



Original Article

MMP9 secreted from mononuclear cell quality and quantity culture mediates STAT3 phosphorylation and fibroblast migration in wounds

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ABSTRACT

Introduction: Intractable ulcers may ultimately lead to amputation. To promote wound healing, researchers developed a serum-free ex vivo peripheral blood mononuclear cell quality and quantity culture (MNC-QQc) as a source for cell therapy. In mice, pigs, and even humans, cell therapy with MNC-QQc reportedly yields a high regenerative efficacy. However, the mechanism of wound healing by MNC-QQc cells remains largely unknown. Hence, using an in vitro wound healing model, this study aimed to investigate MNC-QQc cells and the migratory potential of dermal fibroblasts.

Methods: After separation from a 50 mL blood sample from healthy individuals, mononuclear cells were cultured for 7 days in a serum-free ex vivo expansion system with five different cytokines (MNC-QQc method). The effects of MNC-QQc cells on human dermal fibroblast migration were observed by scratch assay. An angiogenesis array screened the MNC-QQc cell supernatant for proteins related to wound healing. Finally, fibroblast migration was confirmed by observing the intracellular signal transduction pathways via Western blot.

Results: The migration of fibroblasts co-cultured with MNC-QQc cells increased by matrix metalloproteinase-9 (MMP9) secretion, as suggested by the angiogenesis array. Furthermore, the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in fibroblast/MNC-QQc cell co-culture and fibroblast culture with added recombinant human MMP9 protein increased. When fibroblasts were cultured with either an MMP9 inhibitor or a STAT3 inhibitor, both fibroblast migration and STAT3 phosphorylation were significantly suppressed.

Conclusions: MNC-QQc cells promote wound healing by the secretion of MMP9, which induces fibroblast migration via the STAT3 signaling pathway.

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Abbreviations: BM, Bone marrow; BMMNC, Bone marrow mononuclear cells; DMEM, Dulbecco's modified Eagle's medium; EPC, Endothelial progenitor cells; FBS, Fetal bovine serum; HRP, Horseradish peroxidase; MMP, Matrix metalloproteinase; MNC, Monocyte cell; PB, Peripheral blood; PBMNC, Peripheral blood monocyte cells; PBS, Phosphate-buffered saline; QQc, Quality and quantity culture; SE, Standard error; VEGF, Vascular endothelial growth factor.

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1. Introduction

Peripheral blood (PB) and bone marrow (BM) mononuclear cells (PBMNCs and BMMNCs, respectively) are mainly composed of lymphoid cells or myeloid monocytes, with limited stem/progenitor cell types, such as hematopoietic stem/progenitor cells, endothelial progenitor cells (EPCs), or other mesenchymal stem cells [1]. Recently, clinical vascular regenerative therapy for patients with severe ischemic heart or limb diseases has employed autologous total mononuclear cells (MNCs) that are freshly isolated from BM or PB [1–3]. However, freshly isolated MNCs from patients has limited efficacy because of EPC dysfunction [4,5]. To overcome this limitation, we previously established a culture method that increases the

quality and quantity of EPCs derived from BM or PB, named as quality and quantity control culture (QQ culture) of MNCs (MNC-QQc).

The MNC-QQc using PBMNCs yields a remarkable effect on tissue regeneration [1]. The MNC-QQc cells have demonstrated satisfactory results in murine ischemic hindlimb model, pig wound model, and acute kidney injury; MNC-QQc cells indeed yield tissue regeneration effects by promoting blood vessel formation [1,6,7]. Cutaneous regeneration occurs as a cellular response to injury and involves the activation of keratinocytes, fibroblasts, endothelial cells, and macrophages [8]. However, the direct effects of MNC-QQc cells on wounded cutaneous cells and the signaling pathway that is activated by MNC-QQc cells are still poorly understood.

In diabetic mice, tissue regeneration generally fails, mainly because of inadequate cytokine secretion of ischemic tissues [9–12]. In MNC-QQc cells, a phenotype is increased during the expression of genes involved in vessel maturation; these genes include angiopoietin-1 (Ang-1), vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP2), and matrix metalloproteinase-9 (MMP9) [1]. MMP9 is expressed in several types of injured epithelia, including the eye, skin, gut, and lung, and plays an important role in wound healing [13]. Wound healing is reportedly delayed in MMP9-knockout mice [14]. However, studies on the MMP9 release from MNC-QQc cells in the migration of dermal fibroblasts or the involvement of signal transducer and activator of transcription 3 (STAT3) signaling pathways in dermal fibroblast migration are limited. We hypothesized that MNC-QQc cells contribute to MMP9 release following the promotion of dermal fibroblast migration through the STAT3 signaling pathway to reconstitute a skin defect.

This study aimed to investigate whether noncontact co-culture of fibroblasts with MNC-QQc cells promotes fibroblast migration by MMP9 secretion and regulates the STAT3 pathway to induce cell migration and aid in scratch-wound healing.

2. Materials and methods

2.1. QQ culture of MNCs

The Clinical Investigation Committee at Juntendo University School of Medicine approved our protocols for peripheral blood (PB) collection from healthy donors (Ethics approval number: M12-0902; Ethics committee approval date: 11/19/2012). All study participants provided a written informed consent. All study procedures were conducted in accordance with the principles of the World Medical Association Declaration of Helsinki.

We collected PB samples of 50 mL each from healthy volunteers aged 20–45 years by venipuncture at the forearm using vacuum blood collection tubes with ethylenediaminetetraacetic acid disodium salt. Then, MNCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich, St. Louis, MO, USA) [1]. The QQ culture medium was composed of Stemline II medium (Sigma–Aldrich, St. Louis, MO, USA) containing stem cell factor (100 ng/mL), thrombopoietin (20 ng/mL), Flt-3 ligand (100 ng/mL), VEGF (50 ng/mL), interleukin-6 (20 ng/mL) (all from PeproTech, Rock Hill, NJ, USA), and antibiotics (Penicillin at 100 units/mL and Streptomycin at 100 µg/mL, Gibco, NY, USA). After inoculation at a cell density of 2×10^6 cells/2 mL, with QQ culture medium in each well of 6-well Primaria tissue culture plate (BD Falcon; BD Biosciences, San Jose, CA), the MNCs were cultured for 7 days.

2.2. Wound model, cellular therapy, histology, and immunofluorescence

We used euglycemic BALB/cA^jcl-*nu/nu* male mice. Each mouse was anesthetized, and a set of bilateral 6-mm punch biopsy

excisions was performed on the dorsum to yield full-thickness wounds, including the hypodermis and panniculus. India ink was applied intradermally at the margins to mark the wound edge permanently. A silicone stent (Grace Bio-Laboratories, Bend, OR, USA) with an 8-mm inner diameter was sutured with 5-0 nylon (Ethicon, Somerville, NJ, USA) around each wound to minimize skin contracture and to ensure healing by secondary intention. On postoperative day 3, freshly isolated MNCs with 2×10^4 cells/20 µL phosphate-buffered saline (PBS) or MNC-QQc 2×10^4 cells/20 µL PBS was injected into the center of the muscle at the wound base. Wounds were covered with Tegaderm to prevent cellular leakage and drying. Animals were euthanized, and wounds were harvested on postoperative day 14 ($n = 3$ per group). A full-thickness excision was performed, including 3 mm beyond the margin of the original wound edge (demarcated with India ink) [15].

Dorsal wound skin samples were fixed in 4% paraformaldehyde overnight, treated with 5%–30% sucrose in PBS solution overnight, embedded in tissue processing medium (OCT), and stored at -80°C [15]. For histological analysis, frozen wound tissue sections of 8 µm obtained from the central part of the wound were stained with hematoxylin and eosin (H&E). Frozen wound tissue sections were incubated with PBS containing 10% goat serum. Sections were further incubated overnight with a combination of anti-platelet-derived growth factor receptor- α (PDGFR α) antibodies (1:300; Biossusa Inc., Cambridge, MA, USA) at 4°C . Tissue sections were incubated with Alexa 488-conjugated goat anti-rabbit antibody (1:300; Molecular Probes, Eugene, OR, USA) at room temperature for 60 min and mounted with Hoechst 33342 for nuclear staining. Negative controls without primary antibodies were used in each experiment to rule out nonspecific labeling. The wound edge was analyzed using the LSM 510 two-photon laser confocal scanning system (Zeiss, Thornwood, NY, USA) [16].

2.3. Fibroblast culture

Purchased from Lonza Ltd. (Tokyo, Japan), human dermal fibroblasts between 8 and 12 passages were collected from healthy subjects. We then cultured these fibroblasts in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaille, France), in a humidified incubator at 37°C with 5% CO_2 in the atmosphere. DMEM was originally formulated to contain high glucose concentration (4.5 g/L). Subsequently, we plated the fibroblasts at 0.8×10^4 cells per well of a 96-well plate and serum-starved them at 70%–80% confluence with 0.5% FBS/DMEM for 24 h. These cells were used for the following experiments.

2.4. Human angiogenesis array

The levels of angiogenesis-related proteins in the media after the MNC-QQc were detected by Proteome Profiler Human Angiogenesis Array according to the manufacturer's protocol (R&D systems, MN, USA). Stemline II medium without cells under the same culture condition was measured as the negative control. Moreover, we added a cocktail of biotinylated detection antibodies in the cell culture supernatant. Then, the sample mixture was incubated with the antibody array to arrow-bind immobilized captured antibodies on the membrane. The signals were visualized using streptavidin-horseradish peroxidase (HRP) and chemiluminescent detection reagents and then measured on an image analyzer (LAS-3000; Fujifilm Co., Tokyo, Japan).

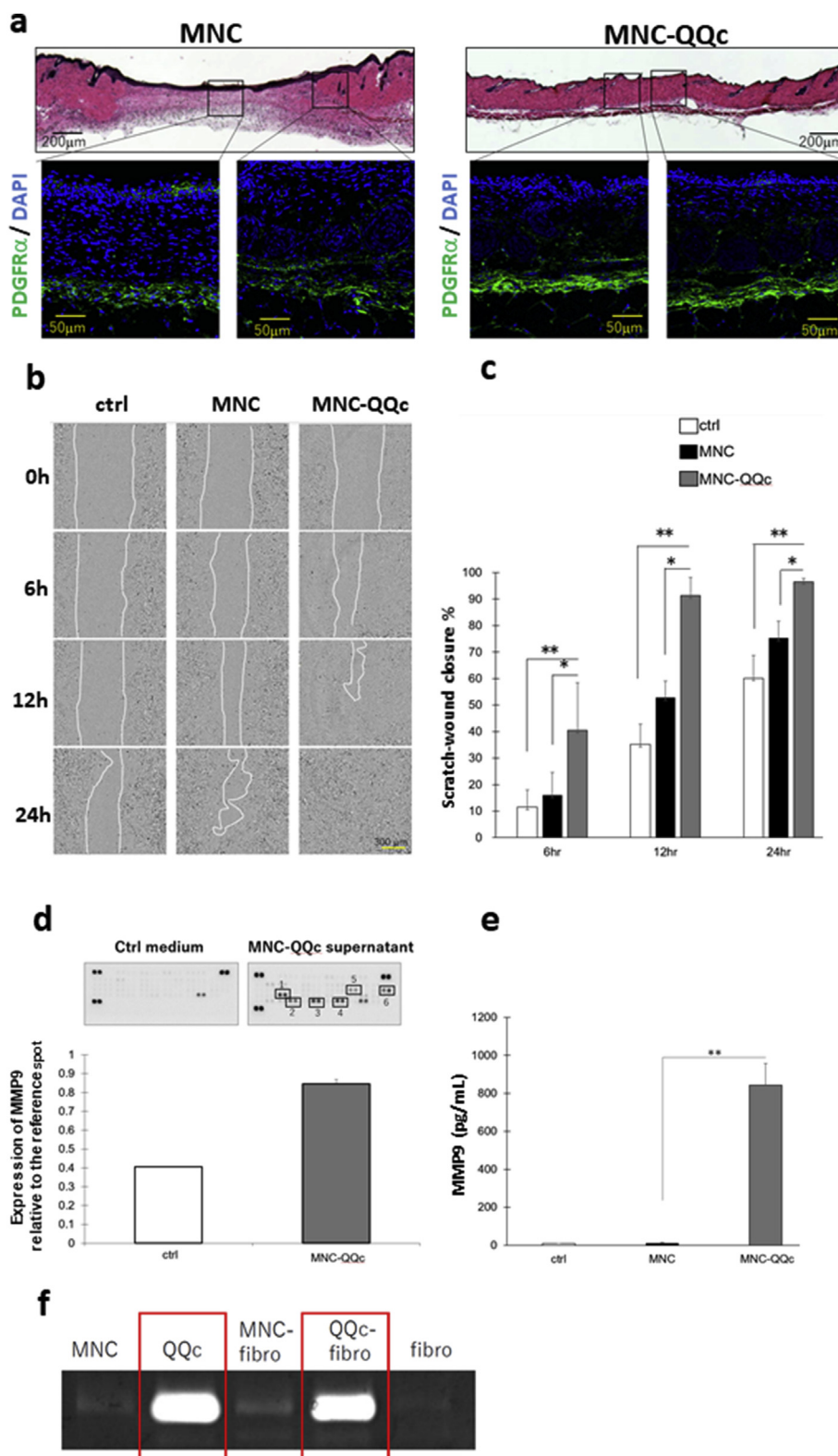


Fig. 1. Effects of mononuclear cell quality and quantity culture (MNC-QQc) cells on fibroblast migration and matrix metalloproteinase-9 (MMP9) release from MNC-QQc cells. **a:** Histological analysis of the wound skin on day 14 during healing. Skin sections stained with hematoxylin and eosin (H&E) revealed wound histology (top row). Scale bar = 200 µm. Representative immunostaining with anti-platelet-derived growth factor receptor-α (PDGFRα; green). Nuclear counterstaining was performed using (4,6-diamidino-2-phenylindole (DAPI; blue)). Scale bar = 50 µm. The left panels show ulcerated skin treated with MNC. The right panels show ulcerated skin treated with MNC-QQc cells. Images are representative of three different experiments. **b:** Photos of fibroblast-seeded plates. The vertical area indicates linear wounds made with a 96-well wound marker. Cells were co-cultured with an equal amount of MNCs or MNC-QQc cells on plate inserts for 0, 6, 12, and 24 h. Vertical scratch width = 300 µm. **c:** The scratch-wound closure in fibroblasts stimulated by MNC or MNC-QQc cells was determined by measuring the acellular area at 0, 6, 12, and 24 h. The data of three different experiments are presented as mean ± standard error (SE); **p < 0.005, compared with the control [ctrl] group, *p < 0.05, compared with the MNC [MNC] group. **d:** Upper panels: Expression of MMP9 relative to the reference spot using human angiogenesis array. Reference spots are included to align the transparency overlay template and demonstrate that the array was incubated with streptavidin-horseradish

2.5. Quantification and activity of MMP9

Culture supernatants were collected from MNC or MNC-QQ cells and stored at -80°C until use. We quantified the MMP9 levels secreted from cells using MMP9 ELISA kit (R&D Systems, MN, USA) according to the manufacturer's protocol. MMP9 levels were quantified by inspecting the samples at 450 nm absorbance on a microplate reader (Molecular Devices, CA, USA). MMP activity was investigated by Novex® zymogram gel according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Scratch assay

In 96-well Essen ImageLock microplates (Essen BioScience, Ann Arbor, MI, USA), fibroblasts were attached with DMEM containing 10% FBS. Then, they were cultured under the abovementioned serum starvation condition. Furthermore, we created wounds by using a 96-well wound maker (Essen BioScience). After removing the detached cells, the wells were added with culture inserts (HTS Transwell 96-Well; catalog No.7369; Corning, NY, USA) containing an equal number of MNCs, MNC-QQc cells, or recombinant human MMP9 protein (500 pg/mL) (Abcam, Cambridge, UK), with or without MMP9 inhibitor 1 (1 μM) (Merck Biosciences, Darmstadt, Germany) or tyrphostin AG 490 (40 μM) (Sigma–Aldrich, St. Louis, MO). The culture insert is a porous membrane made of 0.4 μm polyethylene terephthalate material that allows the easy permeation of cytokines. The culture insert has a suspended design, which allows it to hang in the middle of the well. Fibroblasts were attached to the bottom of the plate well, and MNCs or MNC-QQc cells were seeded in the insert wells. Two types of cells were co-cultured in a non-contact manner using the culture insert device, and only the medium was common. Then, the fibroblasts on each well were imaged using an IncuCyte S3 Live-cell analysis system (Essen BioScience, MI, USA), and the devoid area of cells in the images was measured using the ImageJ software.

2.7. Cell proliferation assay

To demonstrate the effect of MNC-QQc cells or MMP9 on dermal fibroblast proliferation, we counted the viable cells by using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan), which was based on the tetrazolium salt method, applying the manufacturer's instructions. Then, we seeded the fibroblasts with 200 μL of DMEM in each well on a 96-well plate and placed the culture-insert devices with an equal number of MNCs, MNC-QQc cells, or added recombinant MMP9 (500 pg/mL). The media without the cells served as the negative controls. Thereafter, the cells were incubated with the CCK-8 reagent for 2 h at 37°C in a CO_2 incubator and then set the absorbance at 450 nm on a reader. For each experiment, four replicates of each treatment were used.

2.8. Western blot analysis

Using the SDS-PAGE with 4%–12% gel application (Thermo Fisher), we separated the proteins in whole-cell lysates from fibroblasts. We then transferred them electrically on a polyvinylidene fluoride microporous membrane (Life Technologies, MA,

USA). Next, this membrane was incubated at 4°C for 18 h with antibodies against phospho-STAT3 (p-STAT3, 1:1000), STAT3 (1:1000), or β -actin (1:2000, all from Cell Signaling Technology, MA, USA), and then washed. Afterward, it was incubated with HRP-labeled anti-rabbit IgG (1:2000, Cell Signaling Technology) for 30 min at room temperature. We visualized the HRP-labeled antibody-binding antigenic proteins on the membrane as chemiluminescence by using the Lumi-Light^{PLUS} Western blot kit (Roche Diagnostics Co., Basel, Switzerland), followed by image analysis using an image analyzer (LAS-3000; Fujifilm Co., Tokyo, Japan).

2.9. Statistical analysis

All data were expressed as mean \pm standard error (SE). The statistical difference between two groups was analyzed by Student's *t*-test. When more than three groups were compared, we used one-way analysis of variance with Bonferroni multiple comparison tests. All statistical data were analyzed by the SPSS Software (IBM Software, Tokyo, Japan), and $p < 0.05$ indicated statistical significance.

3. Results

3.1. MNC-QQc cellular therapy increased the number of dermal fibroblasts in skin ulcers

We used skin sections from the wound on day 14 and performed H&E staining to study the construction of the entire wound tissue. In the MNC-QQc transplantation group, regeneration of the epidermis, hair follicles, dermis, subcutaneous fat, and muscle layer was nearly complete and the skin returned to its normal state; in contrast, no regeneration of hair follicles, subcutaneous fat, or muscle layer was observed in the skin sections of the MNC transplant group obtained simultaneously (Fig. 1a, upper images). To identify the effect of MNC-QQc cells on dermal fibroblasts, we examined the wound skin tissue transplanted with MNC-QQc cells, and visualized the dermal fibroblast marker, platelet-derived growth factor receptor- α (PDGFR α), which is expressed in the dermis at all stages of development and during the postnatal period [17]. Following treatment of the MNC-QQ group, the number of dermal PDGFR α positive cells (green) increased in comparison with those in the MNC group on day 14 (Fig. 1a). Thus, the number of dermal fibroblasts increased due to the transplantation of MNC-QQc cells into the wounded skin.

3.2. MNC-QQc cells stimulated dermal fibroblast migration and scratch-wound closure via MMP9 secretion

The effect of MNC-QQc cells on fibroblasts was determined by a scratch assay using human dermal fibroblasts. Indirect co-culture was employed to validate the secretory effects of MNC-QQc cells on fibroblasts. Compared with the control and MNC groups, the fibroblasts co-cultured with MNC-QQc cells indirectly exhibited increased migration at 6, 12, and 24 h (Fig. 1b and c).

The cause of fibroblast migration was determined by performing a proteome profile. A markedly high MMP9 protein expression was detected in MNC-QQc cell supernatants (Fig. 1d). Other spots,

peroxidase during the assay procedure. The boxed spots show altered proteins from MNC-QQc cultures. Box 1: MMP9; box 2: TIMP1; box 3: thrombospondin-1; box 4: uPA; box 5: IL-8; box 6: MCP-1. Lower panel: Quantitation of mean pixel density of the spot on the membrane. Control medium, $n = 1$; supernatant of MNC-QQc cells, $n = 7$. e: Quantitation of MMP9 release from the culture medium (control), supernatants from cultured peripheral blood mononuclear cells (MNCs), and supernatants from MNC-QQc cells (MNC-QQc). The data are presented as mean \pm SE in three different experiments (** $p < 0.01$, compared with the ctrl group). f: MMP activity was investigated using Novex® zymogram gel. From the left, the bands shown are the MNC culture supernatant (MNC), MNC-QQc culture supernatant (QQc), MNC and fibroblast co-culture 24-hour supernatant (MNC-fibro), MNC-QQc and fibroblast co-culture 24-hour supernatant (QQc-fibro), and fibroblast single-culture 24-hour supernatant liquid (fibro). Data are representative data of three different experiments.

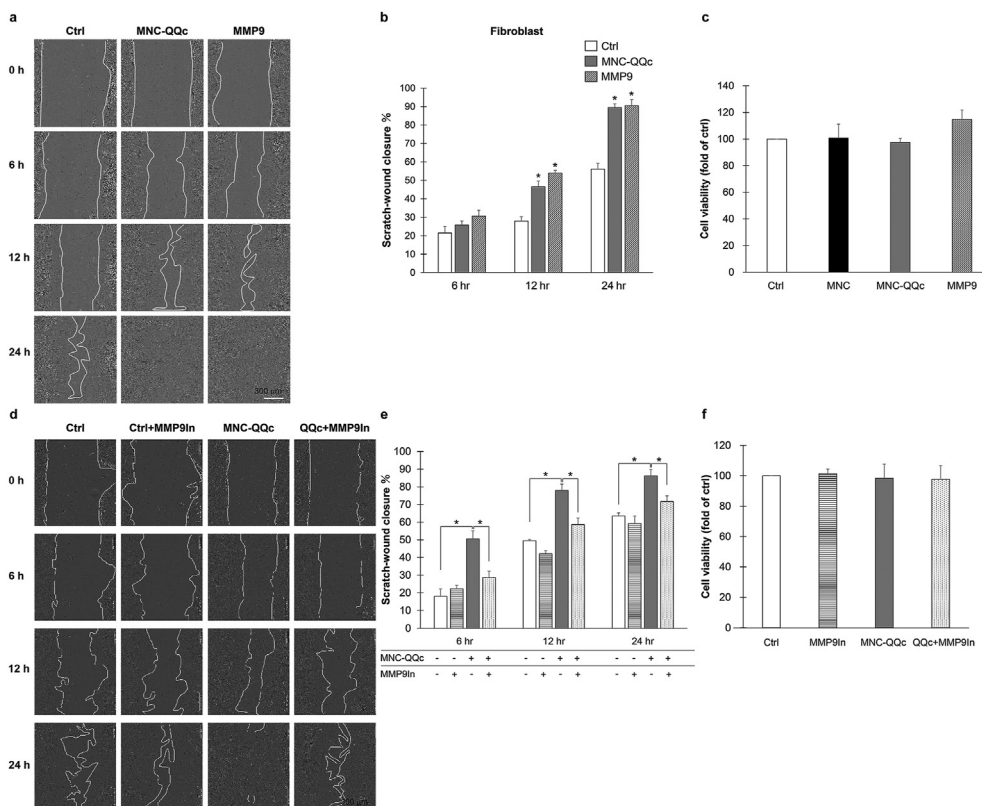


Fig. 2. Mononuclear cell quality and quantity culture (MNC-QQc) cells stimulated dermal fibroblast migration by the matrix metalloproteinase-9 (MMP9) release. a: Photos of fibroblast-seeded plates. The vertical area indicates linear wounds made with a 96-well wound marker. Cells were co-cultured with an equal amount of MNC-QQc cells on plate inserts or treated with 500 pg/mL MMP9 for 0, 6, 12, and 24 h. Vertical scratch width = 300 μ m. b: The scratch-wound closure of MNC-QQc cells and MMP9 stimulated fibroblasts was determined by measuring the acellular area at 0, 6, 12, and 24 h. The data are presented as mean \pm standard error (SE) in three different experiments (* p < 0.05, compared with the control [ctrl] group). c: Viable human dermal fibroblasts were counted using a Cell Counting Kit-. Human dermal fibroblasts cultured for 24 h were assigned to 100% cell viability, and the fold change of differential cell viability is shown in the graph (MNC: fibroblasts were co-cultured with an equal amount of MNC for 24 h; MNC-QQc: fibroblasts were co-cultured with an equal amount of MNC-QQc cells for 24 h; and MMP9: fibroblasts were treated with 500 pg/mL MMP9 for 24 h). The data are presented as mean \pm SE in four different experiments (no significant difference between the groups). d: Photos of fibroblast-seeded plates. The vertical area indicates linear wounds made with a 96-well wound marker. Cells were co-cultured with an equal amount of MNC-QQc cells on plate inserts with or without pretreatment with 1 μ M MMP9 inhibitor (MMP9In) for 0, 6, 12, and 24 h. Vertical scratch width = 300 μ m. e: The scratch-wound closure in fibroblasts stimulated by MNC-QQc cells with or without MMP9 inhibitor (MMP9In) was determined by measuring the acellular area at 0, 6, 12, and 24 h. The data are presented as mean \pm SE in three different experiments (* p < 0.05, compared with the ctrl group). f: Viable human dermal fibroblasts were counted using Cell Counting Kit-8; fibroblasts cultured for 24 h were assigned to 100% cell viability, and the fold change of differential cell viability is shown in graph (ctrl: fibroblasts were cultured for 24 h in DMEM medium; MMP9In: fibroblasts were treated with 1 μ M MMP9 inhibitor for 24 h; MNC-QQc: fibroblasts were co-cultured with an equal amount of MNC-QQc cells for 24 h; and QQc + MMP9In: fibroblasts were treated with 1 μ M MMP9 inhibitor and co-cultured with an equal amount of MNC-QQc cells for 24 h). The data are presented as mean \pm SE in four different experiments (no significant difference between groups).

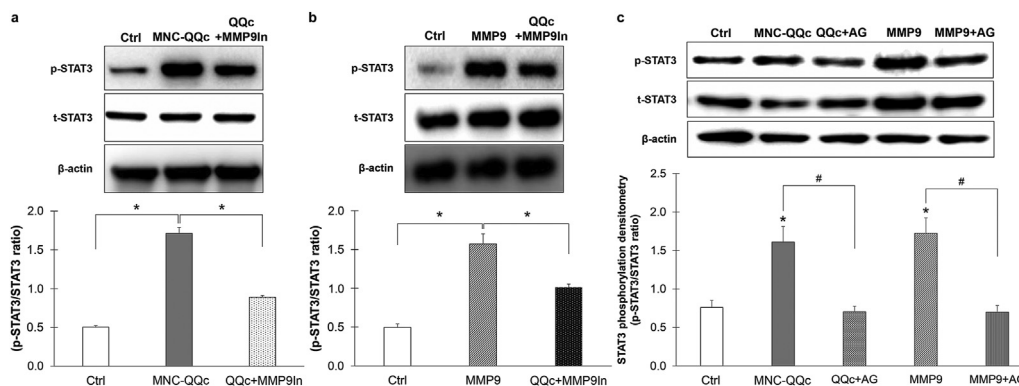


Fig. 3. Effect of mononuclear cell quality and quantity culture (MNC-QQc) cells and matrix metalloproteinase-9 (MMP9) on STAT3 signaling in dermal fibroblasts. Dermal fibroblasts were serum-starved for 24 h. a: Dermal fibroblasts were co-cultured with an equal amount of MNC-QQc cells on plate inserts for 2 h. For inhibitor treatment, after the addition of 1 μ M MMP9 inhibitor (MMP9In), dermal fibroblasts were co-cultured with an equal amount of MNC-QQc cells on plate inserts for 2 h. b: Dermal fibroblasts were treated with 500 pg/mL MMP9 and incubated for 2 h. For inhibitor treatment, after the addition of 1 μ M MMP9In, dermal fibroblasts were treated with 500 pg/mL MMP9 for 2 h. c: Dermal fibroblasts were co-cultured with an equal amount of MNC-QQc cells on plate inserts or treated with 500 pg/mL MMP9 and incubated for 2 h. For inhibitor treatment, after the addition of 40 μ M tyrphostin AG 490 (STAT3 pathway inhibitor), dermal fibroblasts were co-cultured with an equal quantity of MNC-QQc cells on plate inserts or treated with 500 pg/mL MMP9 for 2 h. The data are presented as mean \pm standard error (SE) in three different experiments (* p < 0.05 compared with the control [ctrl] group, # p < 0.05 comparison between two groups).

including interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of metalloproteinase-1 (TIMP1), thrombospondin-1, and urokinase-type plasminogen activator (uPA), appeared on the MNC-QQ supernatant membrane. Of these, we investigated three intense spots: thrombospondin-1, uPA, and MMP9. Thrombospondins are a family of secreted glycoproteins with antiangiogenic functions. They seem to balance angiogenic factors that are secreted from MNC-QQc cells [18,19]. uPA is well known for its thrombolytic effects [20]. As mentioned previously, MMP9 is expressed in injured epithelia of the skin, gut, and lungs, and plays an important role in wound healing [21,22]. These results suggest that the release of MMP9 from MNC-QQc cells promotes dermal fibroblast migration. The amount of MMP9 secretion was significantly higher in the supernatant of MNC-QQ cells than in the supernatant of non-QQ cultured MNCs (Fig. 1e). The indirect co-culture experiment also revealed that fibroblasts co-cultured with MNC-QQ cells showed increased migration (Fig. 1b and c). Therefore, the MMP9 secreted from MNC-QQc cells may be the key factor to promote fibroblast migration. Next, we measured the effect of MMP9 on dermal fibroblast migration. MMP9 activity was similar after 24 h of MNC-QQc or MNC-QQc co-culture with fibroblast culture medium (Fig. 1f). These results suggested that MMP9 protein secreted by MNC-QQc co-culture with fibroblasts can be present without an increase in its enzymatic activity.

3.3. MMP9 stimulated dermal fibroblast migration

The effect of MMP9 on fibroblast activity was determined by scratch assay. The MMP9-treated cells showed an increased migration of dermal fibroblasts at 12–24 h compared with non-MMP9-treated cells (Fig. 2a and b). Moreover, we investigated whether the proliferation or migration was predominantly acted on fibroblasts. When dermal fibroblasts were cultured in DMEM with MMP9 or co-cultured with MNC-QQc cells for 24 h, fibroblast viability remained unchanged (Fig. 2c). Therefore, the MNC-QQc cells and MMP9 promoted cellular migration activity, but the cell number of fibroblasts with co-culture medium remained the same.

3.4. Pharmacologic MMP9 inhibitor suppressed dermal fibroblast migration

To demonstrate the role of MMP9 as a key factor in inducing fibroblast migration, we blocked the MMP9 activity by using its specific inhibitor (MMP9 inhibitor 1). The fibroblast migration in

cells treated with MMP9 inhibitor 1 (1 μM) significantly decreased at 6–24 h compared with that in cells co-cultured with MNC-QQc cells (Fig. 2d and e). The 24 h MMP9 inhibitor treatment did not dramatically alter the cell viability (Fig. 2f). Thus, the scratched fibroblasts co-cultured with MNC-QQc cells demonstrated a marked migratory response, but the stimulated fibroblast migration was blocked by MMP9 inhibition. Cell proliferation was not required for this migratory response. Therefore, MMP9 secreted from MNC-QQc cells is the key factor to promote fibroblast migration.

3.5. MMP9 stimulated migration by dermal fibroblasts via the STAT3 pathway

We identified the important role of MMP9 in dermal fibroblast migration and examined the target molecules of MMP9. STAT3 is a regulatory kinase that apparently promotes fibroblast migration [23,24]. To confirm the role of MNC-QQc cells and MMP9 in STAT3 signaling in fibroblasts, we analyzed the STAT3 phosphorylation. The STAT3 phosphorylation levels significantly increased after 2 h of co-culture with MNC-QQc cells (Fig. 3a) and treatment with 500 pg/mL concentration of MMP9 (Fig. 3b) separately. Conversely, after MMP9 inhibitor treatment, STAT3 phosphorylation significantly decreased (Fig. 3a and b). To verify the function of STAT3 in fibroblast migration, we used a STAT3 pathway inhibitor to suppress the effect of MNC-QQc cells and MMP9. As shown in Fig. 3c, the addition of a STAT3 pathway inhibitor (40 μM AG490) reduced the STAT3 phosphorylation by approximately 50% in both fibroblasts co-cultured with MNC-QQc cells and fibroblasts treated with MMP9. Therefore, MMP9 mediates the effect of MNC-QQc cells on signaling pathways in a STAT3-dependent manner.

3.6. AG490 attenuated the migration of fibroblasts stimulated by MNC-QQc cells and MMP9

To further confirm the function of STAT3 in fibroblast migration, we performed a scratch-wound assay on fibroblasts treated with 40 μM concentration of STAT3 pathway inhibitor (AG490). The migration of dermal fibroblasts co-cultured with MNC-QQc cells or treated with 500 pg/mL MMP9 increased at 12–24 h (Fig. 4a and b). However, this migration was attenuated by AG490 (Fig. 4a and b). Therefore, fibroblast migration stimulated by MNC-QQc cells and MMP9 participates in STAT3 phosphorylation.

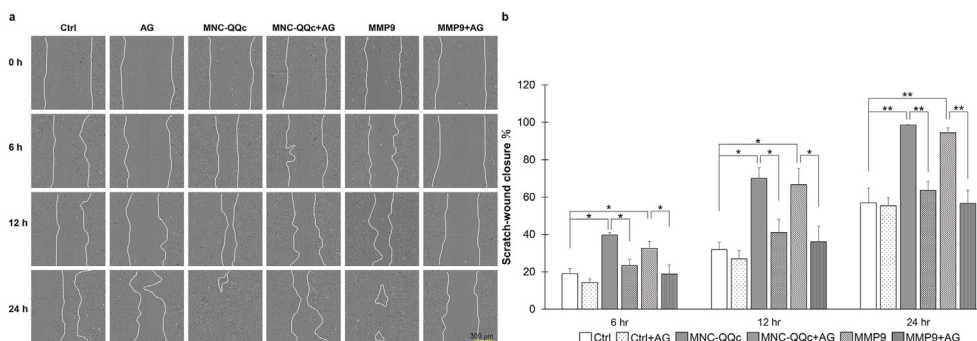


Fig. 4. Effect of mononuclear cell quality and quantity culture (MNC-QQc) cells, matrix metalloproteinase-9 (MMP9), and inhibition of STAT3 phosphorylation on dermal fibroblast migration. a: Photos of fibroblast-seeded plates. The vertical area depicts linear wounds made with a 96-well wound marker. Cells were co-cultured with an equal amount of MNC-QQc cells on plate inserts or treated with 500 pg/mL MMP9 for 0, 6, 12, and 24 h. For inhibitor treatment, after the addition of 40 μM Tyrphostin AG 490 (STAT3 pathway inhibitor [AG]), dermal fibroblasts were co-cultured with an equal amount of MNC-QQc cells on plate inserts or treated with 500 pg/mL MMP9 for 0, 6, 12, and 24 h. The data are presented as mean ± standard error (SE) in three different experiments. Vertical scratch width = 300 μm. b: The scratch-wound closure in fibroblasts stimulated by MNC-QQc cells, MMP9, and tyrphostin AG 490 was determined by measuring the acellular area at 0, 6, 12, and 24 h. The data are represented as mean ± SE in three different experiments (*p < 0.05, **p < 0.01 compared with the ctrl group).

4. Discussion

In several studies, MNC-QQc using peripheral blood MNCs promotes tissue regeneration, but the mechanism underlying this and the effect on wounds are still largely unknown. To the best of our knowledge, this is the first study to investigate the relationship between MNC-QQc cells and dermal fibroblasts. The current study provides the first evidence of the mechanism by which MNC-QQc can promote wound closure. The migration of human dermal fibroblasts is increased when these fibroblasts are co-cultured with MNC-QQc cells and also when they are treated with MMP9, ultimately leading to scratch-wound closure. In addition, the secretion of MMP9 by MNC-QQc cells is mediated by the phosphorylation of the STAT-3 pathway.

The current study also identified that MNC-QQc cells increased the release of chemokine and MMP9 (Fig. 1d). Hence, human dermal fibroblasts co-cultured with MNC-QQc cells or treated with MMP9 promote fibroblast migration. This event may, in part, account for the mechanism of MNC-QQc cells on wound healing. Lobmann et al. revealed an imbalance of MMP and their inhibitors (TIMP-2); this imbalance could be involved in the pathogenesis of nonhealing chronic ulcers [25]. Hattori et al. generated full-thickness cutaneous wounds in MMP9-knockout mice and found that macroscopic wound closure was delayed in these mice compared with that in wild-type mice [14]. During the wound-repair process, MMP9 is upregulated, and it is detected in migrating basal epithelial cells [26,27]. MMP9 plays an important role in the proper migration of keratinocytes and human bronchial epithelial cells in vitro [14,27,28]. Hence, these studies favored that MMP9 is a key factor in cutaneous tissue repair during wound healing process. However, MMP9 on dermal fibroblasts is vaguely known. Our results confirmed that MMP9 secretion is increased by MNC-QQc cells in culture supernatant (Fig. 1c and d) and MMP9 promotes the migration of dermal fibroblasts, as shown in the scratch-wound assay (Fig. 2a and b). We inferred that MMP9 released by transplanted MNC-QQc cells are necessary to coordinate and maintain healing; in addition, wound healing occurs as a cellular response to injury and involves the fibroblast activation.

STAT3 belongs to the family of transcription factors comprising STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STAT3 regulates divergent cellular functions, such as cellular survival and proliferation [29,30], in response to numerous growth factors and cytokines, such as platelet-derived growth factor, VEGF, and IL-6 [24,31–33]. STAT3 is activated by phosphorylation at tyrosine 705, which promotes dimerization, nuclear translocation, and DNA binding [29,34,35]. Numerous cell types express phosphorylated forms of STAT3 such as human dermal microvascular endothelial cells, keratinocytes, and fibroblasts [36,37]. Epidermal cells and keratinocytes that are defective in STAT3 gene expression exhibit impaired growth factor-dependent in vitro migration, indicating that STAT3 is necessary for cell migration in a cell-autonomous manner [38]. Furthermore, Dechow et al. reported that the MMP9 activity determined by human dermal microvascular endothelial cells is increased in the STAT3-containing cell lines and that high protein levels of MMP9 in sequential sections of the tissue microarrays correlate with those of activated STAT3 [36]. In the current study, a STAT3 pathway inhibitor suppressed the MNC-QQc cells and MMP9 by initiating the migration of human dermal fibroblasts (Fig. 4a and b). Consistent with our data, Song et al. reported that the functional cooperation of STAT3 is essential for MMP9 gene transcription [39]. Our data support the role of MNC-QQc cells in increasing fibroblast migration to the wound site through MMP9 release via phosphorylated tyrosine 705 at the STAT3 pathway. Furthermore, the MMP9 promoter contains multiple putative STAT3-binding sites [36]. We infer that STAT3 binding to the MMP-

9 promoter induces cell migration in human skin primary fibroblast cultures. MNC-QQc cells released numerous chemokines (Fig. 1d membrane, the spot enclosed by square number 5 and 6), and chemokines such as IL-8, IL-6, and MCP-1 are reportedly involved in cell migration [24,26,40–42]. Therefore, MMP9 is one of the major factors of MNC-QQc to initiate dermal fibroblast migration. However, further investigation is necessary to identify other components modulated by MNC-QQc cells, including IL-8 and MCP-1 in cutaneous wound healing.

Nerve cell reduction, macrophage infiltration, fibroblast migration inhibition, and vascular endothelial cell reduction are reportedly associated with delayed skin regeneration in diabetes [8,16,43–45]. Although not published, the ongoing preclinical and clinical studies of our groups reveal that MNC-QQc cell transplantation promotes ulcer healing and the MNC-QQc cells may promote wound healing through various mechanisms, including promotion of angiogenesis and differentiation into anti-inflammatory macrophages. Furthermore, we demonstrated the vasculogenic, anti-inflammatory, and wound healing effects of MNC-QQc cell therapy in both in vitro and in vivo models [46]. We developed a serum-free QQc culture system using peripheral blood MNCs that can significantly increase the number of total and differentiated colony-forming EPCs conditioned for anti-inflammatory and regenerative phenotypes [46]. Regarding proliferation of the mononuclear cells, the total cell numbers were decreased by 30% during the culture, but the essential cells for tissue and vascular regeneration, eg EPC, M2-Macrophage, Th2 cells were remained and increased the ratio in the products, The cells related with inflammation such as B cells, NK cells and M1-Macrophage were decreased [46]. These results indicated that under QQc conditions, PBMNCs selectively upregulate the stem cell population for EPCs as well as for anti-inflammatory pro-angiogenic monocytes and T lymphocytes, but drastically eliminates the proinflammatory and anti-regenerative cells [6,15,46]. The mechanisms underlying these changes are currently under investigation. In the present study, the cultured fibroblasts mimicked the cells under hyperglycemic conditions in high glucose medium. This suggests that wound healing for patients suffering from diabetes with refractory ulcers would be enhanced by MNC-QQc cell transplantation. Although we cannot claim that this wound healing effect is entirely dependent on MMP9, the results suggest that MMP9 secreted by the MNC-QQ cells may have a major effect on fibroblasts.

In conclusion, MNC-QQc cells release MMP9 to increase the migration of dermal fibroblasts, and MMP9 is mediated by STAT3 phosphorylation. The relationship between the level of MMP9 expression changes in chronic cutaneous ulcer and STAT3 phosphorylation in dermal fibroblasts in patients with intractable ulcer requires further research.

In this study, we only investigated the mechanism by which MNC-QQc promotes wound healing at the level of dermal fibroblasts in a simulated diabetic environment. The effect of MNC-QQc cells on other cell types such as epidermal cells, vascular endothelial cells localized in ulcerated skin has not been investigated. Finally, it is very important to further elucidate the effects of cell signaling in adult stem cells on tissue maintenance, tissue regeneration, and carcinogenesis. This will be the subject of future research.

Author contributions

T.N.-Y.S. wrote the manuscript and participated in the research. M. K. and H.H. participated in the research. S.F. contributed to the discussion section of the manuscript. H.M. conducted some of the research and contributed to the discussion section of the

manuscript. R.T. contributed to the introduction and discussion sections of the manuscript and reviewed/edited the entire manuscript.

Declaration of competing interest

None.

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