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MIG and I309 Accelerates Proliferation of Human Bone Marrow-Derived Mesenchymal Stem Cells

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Abstract **Background:** Previous studies have shown that mesenchymal stem cells (MSCs) are an important source of somatic stem cells for regenerative medicine. Therefore, the establishment of efficient MSC culture methods to increase their therapeutic value is essential. Here, we focused on cytokines as accelerators of cell proliferation. **Methods:** We evaluated the effect of the supernatant—obtained from a co-culture of bone marrow-derived MSCs (BMSCs) and myeloid cells—on the proliferation of BMSCs. We then screened for cytokines present in high concentrations in the medium. Next, we added the identified cytokines to the culture medium in various combinations, and cultured BMSCs in this medium to evaluate the effects of cytokines on their proliferation and differentiation potential. **Results:** The medium obtained from the co-culture of BMSCs with myeloid cells stimulated BMSC proliferation. Nineteen cytokines were present in high concentrations in the medium. Out of the 19 cytokines, MIG and I309 stimulated BMSC proliferation. Furthermore, the culture medium supplemented with MIG and I309 maintained the differentiation potential of cultured BMSCs. **Conclusions:** MIG and I309 stimulate BMSC proliferation while maintaining their differentiation potential. These results may contribute to the establishment of more efficient MSC culture methods than those currently being used.

Key words: mesenchymal stem cell, MIG, I309, cell proliferation, regenerative medicine

Introduction

Mesenchymal stem cells (MSCs) are somatic stem cells that have the ability to differentiate into adipocytes, osteoblasts, and chondrocytes. Studies have shown that it is also possible for these cells to differentiate into neurons and hepatocytes.[1-3] MSCs also have anti-inflammatory and immunoregulatory functions, and stimulate the regeneration of damaged tissues by means of cytokine secretion. Therefore, MSCs are a promising cell source for regenerative medicine.[4] An increasing body of research is testing the treatment efficacy of MSCs in liver disease, and there are many reports of their effectiveness.[5-7]

Additionally, we have developed a less invasive liver regeneration therapy using bone marrow-derived MSCs (BMSCs) cultured in a small amount of autologous bone marrow fluid. We tested the treatment efficacy,

safety, and optimal cell administration pathways in a canine liver fibrosis model.[8-10]

Human clinical trials have been started based on our previous studies, but a more efficient BMSC culture method is necessary to further enhance the treatment efficacy.

We have simultaneously developed a culture dish that can be used to culture larger amounts of BMSCs through co-culture with fractionated myelocytes (Japan patent JP2012-231788A). In addition, we found that using the supernatant from the culture medium in this culture dish also stimulates BMSC proliferation. Furthermore, using this strategy, we identified two BMSC growth-stimulating factors in the culture supernatant.

Materials and methods

Cells

Human bone marrow mononuclear cells (BMNCs. 2M-125D; Lonza, Basel, Switzerland) and human bone marrow-derived mesenchymal stem cells (BMSCs. PT-2501; Lonza) were used in this study.

Cell-culture

BMNCs were seeded at a density of 1×10^8 cells/cm^2 in either a culture dish coated with nanomaterial gels and inorganic clay (dish X) or in an ordinary uncoated 9.2 cm^2 culture dish (dish Y: Iwaki, Tokyo, Japan). We incubated the cells in Dulbecco's modified Eagle's medium (DMEM: Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS: Sigma-Aldrich, St. Louis, MO, USA) and 100 μg/ml gentamicin (Thermo Fisher Scientific) at 37° C in 5% CO_2. The culture medium was replaced every 2 days.

On day 7, the supernatant from the culture medium was collected from both dishs and separated by centrifugation (2,000 g, 20 min). The supernatant obtained from dish X was called conditioned medium X (medium X) and the supernatant obtained from dish Y was called conditioned medium Y (medium Y). BMSCs (Lonza) were seeded at a density of 5×10^3 cells/well in a 24-well plate (Corning, NY, USA) and cultivated in medium X or medium Y at 37° C in 5% CO_2 for four days (n = 10 for each medium).

MTS assay

Cell proliferation was evaluated via MTS assay using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions.

Reagents

Monocyte chemoattractant protein-2 (MCP2), monocyte chemoattractant protein-3 (MCP3), macrophage inflammatory protein 1α (MIP 1α), macrophage inflammatory protein 1β (MIP 1β), macrophage derived chemokine (MDC), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), macrophage colony stimulating factor (M-CSF), monokine induced by interferon-γ (MIG), I309, epithelial-derived neutrophil-activating protein78 (ENA 78), and neutrophil Activating Protein 2 (NAP 2) were purchased from Wako (Osaka, Japan). Regulated on activation, normal T cell expressed and secreted (RANTES), eotaxin-2, growth related oncogene (GRO), urokinase-type plasminogen activator receptor (uPAR), and soluble glycoprotein 130 (sgp130) were purchased from R&D systems (Minneapolis, MN, USA) and tissue inhibitor of metalloproteinase (TIMP 1) was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA).

Cytokine antibody array analysis

The cytokine levels in medium X and medium Y were measured using a RayBio Human Cytokine Antibody Array G Series 1000 (RayBiotech, Peachtree Corners, GA, USA) according to the manufacturer's instructions. Then, the cytokines in medium X were extracted with a net intensity of ≥1.5 times that in medium Y.

Evaluation of cell proliferation in medium with added cytokines

Medium A consisted of DMEM + 10% FBS + 100 μg/ml gentamicin + 19 cytokines selected for the cytokine antibody array analysis. Medium with only four of these 19 cytokines (MIG, I309, IL-8, and MIP-1α) was called medium B. Medium with only MIG and I309 was called medium C, and medium which did not contain MIG and I309 but contained the remaining 17 cytokines was called medium D. DMEM + 10% FBS + 100 μg/ml gentamicin

Table 1 The addition concentration of cytokines

	Cytokine	Final concentration	ED50
Medium A	MCP3	500 ng/ml	10～100 ng/ml
	MIP-1α	100 ng/ml	1～10 ng/ml
	RANTES	300 ng/ml	20～30 ng/ml
	MDC	500 ng/ml	10～100 ng/ml
	MIP-1β	200 ng/ml	5～20 ng/ml
	IL-6	1 ng/ml	0.1 ng/ml
	MCP2	500 ng/ml	10～100 ng/ml
	I-309	500 ng/ml	10～100 ng/ml
	CCL24	500 ng/ml	10～100 ng/ml
	GRO	1.5 μg/ml	0.15～0.30 μg/ml
	uPAR	100 μg/ml	5～10 μg/ml
	IL-8	500 ng/ml	10～100 ng/ml
	M-CSF	10 ng/ml	1 ng/ml
	NAP-2	100 ng/ml	1～10 ng/ml
	MIG	500 ng/ml	10～100 ng/ml
	IL-10	20 ng/ml	2 ng/ml
	sgp130	1 μg/ml	0.5～1 μg/ml
	TIMP1	1 μg/ml	0.5 μg/ml
	ENA-78	100 ng/ml	5～10 ng/ml
Medium B	MIP-1α	100 ng/ml	1～10 ng/ml
	I-309	500 ng/ml	10～100 ng/ml
	IL-8	500 ng/ml	10～100 ng/ml
	MIG	500 ng/ml	10～100 ng/ml
Medium C	I-309	500 ng/ml	10～100 ng/ml
	MIG	500 ng/ml	10～100 ng/ml
Medium D	MCP3	500 ng/ml	10～100 ng/ml
	MIP-1α	100 ng/ml	1～10 ng/ml
	RANTES	300 ng/ml	20～30 ng/ml
	MDC	500 ng/ml	10～100 ng/ml
	MIP-1β	200 ng/ml	5～20 ng/ml
	IL-6	1 ng/ml	0.1 ng/ml
	MCP2	500 ng/ml	10～100 ng/ml
	CCL24	500 ng/ml	10～100 ng/ml
	GRO	1.5 μg/ml	0.15～0.30 μg/ml
	uPAR	100 μg/ml	5～10 μg/ml
	IL-8	500 ng/ml	10～100 ng/ml
	M-CSF	10 ng/ml	1 ng/ml
	NAP-2	100 ng/ml	1～10 ng/ml
	IL-10	20 ng/ml	2 ng/ml
	sgp130	1 μg/ml	0.5～1 μg/ml
	TIMP1	1 μg/ml	0.5 μg/ml
	ENA-78	100 ng/ml	5～10 ng/ml

NOTE; MCP2: Monocyte chemoattractant protein-2, MCP3: Monocyte chemoattractant protein-3, MIP 1α: Macrophage inflammatory protein 1α, MIP 1β: Macrophage inflammatory protein 1β, MDC: Macrophage derived chemokine, IL-6: Interleukin-6, IL-8: Interleukin-8, IL-10: Interleukin-10, M-CSF: Macrophage colony stimulating factor, MIG: Monokine induced by interferon-γ, ENA78: Epithelial-derived neutrophil-activating protein78, NAP2: Neutrophil Activating Protein 2, RANTES: Regulated on activation normal T cell expressed and secreted, GRO: Growth related oncogene, uPAR: Urokinase-type plasminogen activator receptor, sgp130: Soluble Glycoprotein 130, TIMP1: Tissue inhibitor of metalloproteinase1.

with no added cytokines was used as the control medium. The concentration of the added cytokines was between 1 and 10 times their respective ED_{50} value (Table 1). Next, we seeded BMSCs in a 48-well plate (Corning) at a density of 5×10^3 cells/well, and incubated them in control medium at 37℃ in 5% CO_2 for 24 hours. After 24 hours, we verified that the cells were attached to the plate, then replaced the medium with a cytokine-containing medium—either medium A, B, C, or D (n=6 each). The medium in the control group was replaced with control medium after 24 hours. After this, the cells were cultivated at 37℃ in 5% CO_2, and the percentage of the image area in each well that is occupied by cells was measured every hour using an IncuCyte Zoom (Essen BioScience, AnnArbor, Michigan, USA). The cells were cultivated for five days after cytokine-containing media were added, and the proliferative ability of the cells was evaluated using a growth curve and MTS assay.

Differentiation ability assay

A Human Mesenchymal Stem Cell Functional Identification-Kit was purchased from R&D Systems (Minneapolis, MN, USA). Cell differentiation was performed following the manufacturer's instructions. In brief, cells were seeded at a density of 2×10^4 cells/cm^2. When the cells were 100% confluent, the medium was replaced with Adipogenic Differentiation Medium to induce adipogenesis. The medium was replaced with fresh Adipogenic Differentiation Medium every 3-4 days. After 14 days, the cells were stained with Oil Red O (Sigma-Aldrich) to detect lipid accumulation.

Statistical analysis

Student *t*-tests were used for statistical comparisons between two different groups, whereas one-way ANOVA with a post-hoc Tukey's test was used for statistical comparison of more than two groups. Values of $p < 0.05$ were considered statistically significant. Data are presented as the mean ± standard deviation.

Results

BMSC growth was stimulated by co-culture with myeloid cells

With the surface coating in dish X, it was possible to cultivate not only MSCs but also non-adherent myeloid cells. However, in dish Y—which was uncoated—the culture medium exchange process during cultivation of BMNCs removed the non-adherent myeloid cells so that only the adherent cells, which are mainly MSCs, could be cultivated (Fig. 1a). Furthermore, MTS assays on BMSCs

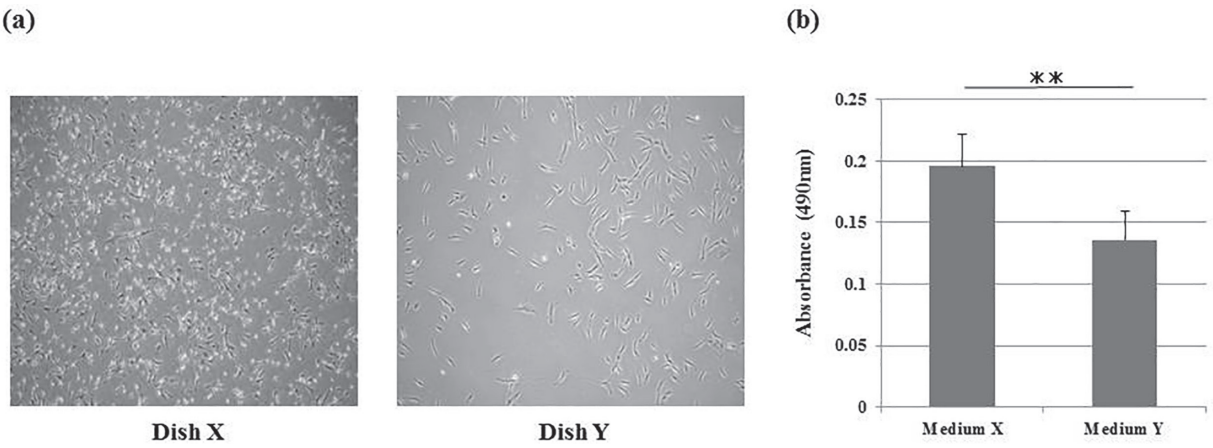

Fig. 1 BMNC culture in dish X or dish Y and BMNC culture using medium X or medium Y.

(a) BMNCs were seeded in dish X and dish Y, and the cells were imaged in the culture dish on day 7 under a microscope (40× magnification). (b) Evaluation of BMSC proliferation using an MTS assay. BMSCs were cultured for four days using medium X or medium Y, then MTS assay was performed. (n=10 each, *$p < 0.05$).

cultivated using medium X or medium Y revealed that BMSCs cultivated in medium X had significantly higher proliferation than those in medium Y. These results suggest that medium X, which was obtained from the co-culture of BMSCs with non-adherent myeloid cells, contains a factor that promotes cell proliferation (Fig. 1b).

Addition of cytokines—that displayed high concentrations in the cytokine array analysis—stimulated BMSC growth

A cytokine antibody array analysis was used to screen for differences in cytokine concentrations between medium X and medium Y. Nineteen cytokines were present in higher concentrations (1.5 times or higher) in medium X than in medium Y (Table 2).

Medium A contained these 19 cytokines at concentrations between 1 and 10 times their ED50. BMSCs cultivated in this medium exhibited significantly faster growth relative to that in the control, and the MTS assay also revealed a significant increase in proliferation (Figs. 2a, 2b). No changes in cell morphology were observed in BMSCs cultivated in medium A (Fig. 2c). Next, we cultured BMSCs in medium without one of these 19 cytokines (one at each time), and identified the cytokines that did not have an effect on proliferation. Then, we confirmed that the growth-stimulating effects of medium B, which contained four of the 19 cytokines (MIG, I309, IL-8, MIP-1α), was the same as that of medium A (Figs. 3a, 3b). We further narrowed down the growth-stimulating cytokines to just two (MIG and I309) using the same method, and added these to a medium (medium C). BMSCs cultured in medium C showed similar proliferation rates as those cultured in medium B (Figs. 3c, 3d) and medium A (Figs. 3e, 3f). Additionally, medium D, which was essentially medium A without MIG and I309, did not promote BMSC proliferation (Figs. 3g, 3h). Furthermore, media with either MIG alone or I309 alone increased cell proliferation compared to that in control medium, but had significantly lower effect on cell proliferation than medium C (Fig. 3i). The above results show that the addition of the cytokines MIG and I309 to the culture medium is sufficient to efficiently culture BMSCs.

Table 2 Cytokine antibody array analysis results

Cytokine	Net intensity (Medium X)	Net intensity (Medium Y)	Net intensity ratio
MCP3	54141.31	619.26	88.74
MIP-1α	45374.43	525.39	86.27
RANTES	954.82	39.54	25.14
MDC	2262.52	114.57	19.71
MIP-1β	25034.14	1672.28	14.96
IL-6	44012.18	4349.78	10.12
MCP2	1087.69	130.60	8.35
I-309	364.86	56.34	6.49
CCL24	712.62	129.55	5.51
GRO	4360.48	934.14	4.67
uPAR	1005.71	247.30	4.05
IL-8	1525.89	539.74	2.82
M-CSF	215.73	89.17	2.48
NAP-2	496.07	200.48	2.47
MIG	678.31	358.74	1.89
IL-10	817.19	460.72	1.77
sgp130	442.00	229.92	1.74
TIMP1	1321.80	825.79	1.60
ENA-78	260.73	166.55	1.55

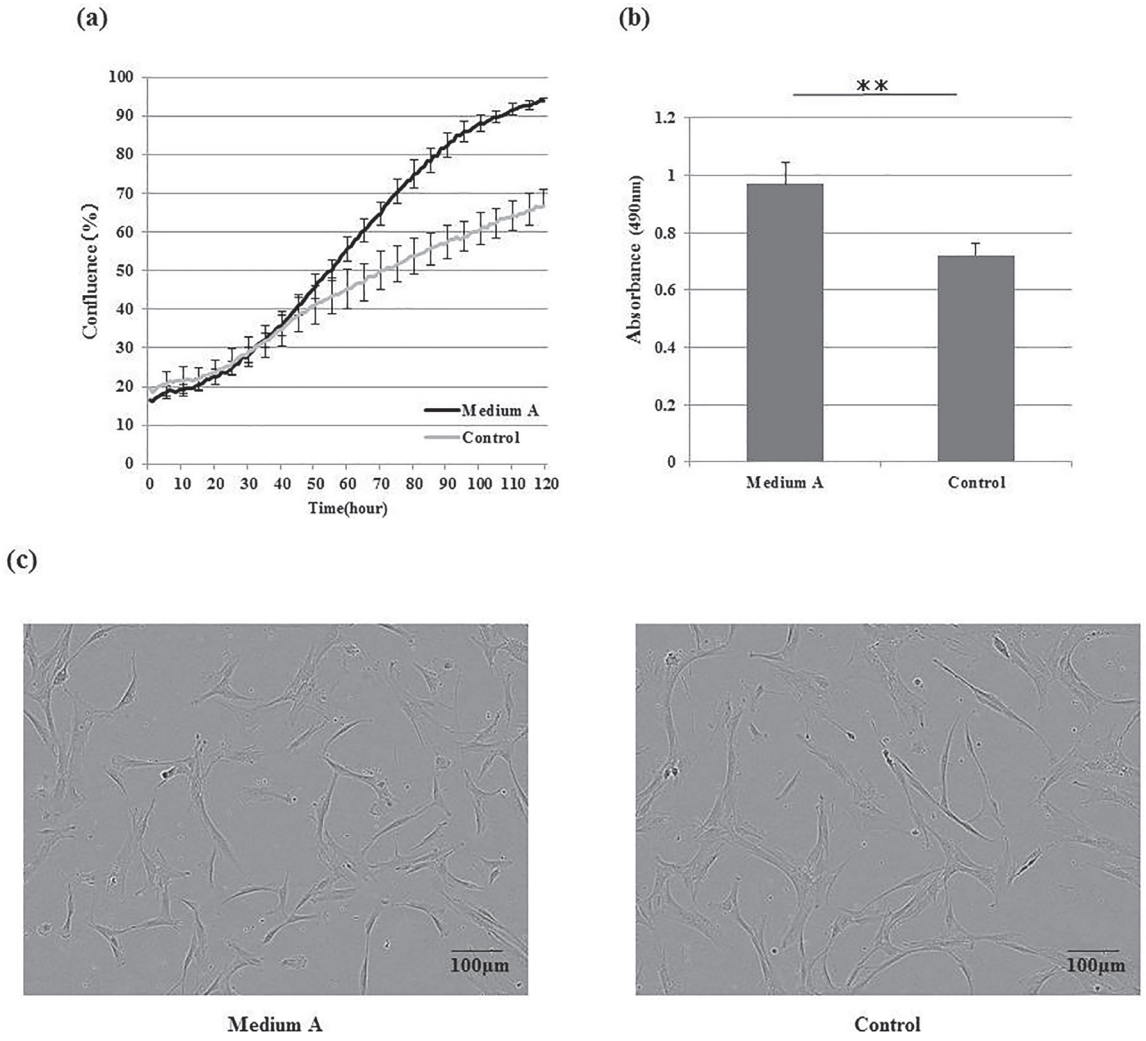

Fig. 2 Evaluation of cell proliferation and morphology of BMSCs cultured in media containing cytokines. BMSCs were cultivated in control medium for 24 hours, and then the medium was replaced with the cytokine-containing medium A. The cells were cultured for five days after the medium was replaced, and their proliferation was evaluated. (a) Growth curve for BMSCs cultured in medium A and in control medium (n=6 each, $^*p < 0.05$). (b) Evaluation of proliferation by MTS assay. The medium was replaced with cytokine-containing medium, and the cells were cultured for five days. After this, an MTS assay was conducted (n=6 each, $^*p < 0.05$). (c) The cells in each well were imaged on day 2 under a microscope (100× magnification).

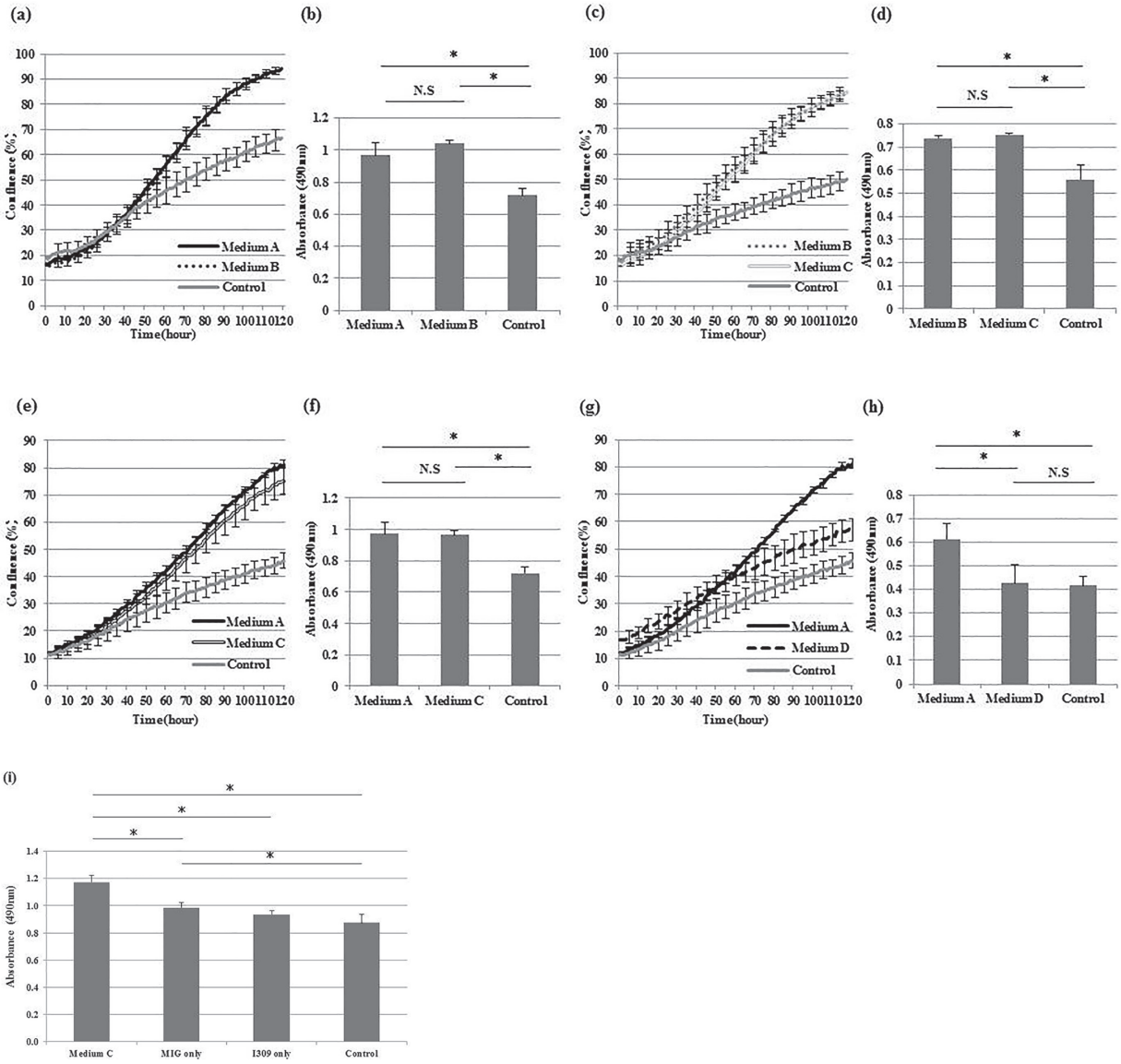

Fig. 3 Evaluation of cell proliferation of BMSCs cultured in media containing cytokines. BMSCs were cultured in control medium for 24 hours, then the medium was replaced with the various cytokine-containing media. The cells were cultured for five days after the medium was replaced, and their proliferation was evaluated (n=6 each, *$p < 0.05$). (a) Evaluation of proliferation via growth curves in medium A, medium B, and control medium. (b) Evaluation of proliferation by MTS assay in medium A, medium B, and control medium. (c) Evaluation of proliferation by a growth curve in medium B, medium C, and control medium. (d) Evaluation of proliferation by MTS assay in medium B, medium C, and control medium. (e) Evaluation of proliferation by a growth curve in medium A, medium C, and control medium. (f) Evaluation of proliferation by MTS assay for cells cultured in medium A, medium C, and control medium. (g) Evaluation of proliferation by a growth curve in medium A, medium D, and control medium. (h) Evaluation of proliferation by MTS assay for cells cultured in medium A, medium D, and control medium. (i) Evaluation of proliferation by MTS assay for cells cultured in medium C, a medium with only MIG added, a medium with only I309 added, and control medium.

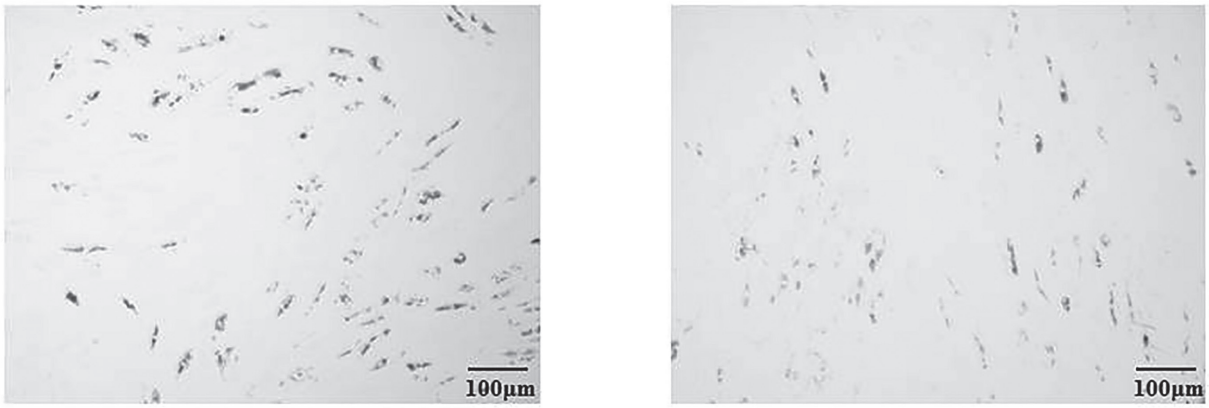

Fig. 4 Differentiation ability assay.
BMSCs were cultured in medium C or control medium until they reached 100% confluence, at which point the medium was replaced with adipocyte differentiation medium. After culturing the cells for 14 days, they were stained with Oil Red O, and the appearance of lipid droplets in the cells was observed using a microscope (100× magnification).

BMSCs cultured in the presence of MIG and I309 were able to differentiate into adipocytes

We assessed whether medium C—which contained MIG and I309—could affect the differentiation of BMSCs into adipocytes. The results showed that BMSCs cultivated in medium C could differentiate into adipocytes similar to those cultivated in the control medium. This demonstrates that adding MIG and I309 to medium does not affect the differentiation potential of BMSCs.

Discussion

In this study, we investigated the stimulation of BMSC proliferation using the culture supernatant resulting from the co-culture of BMSCs and myeloid cells. Additionally, using a cytokine array screen, we identified 19 cytokines with high concentrations in the culture supernatant. We added these cytokines to the culture medium, and discovered that they promoted BMSC proliferation to a similar degree as the culture supernatant obtained from the co-culture of BMSCs and myeloid cells. Furthermore, we cultured BMSCs without each of these 19 cytokines—one by one—and identified the cytokines that affected BMSC proliferation. In the end, two cytokines (MIG and I309) were identified as BMSC growth-stimulating factors. Moreover, we found that the potential of BMSCs to differentiate into adipocytes was maintained on culturing BMSCs in the presence of these cytokines.

I309 is also known as chemokine ligand 1 (CCL1), and is produced by monocytes, activated T-lymphocytes, and endothelial cells.[11,12] CCL1 is thought to induce migration in lymphocytes, especially T-lymphocytes,[13,14] and also promotes regulatory T cell (Treg) proliferation,[15] thereby leading to the suppression of cytotoxic T cells and promotion of tumor progression.[16,17] MIG is also known as CXC chemokine ligand 9. It is produced by macrophages, monocytes, neutrophils, antigen presenting cells, B cells, and eosinophils in response to IFN-γ stimulus.[18] It has also been reported to guide T lymphocytes to sites of inflammation and infection, and to promote T lymphocyte proliferation.[18,19] Our study revealed that MIG and I309 promote MSC proliferation. We believe that this report is the first to identify the potential application of MIG and I309 for promoting MSC proliferation.

The mechanism by which MIG and I309 promote MSC proliferation is not known. It has been reported that NF-kB pathway activity,[20,21] reduced oxidative stress,[22] and the Wnt3a signaling pathway[23] promote MSC proliferation. These mechanisms may also be involved in the actions of MIG and I309.

Additionally, there is likely a synergistic effect between MIG and I309 on the stimulation of BMSC proliferation, because the combination MIG and I309 resulted in significantly increased BMSC proliferation compared to that observed on using either MIG or I309 alone. Further studies are needed to elucidate these mechanisms.

The present study was done *in vitro*, so we cannot extrapolate regarding the therapeutic effect of culturing MSCs in MIG and I309. It is necessary to evaluate the therapeutic effect and safety of these cells *in vivo* before using them in human clinical trials.

Conclusions

In the present study, we showed that MIG and I309 stimulate BMSC proliferation while maintaining their differentiation potential. These results may contribute to the establishment of more efficient MSC culture methods than those used currently.

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Conflict of Interest

The authors declare no conflict of interest.

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