabetic Endothelial Progenitor Cell Vasculogenesis and Accelerates Wound Closure

RUNNING TITLE: Quantity and Quality Cultured Diabetic EPC Therapy

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ABSTRACT

Delayed diabetic (DM) wound healing is, in part, the result of inadequate endothelial progenitor cell (EPC) proliferation, mobilization, and trafficking. Recently, we developed a serum free functional culture system called quantity and quality control culture system (QQc) which enhances the number and vasculogenic potential of EPCs. We hypothesize that QQc restoration of DM EPC function will improve wound closure. In order to test this hypothesis, we measured pre/post-QQc DM KSL (e.g. c-kit^+Sca-1^-lin^-) cell activity in vitro as well as the effect of KSL cell adoptive transfer on the rate of euglycemic wound closure. KSL cells were magnetically sorted from control and streptozotocin-induced Type I DM C57BL6J bone marrow. Freshly isolated control and DM KSL cells were cultured in QQc for 7 days and pre/post-QQc KSL function was tested. The number of KSLs significantly increased post QQc for both DM and controls and also increasing DM -KSL vasculogenic potential above fresh control KSL level. Similarly, fresh DM cells form fewer tubules, but QQc increases DM tubule formation to greater than fresh control cell levels (p<0.05). Adoptive transfer of post-QQc DM KSL cells significantly enhance wound closure compared to fresh DM KSL cells and equaled wound closure of post-QQc control KSL cells. Post-QQc DM KSL enhancement of wound closure is mediated, in part, via a vasculogenic mechanism. This study demonstrates that QQc can reverse DM EPC dysfunction and achieve control-levels of
EPC function. Finally, post-QQc DM EPC therapy effectively improved euglycemic wound closure and may improve DM wound healing.
INTRODUCTION

While blood supply is essential for tissue viability, new blood vessel formation is critical for tissue recovery, regeneration and repair. Postnatal new blood vessel formation was long thought to be restricted to angiogenesis -- the sprouting of new blood vessels from existing vascular structures. However, in 1997, we demonstrated that the de novo formation of new blood vessel derived from bone marrow (BM) derived cells (i.e. vasculogenesis) is an important part of postnatal healing.(1-3) The BM-derived endothelial progenitor cells (EPCs) are precursors of endothelial cells (ECs) and are characterized by their surface expression of KDR, CD133, CD34 for humans and lineage negative (Lin)-, c-kit+, Sca-1+ cells for murine bone marrow cells.(4-6)

After injury, locally-derived circulating factors mobilize EPCs from their endosteal BM niche. Circulating BM-derived EPCs traffic to the site of injury, diapedesis, cluster, tubulize, and canalize to form nascent vessels that inosculate with the existing vasculature.(7)(8) EPCs have been shown to revascularize numerous ischemic tissues including: myocardium (i.e. myocardial infarction), brain (i.e. cerebral infarction), and skin (i.e. cutaneous wounding).(9; 10) While BM-derived EPCs contribute to only 25% of newly formed endothelium in healing tissues, when EPC function is impaired there are marked deficits in tissue repair mechanisms.(11; 12)

Compared with non-diabetic patients, DM EPCs have impaired proliferation, adhesion, migration, and differentiation.(13-15) Although the pathogenesis of impaired diabetic wound healing is multifactorial, EPC dysfunction plays a central role. (16; 17) These intrinsic DM EPC vasculogenic impairments may result in more than 83,000 amputations each year and a post-amputation 3-year mortality rate of 75.9%(18).
preclinical studies, the administration of exogenous EPCs has improved ventricular function after myocardial ischemia,(19; 20) enhanced neuronal recovery after cerebral vascular occlusion, and accelerated restoration of blood flow to ischemic limbs.(13)\textsuperscript{16,17,21,22,23} Based on these exciting results, we have conducted a Phase 3 clinical trial of autologous G-CSF mobilized peripheral blood EPC cell therapy for non-healing diabetic foot patients.(24) The results demonstrated that more successful therapeutic results were seen in patients receiving high vasculogenic EPCs. From these results we hypothesize that successful autologous DM EPC therapy relies on the vasculogenic function of transplanted EPCs and speculate that the intrinsic DM EPC dysfunction will limit the efficacy of the therapeutic strategy.(25; 26)

Recently, our group established a serum-free quality and quantity culture (QQc) system (containing stem cell factor, thrombopoietin, vascular endothelial growth factor, interleukin-6, and Flt-3 ligand) which enhances the vasculogenic potential of EPCs.(27) We hypothesize that QQc can reverse the detrimental effects of DM-induced EPC dysfunction and supply a sufficient number of functional EPCs for adoptive autologous cell based therapy for diabetic patients. In the present study, we test this hypothesis.

**MATERIALS AND METHODS**

*Diabetic Mouse Model*

C57BL/6J male mice aged 8-10 weeks and weighing 20-25g were purchased from Crea Japan Inc. (Kawasaki, Japan) and Jackson Laboratories (Bar Harbor, ME). Obliteration of pancreatic beta cells was achieved with intraperitoneal (i.p.) injections of 50mg/kg streptozotocin (STZ; Sigma-Aldrich; St Louis, MO) in 50mM sodium citrate
buffer (pH 4.5) for 5 consecutive days. Ten days after the initial injection, mice with a blood sugar level above 300 mg/dl were deemed DM, while those with a level below 300 mg/dl received an additional 3 days of STZ injections (50mg/kg). Mice were considered DM if they maintained glucose levels above 300 mg/dl for at least 4 weeks before the date of wounding. Control mice received i.p. injections at the same time points with an equal volume of 50 mM sodium citrate buffer. A total of 200 mice were used in this experiment (n=100 per group). All procedures were conducted in accordance with the guidelines set forth by the committee of Ethical Animal Care and Use at Tokai University School of Medicine and The Institutional Animal Care and Use Committee at New York University Medical Center.

**Bone Marrow Progenitor Cell Isolation**

Bone marrow cells were harvested from DM and control mouse femurs and tibias as previously described. Mononuclear cells were washed with PBS-EDTA and erythrocytes were removed by ammonium chloride hemolyzation and stained with a lineage positive antibody cocktail containing CD45R/B220, TER119, CD3e, CD11b, Ly-6G & Ly6C (Gr-1) for 20 minutes at 4°C (all antibodies obtained from BD PharMingen; San Diego, CA). After labeling the lineage positive antibodies with biotin labeled magnetic beads, cells underwent a negative selection process with a magnetic cell sorting system (MACS). Lin⁺ cells were counted and then incubated with Rat-FITC anti mouse Ly-6A/E (Sca-1) (BD PharMingen) and Rat-PE CD117 (c-kit) (BD PharMingen) for 20min at 4°C, washed three times and resuspended in 20% IMDM (Gibco; Carlsbad,
CA). FITC conjugated Sca-1 and PE conjugated c-kit double positive cells (KSL) were then obtained by fluorescent activated cell sorting (FACS).

**Serum-Free Quantity and Quality Control Culture (QQc)**

Diabetic and control BM KSLs were isolated as described above and 1x10^3 cells were placed in each well of a 24 well plate (BD Falcon; Bedford, MA) and cultured in QQc for 7 days as previously described(27). Briefly, QQc is an optimized growth factor/cytokine combination (20ng/ml TPO, 20ng/ml IL-6, 100ng/ml SCF, 100ng/ml Flt-3 ligand, 50ng/ml VEGF; all from Peprotech; Rocky Hills, NJ) serum free Stem Span (Stem Cell Tec.) media. After 7 days of QQc, control KSLs were termed C_{QQc} and DM KSLs DM_{QQc}. Growth in QQc has been shown to dramatically expand and enhance the vasculogenic potential of EPCs.

**EPC- Colony Forming Assay of KSL population**

The vasculogenic potential of DM and control BM KSL cells was assessed using the EPC-CFA (EPC-Colony Forming Assay) as previously described.(6; 29-31) EPC-CFA is designed to distinguish total EPC-colony forming units (tEPC-CFUs) into two different types: primitive (small cell) and definitive (large cell) EPC-CFUs. The primitive EPC-CFUs (pEPC-CFUs) is a predominantly proliferative population of cells while the definitive EPCs-CFUs (dEPC-CFUs) is a predominantly vasculogenic population with greater adhesion, migration, and differentiation, and tubularization potential. Briefly, a total of 500 BM KSL cells/dish were seeded into a 35 mm hydrophilic tissue culture dish.
Seven days later, EPC-CFUs, pEPC-CFU dEPC-CFUs were counted by two investigators who were blinded to the experimental conditions. Experiments were run in triplicate.

**Tube Formation Assay**

Tubule formation assay was performed by adding Biocoat Matrigel (Becton Dickinson; Franklin Lakes, NJ) into 24-well plates and incubating in a CO\(_2\)-free incubator at 37°C for 30 min. The same lot of Matrigel was used for all of the experiments. The gels were then overlaid with 3 x 10\(^3\) cells of fresh and expanded diabetic and control cells co-cultured with 1x10\(^4\) endothelial cells suspended in culture medium and incubated at 37°C in an atmosphere of 5% CO\(_2\). The well cultured with endothelial cells only was used as a control. After 12 hours of incubation, gels were examined by using a phase-contrast microscope equipped with a digital camera (Nikon eclipse TE2000-U; Melville, NY). A blinded observer measured the total number of tube-like structures per high power field in 5 random fields.

**Quantitative Real-Time PCR**

Total RNA from 2x10\(^4\) DM and control KSL cells was extracted using the RNeasy Micro kit (Qiagen; Basel, Switzerland) based on manufacturer’s protocol and reverse transcription was performed using high capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA). The transcription reaction was carried out at 37°C for 2h. The obtained cDNA amplified using the reaction mixture of Taq Man FAST Universal PCR Master Mix (Applied Biosystems). The TaqMan probes (Applied Biosystems) were used 18SrRNA (Ribosomal RNA control reagents 4308329), EGF
(Mm00438696_m1, Egf), HGF (Mm01135193_m1, Hgf), FGF-2 (Mm00433287_m1, Fgf2), FGF-7 (Mm00433291_m1, Fgf7), vWF (Mm00550376_m1,Vwf), CD29 (Mm0125320_m1,Itgb1), and FLK-1( Mm0122419_m1, Kdr). The PCR mixtures were pre incubated at 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 sec and 62°C for 30 sec by ABI 7500 FAST (Applied Biosystems). The real-time data was analyzed by Delta (Δ) Ct method. ΔCt was calculated as (gene of target Ct)-(18SrRNA Ct). The relative quantity of mRNA of gene of target was calculated by ΔCt calculation as $2^{-\Delta C_t}$.

**Wound Model and KSL Adoptive Cellular Therapy**

In order to reduce the confounding variables that would affect KSL function in a diabetic wound, first we used 8-10 week-old euglycemic C57BL/6J male mice (n = 45; 3 per experimental group in triplicates) as recipients for DM and control KSL therapy.(32) To verify the efficacy of QQc DM cells in diabetic wound healing, a diabetic STZ mouse wound model was similarly prepared. Briefly, each mouse was anesthetized and depilated, and one set of bilateral 6-mm punch biopsies was excised on the dorsum. Excisions were full-thickness, including the hypodermis and panniculus carnosum. India ink was applied intradermally at the margins to permanently mark the wound edge. A silicone stent (Grace Bio-Labs; Bend, OR) with an 8-mm inner diameter was sutured with 5-0 nylon (Ethicon Inc.; Somerville, NJ) around each wound to minimize skin contracture and ensure healing by secondary intention. On post-operative day 3, a 1-cc syringe with a 30 gauge needle was used to inject 25 µl of: saline; or $2 \times 10^4$ freshly-isolated control KSL (FC); or $2 \times 10^4$ freshly-isolated diabetic KSL (FD); or $2 \times 10^4$ post-QQ control KSL (QC); or $2 \times 10^4$ post-QQc diabetic KSL (QD) into the center of the muscle at the base of the
wound. The wounds were covered with Tegaderm® to prevent the cells from leaking and drying.

Wound photographs were acquired with a 7-megapixel digital camera (Canon U.S.A., Inc.; Lake Success, NY) from a distance of 6.5 cm, with the lens oriented parallel to the wound. Wound area was measured digitally (Photoshop CS3, Adobe Systems, Inc.; San Jose, CA) and calibrated against the internal diameter of the silicon stent to correct for magnification, perspective, or parallax effects. Percent wound closure ($1 - \frac{\text{wound area}}{\text{original wound area}}$) was measured photogrammetrically on days 0, 3, 7, 10, 14, 18, and 21.

**Wound Harvest**

Wounds were harvested from euthanized animals at post-operative days 7, 14, and 21 ($n = 4$ per group at each time point). A full-thickness excision including 3 mm beyond the margin of the original wound edge (demarcated with India ink) was performed. Each wound was bisected and one-half of the wound was frozen in OCT for cryosectioning. The other half was fixed in 100% methanol and embedded in paraffin. Sections were cut from the central region of the wound at a thickness of 5 micrometers. Prior to staining, paraffin sections were deparaffinized and rehydrated by successive passages through xylene and decreasing concentrations of ethanol.

**Van Gieson’s Stain for Wound Maturity**

Wound maturity can be quantified with Van Gieson’s staining protocol, which simultaneously stains mature collagen deep red and immature collagen pink.$^{(33)}$
Horizontal sections were cut from each specimen at each time point. Paraffin sections were processed with staining solution as described previously. (34) Sections were imaged and digitized in their entirety at 200X resolution with an Aperio ScanScope GL scanning optical microscope (Aperio Technologies, Inc.; Vista, CA). Images were then analyzed with Adobe Photoshop CS3 (Adobe Systems, Inc.). Percentage mature collagen was quantified by measuring the total pixel area of the wound and the percentage of pixels therein that were consistent in color with mature collagen. Lateral wound margins were identified at the border of the panniculus carnosum layer.

**CD-31 Staining for Vascularity and PCNA Staining for Cellular Proliferation**

Paraffin sections were incubated in either CD31 (an endothelial marker) or PCNA (a nuclear marker for proliferation) (both from Cell Signaling Technology, Inc., Danvers, MA) antibodies, washed, and stained with DAB (Vector Laboratories; Burlingame, CA). Slides were examined under 200X magnification and captured as digital images (Olympus BX51 microscope and DP12 camera). In CD31-labeled sections, patent vessels were tallied, and numeral density was reported as vessels/200X field. Cross-sectional area of each vessel was obtained with Adobe Photoshop CS3 and reported as total cross-sectional area normalized to wound area as well as average cross-sectional area per vessel. In PCNA-labeled sections, nuclei exhibiting positive PCNA staining were tallied and reported as cells/ 200X field.

**GFP and vWF co-staining**
In order to follow the adoptively transferred cell trafficking, BM-KSLs from diabetic and controls were isolated from 8-10 week old green fluorescent protein (GFP)-expressing C57BL6 mouse (Clea, Japan) as described above. The KSL cells were cultured in QQc medium for a week as described above. 2x10^4 GFP-KSL cells were injected into 8-10 week-old euglycemic C57BL/6J male mice as described above. Wounds were harvested from euthanized animals at post-operative days 7, 14, and 21 (n = 4 per group at each time point) as described above. Tissue sections were fixed in 4% paraformaldehyde overnight at 4°C then processed through 100% ethanol, xylene and paraffin embedded. To enhance GFP expression, samples were incubated with a 1:300 dilution of anti-GFP mouse polyclonal antibody (Invitrogen) for 1 hour at room temperature, washed, and stained with DAB (DOTITE). For vWF staining, the sections were further treated with 1:300 dilution anti-vWF rabbit polyclonal antibody (DAKO) for 4°C overnight then washed and blocked in 5% normal sheep serum for 5 minutes followed by anti rabbit IgG alkaline phosphatase-streptavidin complex (NICHIREI). The double stained images with vWF images were obtained with same equipment as described above. Dual filter images were superimposed to illustrate GFP trafficking and wound vascular architecture.

**Statistical Analysis**

All data are presented as the mean ± standard deviation. A Kruskal-Wallis one-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc analysis was performed when comparisons involved more than two groups. Mann Whitney Test was used for pair-wise comparisons. Significance was considered to be p<0.05. The number of animals
in each group was determined with an *a priori* power analysis using a standard for adequacy of 80% to reject the null hypothesis of zero correlation using G*Power (G*Power©; Melbourne, Australia).

**RESULTS**

*QQc restores growth and vasculogenic potential of DM endothelial progenitor cells.*

Since *ex vivo* expansion is an important step in adoptive cellular therapy, we measured the effects of QQc on KSL proliferation. After 7 days of QQc, the C_{QQc} population increased 338.2 ± 260.7-fold (Figure 1a). Similarly, after 7 days of QQc, the DM_{QQc} population increased 329.0 ± 125.7-fold (Figure 1a). There was no significant difference (p=0.6) in the QQc cellular expansion of DM and control KSL cells.

Since QQc restored *ex vivo* DM KSL expansion to control rates, we assessed the effects of QQc on the vasculogenic potential of DM KSL cells using EPC-CFA. Before expansion, DM KSL cells had similar number of _p_ CFU (19.2 ± 4.9 v. 18.6 ± 04.2; p=0.5) but significantly fewer _t_ CFUs (22.7 ± 5.2 v. 25.0 ± 3.8; p<0.01), and _d_ EPC-CFU (4.2 ± 2.3 v. 6.3 ± 2.2; p<0.01) compared to control KSLs. After QQc, not only did the number of DM _t_ EPC-CFUs (4469 ± 1593; p<0.01), _p_ EPC-CFU (1862 ± 842; p<0.01), and _d_ EPC-CFU (2607 ± 1084; p<0.01) increase significantly from pre-QQc levels, but the number of DM _t_ EPC-CFUs (4469 ± 1593 v. 4884 ± 1495; p=0.4) and _d_ EPC-CFU (2607 ± 1084 v. 1839 ± 813; p=0.06) was restored to control levels (Figure 1 b). Importantly, QQc increased the percentage of DM _d_ EPC-CFU (the EPC population that most readily forms new vessels) more than 3-fold from (17.8 ± 8.8% v. 58.2 ± 12.7%; p<0.01) (Figure 1 c,d).
**QQc restores tubular formation of DM progenitor cells.**

Organization of endothelial cells in a three dimensional network of tubes is the final step of angiogenesis. Since QQc increased the rate of DM KSL proliferation as well as differentially increased the proportion of DM dCFUs, we tested the effects of QQc on DM KSL tubule formation *in vitro*. Prior to QQc, on matrigel, DM KSLs had significantly fewer tubules/hpf than controls (17.55±7.4 v. 28.53±15.4; p<0.01) (**Figure 2**). Moreover, the tube formation with DM KSLs demonstrated significantly less tubules/hpf compared to HUVEC with no cell group suggesting that DM s KSL may have a negative effect to augmenting angiogenesis (21.5±6.5 v. 17.55±7.4; p<0.05). Although the number of tubules/hpf with DM QQc KSLs was still significantly lower (33.3±8. v. 47.1±15.3; p<0.05) than CQQc KSLs tubules/hpf, DM KSL tube formation significantly increased (17.55±7.4 v. 33.3±8.0; p<0.01) after QQc and it had significantly increased compared to pre QQc control KSLs (33.3±8.0 v. 28.53±15.4; p<0.05)

**QQc enhances expression of vasculogenic and wound healing factors.**

Since QQc increased the rate of DM KSL proliferation, differentially increased the proportion of DM dEPC-CFUs, and increased the tubule forming potential of DM KSLs, we tested the effects of QQc on DM KSL gene expression. After QQc, DM KSL cells increased their expression of wound healing-related growth factor genes, EGF, FGF-2 and FGF-7, and vasculogenesis-related genes, vWF, CD29 and Flk-1 (**Figure 3**). While all key wound healing-related factors increased after QQc, EGF production increased 3.5 fold in control KSL cells and 8.3 fold in DM KSL. Among the
vasculogenesis-related genes, the expression of vWF increased by 8.3 and 6.7 folds in control and diabetic KSL respectively post QQc. The expression of Flk-1 in C_{QQc} KSL remarkably increased (32.7 fold) and in DM_{QQc} KSL were increased 2.5 fold.

In addition to the upregulation of key wound healing and vasculogenic genes, KSL cells also increase their expression of CD29/integrin β-1, an integrin unit associated with the angioblastic growth cone during vasculogenesis. After QQc, CD29 expression in both of C_{QQc} and DM_{QQc} KSLs increased significantly (2.3 fold for control and 1.9 fold for DM p < 0.05). Moreover, the expression of CD29 in DM_{QQc} KSLs was not significant difference from CD29 expression in C_{QQc} KSLs.

**Post-QQc DM progenitor cell therapy accelerates wound closure.**

In order to reduce the number of confounding variables that might affect EPC function during wound closure, we tested pre/post-QQc DM and control KSL adoptive cellular therapy in wounded euglycemic mice. Adoptive cellular therapy with pre-QQc DM KSL cells had little impact on wound healing compared to PBS treatment on day 7 (26.1±3.0% v.30.7±1.9% p<0.05); day 14 (61.5±5.6% v. 70.1±3.5%; p=0.50); day 18 (70.0±13.6 v.87.4±11.1; p=0.6); or day 21 (83.5±5.0% v. 89.7±5.5% p=0.05) (Figure 4 A,B). In marked contrast, adoptive transfer of DM_{QQc} KSLs accelerated the percent wound closure compare to pre-QQc DM treatment at day 14 (81.3±8.7% v.61.5 ±20.3% p<0.05); or day 18 (70.0±13.6% v. 97.7±4.0% p<0.01) or day 21 (83.5± 5.0% v. 89.7±5.5% p<0.05) (Figure 4 A,B). Moreover, the percent wound closure achieved with adoptive transfer of DM_{QQc} KSLs was not significantly different than the percent wound closure achieved with adoptive transfer of C_{QQc} KSL at day 14 (81.3±
7.2% v. 89.98±7.7%; p<0.05) but it was not significantly different at day 18 (97.7±4.0% v. 99.1±1.5%; p=0.9) or day 21 (94.4±1.6% v.100±0%; p=0.3) (Figure 4 A,B).

**Post-QQc DM adoptive DM progenitor therapy enhances wound vascularization and collagen maturation**

In order to understand how QQc improved DM KSL-mediated wound closure, we measured wound vascularization and collagen maturation. Interestingly, wounds injected with QD KSLs showed significantly higher CD31 counts compared with the freshly isolated diabetic KSL treatment group and PBS (13.8 ± 1.8 v. 8.6 ± 0.9, 7.2 ± 0.8; p<0.01) starting Day 7. At day 21, the vascularity in the post-QQc diabetic KSL treatment groups significantly increased compared with freshly isolated control and diabetic KSL treatment group, as well as compared with the PBS control group (25.5 ± 1.7 v. 15.0 ± 1.8, 18.0 ± 1.2, 11.5 ± 1.0; p<0.05). Interestingly, post-QQc KSL treatment groups showed a rapid increase in vascularity after injection, as compared with the relatively delayed response in the freshly isolated KSL treatment group. (Figure 5a, b)

At day 21, the percentage of mature collagen as assessed by Van Gieson’s staining in the wounds treated with post-QQc diabetic KSLs (58±11 %) was greater compared with wounds treated with freshly-isolated control (43±14 %) and DM KSLs (38±3 %) and PBS (33±7 %, p<0.01 and p<0.01, respectively). **Figure 6 a, b**

**Post QQc DM progenitor cells obtain high potential for direct vasculogenesis.**

In order to identify whether the increased vascularity is due to differentiation of injected post-QQc KSLs or due to increased numbers of resident endothelial cells, we
injected pre and post-QQc GFP control and diabetic KSLs in the wound and identified the GFP and vWF co-staining cells. As a result, GFP positive cells co-stained with vWF were only identified in the post QQc control and diabetic cell treated group at Day 21. Comparing post QQc control and diabetic cell treated group, post QQc control cell treated group showed higher number of GFP cells incorporated into the vasculature suggesting higher vasculogenesis of post QQc control KSLs (Figure 5c).

**Post-QQc DM progenitor cell therapy increases cellular proliferation in the wound.**

In order to study the effects of QQc expanded DM KSLs on native cells in the wound, we measure fibroblastic proliferation *in situ*. After adoptive QD KSL treatment, wound fibroblastic proliferation peaked early and declined significantly from day 7 to day 21 in the groups treated with FC KSLs (795±221 v. 247±86 cells/field, p=0.044), and post-QD KSLs (761±171 v. 238±141 cells/field, p=0.011). Contrarily, there was no increase in cellular proliferation in wounds treated with freshly-isolated diabetic KSLs (664±321 v. 534±116 cells/field, p=0.41) or PBS (621±122 v. 672±278 cells/field, p=0.31) (Figure 7).

**The function of Post QQc Diabetic progenitor cells are deteriorated in glycemic diabetic wounds**

The in vivo efficacy of post QQc DM KSLs was also tested in STZ-induced glycemic murine diabetic wounds. As a result, post QQc KSLs indicated significant percent wound closure compared to pre QQc KSLs and PBS at day 14 (73.60%±3.69 vs 55.02%±3.61; vs 58.98%±5.86; p<0.05) and day 21 (96.34%±1.52 vs 82.29%±4.72 vs
84.01%±2.28; p<0.05) (Figure 8.) However, there was no significant difference between the wound closures between pre CQQc KSL, pre/ post DMQQc KSL and PBS at all times. Although post DMQQc KSL can accelerate wound healing and possess the restored vasculogenic potential in a euglycemic in vivo condition, these data suggests functional limitation of post DMQQc KSL function in glycemic diabetic condition.

**DISCUSSION**

Current diabetic wound treatment hinges on patient education, prevention, and early diagnosis. Once a wound has developed, however, invasive therapies are costly while noninvasive therapies are less effective. Ultimately, since current treatments do not correct the underlying pathophysiology, many patients suffer untoward complications and require amputations. While investigators have long focused on the detrimental effects of elevated blood sugar on diabetic wound healing, recent data suggests that diabetic impairment of EPC function has a secondary effect on diabetic wound healing.(5; 35) This latter point is highlighted in the results of our recent clinical trial.(24) By injecting autologous G-CSF mobilized peripheral blood EPCs into the non-healing wounds of diabetic patients, we found that successful wound healing correlated with the vasculogenic function of transplanted EPCs. Moreover, we discovered that autologous EPC therapy has two inherent limitations: 1) low EPC cell number and 2) low vasculogenic function.

In an effort to overcome the limitations of autologous EPC therapy in diabetic patients, we studied the effect of QQc DM EPC ex vivo expansion on wound healing. In our study mouse BM KSLs were used as EPC enriched population based on recent study
reported by Kwon et al. (6) QQc is a serum free culture system recently developed and reported by our group.(27) QQc is a functional culture system which not only increases the number of EPCs, but also increases the population of differentiated colony forming EPCs (ie. vasculogenic EPCs). Our in vitro experiments demonstrate that QQc significantly increases DM EPC cell number, definitive colony formation, and tubulization. Since QQc not only increased the number of DM EPCs, but also restored their function to the level of control EPC, we tested the effects of QQc DM EPC ex vivo expanded cells on wound closure. We used a stented wound closure model to minimize the effects of wound contracture.(36) In order to focus our investigation on the function and efficacy of post-QQc DM EPCs compared to fresh healthy allogeneic EPCs, we selected a euglycemic wound closure mice model. We hypothesized that the use of a euglycemic recipient would eliminate the effect of confounding variables present in a DM recipient model.

Since new blood vessel formation is crucial for successful wound healing, we hypothesize that DM QQc therapy leads to accelerated wound closure by enhancing vasculogenesis..(37) CD31 staining demonstrated that post-QQc DM EPC treatment increased wound vascularity compared with freshly-isolated DM EPC treatment and control groups at all time points. Moreover, as demonstrated previously by Masuda et al, since QQc increases the number of $d$EPC CFU (i.e. vasculogenic EPCs) and $d$EPCs more readily form new vessels (compared to $p$EPCs), we hypothesize that they are the vitally important EPC fraction mediating the therapeutic vasculogenesis observed in our in vivo experiments. Collectively, our findings suggest a potential mechanism by which DM QQc EPCs accelerate wound closure ─ transplanted post-QQc EPCs accelerate wound closure
by forming tubules and inosculating with existing vasculature. This idea is further supported by the finding that GFP labeled KSL cells incorporated into the native vascular network.

Enhanced new blood vessel formation may accelerate wound closure in a number of different ways. We found that DM$_{QQc}$ therapy significantly enhanced the percentage of mature collagen in the wound. Interestingly, post-QQc DM EPCs exhibit significantly higher CD29mRNA expression compared to fresh DM EPCs. Recently, it was reported that CD29 directly influence growth factor signaling and promotes fibroblast migration. Together with the PCNA data, we hypothesize that DM$_{QQc}$ stimulate fibroblast migration to the wound and accelerate wound closure.

In order to confirm the efficacy of QQc therapy in diabetic mice we injected pre and post QQc control and diabetic KSLs to a full thickness wound in STZ induced glycemic diabetic mice. The results indicated that accelerated wound healing was seen only in post QQc Control KSLs treated group. The healing of pre and post DM QQc KSLs and pre QQc Control KSLs showed and PBS treated group demonstrated same percent wound closure at all times from Day 3 to Day 21. In other words, the healing of post QQc DM KSLs in a euglycemic wounds were not seen in a diabetic condition. We assume from this result that hyperglycemic condition of DM mouse may have deteriorated the function of post QQc DM KSLs. Since post QQc DM KSL have a highly therapeutic effect in euglycemic conditions, we believe that QQc therapy may be effective in euglycemic diabetic patients, patients with controlled blood glucose levels (blood glucose <8mmol/L or <140mg/dl.) according to the practical guidelines on the management and prevention of diabetic foot. The phenomenon seen in our
data is similar to the situation we daily face treating diabetic patients with chronic wounds in patients with high blood glucose. Many previous reports have shown that one of the standard of care for diabetic wounds involves systemic glucose control and effective wound healing cannot be expected for uncontrolled diabetes even with highly effective therapy. Our results on EPC therapy for uncontrolled diabetic mice shows that the condition of the host has a great impact on deterioration of the cells being administered and we believe that “metabolic memory” and epigenetic modification by hyperglycemia is one its possibilities. Although in euglycemic diabetic patients, the efficacy of EPC therapy for wound healing is limited due to autologous EPC vasculogenic dysfunction as shown in our previous report (40). We believe that present EPC therapy with application of autologous dysfunctional EPC may not be effective even for glucose controlled DM patients. The application of QQc in these patients may be the key for highly therapeutic autologous DM EPCs therapy. In order to test our hypothesis we have tried to establish a stented wound healing model of insulin treated STZ diabetic mice with controlled glucose level and treat these mice with pre and post DM QQc KSLs. Unfortunately the model was difficult to be established due to many interventions on the mice. Therefore, this hypothesis remains to be proven.

Another limitations of our study includes not knowing the exact mechanism of how QQc restores the vasculogenic dysfunction of DM EPCs. We have recently looked into effect of QQc on oxidative stress of control and DM BM KSLs and found that QQc relieves oxidative stress on both control and DM BM KSLs (data not shown.) However,
this was not the specific mechanism for restoring DM BM KSL dysfunction. We plan to further investigate for our future study.

In summary, we have demonstrated that QQc not only restores DM EPC function, but also achieves supra-physiologic EPC vasculogenic function in vitro and in vivo. Since QQc is serum-free and it rapidly expands the number of DM EPCs, this system may facilitate cell-based therapies for DM patients. Although we still have limitation of this study for future clinical applications for DM patients, this study can be stated to be the first step in establishing an ideal cell based therapy for diabetic patients. Moreover, the rapidly expanded post-QQc EPC population could be aliquoted, cryopreserved, and used again for metachronous wounds or other ischemic conditions (e.g. myocardial ischemia).

CONCLUSION

Here, we demonstrate that a novel serum free QQc system expands the number of cells and enhances the vasculogenic and therapeutic potential of diabetic EPCs. We hypothesize that adoptive post-QQc diabetic EPC therapy may be an effective cell-based therapy for non-healing diabetic wounds.
AUTHOR CONTRIBUTIONS

RT: Conception and design, financial support, manuscript writing, provision of the study material, collection of data, data analysis and interpretation,
MV: Collection of data, data analysis and interpretation, manuscript writing
HM: Conception and design, manuscript writing
RI: Collection and assembly of data, data analysis and interpretation
MK: Collection and assembly of data, data analysis and interpretation
MM: Administrative support
HM: Manuscript writing
SMW: Financial support, administrative support, manuscript writing, final approval of manuscript.
TA: Financial support, administrative support, manuscript writing, final approval of manuscript

ACKNOWLEDGMENTS

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Kayoko Arita from Juntendo University School of Medicine Department of Plastic and Reconstructive Surgery for their kind assistance in technical assistance. I also like to thank Dr. Yoshinori Okada, Dr. Hiroshi Kamiguchi and Ms. Yoko Kameyama from Tokai University School of Medicine for their outstanding technical support of flow cytometry, real time PCR and immunohistochemistry.
REFERENCES


**Figure Legends**

**Figure 1:** QQc restores growth and vasculogenic potential of diabetic progenitor cells more than the level of controls. (A): Fold increase of control and diabetic post-QQc cells. (B): The frequency of EPC-CFU production from pre and post control and diabetic QQc KSLs. (C): The frequency of DEPC-CFU production from pre and post control and diabetic QQc KSLs. (D): The percent of PEPC-CFU and DEPC-CFU from total EPC-CFU. (A,B,C): n.s, not significant; *p<0.05; **p<0.01. n=3 dishes per group x 4 trials.

Abbreviations: CFU, colony forming unit; QQc, KSL, c-kit^+, Sca-1^+, lin^- cells; QQc = quality and quantity culture; PEPC-CFU = primitive endothelial progenitor cell; DEPC-CFU = definitive endothelial progenitor cell.

**Figure 2.** QQc restores tubular formation of diabetic progenitor cells (A): Representative features of tube formation assay of HUVECs by co-culturing with presence or absence of pre and post control and diabetic QQc cells (4x magnification). The ratio of HUVEC:KSL cells is 1x10^4:3x10^3 (10:3) (B): Graph of the numbers of tubules formed in each group. n=10 wells per group. *p<0.05; **p<0.01. Abbreviations: HUVEC = human umbilical vein endothelial cell; QQc = quality and quantity culture; FC = freshly isolated control cells; FD = freshly isolated diabetic cells; QD = post-QQc diabetic cells; HPF = high power field.

**Figure 3.** QQc enhances expansion of vasculogenic and wound healing factors. The total RNA were prepared from pre- and post-QQc cells from control and diabetic
mice KSL cells. The gene expression levels of proangiogenic growth factors were estimated by real-time PCR and the data were shown as the relative gene expression of the target genes versus 18S rRNA. The target genes of quantitative PCR were EGF, FGF-2, FGF-7, vWF, CD29 and Flk-1. The data are shown by means ± SD (n=4, *p < 0.05, **p < 0.01, ***p < 0.001). Abbreviations: vWF = von Willebrand Factor; Flk-1 = fetal liver kinase-1; QQc = quality and quantity control culture.

**Figure 4.** Post QQc adoptive diabetic progenitor cell therapy accelerates wound healing. (A): Representative images show wound healing in euglycemic mice treated with PBS, pre and post control and diabetic QQc cells. Wounds were photographed at the times indicated, from Day 0 to Day 21. (B): The graph shows the comparison of percent wound closure between PBS and FD treated group (left), QC and QD treated group (top right), QD and FD treated group (bottom right). (A,B): .*p<0.05; **p<0.01. Abbreviations: QQc = quality and quantity culture; PBS = phosphate-buffered saline; FC = freshly isolated control cells; FD = freshly isolated diabetic cells; QD = post-QQc diabetic cells; QC = post-QQc control cells.

**Figure 5.** Post QQc diabetic progenitor cell therapy enhances wound vascularization. (A): Representative immunohistochemistry staining of CD-31 to evaluate vascular density in the wound of PBS, FC, FD, QD treated groups at Day21. (x40) (B): The graph shows the CD31 positive vessels per HPF at Day 7, 14 and 21. Left graph shows the comparison between QD vs FC, FD, PBS treated group. Right graph shows the comparison between QC and QD treated group.
*p<0.05; n.s, not significant. (C) Representative immunohistochemistry double staining of GFP (brown) and vWF (red). The arrow points at the vessels with positive double staining. x60 scale bar = 20µm. The staining demonstrates that GFP/vWF double stained vessels are only observed in the post QQc control and diabetic cell treated wounds. GFP positive cells observed more in the QC treated group. Abbreviations: QQc = quality and quantity culture; PBS = phosphate-buffered saline; FC = freshly isolated control cells; FD = freshly isolated diabetic cells; QD = post-QQc diabetic cells; QC = post-QQc control cells.

**Figure 6.** Post-QQc diabetic progenitor cell therapy enhances the percentage of mature collagen in the wound. (A): Representative Van Gieson’s staining demonstrating mature collagen staining in PBS, FC, FD, and QD treated groups at Day21. (x10) (B): The graph shows the percentage of mature collagen in wounds treated with PBS, FC, FD, and QD cells at Day21. **p<0.01. Abbreviations: QQc = quality and quantity culture; PBS = phosphate-buffered saline; FC = freshly isolated control cells; FD = freshly isolated diabetic cells; QD = post-QQc diabetic cells.

**Figure 7.** Post-QQc diabetic progenitor cell therapy increases cellular proliferation in the wound. The left and middle graph shows the number of PCNA positive cells in the wound per HPF at Day 7, Day 14, and Day21 in the following treated groups: PBS vs. FC (left), QD vs. FC (middle). The right bar graph shows the number of PCNA positive cells in the wound per HPF at day 21. *p<0.05. Abbreviations: QQc = quality and quantity culture; PCNA = proliferating cell
nuclear antigen; PBS = phosphate-buffered saline; FC = freshly isolated control cells; FD = freshly isolated diabetic cells; QD = post-QQc diabetic cells; HPF = high power field.

Figure 8. *Efficacy of Post-QQc Db progenitor cell therapy is deteriorated by Diabetic condition.* (A): Representative images show wound healing in STZ induced diabetic mice treated with PBS, pre and post control and diabetic QQc cells. Wounds were photographed at the times indicated, from Day 0 to Day 21. (B): The graph shows the comparison of percent wound closure between QC vs FC vs PBS treated group. QC indicated significant percent wound closure compared to FC and PBS at day 14 (QC: 73.60% ± 3.69 vs FC: 55.02% ± 3.61; vs PBS: 58.98% ± 5.86; p<0.05) and day 21 (QC: 96.34% ± 1.52 vs FC: 82.29% ± 4.72 vs PBS: 84.01% ± 2.28; p<0.05). The wound closure between FC and PBS were similar without any significance. (C) The graph shows the comparison of percent wound closure between QD vs FD vs PBS treated group. There was no significant difference between the three compared groups at all time points. *p<0.05. Abbreviations: QQc = quality and quantity culture; PBS = phosphate-buffered saline; FC = freshly isolated control cells; FD = freshly isolated diabetic cells; QD = post-QQc diabetic cells; QC = post-QQc control cells.
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256x132mm (300 x 300 DPI)
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272x176mm (300 x 300 DPI)
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288x110mm (300 x 300 DPI)
Efficacy of Post-QQc Db progenitor cell therapy is deteriorated by Diabetic condition.

310x186mm (300 x 300 DPI)