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FULL-LENGTH ARTICLE

Basic Research

Conditioned media from mesenchymal stromal cells and periodontal ligament fibroblasts under cyclic stretch stimulation promote bone healing in mouse calvarial defects

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ABSTRACT

Background aims: When cells are exposed to stresses such as mechanical stimuli, they release growth factors and adapt to the surrounding environment H ere, we demonstrated that mechanical stimulation during culture affects the production of osteogenic and angiogenic factors.
Methods: Human bone marrow derived mesenchymal stromal cells (hMSCs) and human periodontal ligament fibroblasts (HPLFs) were cultured under cyclic stretch stimulation for 24 h. Collected of the cells and conditioned media (CM), the gene and protein expression levels of osteogenic and angiogenic factors were evaluated. CM was also evaluated for angiogenic activity and calc ification ability. In in vivo study, CM was administered to a mouse calvarial defect model and histologically and radiologically evaluated.
Results: Quantitative real time polymerase chain reaction results showed that the expression of bone morphogenetic pro tein 2, 4 (BMP 2, 4), vascular endothelial growth factor A (VEGF A), and platelet derived growth factor AA (PDGF AA) was upregulated in the cyclic stretch stimulation group in comparison with the non stretch group in each cell type. Enzyme linked immunosor bent assay results revealed that the expression of BMP 2,4, VEGF A was upregulated in the cyclic stretch group in comparison with the non stretch group in each cell type. Only HPLFs showed significant difference in PDGF AA expression between the cyclic str etch and the non stretch group. Tube formation assay and Alizarin Red S staining results showed that angiogenic activity and calcification ability of CM was upregulated in the cyclic stretch stimulation group in comparison with the non stretch group in eac h cell type. CM was administered to the mouse calvarial defect model. Histological and radiological examination showed that the bone healing was promoted by CM from the cyclic stretch culture group. Immunohistological staining revealed that CM from cyclic stretch group have greater angiogenic effect than CM from the non stretch group.
Conclusions: These results indicate that osteogenesis was promoted by CM obtained under cyclic stretch stimulation through the increase of angiogenesis in the mouse calvarial defect model.

Introduction

Reconstruction of bone defects resulting from trauma or disease requires a certain amount of autologous bone or other graft substitutes. Autologous bone grafting is considered to be an effective method and the gold standard for the reconstruction of bone defects because of the amount of bone available, its osteogenic potential and the vast amount of previous research on the subject. However, the use of autologous bone has limited the amount of bone that can be harvested [1]. Moreover, invasion of the donor site cannot be ignored [2]. Allograft and xenograft materials are commercially available [3–5]. However, these materials are difficult to mold to fit the recipient site and have a potential risk of inducing infections and immune responses [4,5]. Biomaterials such as β-tricalcium phosphate and hydroxyapatite have the advantage of unlimited availability but carry a risk of infection after implantation and have poor osteoinductivity compared with autologous bone [6].

Stem cell therapy, which uses cells collected from bone marrow, fat and dental pulp as well as other sources, has beneficial effects on

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bone regeneration [7]. Promising results have been demonstrated in both animals and humans; however, the level of engraftment and differentiation of the transplanted cells needs to be improved [8,9]. Recent studies have investigated the role played by paracrine factors produced by stem cells in tissue regeneration and healing. Furthermore, several studies have demonstrated that osteogenesis and angiogenesis are promoted by conditioned media (CM) collected from human bone-marrow-derived mesenchymal stromal cells (hMSCs) [10,11]. CM contain various growth factors related to osteogenesis and angiogenesis, such as bone morphogenetic protein (BMP), insulinlike growth factors (IGF-1, IGF-2), transforming growth factor beta 1 (TGF-β1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor 2. These growth factors promote osteogenesis by cooperating, even at low concentrations [12].

Cells are exposed to various stresses, such as mechanical, hypoxic and chemical stimulation, *in vivo*. It has been reported that these stresses regulate chemotaxis, proliferation and differentiation of the cells through intracellular signal transduction pathways [13–15]. It is well known that the hypoxia-inducible factor pathway senses changes in local oxygen availability and responds via transcriptional activation of angiogenic factors, VEGF and angiopoietins. Mechanical stress is also known to be a stimulus that affects bone metabolism. Furthermore, it has been reported that bone tissue and osteoprogenitor cells respond to mechanical stress *in vitro* and *in vivo*. Moreover, it has been reported that the secretion of osteogenic factors (BMP, TGF-β1, etc.) is enhanced in hMSCs, periosteum and fibroblasts by the addition of cyclic stretch stimulation [16–18]. However, few studies have been conducted on CM derived from cells cultured under stretch stimulation, and no sufficient *in vivo* analysis about CM has been performed.

In this study, we cultured hMSCs and human periodontal ligament fibroblasts (HPLFs) under cyclic stretch stimulation. Cyclic stretch stimulation brought greater osteogenic and angiogenic potential to CM not only *in vitro* but also *in vivo*. These findings suggest that the biological effect of CM from stem cells can be modified by culture conditions. This study indicates more effective therapeutic potential for bone regeneration therapy using CM.

Methods

Cell preparation and cell culture

HPLFs were purchased from ScienCell Research Laboratories, Inc (Carlsbad, CA, USA). The hMSCs were purchased from Lonza, Inc (Walkersville, MD, USA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing an antibiotic-antimycotic cocktail (100 U/mL penicillin G, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B; Gibco, Waltham, MA, USA) and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 and 95% air. After primary culture, the cells were subcultured at a density of 1×10^4 cells/cm^2. Cells at the third to ninth passages were used for the experiments. When the cells reached subconfluency, they were trypsinized and used for cell stretching.

Cell stretching, CM and RNA preparation

A silicone chamber (STB-CH-10; Strex Inc, Osaka, Japan) and a mechanical stretching device (STB-1400; Strex Inc) were used during culture. The silicone chamber was coated with type I collagen (Cellmatrix type I-C; Nitta Gelatin Inc, Osaka, Japan). HPLFs and hMSCs were seeded into a silicone chamber at a density of 1.0×10^5 cells/chamber. After they reached 80% confluency, the chamber was attached to a mechanical stretching device. In the stretch group, cells were given a cyclic uniaxial stretch (5% elongation) for 24 h (0.17 Hz, 37°C, 5% CO_2). In the non-stretch group, cells were not given a cyclic stretch stimulation. After 24 h, the CM (non-stretch CM [N-CM] and stretch CM [S-CM]) and cells were collected from the silicone chamber according to a previously described method [15].

Total RNA was extracted from the cells using TRIzol (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was reverse transcribed for real-time quantitative polymerase chain reaction (qPCR) to measure the expression of messenger RNA (mRNA) related to osteogenesis and angiogenesis (Figure 1A,B). The collected CM were stored at 4°C before being used for the following experiments.

RNA expression assay in HPLFs and hMSCs

RNA purity and quantification were determined by spectrophotometry (NanoDrop spectrophotometer; Thermo Scientific NanoDrop Technologies, Wilmington, DE, USA). First-strand complementary DNA synthesis was carried out using a SuperScript IV first-strand synthesis system (Thermo Fisher Scientific, Waltham, MA, USA) with 1 μg total RNA.

A qPCR was performed using THUNDERBIRD SYBR qPCR mix (Toyobo Co, Ltd, Osaka, Japan) and Mx3000P (Agilent Technologies, Santa Clara, CA, USA). The data of these targeted genes were normalized to the internal control glyceraldehyde 3-phosphate dehydrogenase to obtain ΔCT. The final quantities of the genes of interest, relative to those seen in samples from the non-stretch cells, were reported using $2^{-\Delta\Delta CT}$. The primer sets are listed in Table 1.

Enzyme-linked immunosorbent assay for CM

The levels of BMP-2, BMP-4, VEGF-A and PDGF-AA in the CM collected from HPLF and hMSC cultures under stretch or non-stretch conditions for 24 h were investigated using enzyme-linked immunosorbent assay (ELISA). The concentration of these growth factors was measured using a human Quantikine ELISA kit, SimpleStep ELISA kit and traditional ELISA kit (Abcam plc, Cambridge, UK) according to the manufacturer's instructions.

Tube formation assay

Human umbilical vein endothelial cells (HUVECs) (PromoCell, Heidelberg, Germany) at passage two or three and 80% confluency were suspended in HPLF N-CM, HPLF S-CM, hMSC N-CM or hMSC S-CM. The cells were cultured at a density of 4.0×10^5 cells/mL, in triplicate, in 96-well plates coated with growth factor reduced Matrigel

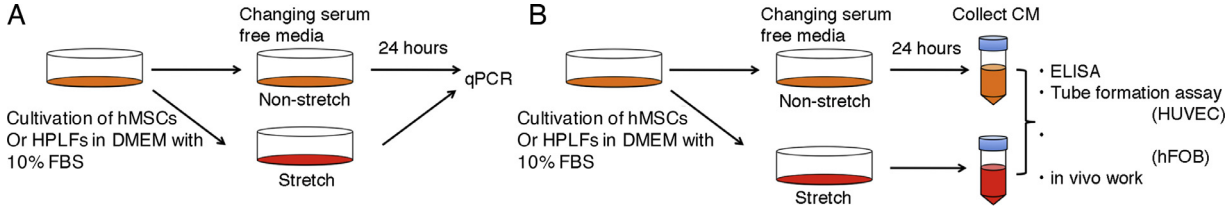

Fig. 1. Outline of the study: *in vitro* experiments and analyses. After incubation for 24 h under stretch/non-stretch stimulation, harvested cells were subjected to qPCR (A), and harvested CM were subjected to ELISA, Alizarin Red S staining, tube formation assay and *in vivo* analysis (B). (Color version of figure is available online).

Table 1
Primer sets.

Origin	Primer	Sequence (forward5′-3′)	Sequence (reverse5′-3′)
Human	GAPDH	AGCAAGAGCACAAGAGGAAGAG	TCTACATGGCAACTGTGAGGAG
Human	BMP-2	CGACACTGAGACGCTGTTCC	TGGCGCAAGGACCGAATGTC
Human	BMP-4	CCACTGGCTGACCACCTCAA	ACAGCATGGAGATGGCACTCA
Human	VEGF-A	GCCACCACACCATCACCATC	AATGCTTCCGCCGGAGTCTC
Human	PDGF-AA	ATGCGTCAACCAATCGTACG	AAGCGGCAATGCATCTCACG

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(356230; BD Biosciences, San Jose, CA, USA). Endothelial growth medium 2 BulletKit (CC-3162; Lonza) and serum-free DMEM were used as positive and negative controls, respectively. After 8 h, the formation of tube-like structures was examined using a microscope. Photographs were taken, and the number of branching points, total tube length and tube formation area (see supplementary Figure 1) were measured and assayed using a BZ-9000 fluorescence microscope and BZII analyzer (KEYENCE, Osaka, Japan).

Alizarin Red S staining

Human fetal osteoblasts (hFOBs) (CRL-11372; American Type Culture Collection, Manassas, VA, USA) were cultured in six-well plates at a concentration of 4.0×10^5 cells/mL with osteogenic supplements (10% FBS, 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid and 100 nM dexamethasone) in DMEM at 34°C in an atmosphere containing 21% O_2 and 5% CO_2. When the cells reached 80% confluency, media were replaced with HPLF N-CM, HPLF S-CM, hMSC N-CM or hMSC S-CM. All media were supplemented with 1% FBS. After 14 days of culture, cells were stained with a saturated solution of Alizarin Red S adjusted to pH 4.2 (A5533; Sigma-Aldrich, St Louis, MO, USA). The calcification spots stained by Alizarin Red S were extracted by 5% formic acid, and the absorbance (at 405 nm) of the clear yellow extracts was measured using Infinite 200 PRO NanoQuant (Tecan Group Ltd, Mannedorf, Switzerland). DMEM with osteogenic supplements and serum-free DMEM were used as positive and negative controls, respectively.

Mouse calvarial defect model

All animal experiments were performed following the Guidelines for Animal Experimentation of the Nagoya University School of Medicine. We used 8- to 10-week-old male Institute for Cancer Research mice (Japan SLC, Hamamatsu, Japan). Anesthesia was induced via an intraperitoneal injection of medetomidine hydrochloride at 0.3 mg/g, midazolam at 4 mg/kg and butorphanol tartrate at 5 mg/kg of body weight. To determine the effect of administering N-CM or S-CM, we made a mouse calvarial defect model diameter of 2 mm with a disposable biopsy punch (Kai Industries, Gifu, Japan). CM 2 μL was infiltrated into the collagen sponge (KOKEN Co, Ltd, Tokyo, Japan) and applied to the calvarial defect immediately [18]. We defined the following groups according to the implanted materials: (i) defect: unfilled defect, (ii) DMEM: serum-free DMEM/collagen sponge, (iii) HPLF N-CM: HPLF N-CM/collagen sponge, (iv) HPLF S-CM: HPLF S-CM/collagen sponge, (v) hMSC N-CM: hMSC N-CM/collagen sponge and (vi) hMSC S-CM: hMSC S-CM/collagen sponge. Mice were killed 2 or 4 weeks after surgery.

Radiographic and histological analyses

Mouse calvarial bones were harvested 28 days after surgery. The samples were subjected to microcomputed tomography analysis using a laboratory x-ray computed tomography device (SkyScan 1176; Bruker, Billerica, MA, USA). Images were compiled into 3-dimensional images and analyzed using CTAn (Bruker). We then compared the area (px) of newly regenerated bone. After the radiological assessment, the samples were fixed with a 4% paraformaldehyde solution for 24 h at room temperature. They were then decalcified with Morse's solution (10% sodium citrate, 20% formic acid) overnight at 4°C and then dehydrated using graded ethanol, cleared with xylene and embedded in paraffin. The specimens were then cut in a sagittal direction to make 5-μm thick histological sections and stained with hematoxylin and eosin. Histological analysis was performed using a light microscope.

Immunofluorescence histology

Samples sacrificed 2 weeks after surgery were washed in phosphate-buffered saline and then embedded with O.C.T. compound (Sakura Finetek, Tokyo, Japan), after which 2-μm sections were made on a cryostat (Leica CM3050 S; Leica Microsystems, Wetzlar, Germany). The sections were then blocked with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) for 10 min and incubated with the primary antibody (rabbit polyclonal antibody CD31, ab28364; Abcam plc) in a blocking buffer for 1 h. The sections were then incubated with the secondary antibody and conjugated with goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor 488 (A11008; Thermo Fisher Scientific) for 1 h. Cell nuclei were labeled with diamidinophenylidole (D1306; Life Technologies, Carlsbad, CA, USA). The sections were then mounted with ProLong gold antifade reagent (P36934; Life Technologies). Vascular density was assessed morphometrically by examining five fields per section of the calvarial defect, in three successive sections, after immunofluorescence staining using anti-CD31 Ab. Images were captured using a BZ-9000 fluorescence microscope (KEYENCE).

Statistical analysis

All statistical analyses were performed using SPSS Statistics 19.0 (IBM Corporation, Armonk, NY, USA). Paired Student's *t*-test was used for comparisons. One-way analysis of variance was applied to compare two or more means, followed by Tukey's honestly significant difference test. The results were expressed as the mean ± standard deviation. $P < 0.05$ was considered statistically significant.

Results

Effect of stretch stimulation on osteogenic and angiogenic properties

The results of the qPCR showed that the osteogenic (BMP-2, BMP-4) and angiogenic (VEGF-A, PDGF-AA) factors were significantly upregulated in cells cultured under cyclic stretch stimulation (HPLFs/hMSCs). In HPLFs, the mRNA expression of BMP-2, BMP-4 and VEGF-A cultured under cyclic stretch stimulation was approximately 3- to 5-fold higher when compared with those under normal conditions ($P < 0.05$). The expression of PDGF-AA also increased 2-fold (Figure 2A). In hMSCs, the mRNA expression of BMP-2, VEGF-A and PDGF-AA in cells cultured under cyclic stretch stimulation was approximately 10- to 15-fold higher when compared with those under normal conditions ($P < 0.05$). BMP-4 also showed a 2-fold increase in expression ($P < 0.05$) (Figure 2B).

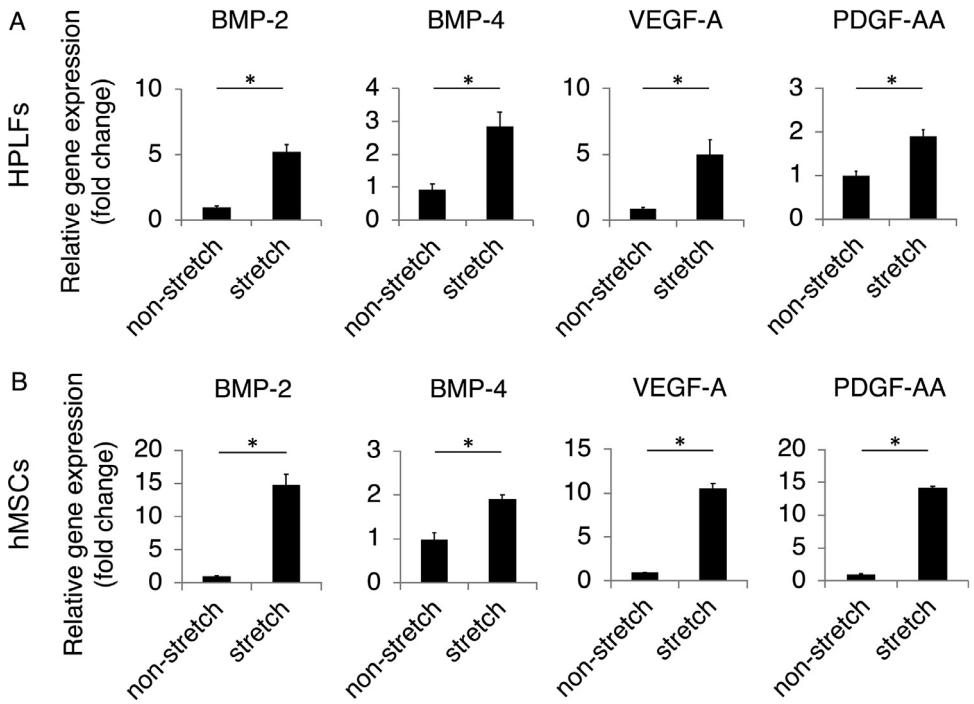

Fig. 2. Expression of osteogenic and angiogenic genes from HPLFs and hMSCs under non-stretch and stretch conditions. The qPCR results of mRNA extracted from HPLFs and hMSCs 24 h under non-stretch and stretch conditions (n = 6). BMP-2, BMP-4, VEGF-A and PDGF-AA were upregulated under stretch conditions in both cell types (A,B). Data are represented as the mean ± SD; $^{*}P < 0.05$. SD, standard deviation.

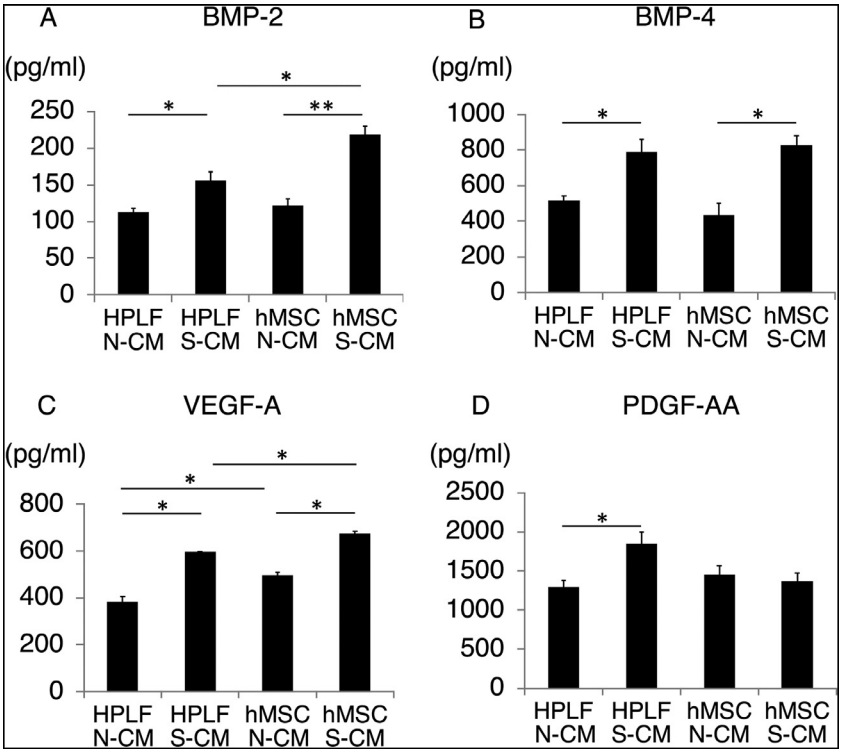

Fig. 3. CM were collected after 24 h of incubation under non-stretch and stretch conditions (n = 6). (A) BMP-2, (B) BMP-4 and (C) VEGF-A were upregulated under stretch conditions in HPLFs and hMSCs. (D) PDGF-A was upregulated under stretch conditions in HPLFs. Data are represented as the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$. SD, standard deviation.

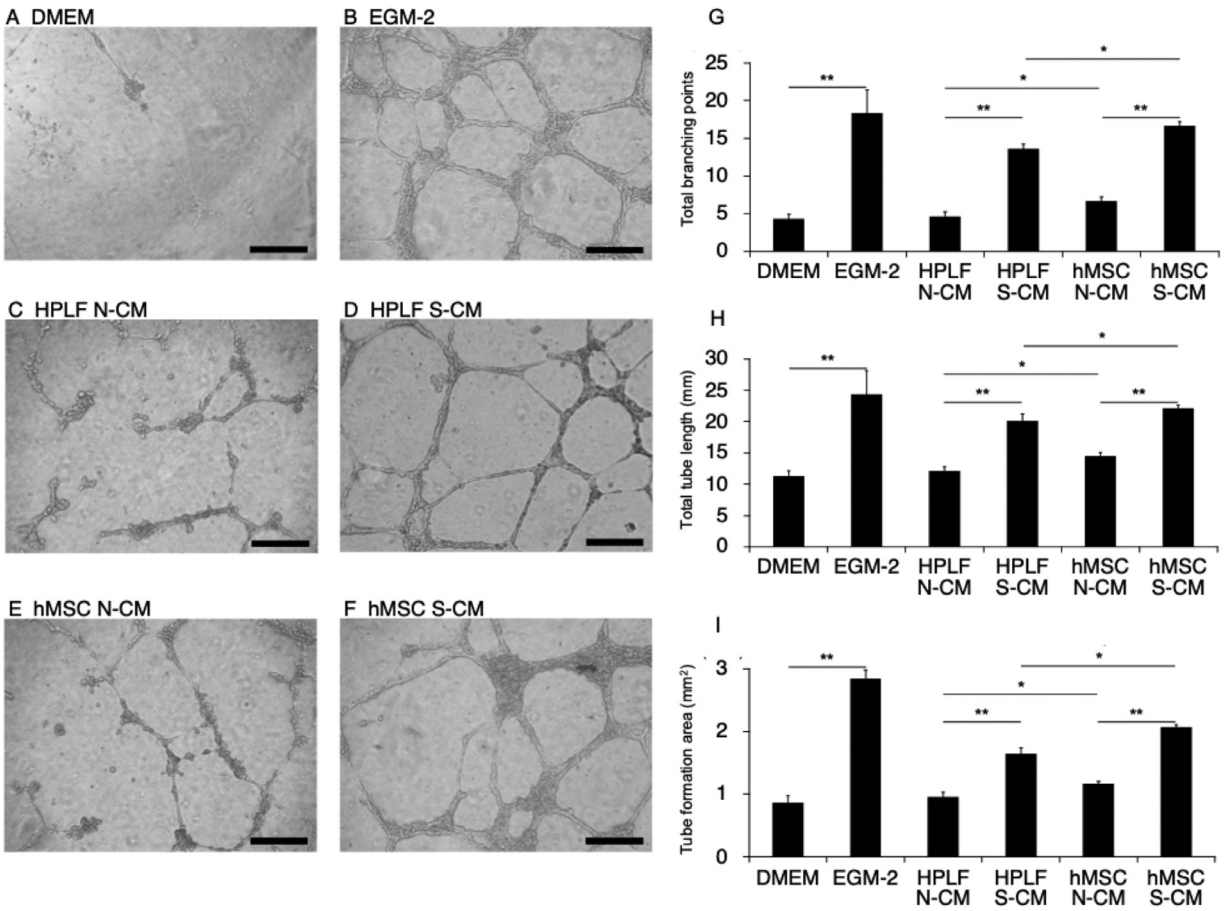

Fig. 4. CM from HPLFs and hMSCs enhance tube formation *in vitro*. The angiogenic potential of the CM was tested using a Matrigel tube formation assay (n = 6). HUVECs were suspended in N-CM or S-CM in triplicate. Photographs are from (A) DMEM, (B) EGM-2, (C) HPLF N-CM, (D) HPLF S-CM, (E) hMSC N-CM and (F) hMSC S-CM cultures. (G) The total branching points, (H) total tube length and (I) area of tube formation were calculated using a BZ-9000 fluorescence microscope. The S-CM cultures showed increased tube formation compared with the N-CM cultures. Data are represented as the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$. Bar = 200 μm. EGM-2, endothelial growth medium 2; SD, standard deviation.

ELISA was performed with the CM to measure protein expression, and the qPCR results were confirmed with RNA. The expression of BMP-2, BMP-4, VEGF-A and PDGF-AA in the HPLF S-CM was significantly increased by 1.3- to 1.5-fold compared with the standard condition group HPLF N-CM ($P < 0.05$) (Figure 3A–D). The expression of BMP-2, BMP-4 and VEGF-A in the hMSC S-CM was significantly increased by 1.3- to 2.0-fold compared with those in the hMSC N-CM (BMP-2, $P < 0.01$; BMP-4, VEGF-A, $P < 0.05$) (Figure 3A–C). There was no significant difference in the expression of PDGF-AA (Figure 3D). A higher expression of BMP-2 and VEGF-A was observed in the hMSC group compared with the HPLF group (Figure 3A,C).

Effect of CM from HPLFs/hMSCs on angiogenesis and mineralization in vitro

A tube formation assay was carried out using HUVECs to evaluate the angiogenic activity of the CM. In each CM sample from HPLFs and hMSCs, HUVECs showed increased tube formation in the N-CM group compared with DMEM (Figure 4A,C,E), and the S-CM group showed increased tube formation when compared with N-CM (Figure 4D,F). The number of branching points (2.5- to 2.9-fold), total tube length (1.5- to 1.7-fold) and tube formation area (1.7- to 1.8-fold) were significantly larger in the S-CM group than in the N-CM group in each cell group ($P < 0.01$) (Figure 4G–I). The number of branching points, total tube length and area of tube formation were significantly larger in the hMSC group than in the HPLF group ($P < 0.05$).

To evaluate the calcification potential of CM, we performed Alizarin Red S staining with hFOBs, which have a high calcification potential. Cultures of hFOBs in N-CM and S-CM from each cell group produced more mineral deposits compared with DMEM (Figure 5A,C,E). The calcification ability of S-CM was higher by 1.8- to 2.6-fold than N-CM in each cell group ($P < 0.01$) (Figure 5C–G). Calcification was increased in the hMSC group compared with the HPLF group (Figure 5G).

Effect of CM on bone healing in the mouse calvarial defect model

Hematoxylin and eosin staining was performed on postoperative day 28 to evaluate osteogenesis. In the defect and DMEM groups, the defect was filled with fibrous tissue and collagen scaffolds, respectively, and there was almost no osteogenesis (Figure 6A,B), whereas osteogenesis was observed in the N-CM and S-CM groups of each cell type. The CM from each cell type showed greater osteogenesis in the S-CM group than in the N-CM group (Figure 6C–F). New bone was also evaluated radiologically on day 28. The defect and DMEM groups showed almost no calcification (Figure 6G,H), whereas the N-CM group showed new bone with weak calcification at the CM implant site in each cell type on day 28 (Figure 6I,K). By contrast, the S-CM group had strongly calcified bone at the application site, and the bone volume ratio was higher by 1.8- to 2.0-fold in the S-CM group than in the N-CM group ($P < 0.05$), although there was no significant difference between cell types (Figure 6J,L,M).

The tissues were subjected to fluorescent immunostaining on postoperative day 14 to clarify the effect of CM on bone healing and evaluate angiogenesis. In the HPLF and hMSC cell groups, the

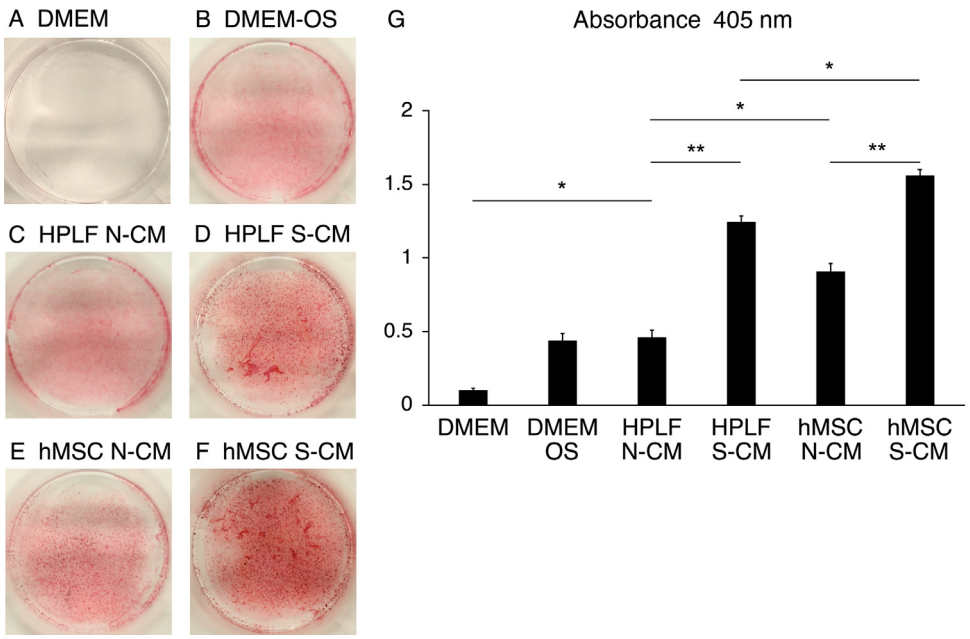

Fig. 5. Alizarin Red S staining of hFOBs. HPLF S-CM and hMSC S-CM increased the staining intensity. Triplicate wells were used for each treatment. Representative images of hFOBs cultured in (A) DMEM, (B) DMEM-OS, (C) HPLF N-CM, (D) HPLF S-CM, (E) hMSC N-CM and (F) hMSC S-CM. (D) HPLF S-CM and (F) hMSC S-CM were stained more strongly by Alizarin Red S compared with (C) HPLF N-CM and (E) hMSC N-CM. (E) The calcified spots stained with Alizarin Red S were extracted with 5% formic acid, and the absorbance of the clear yellow extract (at 405 nm) was measured and graphed. (G) The calcification ability of S-CM was higher than that of N-CM in each cell group. Calcification was increased in the hMSC group compared with the HPLF group. Data are represented as the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$. DMEM-OS, DMEM with osteogenic supplements; SD, standard deviation. (Color version of figure is available online).

N-CM and S-CM groups showed vascular endothelial cell marker CD31-positive cells with angiogenesis; and in each cell group, more angiogenesis by 2.6- to 3.6-fold was observed in the S-CM group compared with the N-CM group ($P < 0.05$). By contrast, there was no significant difference between HPLFs and hMSCs (Figure 7A–E).

Discussion

Stem cell therapy has shown promising results in bone healing. However, when cells are used for bone regeneration, some problems remain, such as high cost, safety issues (e.g., infectious diseases), cell manipulation and invasion during cell collection [2]. It has also been reported that hMSCs do not settle and disappear within a few weeks [8]. It has been proposed that the main part of the regeneration process is due to the paracrine effect caused by growth factors and cytokines secreted from the administered cells [10,19].

We previously reported that hMSC-derived CM promote bone regeneration in various animal models [11,20,21]. Cytokines contained in the CM are marketed and used as single agents, but their concentrations are several thousand times higher than those seen *in vivo* and can cause an inflammatory response (e.g., edema, erythema) at the administration site. We have previously reported that osteogenesis is promoted by the interaction of low-concentration cytokines in the CM [12]. In this study, cyclic stretch stimulation increased the content of osteogenic and angiogenic factors in the CM, but the concentration was as low as several hundred to several thousand picograms. This study clarified that CM are useful for bone regeneration when obtained by cyclic stretch stimulation. Additionally, we found that low concentrations of cytokines interacted to induce osteogenesis and angiogenesis in a mouse calvarial defect model.

In this study, we used HPLFs, cells derived from the periodontal ligament, along with hMSCs. In the field of orthodontics, it has been reported that osteogenesis occurs on the traction side of the periodontal ligament (the side opposite the direction in which the tooth moves) during tooth movement and that the secretion of various cytokines increases when mechanical stimulation is applied [17]. Therefore, CM obtained from stretched HPLFs is considered to be useful for bone regeneration. Although this study was inferior to hMSCs, extension stimulation increased the secretion of osteogenic and angiogenic factors, and *in vivo* osteogenesis and angiogenesis were more significant than that seen in the N-CM group.

We evaluated the expression of BMP-2 and BMP-4, which are potent osteogenic factors, and VEGF-A and PDGF-AA, which are related to angiogenesis and cell proliferation. It is known that bone regeneration is closely related to angiogenesis [22–24]. Blood vessels supply the oxygen, nutrients and cells needed for tissue regeneration. VEGF plays a central role in angiogenesis and was elevated in the stretch group. Previous studies have reported the upregulation of monocyte chemotactic protein-1 (MCP-1), which is important for the accumulation of stem cells [25], as well as that of IGF-1 and TGF-β1, which are important for osteogenesis in the periodontal ligament and vascular endothelial cells via cyclic stretch stimulation [17,26]. However, none of these had increased gene expression in our experiments (data not shown). In addition, there was a significant difference in the content of osteogenic and angiogenic factors in the CM among each cell group, but there were no significant differences between the HPLF S-CM and hMSC S-CM groups or between the HPLF N-CM and hMSC N-CM groups *in vivo*. It is possible that something other than the growth factors examined in this study is involved. Comprehensive analyses, such as cytokine arrays and microarrays, will be necessary in future studies.

Fujio *et al.* [15] obtained CM rich in angiogenic factors that were increased by culturing cells under hypoxic conditions and reported that administering CM promoted bone healing. Cells are regularly exposed to various types of mechanical stimulation (e.g., stretch, pressure, or shear stress) [27]. There have been many reports that mechanical stimulation affects cell differentiation and proliferation, including increased expression of factors involved in osteogenesis [16–18]. Stretch stimulation is clinically considered to stimulate osteogenesis, as shown by distraction osteogenesis [28,29] and osteogenesis on the traction side of the periodontal ligament during orthodontic treatment [30].

In this study, we focused on cyclic stretch stimulation and attempted to modify the CM. In each cell type, the expression of

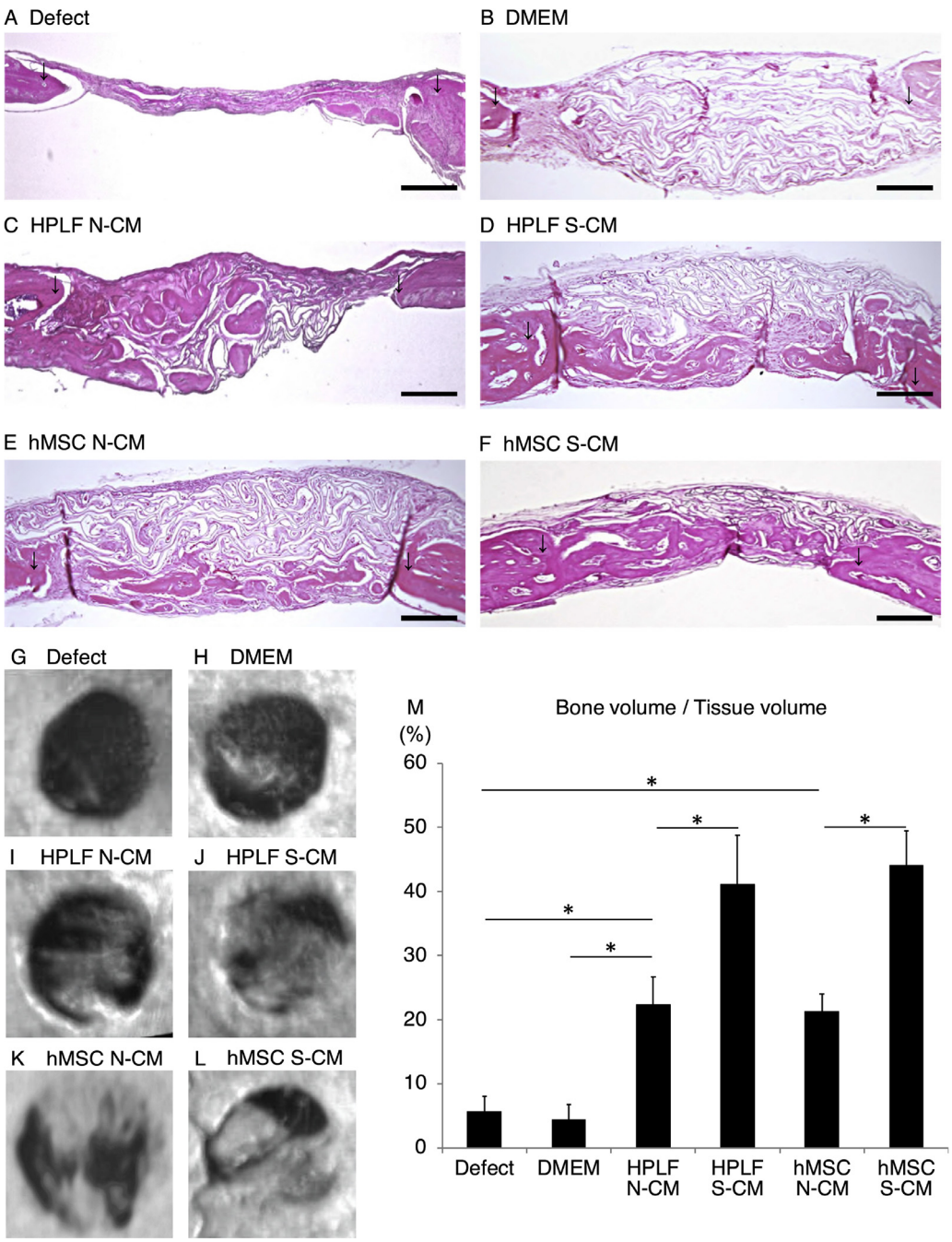

Fig. 6. H&E staining on day 28. (A,B) The defect and DMEM groups showed almost no osteogenesis, whereas trabecula formation was observed in the N-CM and S-CM groups of both cell types. (C–F) All cells showed more prominent osteogenesis in the S-CM group compared with the N-CM group. However, there were no differences among cell types. Newly formed bone was assessed radiologically on day 28 (n = 6). (G,H,M) The CM on day 28 showed minimal calcification in the defect and DMEM groups. (I,K,M) The HPLF N-CM and hMSC N-CM groups showed little osteogenesis in the bone defect. (J,L,M) The HPLF S-CM and hMSC S-CM groups showed strongly calcified osteogenesis with high x-ray density in the calvarial bone defect compared with the HPLF N-CM and hMSC N-CM groups. (M) There was no significant difference between the HPLF and hMSC groups. Data are represented as the mean ± SD; $^{*}P < 0.05$. Bar = 200 μm. Arrow = bone edge. H&E, hematoxylin and eosin; SD, standard deviation. (Color version of figure is available online).

genes related to osteogenesis and angiogenesis was increased by stretching, and the content of cytokines in the CM was also increased. Furthermore, we demonstrated that calcification and tube-forming abilities were improved. We suggest that it is possible to stimulate the secretion of more growth factors from the cells by placing them in an austere environment and that various characteristics of the CM could be created by changing the stimulation delivered to the cells.

In consideration of the conditions of cyclic stretch stimulation, the stretch rates were set at 5%, 10% and 20%. However, 20% of each cell type detached during the stretch. At 10%, there was no significant increase in gene expression compared with 5% (data not shown). Stretched cell cycles of 0.17 and 0.34 Hz were tested; however, at 0.34 Hz, the cells detached, so the experimental conditions were used. Previous studies have shown that stretching cardiac stem cells 20% at 1 Hz increased the expression of angiogenic factors such as VEGF and fibroblast growth factor 2 as well as pro-inflammatory cytokines, which promoted muscle differentiation [31]. When tendon-derived stem cells were stretched 8% at 0.5 Hz, increased expression of BMP-2 was observed and bone differentiation was promoted [32]. These studies suggest that the optimal stretching conditions differ between cell types. Changing the stretching condition or cell type allows for obtaining useful CM for bone regeneration.

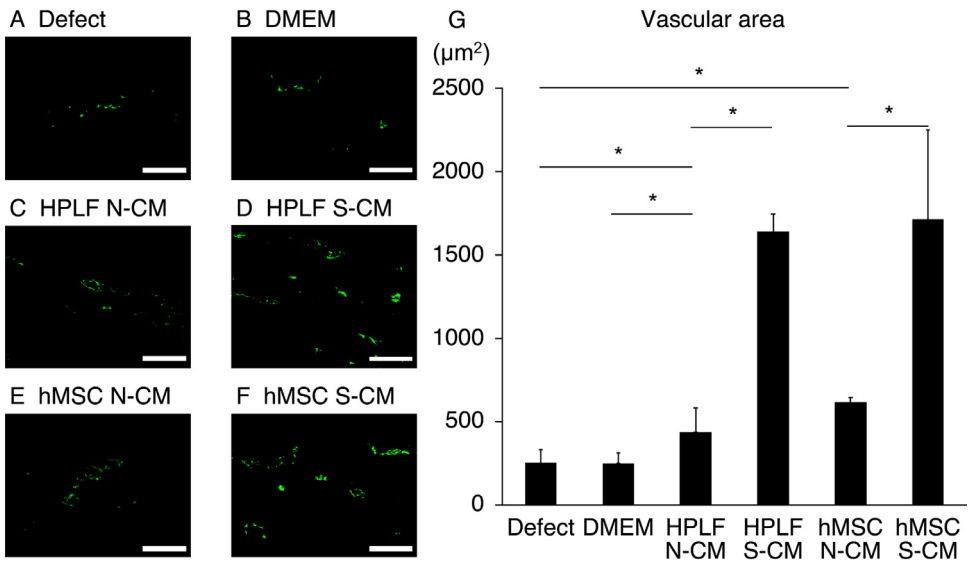

Fig. 7. Administration of CM (C, D, E, F) promoted blood vessel regeneration compared with Defect (A) and DMEM (B). In addition to, administration of S-CM promotes blood vessel regeneration more than that seen with N-CM. Blood vessel regeneration was assessed by immunofluorescence staining for CD31 (n = 5). HPLF S-CM (D) and hMSC S-CM (F) showed a significantly higher vascular density compared with HPLF N-CM and hMSC N-CM (E). C, However, there was no significant difference between HPLFs and hMSCs (E). Data are represented as the mean ± SD; $^*P < 0.05$. Bar = 100 μm. SD, standard deviation. (Color version of figure is available online).

In summary, the biological effects of the CM obtained from HPLFs and hMSCs can be altered by varying the culture conditions. Cyclic stretching stimulated the upregulation of osteogenic and angiogenic factors secreted by each cell type. Administration of CM from HPLFs and hMSCs cultured under stretch conditions promoted angiogenesis and osteogenesis in mouse calvarial defects. Therefore, paracrine factors secreted from cells can be optimized for use in therapy for osteogenesis by changing the culture conditions.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: Hideharu Hibi, Masahito Fujio, Kota Ogisu. Acquisition of data: Masahito Fujio, Kota Ogisu, Makoto Tsuboi, Chang Qi. Analysis and interpretation of data: Kota Ogisu, Makoto Tsuboi, Chang Qi, Naoto Toyama, Hisanobu Kamio. Drafting or revising the manuscript: Masahito Fujio, Kota Ogisu. All authors have approved the final article.

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Supplementary materials

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