



■ RESEARCH

Angiogenic conditioning of peripheral blood mononuclear cells promotes fracture healing

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Objectives

The objective of this study was to investigate the therapeutic effect of peripheral blood mononuclear cells (PBMNCs) treated with quality and quantity control culture (QQ-culture) to expand and fortify angiogenic cells on the acceleration of fracture healing.

Methods

Human PBMNCs were cultured for seven days with the QQ-culture method using a serum-free medium containing five specific cytokines and growth factors. The QQ-cultured PBMNCs (QQMNCs) obtained were counted and characterised by flow cytometry and real-time polymerase chain reaction (RT-PCR). Angiogenic and osteo-inductive potentials were evaluated using tube formation assays and co-culture with mesenchymal stem cells with osteo-inductive medium *in vitro*. In order to evaluate the therapeutic potential of QQMNCs, cells were transplanted into an immunodeficient rat femur nonunion model. The rats were randomised into three groups: control; PBMNCs; and QQMNCs. The fracture healing was evaluated radiographically and histologically.

Results

The total number of PBMNCs was decreased after QQ-culture, however, the number of CD34+ and CD206+ cells were found to have increased as assessed by flow cytometry analysis. In addition, gene expression of angiogenic factors was upregulated in QQMNCs. In the animal model, the rate of bone union was higher in the QQMNC group than in the other groups. Radiographic scores and bone volume were significantly associated with the enhancement of angiogenesis in the QQMNC group.

Conclusion

We have demonstrated that QQMNCs have superior potential to accelerate fracture healing compared with PBMNCs. The QQMNCs could be a promising option for fracture nonunion.

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Keywords: Endothelial progenitor cell, CD34+ cell, Peripheral blood mononuclear cell, Quality and quantity control culture, Fracture healing

Article focus

- Peripheral blood mononuclear cells treated with the quality and quantity culture method are a potential cell candidate for clinical application in cases of bone nonunion.
- Peripheral blood mononuclear cells have no serious ethical or technical problems associated with their use, in contrast to human embryonic stem cells, neural stem cells, or mesenchymal stem cells.
- Angiogenic conditioning culture of PBMNCs enhances the radiological and histological repair of bone fractures.

Key messages

- The angiogenic and osteogenic potential of PBMNCs could be amplified by *ex vivo* conditioning culture.
- QQMNCs accelerate bone fracture healing.

Strengths and limitations

- Strengths: This is the first study to demonstrate the potential use of PBMNCs cultured with a special medium for fracture healing as a more feasible and promising cell candidate in the clinical setting.

- Limitations: We used a small number of animals to evaluate fracture healing and we did not compare other cell fractions such as mesenchymal stem cells, CD34+ cells or bone marrow CD34+ cells.

Introduction

Fractures may fail to heal for a variety of reasons, including both mechanical and biological factors. A key mechanical factor is stability of the fracture site, while reduced osteogenic potential and loss of vascularity are important biological factors leading to nonunion.¹⁻³ Standard treatment involves autologous bone grafting, but graft harvesting is associated with morbidity while graft supply is limited, and grafts have unpredictable reparative potential.⁴

The cell therapy approach has been adopted to augment the effectiveness of biological factors. Mesenchymal stem cells (MSCs), bone marrow mononuclear cells (BMMNCs) and CD34+ cells have all been proposed as cell candidates.⁵⁻⁷ In particular, CD34+ cells (the cell fraction containing endothelial progenitor cells (EPCs)) are used for cell-based angiogenic therapy, and their therapeutic effect on fracture healing was demonstrated in preclinical and clinical studies.^{7,8} Thus, CD34+ cells have been shown to have a potential for acceleration of bone healing by improving angiogenesis. However, CD34+ cell therapy has limitations. First, the number of these cells is limited, comprising < 0.01% of the cells in peripheral blood and 0.1% in bone marrow.⁹ Second, ageing or disease attenuates the therapeutic potential of the cells.¹⁰ Third, to obtain this cell fraction, invasive procedures are required such as apheresis and the administration of granulocyte colony-stimulating factor (G-CSF).⁸ To overcome these problems, Masuda et al¹⁰ developed a serum-free culture system with added cytokines and growth factors to increase the angiogenic cell fraction using stem cell factor (SCF), thrombopoietin (TPO), Flt-3 ligand, interleukin-6 (IL-6), and vascular endothelial growth factor (VEGF). With the use of this culture method, CD34+ cells can be expanded while maintaining their CD34 positivity and increasing their angiogenic potential. Based on these effects, this approach was named 'quality and quantity culture' (QQ-culture).¹¹ To minimise the invasiveness and cost of the cell isolation procedure, this QQ-culture method was applied to PBMNCs. A preclinical study demonstrated that this approach upregulated the cells' angiogenic and anti-inflammatory potentials, and showed that cultured cells had greater therapeutic effect in a mouse hind limb ischaemia model.¹² Based on these preliminary results, we hypothesised that human PBMNCs treated by the QQ-culture method (QQMNCs) could be favourable candidates for fracture healing.

In this study, we aimed to evaluate the angiogenic and osteogenic potential of QQMNCs, and to investigate whether QQMNCs accelerate fracture healing in a femoral fracture nonunion model in immunodeficient rats.

Materials and Methods

This study was approved by the institutional animal committee of Hiroshima University (A14-52). All animals were treated according to the guidelines stipulated by the Institutional Animal Care and Use Committee.

Quality and quantity culture. Samples of human peripheral blood (20 mL) were collected from healthy volunteers, and PBMNCs were isolated by density gradient centrifugation using Lymphocyte Separation Solution (LSM) ($d = 1.077$, Histopaque-1077 sterile-filtered; Sigma-Aldrich, St Louis, Missouri), as previously reported.⁸ Informed consent was obtained from all volunteers. Isolated PBMNCs were treated by the QQ-culture method for seven days without any change of medium at a density of 2×10^6 cells/2 mL/well in six-well culture plates (BD Biosciences, #353846, Primaria, BD Falcon, San Jose, California). The complete QQ-culture medium was prepared using serum-free medium (Stem Line; Sigma-Aldrich) supplemented with human stem cell factor (SCF) (#130-096-692, concentration 100 ng/mL; Miltenyi Biotec, Bergisch Gladbach, Germany), human thrombopoietin (TPO) (#130-094-011, concentration 20 ng/mL; Miltenyi Biotec), human Fms-related tyrosin kinase (Flt)-3 ligand (#130-096-474, concentration 100 ng/mL; Miltenyi Biotec), human vascular endothelial growth factor (VEGF) (#100-20, concentration 50 ng/mL; Peprotech EC Ltd, London, United Kingdom), and human interleukin (IL)-6 (#130-095-365, concentration 20 ng/mL; Miltenyi Biotec). Cell density in QQ culture corresponded to approximately 1×10^6 PBMNCs in 1 mL as previously reported (Supplementary Fig. a).^{12,13} After culture, QQMNCs were counted and used for further analysis as described below.

Cell characterisation by flow cytometry. To characterise PBMNCs and QQMNCs ($n = 6$ per group), fluorescence-activated cell sorting (FACS) analysis was performed using a BD LSRFortessa Cell Analyzer (BD Biosciences) and CellQuest software (BD Biosciences) after staining with mouse anti-human monoclonal antibodies against the following surface markers: Cluster of Differentiation (CD)34-Brilliant Violet (BV) 421 (clone 581; BD Biosciences); Vascular Endothelial Growth Factor Receptor(VEGFR)2-Phycoerythrin(PE) (clone 7D4-6; BioLegend, San Diego, California); Cluster of Differentiation (CD206)-fluorescein(FITC) (clone 15-2; BioLegend); and C-C chemokine receptor type 2 (CCR2)- Brilliant Violet (BV)605 (clone K036C2; BioLegend). Dead cells were excluded on the basis of 7-Amino-Actinomycin D (7-AAD) staining (BD Biosciences). Cells were stained with monoclonal antibodies for 20 minutes at 4°C following Fc receptors (FcR) blocking, washed twice using Hank's buffered salt solution containing 2% fetal bovine serum (FBS), and then analysed. Relevant isotype controls (Immunoglobulin G(IgG)1-BV421 isotype control (BD Biosciences), IgG1-PE (BD Biosciences), IgG1-FITC (BD Biosciences), and IgG2a-BV605 (BioLegend)) were also used. In all samples, 20 000 events were acquired.

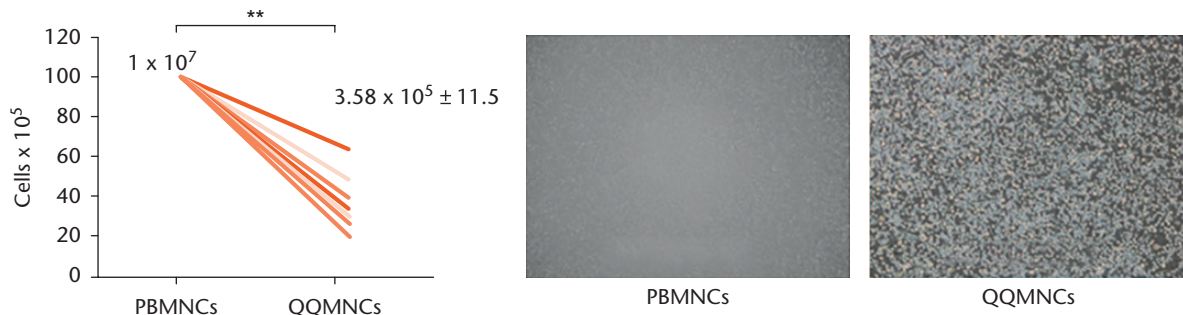


Fig. 1a

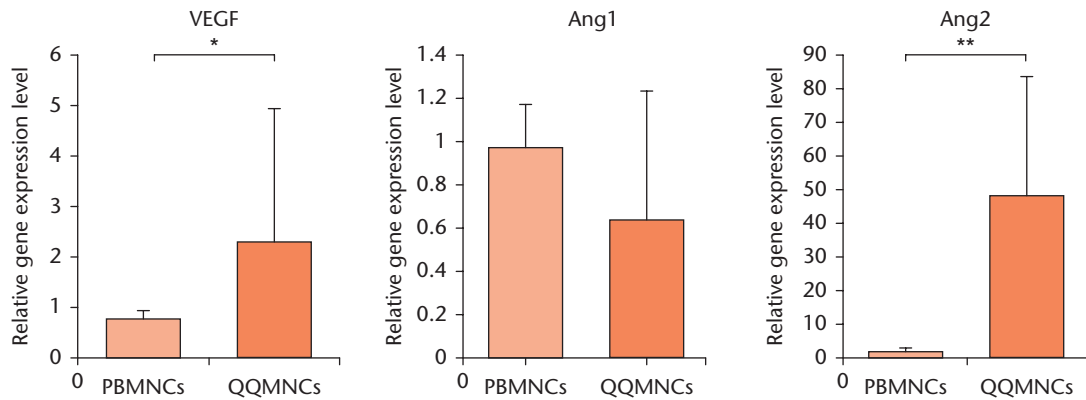


Fig. 1b

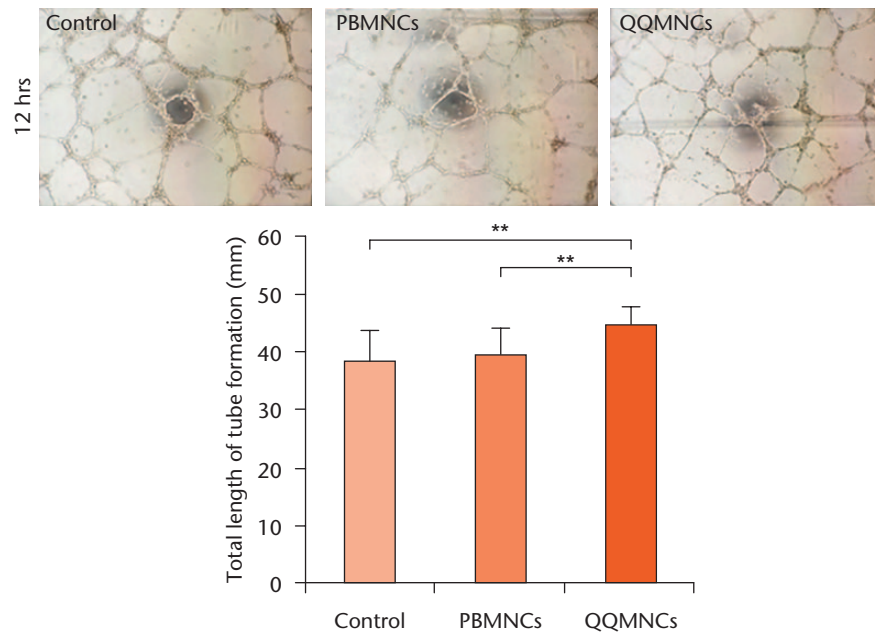


Fig. 1c

Cell characterisation of quality and quantity control-cultured mononuclear cells (QQMNCs). (a) Total cell counts of peripheral blood mononuclear cells (PBMNCs) and QQMNCs and their images: 2×10^6 cells/2 mL/well were cultured in Quality and Quantity (QQ) culture using five wells in a six well-plate. After culture, QQMNCs were counted. The graph shows total counts of PBMNCs and QQMNCs in five wells. This graphs show that total cell number decreases after culture (** $p < 0.01$, $n = 12$). Phase contrast image of PBMNCs and QQMNCs ($\times 40$ magnification); b) gene expression of angiogenic markers relative to glyceraldehyde 3-phosphate dehydrogenase: quantitative real-time polymerase chain reaction (qRT-PCR) assay of PBMNCs and QQMNCs. Each value indicates a mean SD standard deviation (* $p < 0.05$, ** $p < 0.01$; $n = 6$ volunteers). The expression of both vascular endothelial growth factor (VEGF) and angiotensin (Ang) 2 were significantly greater in QQMNCs than in PBMNCs; c) enhanced *in vitro* tube formation by human umbilical vein endothelial cells (HUVECs) when co-cultured with QQMNCs. Representative images of each group are shown ($\times 40$ magnification). The total length of tube formation was calculated by ImageJ Software version 1.48 (National Institutes of Health, Bethesda, Maryland). The overall length in the QQMNC group was significantly longer than in other groups (** $p < 0.01$).

Table 1. List of primers used in real-time polymerase chain reaction

Type			
Human probes	Angiogenic	VEGFA	Hs 00900055_m1
		angiopoetin1	Hs 00375822_m1
		angiopoetin2	Hs00169867_m1
	Osteogenic	RUNX2	Hs 00231692_m1
		Type1 collagen	Hs 00164004_m1
		osteocalcin	Hs 01587814_g1
Internal control	GAPDH	Hs 02758991_g1	
Rat probes	Angiogenic	VEGF	Rn 0511601_m1
		FGF2	Rn 00570809_m1
		RUNX2	Rn 01512298_m1
	Osteogenic	Type1 collagen	Rn 01463848_m1
		osteocalcin	Rn 00566386_g1
	Internal control	GAPDH	Rn 01749022_g1

VEGFA, vascular endothelial growth factor A; RUNX2, Runt-related transcription factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FGF2, fibroblast growth factor 2

Cell characterisation by quantitative real-time polymerase chain reaction. Using TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts), total RNA was extracted from PBMNCs or QQMNCs ($n = 6$ per group). Complementary DNA (cDNA) was synthesised using 10 ng total RNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). A real-time PCR assay was performed using TaqMan Gene Expression Assay (Applied Biosystems, Foster City, California). Relative expression was calculated using the $\Delta\Delta C_t$ values and results are expressed as $2^{-\Delta\Delta C_t}$. The value of each control sample was set at one and was used to calculate the fold difference in each target gene. All primers and probes used are listed in Table 1.

Tube formation assay. PBMNCs and QQMNCs were evaluated by tube formation assay by co-culturing with human umbilical vein endothelial cells (HUVECs) on ECMatrix gel (Merck-Millipore, Darmstadt, Germany) to confirm their angiogenic potential. To solidify the matrix solution, 150 μ L was incubated at 37°C for one hour in a 96-well plate. Then 1×10^3 cells from each group (PBMNCs, QQMNCs) were mixed with 1.5×10^4 HUVECs in 50 μ L of Endothelial Basal Medium -2 (EBM-2) complete medium containing 2% FBS, seeded onto the surface of the polymerised ECMatrix and co-cultured. As a control, HUVECs were cultured alone. After incubation for 12 hours, photomicrographs of each well were taken under a light microscope (IX71N-22FL/PH; Olympus, Tokyo, Japan), and then the length of tube formation was calculated using ImageJ Software version 1.48 (National Institutes of Health, Bethesda, Maryland) ($n = 16$ in each group four donors, repeated four times).

Osteogenic induction of MSCs *in vitro*. Commercially available primary human bone marrow MSCs (BMMSCs) from healthy donors (Lonza Ltd, Basel, Switzerland) were cultured and expanded in a specific medium, mesenchymal stem cell growth medium (MSCGM Bulletkit; Lonza Ltd). MSCs at passage four were seeded into 12-well

plates with an initial seeding density of 10 000 cells/cm² and preconditioned in osteogenic induction medium (StemPro Osteogenesis Differentiation Kit; Thermo Fisher Scientific) at 37°C for one day. After preconditioning, the MSCs were co-cultured with PBMNCs or QQMNCs (1×10^5 cells, $n = 12$ in each group; three donors, repeated four times) using cell culture inserts (Falcon Cell Culture Inserts: For Use With 12-Well Plates#353180) for seven days. As a control, MSCs alone were cultured in osteogenic induction medium. Total RNA was extracted from the MSCs at days 3 and 7 of co-culture using TRIzol, and cDNA was synthesised. A real-time PCR assay was performed in the same manner as described above. All primers and probes used in this analysis are listed in Table 1. For evaluation of calcium deposition, the MSCs were stained with Alizarin Red S (CAS No. 130-22-3; Sigma-Aldrich) at day 14 of co-culture, and the stained area was measured using ImageJ Software.

Animal study

Animals and surgical procedure. Male athymic nude rats (F344/NJcl-rnu/rnu, nine to ten weeks old, CLEA Japan, Inc, Tokyo, Japan) were used to evaluate the therapeutic potential of QQMNCs *in vivo*. All surgical procedures were performed under normal sterile conditions. The rats were anaesthetised using ketamine (100 mg/kg) and xylazine (12 mg/kg) by intraperitoneal administration. A transverse femoral fracture was created in the right femur of each rat and nonunion was induced by cauterising the periosteum to a distance of 2 mm on each side of the fracture, as previously described.^{14,15} In order to avoid significant displacement of the fracture and achieve well-aligned stability, a 1.2 mm diameter Kirschner wire was inserted from the trochlear groove into the femoral canal in a retrograde manner. Finally, the wound was tightly closed in layers. Rats were then randomised to three groups: control; PBMNC; and QQMNC. In the PBMNC and QQMNC groups, 2×10^5 cells were injected into the site immediately after surgery while the control group received phosphate-buffered saline (PBS) alone.

Evaluation of fracture healing. Rats were anaesthetised before imaging. Serial radiographs were taken at zero, two, four, six and eight weeks after surgery, and fracture healing was evaluated by Qui's radiographic scoring system ($n = 8$ in each group and at each time point).¹⁶

For micro-CT imaging (μ CT), rats were killed at two, four and eight weeks, and the right femurs were harvested. The femurs were fixed in 4% paraformaldehyde (Wako Pure Chemical Industries Ltd, Osaka, Japan) at 4°C for 24 hours, wires were removed, and μ CT images were acquired (SkyScan 1176; Bruker, Kontich, Belgium). The imaging settings were as follows: acceleration voltage: 40 kV; beam current: 800 μ A, no filter; resolution: 18 μ m; and rotation: 360° in 1° steps. Acquired images were reconstructed using the manufacturer's software (NRecon version 1.6.4.1; Skyscan), and total callus volume (TV),

Table II. Percentage of positives among the cell population in 20 000 cells of PBMNCs and QQMNCs

	PBMNCs %	QQMNCs %	p-value
CD34	1.20 (SE) 0.24	12.1 (SD) 4.04	< 0.05
VEGFR-2	15.0 (SE) 1.86	5.02 (SD) 1.01	< 0.01
CD206	0.434 (SE) 0.065	11.3 (SD) 3.93	< 0.05
CCR2	25.9 (SE) 2.71	12.9 (SD) 4.10	< 0.01

% in PBMNCs and % in QQMNCs indicate the % positivity of each cell population in a sample of 20 000 PBMNCs or QQMNCs, data compared with the value of PBMNCs n = 6 volunteers
 CD34: Cluster of Differentiation 34, VEGFR2: Vascular Endothelial Growth Factor Receptor 2. CD206: Cluster of Differentiation 206, CCR2: C-C chemokine receptor type 2 (all p-values Student's t-test)

mineralised bone volume (BV), and bone volume fraction (BV/TV) were analysed on either side, 2 mm away from the fracture site using a CT Analyzer, version 1.11.8.0 (Skyscan) (two weeks: n = 6 per group; four weeks: n = 6 per group; and eight weeks: n = 8 per group).

Histological evaluation. After µCT scanning, the right femurs were decalcified with 0.5 M ethylenediamine-tetraacetic acid (EDTA) solution for three weeks. The samples were embedded in paraffin, and cut into 5 µm thick sections through the site of the fracture. The sections were stained with Toluidine blue and the process of endochondral ossification was evaluated at two, four and eight weeks after surgery. The degree of fracture healing was evaluated using the five-point scale (grades 0 to 4) proposed by Allen, Wase and Bear.¹⁷ The assessment was blinded. In addition, the sections at two weeks after surgery were stained for smooth muscle actin to assess angiogenesis. Researchers blinded to the identity of each group counted the number of capillary structures in five random areas around the fracture sites using a light microscope (×200 magnification). The capillary density was then calculated as the number of vessels/mm² as previously described.¹⁸

Gene expression analysis (around the fracture site). From the two-week old rat model, the tissue around the fracture site was harvested after death, and RNA was isolated (n = 6 per group). Real-time PCR was performed to evaluate fracture healing-related gene levels as described above. All primers and probes used are listed in Table I.

Statistical analysis. All values are expressed as means and standard error. Student's t-test (for two groups), analysis of variance (ANOVA) and Tukey's *post hoc* test (more than two groups) were used. Probability (p) values of less than 0.05 were considered statistically significant.

Results

Cell number and population transition after QQ culture. The fold decrease of QQMNCs to PBMNCs per well in all subjects was an average of 0.358-fold (Fig. 1a).

Based on fluorescence-activated cell sorting, QQMNCs exhibited enrichment of CD34+ and CD206+ cells

compared with PBMNCs (10.01-fold in CD34+ cells, 26.04-fold in CD206+ cells, Table II). In contrast, the population of Vascular Endothelial Growth Factor Receptor(VEGFR)-2+ and C-C chemokine receptor type 2 (CCR2+ cells) was significantly lower in QQMNCs than in PBMNCs (0.33-fold in VEGFR-2+ cells, 0.50-fold in CCR2+ cells) (Supplementary Fig. b).

Enhanced angiogenic gene expression in QQMNCs. Expression of genes encoding angiogenic factors, including VEGFA and angiopoietin-2 (Ang2), was significantly upregulated in QQMNCs compared with PBMNCs (3.58-fold for VEGF-A, 25.6-fold for Ang2). In contrast, there was no significant difference in Ang1 expression between QQMNCs and PBMNCs (Fig. 1b).

QQMNCs promote tube formation *in vitro*. In an *in vitro* tube formation assay involving co-culture with HUVECs, QQMNCs showed greater angiogenic potential at 12 hours. Total tube length in the QQMNC group was significantly longer than in the PBMNC and control groups (tube length 44.5 mm, SD 3.4 for HUVECs and QQMNCs; 39.4 mm, SD 4.7 for HUVECs and PBMNCs; and 38.5 mm, SD 5.1 for HUVECs alone (Fig. 1c).

QQMNCs enhance osteo-induction of MSCs *in vitro*. At day 3 of co-culture, real-time PCR revealed that the expressions of RUNX2 and Type 1 collagen were significantly upregulated in the QQMNC group compared with the PBMNC and control groups. At day 7, the expression of osteocalcin was significantly higher in the QQMNC group compared with the PBMNC and control groups (Fig. 2a). Alizarin Red staining of co-cultures at day 14 showed a significantly larger stained area in the QQMNC group compared with the control group. However, there was no significant difference between the PBMNC and QQMNC groups (Fig. 2b).

QQMNCs accelerate fracture healing. Fracture healing was evaluated by radiographic, µCT, and histological examinations. Rats which received QQMNCs demonstrated the highest rate of fracture healing, with bridging callus formation observed radiographically (50% (3/6) at four weeks, 62.5% (5/8) at eight weeks). In the group of rats receiving PBMNCs, only one achieved radiographic fracture healing at eight weeks (12.5% (1/8)), while none of the rats in the control group achieved radiographic fracture healing (Fig. 3a, Table III). Qi's radiographic fracture healing score was significantly higher in the QQMNC group than in the other groups at eight weeks (QQMNCs: 5.88, SD 0.64; PBMNCs: 3.0, SD 0.33; control: 2.63, SD 0.182) (Fig. 3a). The µCT analysis confirmed the same rates of fracture healing at eight weeks (QQMNCs: 62.5%; PBMNCs: 12.5%; control: 0%) (Fig. 3b). In the quantitative evaluation of bone formation, the value of BV/TV was significantly higher in the QQMNC group compared with the other groups at eight weeks (QQMNCs: 48.81%, SD 0.90%; PBMNCs: 37.04%, SD 9.13%; control: 34.61%, SD 6.21%) (Fig. 3b).

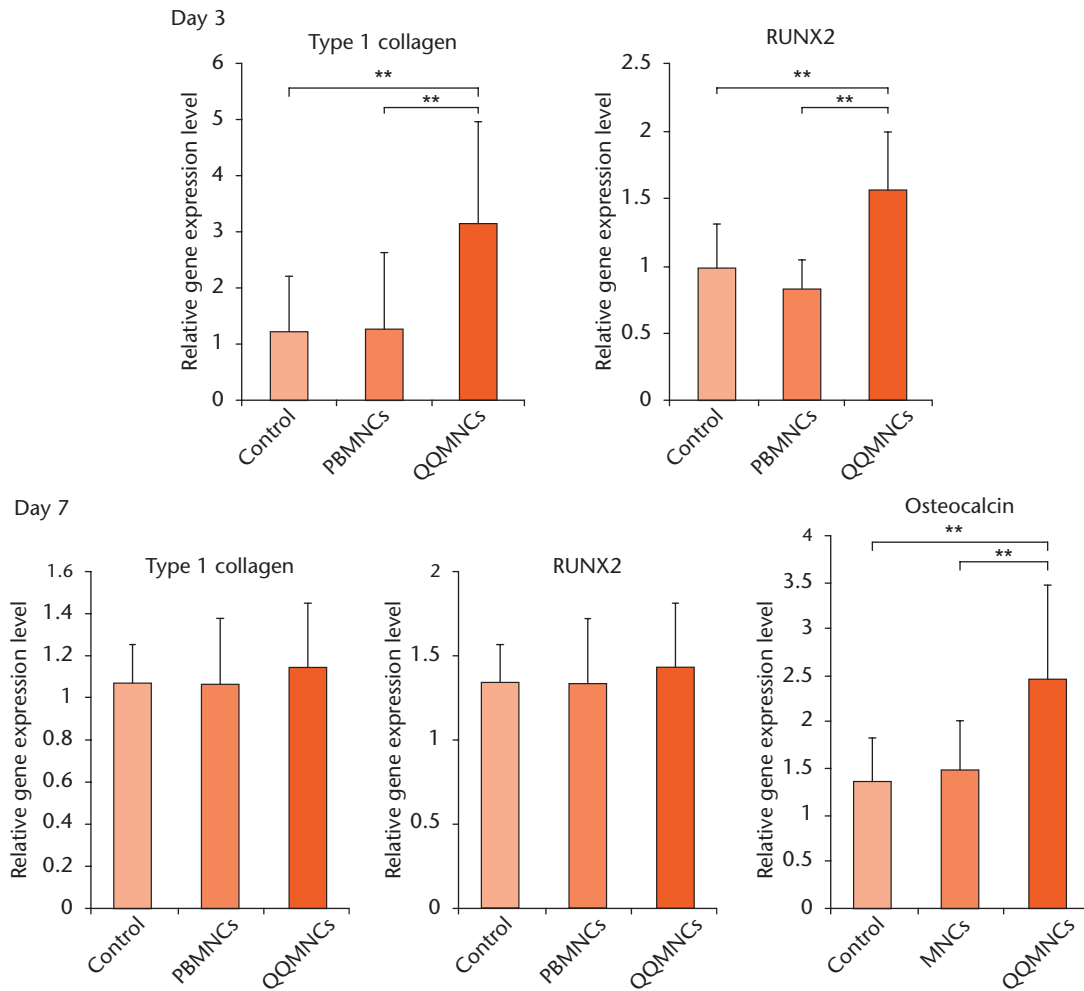


Fig. 2a

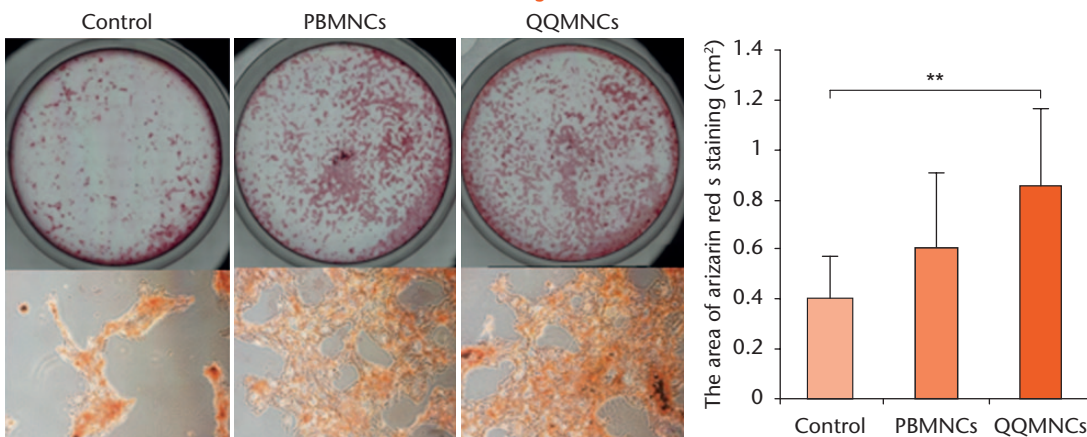


Fig. 2b

Real-time polymerase chain reaction (RT-PCR) assay of osteogenic induction of mesenchymal stem cells (MSCs) co-cultured with peripheral blood mononuclear cells (PBMNCs) or quality and quantity control-cultured mononuclear cells (QQMNCs): a) the graphs show the relative gene expression levels of osteogenic factors (Type 1 collagen, RUNX2, and osteocalcin) (** $p < 0.01$, Tukey's *post hoc* test). Expression of osteocalcin was undetectable at day 3. The expression levels were compared between the QQMNC group and the control and PBMNC groups. Each graph column represents a mean standard deviation (SD); $n = 12$ per group; b) quantification of Alizarin red S staining: MSCs were stained with alizarin red at day 14 of co-culture. Microscopic image of each well of 12-well plate (upper panel: $\times 40$ magnification in 12 wells and lower panel: $\times 200$ magnification) showed enhanced alizarin red staining of MSCs in co-culture with QQMNCs compared with control.

Histological evaluation demonstrated that nonunion sites in the PBMNC and QQMNC groups showed enhanced endochondral ossification consisting of

numerous chondrocytes and newly formed trabecular bone at two and four weeks after surgery. In five rats of the QQMNC group and one in the PBMNC group,

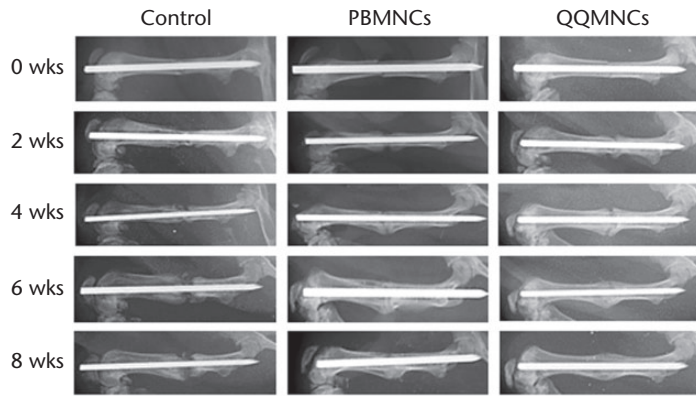


Fig. 3a

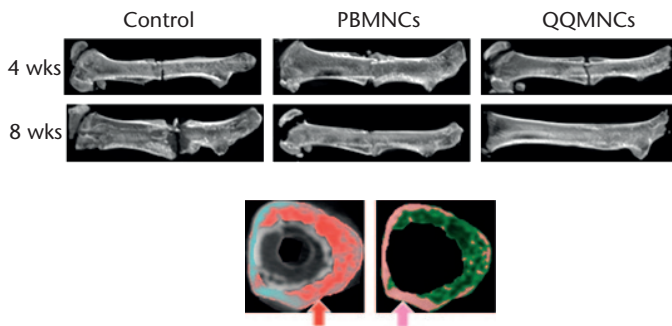
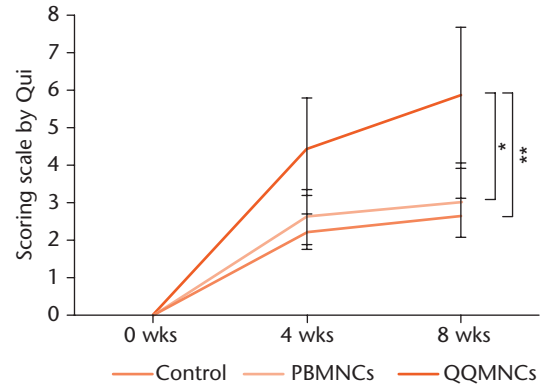
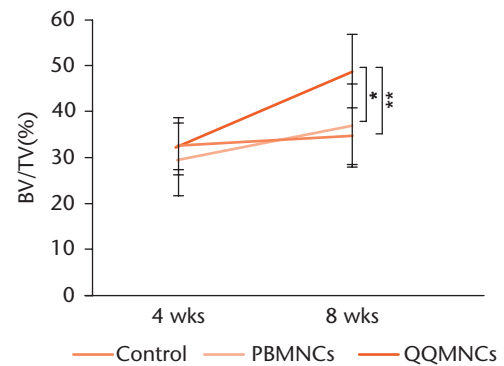


Fig. 3b



Evaluation of bone union by radiography and μ CT: a) representative radiographs of each group at 0, two, four, six, and eight weeks post-operatively and the radiographic scores by Qui in each group at 0, four, and eight weeks. The score in the quality and quantity control-cultured mononuclear cell (QQMNC) group was significantly higher than in the other groups (* $p < 0.05$, ** $p < 0.01$, Tukey's *post hoc* test; $n = 8$); b) representative μ CT images from each group at four and eight weeks post-operatively. The union rates reflected the radiographic images. Mineralised callus volume (BV)/total callus volume (TV) (%) was calculated using μ CT. TV is shown as the red area in the left picture. BV is shown as the pink area in the right picture. The BV/TV ratio was calculated from the fracture site to 2 mm in each case. BV/TV in the QQMNC group was significantly higher compared with in the peripheral blood mononuclear cells (PBMNC) and control groups. The graph represents the mean \pm SD standard deviation (* $p < 0.05$, ** $p < 0.01$, Tukey's *post hoc* test; $n = 6$).

Table III. Bone union rate in a nonunion model

	2 wks	4 wks	6 wks	8 wks
Control (n, %)	0/8, 0	0/8, 0	0/8, 0	0/8, 0
PBMNCs	0/8, 0	1/8, 12.5	1/8, 12.5	1/8, 12.5
QQMNCs	0/8, 0	4/8, 50	4/8, 50	5/8, 62.5

PBMNCs, peripheral blood mononuclear cells; QQMNCs, quality and quantity control-cultured mononuclear cells

bridging callus formation was observed at eight weeks. The histological score of fracture healing was significantly greater in the QQMNC group than in the other groups at eight weeks (QQMNCs: 3.50, $SD 0.75$; PBMNCs: 2.12, $SD 0.99$; control: 1.87, $SD 0.83$) (Fig. 4a).

QQMNCs enhance angiogenesis and osteogenesis in vivo. Capillary density was significantly greater in the QQMNC group than in the PBMNC and control groups in the tissue harvested at two weeks after transplantation (QQMNCs: 21.67 vessels/ mm^2 , $SD 4.96$; PBMNCs: 10.95 vessels/ mm^2 , $SD 2.56$; control: 10.62 vessels/ mm^2 , $SD 3.02$) (Fig. 4b). In addition, in the QQMNC group, several bone healing-related genes were significantly upregulated

(Fig. 5). There were no significant differences in expression of type 1 collagen and osteocalcin among the three groups (Fig. 5).

Discussion

Transplantation of CD34+ cells has been shown to promote fracture healing in a nonunion model.¹⁷⁻¹⁹ However, difficult and costly isolation procedures are critical drawbacks to their clinical application as they require granulocyte-colony stimulating factor (G-CSF) administration, apheresis, and magnetic cell sorting.^{18,20-22} On the other hand, PBMNCs constitute a very accessible cell source which can be obtained less invasively at a lower cost compared with CD34+ cells and BMMNCs. Masuda et al cultured PBMNCs in serum-free medium with cytokines and growth factors in an effort to fortify the cells' angiogenic potential^{10,12,23} and generated QQMNCs. Our results confirm that cultured PBMNCs have superior potential to accelerate fracture healing compared with freshly isolated PBMNCs in a nude rat model of fracture nonunion.

The presence of circulating Endothelial progenitor cell (EPC)s in adult human peripheral blood was first reported

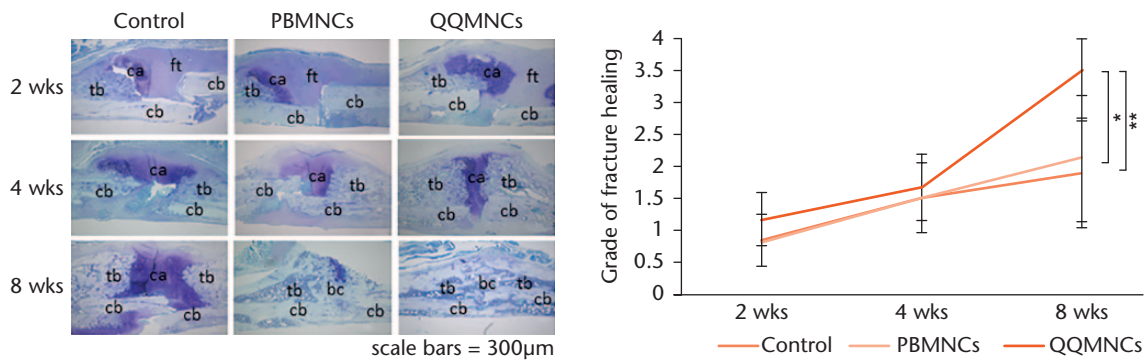


Fig. 4a

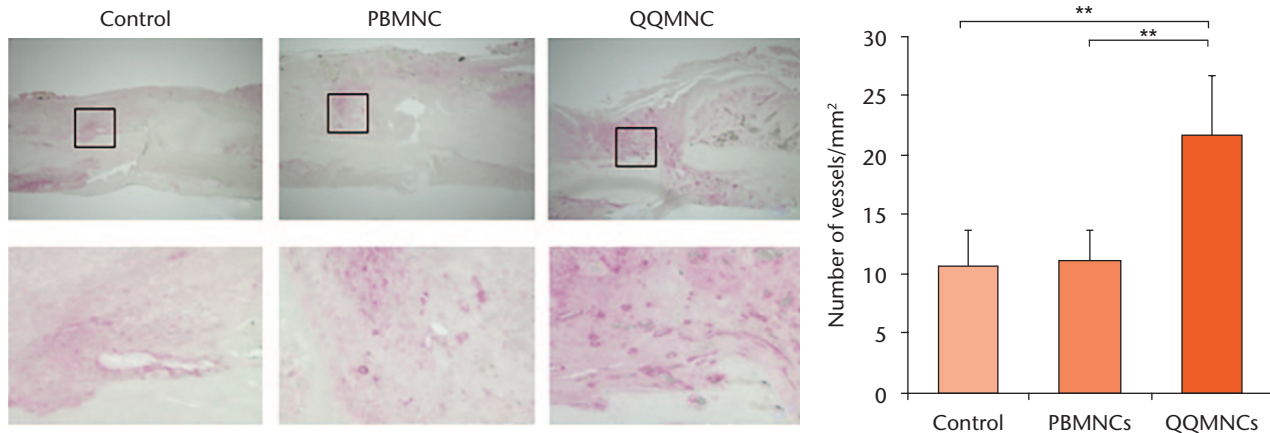


Fig. 4b

Histological evaluation of bone healing after cell transplantation: a) representative histological image ($\times 40$ magnification) of each group stained with toluidine blue at two, four, and eight weeks post-operatively (bc, bridging callus; cb, cortical bone; ca cartilage; ft, fibrous tissue; tb trabecular bone). The nonunion sites of the peripheral blood mononuclear cell (PBMNC) group and the quality and quantity control-cultured mononuclear cell (QQMNC) group demonstrated enhanced endochondral ossification consisting of numerous chondrocytes and newly-formed trabecular bone. The degree of bone healing evaluated by Allen's scoring scale was significantly higher in the QQMNC group than in the other groups at eight weeks post-operatively. The graph represents the mean SD standard deviation ($*p < 0.05$, $**p < 0.01$; Tukey's *post hoc* test $n = 6$ at two, and four weeks, $n = 8$ at eight weeks); b) immunohistochemical evaluation of neovascularisation by α smooth muscle actin staining at two weeks post-operatively: representative images of each group are shown (top images; $\times 40$ magnification, bottom images; $\times 200$ magnification). The density of capillaries was calculated and can be seen in the graph. The density was significantly higher in the QQMNC group than in the control or PBMNC groups. Each column represents the mean SD standard deviation ($**p < 0.01$, Tukey's *post hoc* test; $n = 6$) (PBS, phosphate-buffered saline).

in 1997 by Asahara et al.²⁴ These cells can be obtained as CD34⁺ cells from adult bone marrow.²⁴⁻²⁶ Adult EPCs are mobilised from the bone marrow into peripheral blood under tissue ischaemia, where they integrate into ischaemic lesions, differentiate into mature endothelial cells, and contribute to neovascularisation.^{24,25,27} Masuda et al.¹² previously demonstrated that QQMNCs cultured from PBMNCs also contributed to neovascularisation, contributing extensively to therapeutic effects on vascular regeneration and tissue repair. When we investigated angiogenic vessel formation by tube formation assay and angiogenic gene expression by RT-PCR *in vitro*, we found the same result. Furthermore, we showed that QQMNCs promoted the osteogenic induction of MSCs in co-culture experiments. In addition to angiogenesis, CD34⁺ cells can also differentiate into osteoblasts *in vitro* without losing their expression of haematopoietic stem cell markers.^{7,28,29} These findings suggest that CD34⁺ cells,

which form part of the cell fraction of QQMNCs, may also differentiate into osteogenic cells themselves.

Flow cytometry showed that QQMNCs were enriched in CD206⁺ and impoverished in CCR2⁺ cells. We speculated that the QQMNC fraction might contain fewer M1 macrophages and more M2 macrophages than PBMNCs. Schlundt et al.³⁰ demonstrated that M2 macrophages, which express the cell surface antigen CD206, enhance bone fracture healing. These results support the hypothesis that the enrichment of M2 macrophages in QQMNCs may play a key role in accelerating bone healing.

Many researchers have reported studies on cell therapy for bone fracture healing in the clinical setting. Above all, good results have been reported concerning transplantation of CD34⁺ cells as EPCs, and a phase I/II clinical trial of autologous CD34⁺ cell transplantation in patients with tibial or femoral nonunion was performed.^{8,21,22} Fukui et al.¹⁹ reported observation of

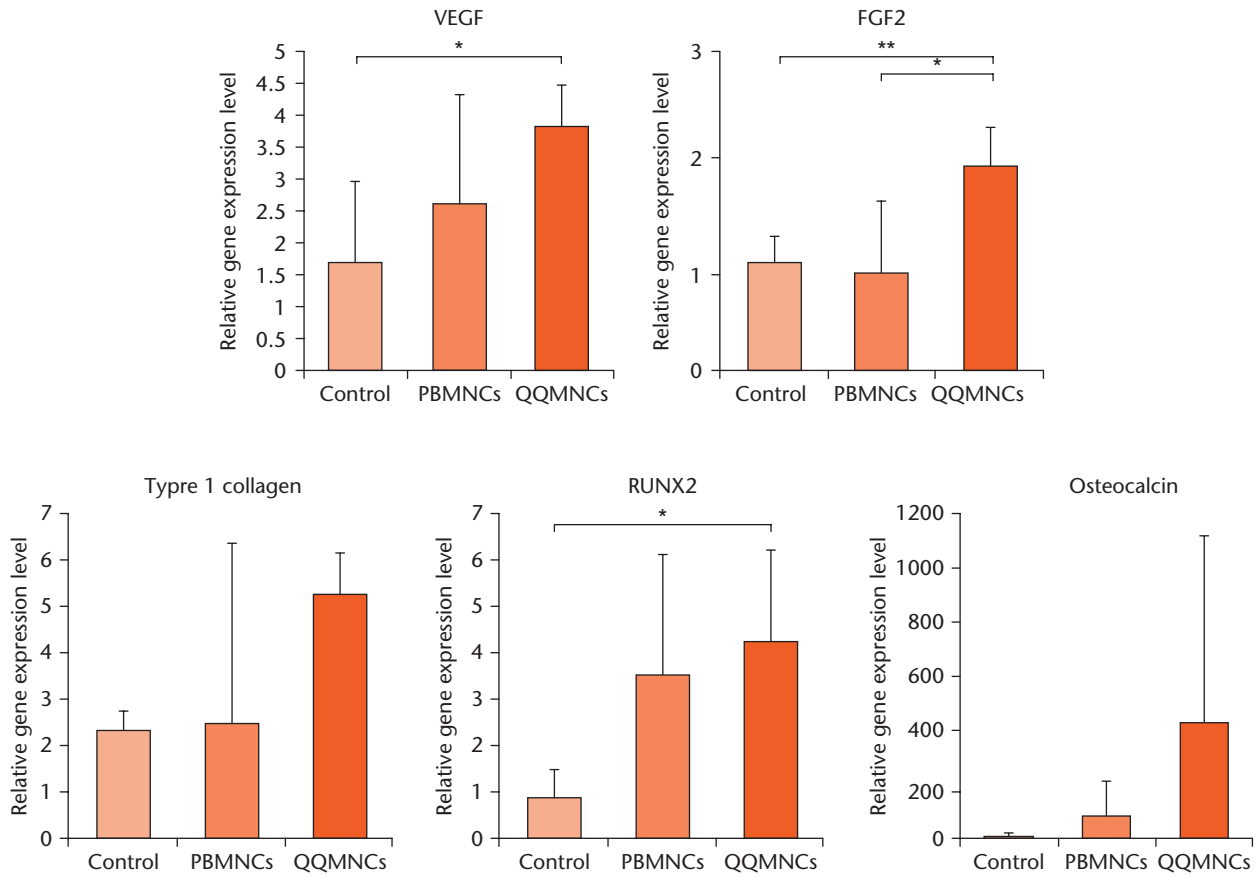


Fig. 5

quantitative real-time polymerase chain reaction (qRT-PCR) of callus around the fracture site at two weeks after implantation. The relative gene expression level relating to angiogenic factors (vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2)). The expression of VEGF was significantly greater in the quality and quantity control-cultured mononuclear cell (QQMNC) group than in the control group and the expression of FGF2 was significantly higher in the QQMNC group than in the peripheral blood mononuclear cell (PBMNC) and control groups. The relative gene expression levels of osteogenic factors (Type 1 collagen, RUNX2, osteocalcin) are shown in the second row of graphs. The expression of RUNX2 was significantly greater in both experimental groups than in the control group (* $p < 0.05$, ** $p < 0.01$, Tukey's *post hoc* test; $n = 6$ per group). Each graph column represents a mean SD standard deviation.

bridging callus formation and complete union in 90% of fracture nonunion models of nude rats at weeks 8 and 12 in the CD34+ group when treated with atelo-collagen gel. Our results showed 62.5% bridging callus formation in nonunion models at eight weeks in the QQMNC group. These results may indicate that the therapeutic effect of QQMNCs is less than that of CD34+ cells but may reflect differences in the delivery methods: local injection of a cell suspension *versus* a 3D carrier. Fukui et al¹⁹ also concluded that the transplantation of PBMNCs contributes to bone repair in nonunion models but is less effective than the transplantation of CD34+ cells. In the current study, using our method, we demonstrated that the therapeutic effect of PBMNCs could be fortified. QQMNCs can be prepared easily and at a lower cost than CD34+ cells, rendering them more feasible for application in the clinical setting than CD34+ cells.


This study has some limitations. First, our nonunion models are different from the clinical condition of fracture nonunion. However, we created the model

following previous reports^{14,15} to evaluate whether QQMNCs promote fracture healing. Second, we speculated concerning the role of M2 macrophages, however, we did not show direct evidence of the influence of QQMNC transplantation on these cells. The issue of whether QQMNCs include more M2 macrophages and how the macrophages affect fracture healing will need to be investigated in the future. Finally, the safety of QQMNCs cannot be confirmed, as adverse effects of QQMNCs were not addressed. In our conditioning culture of PBMNCs, high concentrations of five cytokines and growth factors are used. Therefore, a careful safety study of these cells for future clinical application will be necessary.

In conclusion, we have shown that QQMNCs present unique cell fractions with anti-inflammatory phenotype and have more angiogenic and osteogenic potentials than PBMNCs *in vitro*. Also, we have demonstrated that transplantation of QQMNCs is more effective than PBS or PBMNCs in accelerating bone fracture healing in a fracture nonunion model of nude rats. The QQMNCs can be prepared easily at a low cost and therefore could be a

promising option for the treatment of nonunion fractures in the future.

Supplementary material

 The QQ culture method of acquiring PBMNCs from peripheral blood, the method of CC culture, as well as Graphs showing PBMNCs and QQMNCs in flow cytometry and positive cell population in 20,000 events in PBMNCs and QQMNCs are available alongside this article at www.bjr.boneandjoint.org.uk

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Author Contribution

- K. Mifuji: Study inception, Manuscript preparation, and Statistical analysis.
- M. Ishikawa: Designed the study, Study inception, Manuscript preparation.
- N.Kamei: Assisted in the study process and preparation of the manuscript.
- R. Tanaka: Assisted in the design of the study.
- K. Arita: Assisted in the design of the study.
- H. Mizuno: Assisted in the design of the study.
- T. Asahara: Assisted in the design of the study.
- N. Adachi: Assisted in manuscript preparation, Critically reviewed the manuscript.
- M. Ochi: Critically reviewed the manuscript.

Conflicts of Interest Statement

- None declared

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