treatment with the FOXP3 Fix/Perm Buffer Set (BioLegend), cells underwent intranuclear staining with Foxp3-FITC. The intracellular or intranuclear staining was performed, according to the supplemental protocol for each buffer. The cellular frequency of CD4+/INF-γ+/IL4−, CD4+/INF-γ−/IL4+, or CD4+/CD25+/Foxp3+ in CD4+ helper lymphocyte was evaluated as that of Th1, Th2, or regulatory T cell.

3. Flow cytometry analysis: The scatter diagram of each PBMNC or the QQc cell (QQMNC) population in an individual, was gated into three cell sized populations of lymphocyte, monocyte, and the larger cell. The % positivity of a hematopoietic cell population per each gate in PBMNCs or QQMNCs, was evaluated, and then calculated to that in the whole cells of the three gates. The ratio of the % positivity in the whole cells of QQMNCs to that in PBMNCs was further calculated for each cell population. Similarly, the % positivity of each helper T subset (Th1, Th2, or regulatory T cell) was calculated in CD4+ T cells of the three gates of PBMNCs or QQMNCs; the ratio of the % positivity in CD4+ T cells of QQMNCs to that in PBMNCs was calculated.

Quantitative real-time PCR *in vitro.*

Using Trizol (Invitrogen), total RNA was isolated from PBMNCs or QQMNCs. Contaminated genomic DNA was digested by DNase I treatment (Invitrogen) at 37°C for 15 min. DNase I-treated total RNA was purified by phenol extraction and ethanol precipitation. One hundred ng of purified total RNA was used for cDNA synthesis with SuperScript VILO cDNA synthesis kit (Invitrogen). cDNA mixture was diluted by 10 fold after first-strand cDNA synthesis. Using ABI Prism 7700 (Applied Biosystems), quantitative real-time PCR (qRT-PCR) for diluted cDNA was performed with EagleTaq Master Mix (Roche), 0.3 μM of forward and reverse primers used for cDNA amplification, and 0.25 μM of probe (Sigma Aldrich), according to the manufacturer's protocol. The relative mRNA expression was calculated by ΔΔCt method with normalization against human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH). All primers and probes used were listed in **TABLE 7**.

***In vitro* angio-vasculogenic assay using Matrigel.**

As previously reported,[12] PBMNCs and QQMNCs were respectively incubated in 500 μL of 2% FBS/EBM-2 with 20 μg/mL of acetylated low density lipoprotein, labeled with 1,1'-dioctadecyl–3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (acLDL-DiI) (4×10^4 cells/500 μL) in a 1.5 mL tube for 30 min at 37°C in a CO_2 incubator. Following centrifugation at 400 *g* for 10 min at 4°C, and aspirating the supernatant, the cell pellets were washed by 1 mL PBS and suspended

with EBM-2/2%FBS (1.0×10^3 cells/50 μL). The labeled cells were resuspended together with HUVECs (EPCs : HUVECs = 1 x 10^3 : 1.5x10^4 in 100 μL of 2% FBS/EBM-2). The mixed cell suspension was incubated at 37°C in a water bath, and applied at 100 μL each onto pre-incubated Matrigel (BD Falcon)(50 μL/well) in each 96 well plate (BD Falcon). After incubation for 12 h, the numbers of closed areas formed by HUVECs were counted using Photoshop software in the pictures taken by phase-contrast light microscopy (x 2 HPF)(Eclipse TE300, Nikon). Furthermore, acLDL-DiI labeled PBMNCs or QQMNCs incorporated into tube, were also counted using Image J software in the pictures (x 4 HPF) taken by a fluorescence microscope (IX70, Olympus). The tube and cellular numbers were counted independently by two blinded investigators.

***In vivo* assessment of blood flow, histology and gene expression in murine ischemic hindlimb model transplanted PBMNCs or QQMNCs.**

1. Guideline for animal experiment: All animal studies conformed to national and institutional guidelines. The protocols were approved by the guidelines of the Institutional Animal Care and Use Committee of the Isehara Campus, Tokai University School of Medicine, based on Guide for the Care and Use of Laboratory Animals, National Research Council. The experimental animal protocols for making ischemic models and Laser Doppler Perfusion Imaging (Moor Instrument) are performed under adequate anesthetization by 1.5 to 2.0% isoflurane (Dainippon Sumitomo Pharma Co.) to minimize pain to mice by regarding the 3R's (replacement, reduction and refinement). Following surgery, the mice were subcutaneously injected with buprenorphine (Repetan, 0.1 mg/kg body weight; Otsuka Pharmaceutical Co.) once a day for 3 days to relieve pain or discomfort. At sacrifice, pentobarbital sodium (Somnopentyl, 60 to 70 mg/kg body weight; Kyouritu Seiyaku Co.) was intraperitoneally injected.

2. Making ischemic hindlimb model and cell transplantation: Eight- to ten-week-old male BALB/c nu/nu mice (CAnN.Cg-Foxn1^{nu}/CrlCrljCharles River, Japan) were used, as reported elsewhere.[26] The proximal portion of the left femoral artery including the superficial and the deep branch was suture-ligated, and the proximal and distal portions of the saphenous artery were occluded with a bipolar forcep electric coagulator (MERA N3-14, Senko Medical Instrument Mfg Co.). The overlying skin was closed with 4-0 silk suture. The next day, the cells were suspended in IMDM medium, and intramuscularly injected into ischemic hindlimb.

The cell injection sites and the doses for assays were as follows: each one site of anterior tibial muscle (ATM) and gastrocunemius muscle (GCM) for blood flow analysis and histology, i.e., HE

staining, Azan staining and iNOS immunohistochemistry (5.0 x 10^3 cells/20 μL/site: total 1x10^4 cells/mouse), two sites of ATM for qRT-PCR (5.0 x 10^3 cells/20 μL/site: total 1x10^4 cells/mouse), or for histological assessment of vasculogenesis (1.0 x 10^5 cells/20 μL/ site: total 2 x 10^5 cells/mouse).

3. Assessment of blood flow: Laser Doppler Perfusion Imaging was used to record serial blood flow measurements for three weeks after surgery; these data were analyzed using Moor ldi Main software (Laser Doppler Imager ver 5.2, Moor Instrument). The blood flow in toe region of interest (ROI) of ischemic and contralateral limbs per mouse was measured by Laser Doppler Perfusion Imaging; the blood flow ratio of ischemic versus contralateral hindlimb was calculated. The mice with toe necrosis or limb salvage were only involved in the calculation, but not those with foot necrosis or autoamputation.

4. Tissue preparation of histochemical assessment: Three weeks after surgery, forty μL of Isolectin B4-FITC (Fluorescein Griffonia Simplicifolia Lectin I, Isolectin B4, Vector Lab) was injected into tail vein using a insulin syringe, and then 20 min later, the mice were sacrificed under the adequate anesthetization. Immediately after sacrifice, the mice were perfused with 20 mL of PBS and then with the equivalent volume of 4% paraformaldehyde/PBS by heart puncture. Subsequently, hindlimbs were excised, and incubated in 4% paraformaldehyde/PBS at 4°C overnight. Thereafter, the ATMs were embedded into paraffin for the tissue samples of QQMNC transplantation (Tx) and PBMNCTx. Alternatively, the tissues were coated with O.C.T. compound (Tissue-Tek), immediately frozen in liquid nitrogen, and refrigerated until use at -80°C for those of QQMNCTx, GmCD34Tx and eEPC Tx. Cross sectional tissue samples with the thickness at 6 to 8 μm for paraffin section or at 10 μm for frozen section, were sliced from the tissue blocks of the muscles, and then subjected to the assessments, described below.

5. Microvasculartur and pericyte recruitment: For smooth muscle α actin (SM α actin) staining, the paraffin tissue sections after deparaffinization or the frozen ones were washed with PBS, blocked with 10% goat serum for 30 min at room temperature (RT), then, incubated with Cy3- conjugated anti- SM α actin antibody (Sigma-Aldrich) pre-diluted (1 : 200 ratio) with 1% BSA/PBS for 2 h at RT. After washing with PBS, the sections were mounted with 1.25% (w/v) DABCO (Sigma-Aldrich)/90% (v/v) glycerol /10% (v/v) PBS, then observed under fluorescent microscopy (Biorevo, Keyence). The same protocol without using the antibody, was performed as the negative control. Also, the negative control for Isolectin B4-FITC staining was in advance, prepared from the mice without *in vivo* injecting Isolectin B4-FITC into tail vein.

Using a software (VH analyzer, Keyence), microvascular densities were evaluated by counting

the microvessels stained with Isolectin B4-FITC. Simultaneously, pericyte recruitment to the vasculartures was evaluated by counting the SM α actin-positive microvessels.

6. Myogenesis and interstitial fibrosis: Centrally nucleated muscle fibers stained with hematoxylin-eosin (HE) staining were photographed with an automatic research photomicroscope (AX80, Olympus), and were then counted as myoblasts[27] by VH analyzer. Limb interstitial fibrosis detected by Azan staining was morphometrically assessed by the same photomicroscope and software.[28, 29]

7. Tissue inflammation: The paraffin tissue sections were deparaffinized; autoclaved in citrate buffer at 121°C for 10 min to retrieve the target antigen; incubated with the primary antibody of rabbit polyclonal anti-iNOS antibody (Abcam) pre-diluted with 1% BSA/PBS (1:100 ratio) at 4°C overnight (**TABLE 8**). Thereafter, the samples were treated with 0.3%H_2O_2/Methanol and Histofine Simple Stain Mouse MAX PO(R)(Nichirei Bioscience Inc.), and then dyed with 3,3'-Diaminobenzidine, tetrahydrochloride (DAB)(DOJINDO). Further, the samples were stained with hematoxylin, dehydrated, and then mounted with malinol. The frozen tissue sections were also stained with the anti-iNOS antibody in the same way, after similar autoclave and subsequent treatment with 0.5% TritonX-100/PBS. The control samples were prepared under the same procedure, using Rabbit Immunoglobulin Fraction (DAKO).

8. Vasculogenesis by transplanated cells: The cross sectional tissues at 10 μm, after washing with PBS, were microwaved in Target Retrieval Solution (DAKO) diluted with distilled water (1 : 10 ratio) at 98°C for 10 min. Then, after treatment with STREPTAVIDIN/BIOTIN BLOCKING KIT (Vector Lab) to block endogenous biotin, the sections were incubated with 5% goat serum/1% BSA/PBS for 30 min at RT. For the preparation of primary antibody to human CD31, mouse anti-human CD31 antibody and biotinylated goat anti-mouse IgG (Fitzgerald) were respectively diluted in 1% BSA/PBS (1 : 8 and 1 : 48 ratio), i.e., adjusted to the concentration of 25 μg/mL and 60 μg/mL. The pre-diluted reagents were mixed in the equal volume and reacted at RT for 1 h. The pre-reacted reagent was further mixed with mouse serum (Rockland) (2 : 1 ratio) and incubated at RT for 1 h. The primary antibody reagent for human CD31 was incubated with the tissue sections at 4°C overnight. The sections were washed with PBS and subsequently incubated with streptavidin, Alexa Fluor 594 conjugate pre-diluted in 1% BSA/PBS (1 : 90 ratio) at RT for 1 h. The tissue sections were washed with PBS and finally mounted with 1 □M TOTO-3 iodide (Invitrogen) in 1.25% (w/v) DABCO/90% (v/v) glycerol /10% (v/v) PBS. The tissue specimens were observed by a confocal laser-scanning microscope (LSM510META, Carl Zeiss). Mouse anti- human CD31 antibody and the reagents were detailed in **TABLE 9**. The acquired

images at 0.8 μm z interval (11 sliced images by 10 intervals at total 8 μm thickness) were reconstructed 3D structures using the function of 3D spectrum analysis. Further, to quantify 'vasculogenic properties' in their images, the surface of a volume object in the ROI, was visualized, using an Imaris iso-surface function of 4D Image analysis software (Imaris 6.2.0, Carl Zeiss); murine vessels stained with Isolectin B4-FITC (green) and transplanted cell derived microvessels stained with Alexa 594 conjugated human specific anti- CD31 antibody (red). The values of microvascular density in 2D image and % volume per image cube in 3D were respectively calculated for the green or red coloured microvessels.

9. Murine qRT-PCR of ischemic hindlimb: Mice were sacrificed under adequate anesthetization day 6 after cell Tx: day 7 after surgery. GCMs of ischemic hindlimbs were harvested for total RNA isolation; the mice were perfused with 20 mL of PBS by heart puncture to remove circulating blood; GCMs were resected out and incubated into 1 mL of RNA later at 4°C overnight. After homoginization of GCMs with 1 mL of Trizol (Invitrogen), total RNA was isolated and genomic DNA was digested by DNase I treatment (Invitrogen) at 37°C for 15 min. DNase I-treated total RNA was purified by phenol extraction and ethanol precipitation. Two μg of purified total RNA was used for cDNA synthesis with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA mixture was sequentially diluted by 20 to 160 fold with MiliiQ water after first-strand cDNA synthesis. Using ABI Prism 7700 (Applied Biosystems), TaqMan gene expression assays for diluted cDNA were performed with TaqMan Fast Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol. The amplification of cDNA was as follows: denaturation at 95°C for 3 sec, annealing/extension at 62°C for 30 sec, 40 cycles. The relative mRNA expression was calculated by ΔΔCt method with normalization against mouse 18S rRNA. All primers and TaqMan probes used were listed in **TABLE 10**.

Statistical analysis.

Prism5 software (GraphPad Inc.) was used to conduct all statistical analyses. Wilcoxon Signed rank-test or linear regression analysis was used to analyse quantitative variation or correlation of cells and EPC-CFUs between PBMNC and QQMNC in each individual. To assess the variation of each hematopietic cell or helper T subset through QQc, Wilcoxon Signed rank-test was also applied for comparison of the ratio of % cell positivity in the whole cells or CD4+ T cells of QQMNC to that of PBMNC in each individual. Mann Whitney U-test and by Kruskal-Wallis test were applied to compare the data between two groups and among three to four groups. The

395 experiment to assess angio-vasculogenic properties by transplanted human cells using confocal
396 fluorescent microscope were performed simultaneously on the whole groups: IMDM control,
397 PBMNCTx, eEPCTx, QQMNCTx,and GmCD34Tx. The data were separately analysed in the
398 following comparisons: QQMNCTx versus PBMNCTx or IMDM control; QQMNCTx versus
399 GmCD34Tx, eEPCTx or IMDM control. Especially, vasculogenic properties by transplanted
400 human cells were compared in the groups excluded IMDM control. qRT-PCR assay to evaluate
401 mRNA expression in ischemic hindlimbs was also implemented simultaneously on the whole
402 groups; the data were analysed and compared in the same manner as those in the former
403 experiment. Furthermore, in the assay, the ratio of relative mRNA expression of ischemic- to
404 contralateral (healthy) hindlimbs in IMDM control, was compared by Mann Whitney U-test to
405 confirm the influence of hindlimb ischemia. In histological assays, two tissue sections per mouse
406 were prepared, and four to six fields per tissue section were evaluated. Probability values of $P <$
407 0.05 were deemed statistically significant. All values were expressed as mean ± SE.
408

RESULTS

Decrease in cell counts in QQMNCs.

The fold increase of QQMNCs to PBMNCs per well declined in the whole subjects with the average of 0.54 fold (**TABLE 11**). The calculated total QQMNCs derived from 100 mL PB decreased from original cells, 831.3 ± 75.3, to 399.2 ± 43.1 x 10^5, averagely by 0.48 fold decrease. (**Figure 1a, TABLE 11**).

Interestingly, the fold increase of QQMNCs per well exhibited the negative correlation with total cells of PBMNCs from 100 mL PB in the healthy subjects (**Figure 1b- left**). Taken together, these findings indicate that even using higher PBMNC densities per 100 mL PB resulted in constant relative yields of QQMNCs per the PB volume (**Figure 1b- right**).

Increase of colony forming EPCs in QQMNCs.

To assess vasculogenic activities between PBMNCs and QQMNCs, EPC-CFA was used to monitor two different types of EPC-colony forming units (EPC-CFUs), pEPC-CFUs and dEPC-CFUs, which comprised small cells and large cells, respectively. The pEPCs had high cell proliferation activity; in contrast, the dEPC had high vasculogenic potential.[12]

In *in vitro* assays, dEPCs had higher cell adhesion activity than did pEPCs, and dEPCs formed tube-like structures; additionally, dEPCs extensively formed blood vessel *de novo* following transplantation into ischemic hindlimbs of mice, but pEPCs did less.

Therefore, pEPCs derive from relatively immature and highly proliferative EPCs, while dEPCs are relatively mature, differentiated, and able to promote EPC-mediated cell functions required for vasculogenesis.

The pEPC and dEPC -colony-forming cells each constituted a small proportion of the cells in primary PBMNC populations, 8 x10^{-4} % and 3.5 x10^{-4} % respectively (**Figure 1c, d- left, TABLE11**). These colony assays demonstrated that QQMNCs have much greater vasculogenic potential than do PBMNCs. Following QQc, the frequency of total EPC-CFUs from 2 x 10^5 cells per dish in QQMNCs was significantly enhanced 13.7 fold of that in PBMNCs. Especially, the frequency of dEPC-CFUs increased 41.4 fold of that in PBMNCs.

The present data indicate that the vascular regenerative capacity of QQMNCs was superior to that of PBMNCs (**Figure 1d- left**).

Although QQc reduced the count of cells in each individual culture, dEPC colony forming cells, and total EPC colony forming cells in QQMNCs derived from the even blood volume were

enriched 19.0 and 6.2 fold relative to those in PBMNCs (**Figure 1d- middle, TABLE11**). Not only were QQc enriched with dEPC colony forming cells, the EPC colony forming cells in QQMNCs had a 2.7 fold greater differentiation potential than those in PBMNCs, considering the percentage of dEPC-CFUs to total EPC-CFUs per dish in PBMNCs and QQMNCs (**Figure 1d right**).

We also used a linear regression analysis to compare between PBMNCs and QQMNCs with regard to both the quantity and quality of EPC-CFUs (**Figure 1e**).

Notably, dEPC-CFU and total EPC-CFU counts in QQMNCs were positively correlated with pEPC-CFU counts in PBMNCs, but pEPC-CFU counts in QQMNCs were not (**Figure 1e- left**). In contrast, neither pEPC-CFU counts nor total EPC-CFU counts in QQMNCs were positively correlated with dEPC-CFU counts in PBMNCs (**Figure 1e- middle**).

In summary, the frequency of EPC colony forming cells in QQMNCs depended on that in PBMNCs (**Figure 1e- right**). Importantly, the frequency of dEPC colony forming cells in QQMNCs was positively correlated with that of pEPC colony forming cells in PBMNCs; these correlations indicate that the differentiation during QQc of pEPC colony forming cells in PBMNCs, contributed to formation of dEPC colony forming cells in QQMNCs.

Collectively, assays of colony formation demonstrated that the quantitative and qualitative vasculogenic potential of QQMNCs is drastically enhanced relative to that of PBMNCs, when evaluated even by blood volume.

Moreover, we assessed the functional relationship between CD34+ cells and CD34+ cell depleted MNCs (CD34–MNCs) in PBMNCs during QQc (**Figure 1f**). CD34–MNCs after QQc (QQ-34–MNCs) did not yield EPC colony, while CD34+ cells after QQc (QQ-34+ cells) encompassed pEPC colony forming cells. This means that EPC colony forming cells were basically derived from CD34+ cells. When CD34+ cells were co-cultured with CD34–MNCs (QQ-34+/34–MNC) at a 1 : 500 ratio mimicking the proportion of CD34+ cell involved in naïve PBMNC, the dEPC population expanded significantly relative to the QQ-34+ population, even though the original CD34+ cell count was equal. The result suggests that CD34–MNCs included some cell population to accelerate EPC expansion and differentiation in CD34+ cells through QQc.

Cell population transition in QQMNCs.

Based on microscopy and fluorescent cell sorting, large cells were proportionally more common in QQMNC samples than in PBMNC samples (**Figure 2a, b**). In flow cytometry, the proportion

of each positive cell involved in the whole cells of (A), (B) and (C) gates separated with red lines was estimated (**Figure 2b**).

QQMNCs exhibited the enrichment of CD34+ and CD133+ stem cell populations, compared to PBMNCs: 5.97 fold in CD34+ cells; 3.59 in CD133+cells (**Figure 2c, TABLE 12**).

In contrast, the proportion of many hematopoietic cell types including B lymphoid cells (CD19+ cells), pro-inflammatory monocytes/macrophages (chemokine receptor 2+ cells= CCR2+ cells), and NK cells (CD56+ cells) was lower in QQMNC samples than in PBMNC samples.

The proportion of endothelial lineage cells was slightly increased in CD105, while not changed in CD31 and slightly decreased in vWF. Although not significant, the proportion was slightly increased in CD146, while decreased in VEGFR-2.

In the T-lymphoid cell population, and particularly in the angiogenic T cells subpopulation, CXCR-4+/CD31+/CD3+ cells[30] increased significantly following QQc. Notably, the proportion of anti-inflammatory M2 type (CD206) cells increased significantly 4.95 fold in the two categories of macrophages; inversely, the proportion of pro-inflammatory M1 type (CCR2) cells decreased significantly 0.01 fold.[31 32]

More interestingly, when activated T lymphocytes of QQMMC or PBMNC by phorbol 12-myristate 13-acetate (PMA) and ionomycin, the proportion of Th1 cells (CD4+/INF-γ+□□□−) in helper T (CD4+) cells in QQMNC declined 0.55 fold of that in PBMNC. In contrast, the proportions of Th2 cells (CD4+/INF-γ/IL-4+) and regulatory T cells (CD4+/CD25+/Foxp3+) increased 6.04 and 5.82 fold (**Figure 2d, TABLE 13**).

These data indicate that QQc conditions specifically selected for and/or promote proliferation of stem/progenitor cell populations of EPCs, anti-inflammatory and angiogenic monocytes/T-lymphocytes in primary PBMNC cultures.

Enhanced expression of angiogenic and anti-inflammatory factors in QQMNCs.

Expression of genes encoding five angiogenic and myogenic cytokines (VEGF-B, angiopoietin-1 (Ang-1), leptin, IL-8, IL-10, and insulin-like growth factor-1 (IGF-1) was much higher in QQMNCs than in PBMNCs; the fold increases in QQMNCs versus PBMNCs were 4.2 for VEGF-B, 2.4 for Ang-1, 35.9 for leptin, 6.3 for IL-8, 5.4 for IL-10, 21.2 for IGF-1 (**Figure 3a, b**). While each factor is pro-angiogenic, VEGF-B and Ang-1 also induce vascular maturation, [33, 34] and IGF-1 is myogenic. [35]

Additionally, expression of the gene encoding IL-1β, a pro-inflammatory cytokine, in QQMNCs decreased by 0.23 fold of that in PBMNCs, with the increase of the gene encoding the

anti-inflammatory IL-10 (**Figure 3c**). These expressional profiles indicate that QQMNCs preferred to arrange anti-inflammatory environment in injured tissue. Furthermore, the expression of genes encoding to matrix metalloproteinases (MMPs) of MMP-2 and MMP-9 was significantly higher in QQMNCs than in PBMNCs; the fold increases were 22.1 for MMP-2and 189.4 for MMP-9 (**Figure 3d**). MMP-2 and MMP-9 have anti-fibrotic activity that plays critical roles during neovascularization and tissue remodeling.[36, 37]

QQMNCs promote angiogenesis *in vitro*.

Using an *in vitro* Matrigel assay, we found that QQMNCs promoted tube formation of co-cultured human umbilical vein endothelial cells (HUVECs) for 12 h, but PBMNCs did not (Tube counts/x2HPF= 63.3 ± 1.43 for HUVEC QQMNC vs. 55.1 ± 1.45 for HUVEC + PBMNC or 55.3 ± 1.39 for HUVEC alone) (**Figure 4a, b**).

Moreover, QQMNCs were readily incorporated into the tubes formed by HUVECs; in contrast, PBMNCs were rarely incorporated into such tubes (incorporated DiI-uptaking cells in tubes/x4HPF□ 38.5 ± 8.30 for QQMNC vs. 8.72 ± 1.89 for PBMNC) (**Figure 4c, d**).

These findings indicate that *in vitro* QQMNCs had more angiogenic and EPC incorporating activity than did PBMNCs.

Physiological evidence of therapeutic vasculogenesis in QQMNCs *in vivo* in ischemic hindlimb.

We investigated the potential of QQMNCs to treat ischemia, using a mouse ischemic hindlimb model; the effect was evaluated by blood flow measurement for 21 days after ischemic surgery. We compared the effect of QQMNCTx with that of no-cell Tx (IMDM medium injected mice: IMDM control) or PBMNCTx **(Figure 5a).**

For each mouse, we measured blood flow in the ischemic and contralateral hindlimb; we then calculated the ratio of ischemic blood flow to contralateral flow for the QQMNCTx, PBMNCTx, and control groups: these values were % blood flow ratio = at day 14, 48.8 ± 4.25, 32.3 ± 5.53, and 28.9 ± 4.52: at day 21, 50.7 ± 5.50, 28.1 ± 6.19, and 27.4 ± 6.98, respectively. The ratio in the QQMNCTx was significantly higher after day 14 than that in PBMNCTx and control, indicating that QQMNCTx recovered ischemic blood flow greater than the others.

Comparing to other EPC transplantations (**Figure 5b**), QQMNCTx improved the blood flow ratio earlier than GmCD34Tx, eEPCTx and control: these values in QQMNCTx, GmCD34Tx,

eEPCTx, and control were % blood flow ratio = at day 14, 52.8 ± 6.13, 37.6 ± 3.48, 24.8 ± 2.83, and 24.1 ± 5.13; at day 21, 62.1 ± 6.61, 53.9 ± 6.18, 31.8 ± 1.57, and 27.7 ± 3.86, respectively.

The percentages of autoamputation in the QQMNCTx, PBMNCTx, and control groups were 4.3%, 9.5%, and 15.8%, respectively; conversely, the percentages of limb salvage were 21.7%, 9.5%, and 10.5%, respectively. These findings indicate that QQMNCTx potentiated physiological recovery in hindlimb ischemia more so than did PBMNCTx or control (**Figure 5c**).

When compared to GmCD34Tx and eEPCTx, the percentages of autoamputation in QQMNCTx, GmCD34Tx, and eEPCTx were 4.5, 26.3, and 37.5, respectively. In contrast, the percentages of limb salvage in QQMNCTx, GmCD34Tx, and eEPCTx were 27.3%, 21.1%, and 25.0%, respectively.

Regarding these findings, the earlier blood flow recovery by QQMNCTx mighore favorably rescue ischemic foot from injury, compared to other treatments (**Figure 5d**).

Histological evidence of tissue regeneration by QQMNC transplantation.

Angiogenesis and arteriogenesis

For each animal, we used histological methods to measure Isolectin B4–□□□□ □□□□□□□ microvessel density and assess angiogenic capacity in the ATM of ischemic hindlimbs; the mean densities (microvessel counm^2) for the QQMNCTx, PBMNCTx, and control groups were 400.7 ± 37.9, 118.9 ± 20.1, and 98.7 ± 15.8, respectively (**Figure 6a, b**).

We also evaluated pericyte recruited (SMα actin+) microvessel densitiy in the ATM to assess arteriogenesis for vascular maturation: the mean densities (pericyte recruited microvessel counts/mm^2) for the QQMNCTx, PBMNCTx, and control groups were 38.7 ± 5.5, 19.8 ± 4.3, and 15.0 ± 2.7, respectively (**Figure 6a, c**).

Likewise, the mean microvessel densities (microvessel counts/mm^2) for the QQMNCTx, GmCD34Tx, eEPCTx and control groups were 510.7 ± 30.0, 430.6 ± 29.8, 347.9 ± 36.5, and 210.5 ± 16.8 respectively (**Figure 6d, e**). The mean pericyte recruited microvessel densities (pericyte recruited microvessel counts/mm^2) for the QQMNCTx, GmCD34Tx, eEPCTx and control groups were 42.6 ± 4.3, 39.5 ± 3.8, 29.8 ± 2.0, and 23.2 ± 2.9 respectively (**Figure 6d, f**). These findings demonstrate that QQMNCTx promoted angiogenesis and arteriogenesis for vascular maturation.

Vasculogeensis

We performed *in vivo* experimenents to assess vasculogenic properties of transplanted cells (2 x10^5 cells/mouse): to investigate whether and to what extent transplanted cells differentiate into

573 endothelial cell forming vascular structure in the host tissue, using confocal fluorescenicroscope
574 (**Figure 7a, Online Supplemental Video-1**).
575 The immunohistochemical stainings demonstrated the significantly higher vasculogenic
576 microvessel counts in QQMNCTx muscles than those in PBMNCTx (**Figure 7b, c, d, TABLE**
577 **14**).
578 The average vasculogenic microvessel densities in 2D image (vasculogenic microvessel
579 counts/mm^2) for the QQMNCTx and PBMNCTx groups, were 811.6 ± 178.6 and 202.9 ± 97.3
580 (**Figure 7c**); the percentages of vasculogenic microvessel volume per image cube for the
581 respective groups, were 0.76 ± 0.17% and 0.05 ± 0.04% (**Figure 7d**).
582 The potential of QQMNCTx was also markedly superior to that of eEPCTx, while it was equal to
583 or greater than that of GmCD34Tx. The average vasculogenic microvessel densities
584 (vasculogenic microvessel counts/mm^2) for the GmCD34Tx and eEPCTx groups, were 662.2 ±
585 98.6 and 203.8 ± 50.8, respectively (**Figure 7e, f, TABLE 14**); the percentages of vasculogenic
586 microvessel volume per image cube for the groups, were 0.53 ± 0.15% and 0.06 ± 0.02% (**Figure**
587 **7e, g, TABLE 14**).
588 Similarly, the angiogenic properties of transplanted cells, here evaluated by the confocal
589 microscopic analysis, exhibited the compatible feature with those in the aforementioned
590 experiments of the low dose cellular Tx (1 x10^4 cells/mouse).
591 The findings indicate that transplanted cells of QQMNC as well as GmCD34, exerted
592 vasculogenic properties in ischemic hindlimb, superior to those of PBMNC or eEPC.
593 ***Myogenesis***
594 Muscle fibers with centrally located nuclei indicate myogenesis mediated by fusion of myoblasts
595 in ATM of ischemic hindlimbs; therefore, we determined that the average densities of such
596 regenerating muscle fibers (regenerating muscle fibers/mm^2) for the QQMNCTx, PBMNCTx,
597 and control groups, were 775.6 ± 113.3, 424.2 ± 47.12, and 398.6 ± 48.42, respectively (**Figure**
598 **8a, b**).
599 In the respective experiments to compare myogenesis among the QQMNCTx, GmCD34Tx,
600 eEPCTx and control groups, we also determined that the average densities of regenerating muscle
601 fibers (regenerating muscle fiber counts/mm^2) for the respective groups, were 790.7 ± 110.1,
602 811.8 ± 63.6, 553.6 ± 69.25, and 209.4 ± 44.01, respectively (**Figure 8c, d**). These findings
603 indicate that QQMNCTx potentiated myogenesis greater than PBMNCTx and control, or equal to
604 GmCD34Tx in ischemic hindlimbs, although did not compared to eEPCTx.
605 **Histological evidence of anti-fibrosis and anti-inflammation in QQMNCs in hindlimb**

ischemia.

To evaluate anti-inflammatory and anti-fibrotic potential of QQMNCTx, fibrotic area in ischemic ATM was detected by Azan staining day 21 after treatment. The mean fibrotic area (% fibrotic area/x 40 HPF) for the QQMNCTx, PBMNCTx, and control groups, were 2.78 ± 0.61, 8.41 ± 1.51, and 11.94 ± 3.59. These findings indicate that QQMNCTx exerted greater anti-fibrotic effects than did PBMNCTx (**Figure 9a, b**).

In the respective comparison among the QQMNCTx, GmCD34Tx, eEPCTx and control groups, the mean fibrotic areas (%fibrotic area/x 40 HPF) for each group, were 5.89 ± 1.12, 8.06 ± 1.05, 11.07 ± 1.19, and 17.04 ± 1.75 (**Figure 9c, d**). QQMNCTx as well as GmCD34Tx featured superior anti-fibrotic effects to did control, and further exerted the greater effects than did eEPCTx.

We performed immunohistocehmistry of inducible nitric oxide synthase (iNOS) in ischemic ATM to assess inflammation. The mean iNOS expressing areas (%iNOS expressing area /x 20 HPF) for the QQMNCTx, PBMNCTx, and control groups, were 3.16 ± 0.58, 6.26 ± 0.89, 21.31 ± 2.26 (**Figure 10a, b**). These findings indicate that QQMNCTx inhibited inflammation more markedly than did PBMNCTx.

In the respective comparison among the QQMNCTx, GmCD34Tx, eEPCTx and control groups, the mean iNOS expressing areas (%iNOS expressing area /x 20 HPF) for the respective groups, were 4.97 ± 0.95, 6.07 ± 0.81, 9.75 ± 1.09, and 18.15 ± 1.44 (**Figure 10c, d**). In the same manner as anti-fibrotic aspects, QQMNCTx as well as GmCD34Tx featured superior anti-inflammation effects to did control, and further displayed the greater effects than did eEPCTx.

These findings indicate that QQMNCTx, similarly to GmCD34Tx, potentiated protective effects against inflammation and fibrosis in ischemic hindlimb.

qRT-PCR of gene expression for tissue regeneration in ischemic muscle post cell Tx.

qRT-PCR assay was implemented for the murine mRNA transcripts encoding following factors: IL-1β (pro-angiogenic as well as pro-inflammatory), TGF-β (anti-inflammatory)and IGF-1 (pro-angio-myogenic), or myogenic transcriptional factors: MyoD1 and myogenin.

In the comparison of the murine transcripts among the QQMNCTx, PBMNCTx, and control groups, gene expression of MyoD1, myogenin, and IGF-1, was significantly augmented by responding to QQMNCTx, but not to PBMNCTx.

637 Likewise, in the comparison among the QQMNCTx, GmCD34Tx and eEPCTx groups, gene
638 expression of MyoD1, myogenin, and IGF-1 was significantly enhanced by responding to
639 QQMNCTx, equally to GmCD34Tx, but not to eEPCTx.
640 The relative ratio of each gene expression in PBMNCTx, QQMNCTx, GmCD34Tx, and
641 eEPCTx versus that in control, was as follows: 1.22 ± 0.05, 2.58 ± 0.63, 2.94 ± 0.61 and 1.06 ±
642 0.08 in MyoD1; 1.90 ± 0.10, 2.94 ± 0.49, 2.69 ± 0.41, and 0.84 ± 0.06 in myogenin; 1.17 ± 0.11,
643 2.06 ± 0.40, 2.20 ± 0.56, and 1.00 ± 0.11 in IGF-1, respectively (**Figure 11a, b**).
644 The gene expression of TGF-βwas fairly promoted by responding to QQMNCTx, rather than to
645 PBMNCTx, and also enhanced equal to or higher than that in GmCD34Tx, although not in
646 eEPCTx. The relative ratio of TGF-βgene expression in PBMNCTx, QQMNCTx, GmCD34Tx,
647 and eEPCTx , control, was 3.14 ± 0.42, 4.35 ± 0.60, 3.52 ± 0.36, and 0.52 ± 0.04 (**Figure 11 a, b**).
648 Thus, gene expressions of the potent factors for angiogenesis, myogenesis, and anti-inflammation
649 reacting to QQMNCTx as well as GmCD34Tx, were similarly upregulated, when compared with
650 those to PBMNCTx or eEPCTx.
651 Above all, a gene expression encoding IGF-1 to QQMNCTx was unambiguously enhanced,
652 compared with that to the other Tx groups of PBMNCTx and eEPCTx. The findings correspond
653 with the histological results to show the multi-functional mechanisms of QQMNCTx superior to
654 those of PBMNCTx or eEPCTx.
655 Notably, Tx of all cell sources, more or less, upregulated gene expression of IL-1β, a
656 pro-inflammatory factor, while unpurified EPC Tx of QQMNC as well as PBMNC tended to
657 produce the transcript more drastically than the purified EPC Tx of GmCD34 or eEPC. The
658 relative ratio of IL-1βgene expression in QQMNCTx, GmCD34Tx, and eEPCTx versus that in
659 IMDM control, was 32.00 ± 12.74, 10.47 ± 2.27, and 19.53 ± 7.78, respectively.
660 In the present experiments, PBMNCTx, despite the fairly high expression of IL-1β, did not exert
661 angiogenic ability, whereas GmCD34Tx, even in the lesser expression, did favorably. –In
662 other words, IL-1β □□□□□□□□□□ in ischemic tissue responding to any cell Tx did not
663 seemingly contribute to angiogenesis for vascular regeneration.
664
665

DISCUSSION

Here, we developed and characterized QQMNCs, novel therapeutic cells; QQMNCs derived from PBMNCs that were subjected to QQc; this QQc promoted expansion of EPCs and adoption of regenerative phenotypes by macrophages and T lymphocytes. The QQc that we used to generate QQMNCs was based on a well-established QQc that increases the quality and quantity of EPCs from enriched EPC populations, such as CD34+ and CD133+ cells; this established QQc was used to generate therapeutic stem cells for cardiovascular regeneration in rat infarcted myocardia.[23]

We found here that the vasculogenic signaling condition of this QQc potentiated the vascular regeneration ability of naïve PBMNCs to produce QQMNCs. QQMNCs were superior to PBMNCs, and equal to or greater than G-CSF mobilized CD34+ cells, in terms of regeneration abilities; vascular regeneration (angiogenesis, arteriogenesis and vasculogenesis), myogenesis, anti-inflammation, and anti- fibrosis.

1) EPC expansion and differentiation in QQMNC

EPC-CFA of QQMNC demonstrated intensive expansion potential of colony forming EPCs, especially dEPCs (**Figure 1d**). The concept of colony forming EPCs was recently introduced.[12, 38] pEPCs in small-sized EPC colonies showed a higher rate of proliferation and a higher percentage of cells in S-phase, when compared to dEPCs in large-sized EPC colonies. In contrast, dEPCs had a significantly higher capacity for vasculogenic activity than did pEPCs; similarly, dEPCs also had a greater overall potential for cell adhesion and for formation of tube-like structures *in vitro*; importantly, dEPCs had a greater capacity than pEPCs to support *de novo* blood vessel formation *in vivo* following transplantation into ischemic models.

Therefore, pEPCs are defined as very immature and highly proliferative EPCs; in contrast, dEPCs are believed to derive from pEPCs and represent cells prone to differentiation and promotion of vasculogenesis. These dEPCs are proven to play a key role in vasculogenesis, and to be suitable for vascular regeneration therapy. In this regard, QQc promoted significant expansion and commitment of colony forming EPCs with vasculogenic potential.

To confirm colony forming EPC expansion in QQc of naïve PBMNCs, we evaluated EPC colony forming activity before and after QQc (**Figure 1e**). Total EPC-CFU count in QQMNC samples was correlated with that in PBMNC samples. For example, pEPC-CFU count in PBMNC samples correlated with dEPC-CFU count and with total EPC-CFU count in QQMNC

699 samples, but not with pEPC-CFU count in QQMNC samples. The dEPC-CFU count in PBMNC
700 samples did not correlate to any EPC-CFU count in QQMNC. These data indicate that signals in
701 QQc probably induced concurrent expansion and differentiation of pEPC-CFUs, and this
702 expansion and differentiation resulted in increase in the dEPC-CFU population instead of the
703 pEPC-CFU population. Consequently, the enhanced vasculogenic potential of post QQc cells,
704 compared with the precursor PBMNCs, explained the preferential vascular regeneration.
705
706 ***2) Cell populations in QQ Cultures***
707 The cell numbers of QQMNCs were on average about half of those in the respective
708 PBMNCs.
709 The cellular density of PBMNCs per blood volume is inversely correlated with the ratio of
710 cellular density per well between PBMNCs and QQMNCs. Therefore, the QQMNC numbers are
711 rather dependent of the original blood volume *per se* (**Figure 1b**).
712 From the view of cell populations, the decrease in total cell count is mainly derived from
713 significant reduction of B-lymphocytes (CD19+), NK cells (CD16+, CD56+), cytotoxic T cells
714 (CD8+) and pro-inflammatory monocytes/macrophages (CD14+, CCR2+).
715 In contrast, populations of progenitor cells (CD34+, CD133+) and of anti-inflammatory
716 monocytes/macrophages (CD206+) expanded greatly, but populations of endothelial cells
717 (CD105+, CD146+ and helper T cells (CD4+) expanded only moderately.
718 The increase in CD34+ or CD133+ cell populations indicates the expanded population
719 of immature EPCs. The increase in CD105+ or CD146+ cell populations was also indicative of
720 EPC expansion and differentiation; notably, differentiating EPCs express these markers.[39, 40]
721 The extent of the increase in CD206+ cells and of the decrease in CCR2+ cells
722 indicates the conversion of the monocyte/macrophage phenotype from M1 to M2 type.
723 Monocytes/macrophages differentiate towards a pro-inflammatory, classically activated M1 state
724 or toward an anti-inflammatory, alternatively activated M2 state according to different
725 environments and stimuli. M1 macrophages are induced by pro-inflammatory cytokines and
726 microbial products, such as INF-γ, TNF-α, and lipopolysaccharide (LPS); these macrophages are
727 mainly associated with pathologic inflammations. M2 macrophages are induced by
728 anti-inflammatory cytokines, such as IL-4, IL-13, and IL-10 to ameliorate type 1 inflammatory
729 responses and to control adaptive immunity. Further, their anti-inflammatory cytokines promote
730 and regulate type 2 immune responses, angiogenesis, and tissue repair. [41] In this regard,
731 monocyte/macrophages in QQMNCs mainly adopt angiogenic and anti-inflammatory

phenotypes, and are contributing to regenerative process in ischemic organs.

Among lymphocyte lineage cells, B-lymphocytes, NK cells, and cytotoxic T cells significantly decrease or fade away. Instead, helper T cells are the last surviving lymphocyte population in QQ cultures. The phenotype identification of CD4+ cells disclosed the significant increase in CD4+/CD25+/Foxp3+ regulatory T lymphocytes, and CD4+/IL4+ Th2 lymphocytes.

In recent years, the interaction between monocytes/macrophages and T lymphocytes have been investigated. IFN-γ produced by Th1 lymphocytes induce monocytes to become classical activated M1 macrophages, while IL-4, IL-13 and IL-10 that are produced by Th2 and regulatory T lymphocytes induce differentiation of regenerative M2 macrophages. IL-12 and IL-6 produced by M1 macrophages activate Th1 lymphocytes, while IL-10 and TGF-β□□□. [41 42] Therefore, the cell- cell interactions among M2 macrophages, Th2 and regulatory T cells are considered to accelerate the QQMNCs function as angiogenic and anti-inflammatory tools.

Of note, the majority of T lymphocytes in QQ cultures are CXCR-4+/CD31+/CD3+ cells. This population is called “angiogenic T cells” in the vascular biology field; these T cells deliver angiogenic cytokines in tissues for neovascularization.[30] The finding also encourages that the phenotype of T lymphocytes is conducible to angiogenic preference of QQMNCs.

Therefore, QQMNCs signal regenerative switches on PBMNCs not only by EPC expansion and differentiation, but also through collaborative M2 macrophage polarization and Th2 and regulatory T cell activation in QQ culture.

3) Factors expressed from QQMNCs

In order to further examine the vasculogenic potential of QQMNCs, qRT-PCR was used to determine gene expression profiles. In QQMNCs, the expression of mRNAs encoding anti-inflammatory and pro-angiogenic factors was enhanced, while that of mRNAs encoding pro-inflammatory cytokines declined (**Figure 3**).

The expression of mRNAs encoding pro-angiogenic cytokines/growth factors (e.g., IL-10,[43] leptin,[44, 45] IGF-1,[46] and IL-8[47]) was greatly elevated. Leptin[45] and IL-10[48] promote the vasculogenic and angiogenic potentials of EPCs and endothelial cells. IGF-1[49] and IL-8[50] are also related factors responsible for angiogenic properties of EPCs. IGF-1 also promotes muscle fiber regeneration[35] ; this phenomenon may be reflected in the findings of enhanced myogenesis following QQMNC transplantation. VEGF-B and angiopoietin-1, were upregulated in QQMNCs,

765 and they potentiate vascular survival and maturation relating to arteriogenesis induced by pericyte
766 recruitment. [33] [34] However, expression of mRNA encoding VEGF-A, one of the main
767 pro-angiogenic growth factors, was not elevated, but rather reduced in QQMNCs relative to
768 PBMNCs. The reduction of VEGF-A expression might be explained by negative feedback
769 mechanism through high dose of VEGF-A protein in QQc conditioning.
770 In contrast, expression of mRNAs encoding IL-1β and TNF-α, pro-inflammatory
771 factors, was not upregulated; lower in QQMNCs than in PBMNCs for IL-1β and similar in both
772 cells for TNF-α, although that encoding TGF-β, an anit-inflammatory factor, was lower in
773 QQMNCs. The findings indicate that QQMNCs may not, at least, bring pro-inflammatory cells
774 more than PBMNCs.
775 Moreover, the expression of mRNAs encoding MMP-2 or MMP-9, which playing a
776 critical role in neovascularization and tissue remodeling for anti-fibrosis,[36] were highly
777 upregulated.
778 Taken together, transplantation of QQMNCs, relative to that of PBMNCs, resulted in
779 more favorable conditions for vascular regeneration or tissue repair because of the orchestration
780 of dynamic expression of multiple cytokines and growth factors.
781
782 ***4) Therapeutic potential of QQMNCs for hindlimb ischemia models***
783 QQMNCs have therapeutic potential because transplantation of these cells into
784 ischemic hindlimb tissue was associated with increased blood flow, limb survival, and
785 neovascularization in tissues (**Figure 5**). Moreover, histological findings indicate that
786 transplanted human QQMNCs contributed to new microvessel formation composed of human
787 cells derived from EPCs in QQMNCs, as well as mouse microvessel formation and arteriogenesis
788 supported by pericytes (**Figure 6, 7**). The latter effects were presumably due to angiogenic
789 paracrine effects by accelerated phenotypes of macrophages and T lymphocytes as well as EPCs.
790 The other categorical finding of this transplantation treatment was decreased fibrosis
791 and inflammation, and enhanced myogenesis (**Figure 8, 9, 10**). As indicated by cell population
792 study and gene expression analyses, QQMNCs included many anti-inflammatory M2
793 macrophages; the cells had enhanced expression of the anti-inflammatory cytokine IL-10 and the
794 anti-fibrotic proteases, MMP-2 and -9, as well as decreased expression of the pro-inflammatory
795 cytokine, IL-1β. This anti-inflammatory effect by QQMNCs protected against fibrosis even in
796 severe ischemic muscles. The skeletal myogenesis was also augmented by QQMNC
797 transplantation. Induced vascularization and anti-inflammatory effects and enhanced expression

798 of myogenic factor, IGF-1, by QQMNCs may contribute to skeletal muscle regeneration *in situ*.
799 As recently reported,[7] autologous PBMNCs isolated by apheresis and then
800 transplanted into patients had vascular therapeutic potential, when the largest cell dose (over 1x
801 10^{10} cells of human subject) was implanted. Here, we transplanted only 1 x10^4 cells (PBMNCs
802 or/and QQMNCs) per mouse subject; this dose corresponds to approximately 2.0 to 2.5 x 10^7 cells
803 in a human subject of 50 kg body weight. We implanted far fewer cells than are generally used for
804 clinical treatments. Therefore, the effect of PBMNCs on ischemic hindlimbs was minimal, while
805 QQMNC transplantation had extensive therapeutic effects on vascular regeneration and tissue
806 repair.
807 On the other hand, the cell dose of 1 x10^4 cells per mouse subject also corresponds to that used at
808 GmCD34+ cell implantation in the patients with critical limb ischemia.[17, 19] Of note, at the present
809 study, QQMNCTx exerted the experimental efficacy equal to or in part greater than that of
810 GmCD34Tx.
811 The count of transplanted cell QQMNCs (1x10^4 cells/mouse) for therapeutic activity
812 of tissue regeneration corresponds to cell quantity averagely acquired from less than 100 mL PB
813 of human subjects. The isolation and preparation of QQMNCs require only the MNC isolation
814 and a week culture in QQc conditions that included recombinant factors without any
815 manipulation. Furthermore, the process avoids invasive procedures for isolation, such as BM cell
816 isolation or leukapheresis, and expensive costs for mobilization and target cell isolation, such as
817 G-CSF administration or CD34+/CD133+ cell isolation using magnetic beads.
818 Collectively, QQMNC is expected to be as an advantageous and feasible cell source for cell-based
819 therapy targeting ischemic diseases.
820
821 ***5) Mechanism***
822 The finding of **Figure 1f** indicates that CD34+ cells depleted PBMNCs included some
823 cell population to accelerate EPC expansion and differentiation in CD34+ cells through QQ
824 culture. As demonstrated in **Figure 2c**, **d**, EPCs, M2 macrophage, Th2, and regulatory T
825 lymphocytes were mainly activated in QQMNCs. Increasingly, researches are focused on the
826 interaction between macrophages and T lymphocytes to elucidate the collaborative mechanism of
827 inflammation and immunity. Although we do not have any evidence to indicate that EPCs are
828 involved in this collaboration, the developed culture for EPC expansion, QQ culture, regulates
829 phenotypes of macrophages and T lymphocytes, and consequently exerts EPC expansion and
830 differentiation. Therefore, any cellular or molecular mechanism responsible for the effects of

CD34+ cell depleted PBMNCs needs to be identified in the future for scientific and therapeutic interests.

On the other hand, histochemistry (**Figure 8, 9, 10**) and qRT-PCR assay (**Figure 11**) demonstrated that QQMNCTx as well as GmCD34Tx provides the preferential environment for tissue regeneration of myogenesis, anti-fibrosis, and anti-inflammation in ischemic hindlimb.

With respect to qRT-PCR assay, IL-1β, a pro-inflammatory cytokine, has been reported to function as an angiogenic factor derived from regenerating myoblasts responding to PBMNC implantation in ischemic hindlimb.[51] At the present study, the implantation of PBMNC highly induced the expression of mRNA encoding IL-1β in ischemic tissue, similarly to that of QQMNC, whereas did not demonstrate so much angiogenic potential. The causes of the dissimilar response might be presumably attributed to the distinct animal experiments using different murine strains with various cell doses for transplantation: immunodeficient BALB/c nu/nu nude mice ($1x10^4$ cells /mouse) in our study and C57/BL6 mice ($1x10^6$ cells /mouse) elsewhere.[51]

Notably, QQMNCTx or GmCD34Tx not only upregulated the gene expression of mRNAs encoding IGF-1, a potent myogenic factor[52], MyoD1 and myogenin in ischemic hindlimb, but also histologically promoted myogenesis as well as angiogenesis.

This means that the effective myogenesis by cell transplantation may primarily require *in situ* IGF-1 production, which also signifies a myogenic biomarker in ischemic hindlimb

Regarding the results, skeletal muscle-restricted expression of IGF-1 in the transgenic mice, has been reported to not only accelerate muscle regeneration, but also exert the protective effects against inflammation and fibrosis in the injured skeletal muscle[53]. Moreover, IGF-1 has been reported to inhibit NF-□B activation via TNF-α[54] or pro-apoptotic miRNA expression[55] in ischemic cardiomyocytes. Therefore, IGF-1 supplied to ischemic tissue by local QQMNC transplantation is adequately conceived to exert the protective effect on inflammation, fibrosis or tissue injury. Also, QQMNC *per se* exhibited the enhanced expression of mRNA encoding human IGF-1 (**Figure 3a**).

QQMNCTx or GmCD34Tx also upregulated the expression of mRNA encoding TGF-β□potentinhibitory factor of inflammation, in the transplanted tissue, although QQMNC *in vitro* exhibited the lesser expression than PBMNC. Different from the aspect of IGF-1, the responsive tissue expression *in situ* of TGF-βfollowing cell transplantations, might contribute to protecting against inflammation, rather than the expression by transplanted cells.