Original article

Effects of growth differentiation factor-5 (GDF-5) on cell migration in high-density mesenchymal cell cultures

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Key words : GDF-5, cell migration, mesenchymal cell, high-density culture

Abstract : Growth differentiation factor-5 (GDF-5) expression is localized in the condensing mesenchyme of the developing limb. However, the effect of GDF-5 on mesenchymal condensation remains unclear. In this study, the role of GDF-5 and its signaling pathway during cell migration in micromass cultures were evaluated. Mouse limb mesenchymal cells were dissociated into single-cell suspensions for micromass culturing. GDF-5 added to the culture promoted the formation of cartilaginous nodules in a low cell density micromass culture, as defined by condensed Alcian blue staining. Cell scratch assays showed that treating cells with GDF-5 causes a significant reduction in the cell-free area compared to the control. GDF-5 reduced the cell-free area in cultured murine mesenchymal C3H10T1/2 cells but not in prechondrogenic ATDC5 cells. In C3H10T1/2 cells, cell migration promoted by GDF-5 was suppressed by the addition of a bone morphogenetic protein receptor (BMPR) inhibitor or a p38 MAP kinase inhibitor. Expression of N-cadherin and Sox11 genes were altered by GDF-5 after 1 h but were unaltered by either the BMPR or p38 MAP kinase inhibitor. These data suggest that the BMPR/p38 MAP signaling pathway is involved in undifferentiated mesenchymal cell migration promoted by GDF-5 in micromass cultures.

Introduction

Chondrogenesis is a multistep process in which pluripotent cells differentiate into chondrocytes that proliferate and condense before their cell fate is determined. Cell condensation results from increased cellular density caused by cell migration and aggregation and is an essential step in determining the shape, size and position of future skeletal elements[1]. Various molecules are involved in the condensation stage, including Msh homeobox-1 (Msx-1), Msx-2, paired box-1 (Pax-1), Hox genes, N-cadherin, chondroitin sulfate, syndecan and transforming growth factor-beta (TGF-β)[2]. Growth differentiation factors (GDFs) are members of the bone morphogenetic protein (BMP) family that includes the TGF-β superfamily. GDF-1 through -15 are involved in various functions, including embryonic development, iron metabolism, skeletal

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development, inflammation and apoptotic pathways[3]. GDFs include a subgroup containing GDF-5 (BMP-14, CDMP-1), GDF-6 (BMP-13, CDMP-2) and GDF-7 (BMP-12) based on amino acid sequence similarity[4]. GDF-5, -6 and -7 expression in bones and joints are required for these structures[5-7]. In the developing skeletal elements of the limb, the GDF-5 gene is strongly expressed in most joints, whereas GDF-6 and GDF-7 are expressed in restricted joints[8]. The GDF-5 gene is expressed during the initial cell condensation stage[9,10], and implanting GDF-5 protein-soaked beads in the limb promotes cell aggregation and cartilage formation rather than joint formation, even at later stages[11,12]. Thus, GDF-5 appears to have multiple functions during cartilage development. Regarding the effect of GDF-5 on cell condensation, an increase in the number of chondroprogenitor cells was observed because of mesenchymal cell recruitment, but joint formation failed in GDF-5 (CDMP-1) transgenic mice[13,14].

The GDF-5 signal is transmitted by binding to two different receptors: activin and BMP receptors (BMPRs), which are membrane-bound serine/threonine kinases and can be either type I or type II receptors. GDF-5 binds type I receptors: BMPR-IA [also called activin receptor-like kinase (ALK)-3] and BMPR-IB (ALK-6); or type II receptors: BMPR-II and activin receptor-II (ActR-II)[15-18]. The GDF-5-induced cell signaling cascade can be distinguished into a canonical signaling pathway, i.e., the Smad-pathway, or a non-canonical pathway, i.e., the mitogen-activated protein kinase (MAPK) pathway[3]. In the Smad-pathway, GDF-5 signals through receptor-regulated Smad (R-Smad) 1/5/8. This phosphorylated complex binds to the common mediator Smad (Co-Smad) 4, translocates to the nucleus and activates or suppresses gene expression with or without assistance from other transcription factors[19]. In addition, there are three major classical MAPK extracellular signal-regulated kinases: ERKs, c-Jun N-terminal kinases (JNKs) and p38 MAPKs[20]. Among these MAPK proteins, p38 MAPK plays a key role in MAPK crosstalk signaling networks during TGF-β1-induced chondrogenic differentiation of mesenchymal stem cells[21]. The p38 MAPK pathway promoted by BMP-2 also regulates SRY-box 9 (SOX9) transcription factor activity, which plays a critical role in chondrogenesis[22]. In cells derived from the mesenchyme, GDF-5 activates p38 MAPK signaling in myoblasts[23], a mesenchymal precursor cell line[24] and mesenchymal stem cells derived from bone marrow[25] and the periodontal ligament[26]. GDF-5 signaling regulates chondrogenesis in prechondrogenic cells through ALK-6 (BMPR-IB) to p38 MAP kinase[27]. During chondrogenic cell condensation, GDF-5 promotes chondrogenic cell condensation via p38 MAPK signaling[16]. Conversely, it has been suggested that p38 MAP kinase signaling is not required for mesenchymal condensation[28]. GDF-5 was reported previously to promote mesenchymal cell condensation[29], and studies using a scratch assay revealed that mesenchymal cell migration directly modulates cell condensation[30,31]. However, the detailed mechanism of cell condensation promoted by GDF-5 remains unclear. In this study, the effects of GDF-5 on cell migration, cell condensation and signaling in mesenchymal cells were evaluated.

Materials and methods

Animals, cell culturing and reagents

All animal studies conformed to guidelines approved by the Animal Experiment Committee of Fukuoka Dental College, Fukuoka, Japan (approval #18018, 22001). Timed-pregnant ICR mice (Kyudo Co. Ltd. Saga, Japan) were obtained, and embryos were collected on embryonic day 10 (E10). Micromass culturing of embryonic limb bud mesenchymal cells was performed as described previously[29], with minor modifications. Briefly, whole limb buds were collected under a dissecting microscope using two pairs of sharp forceps. Collected limb buds were incubated at 37℃ for 10 min in dissociation buffer [0.125% trypsin－0.5 mM EDTA (Wako-Fuji Film Chemicals, Tokyo, Japan) and1mg/mL collagenase (Wako-Fuji Film Chemicals) as final concentrations in Dulbecco's phosphate-buffered saline (D-PBS) (Wako-Fuji Film Chemicals)]. Incubated limb buds were dissociated into single-cell suspensions by repeatedly pipetting with Pasteur glass pipettes.

Cells were collected and diluted to $0.125-2.0\times10^7$ cells/mL, plated as $10-30\mu$L drops and cultured in chondrogenic differentiation medium [45% Dulbecco's modified Eagle medium (DMEM), 45% F12 medium (Wako-Fuji Film Chemicals), 10% fetal bovine serum (FBS) supplemented with 100μg/mL ascorbic acid and 1 mM sodium pyruvate]. Next, 24 h after initial plating, the cultures were treated with various recombinant mouse GDF-5 (R&D Systems, Minneapolis, MN, USA) concentrations diluted in 0.1% bovine serum albumin in Hank's balanced salt solution. The BMP receptor inhibitor K02288 (Funakoshi, Tokyo, Japan) and the p38 MAP kinase inhibitor SB202190 (Funakoshi) were used for inhibition experiments. Each inhibitor was dissolved in dimethyl sulfoxide (DMSO) and diluted in a differentiation cell culture medium.

C3H10T1/2 (clone eight) murine pluripotent mesenchymal cells and ATDC5 murine prechondrogenic cells were obtained from the Riken Bioresource Center Cell Bank. Cells were cultured to an appropriate cell number in a medium of DMEM and 10% FBS (Equitech-Bio Inc., Tokyo, Japan). ATDC5 cells were cultured in 1:1 DMEM and Ham's F12 medium (Invitrogen/Life Technologies, Tokyo, Japan), 5% FBS (Equitech-Bio Inc), 3×10^{-8} M sodium selenite (Wako-Fuji Film Chemicals) and 10μg/mL holoform bovine transferrin (Invitrogen). All growth media included antibiotics (100U/mL penicillin-G and 100mg/mL streptomycin). All cells were incubated at 37℃ in a humidified 5% CO_2 atmosphere. Micromass cultures of both cell lines were treated similarly to those derived from embryonic limb buds.

Alcian blue staining

The presence of sulfated proteoglycans, indicative of cartilaginous matrix production, was detected using Alcian blue staining. Mouse limb mesenchymal cells were seeded in 48-well plates and cultured in the absence or presence of GDF-5. After culturing for 4 days, Alcian blue staining was performed as described previously[29], with minor modifications. Briefly, cultures were fixed in 2% acetic acid in ethanol for 15 min at room temperature, rehydrated and stained with 0.5% Alcian blue 8GX (Wako-Fuji Film Chemicals) in 0.1 M HCl at room temperature. Cell images were captured using a scanner (CanoScan 9000FMKII, Canon, Tokyo, Japan). The intensity of Alcian blue staining in mouse limb mesenchymal cell micromass cultures was measured based on the absorbance at 600 nm (1420 Multilabel counter ARVO MS, PerkinElmer, Yokohama, Japan) after the Alcian blue-stained cartilage matrices were solubilized with 4 M guanidine hydrochloride. The diameter of the Alcian blue-positive formation area was measured and defined as including all cartilaginous nodules.

Cell migration assays

The cell migration assay was performed as described previously[32], with minor modifications. Briefly, mouse limb mesenchymal cells, C3H10T1/2, or ATDC5 cells were plated in 30μL cell suspensions in 12-well cell culture dishes. After 24 h, the cultures were scratched using a 200μL pipette tip. Immediately after washing three times with the culture medium, 500 ng/mL GDF-5 or the vehicle was added to the cells. In addition, cells were cultured with or without K02288 (25nM) or SB202190 (20μM)[33,34] in combination with or without GDF-5 in the reduced serum medium (2.5%). Phase contrast images of the scratches were taken using a microscope every hour for up to 5 h. The cell-free areas were measured using Adobe Photoshop Elements 2020 (Adobe, San Jose, CA, USA).

Reverse transcription and real-time PCR

Total RNA was isolated from each micromass culture using the RNeasy Plus Mini kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions. Reverse transcription and real-time- PCR were performed as reported previously[35], with minor modifications. Briefly, reverse transcription was performed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Tokyo, Japan), according to the manufacturer's instructions. RT-PCR was performed using iTaq SYBR Green Supermix with ROX (Bio-Rad) for 45 cycles of 95℃ for 30 s, 60℃ for 45 s and

72℃ for 30s. Each primer pair was synthesized by Takara Bio: N-cadherin (NM_007664.5), 5′-CAG CAC ACC TTC ACC CAA CA-3′ and 5′-CCA CTG ATT CTG TAT GCC GC-3′; GAPDH (NM_007664.5), 5′-GTG ACG ACT GAA CGG CAG GA-3′ and 5′-GCA CGG TGC TAG TGG ACT ACA GA-3′; myeloid zinc finger-1 (Mzf-1) (NM_001290453.1), 5′-AGC TTG ATG GAC CCT CGG AAA TG-3′ and 5′-CAG GGT CTG GAG TCT GAG GC-3′; and Sox11 (NM_009234.6), 5′-TTC CCCACT TTG GGT AAC TA-3′ and 5′-TTC CCA CTT TGC ATT GGG TAA CT-3′. Gene expression was measured in the four samples and normalized to GAPDH expression. The relative expression of each gene compared to expression after initial cell attachment was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Each experiment was performed using five or eight samples. Statistical significance was determined using Kruskal–Wallis one-way analysis of variance followed by post-hoc t-tests. Differences were considered statistically significant at $p < 0.05$.

Results

Morphological analysis of cartilaginous nodule formation using a mouse limb mesenchymal micromass culture was initially performed to assay the function of GDF-5 in regulating cartilage formation in vitro. Cartilaginous nodules were detected using Alcian blue staining, which revealed the presence of sulfated proteoglycans. In the absence of GDF-5, the spontaneous formation of cartilaginous nodules was observed after 3 days in the micromass culture using 2.0×10^7 cells/mL (Fig. 1A, E). The nodules were enlarged in the presence of 10－100 ng/mL GDF-5 (Fig. 1B, C, F, G). In the presence of 500 ng/mL GDF-5, all micromass culture areas were stained with Alcian blue (Fig. 1D, H). This result indicated that GDF-5 promotes the enlargement of cartilaginous nodules by promoting mesenchymal cell aggregation around the nodule during chondrogenic cell differentiation.

Because cartilaginous nodules were formed in high-density micromass cultures and were challenging to identify morphometrically in the presence of GDF-5, micromass culturing was performed using gradually lower cell densities in the absence or presence of GDF-5. Cartilaginous nodule formation was observed in cultures using 1.0×10^7 or 0.125×10^7 cells/mL (Fig. 2B-E), which were 1/2 or 1/16 of the cell density at 2.0×10^7 cells/mL (Fig. 2A), respectively. In the presence of GDF-5, cartilaginous nodule formation was promoted in a dose-dependent manner at 2.0×10^7 cells/mL (Fig. 2A, F, K, P). Similarly, cartilaginous nodule formation was observed at 1.0×10^7 (Fig. 2G, L, Q) to 0.25×10^7 cells/mL (Fig. 2I, N, S) in the presence of GDF-5. The

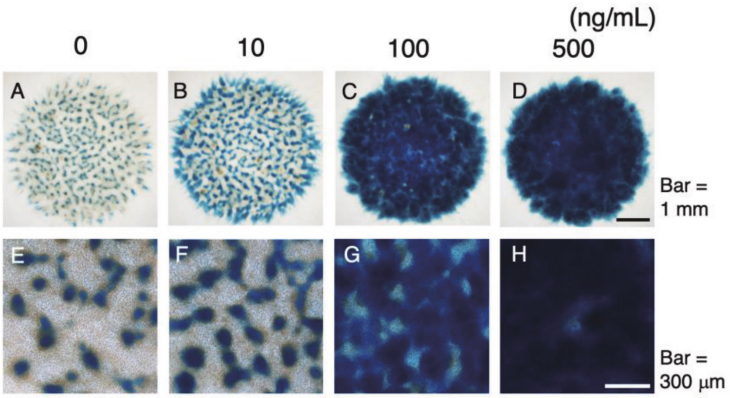

Fig. 1 GDF-5 enlarges cartilaginous nodules in the mouse limb bud mesenchymal cell micromass culture. Limb bud mesenchymal cells were cultured for 3 days in the absence or presence of three doses of GDF-5. Cartilaginous nodules were identified using Alcian blue staining. Cartilaginous nodules formed in the absence of GDF-5 (A, E) or were enlarged in the presence of GDF-5 (B-D, F-H). Enlarging cartilaginous nodules was promoted by increasing the GDF-5 dose (10－500 ng/mL) (B-D, F-H). Scale bar in (D): 1 mm. Scale bar in (H): 300μm.

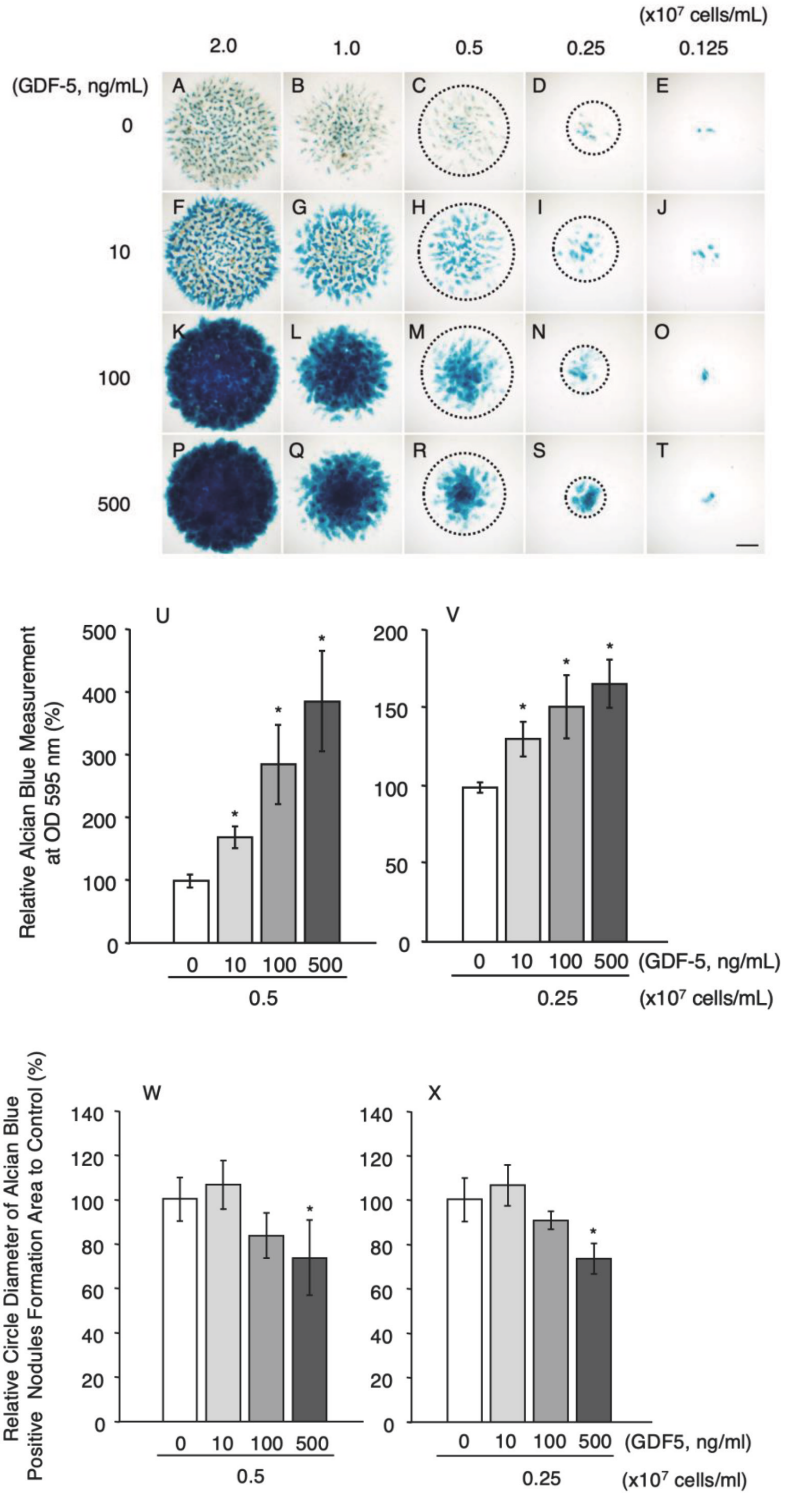

Fig. 2 The cartilaginous nodule formation area in a mouse limb bud mesenchymal cell micromass cultured at a low cell density is reduced in the presence of GDF-5. Limb bud mesenchymal cells from 0.125 to 2.0×10^7 cells/mL were cultured for 3 days in the absence or presence of 10－500 ng/mL GDF-5 (A-T). Cartilaginous nodules were identified using Alcian blue staining. All cell densities promoted cartilaginous nodule formation in the presence of GDF-5 (F-T). Results from Alcian blue staining are expressed as a percentage of staining intensity compared to the untreated controls at 0.5 (U) and 0.25 (V) $\times10^7$ cells/mL density. GDF-5 enhanced Alcian blue stain at both cell densities (U, V). The diameter of the cartilaginous nodule formation area at 0.5 (W) and 0.25 (X) $\times10^7$ cells/mL was measured and expressed as a percentage of the diameter compared to untreated controls. GDF-5 reduced the nodule formation area in both cell densities (W, X). The circle (dotted line) in (C) to (R) indicates the cartilaginous nodule formation area. Scale bar in (T) : 1 mm. *$p<0.05$ compared to the control.

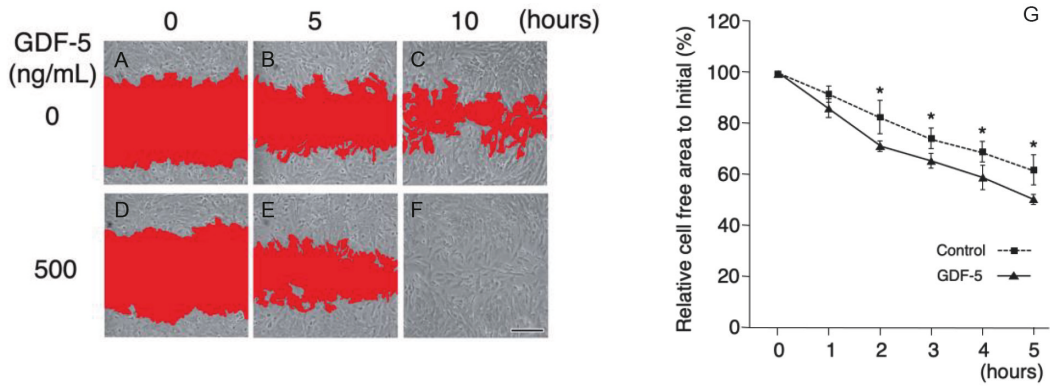

Fig. 3 GDF-5 promotes cell migration in mouse limb mesenchymal micromass cultures. After plating cells at 0.5×10^7 cells/mL, they were scraped and cultured in the absence or presence of 500 ng/mL GDF-5. Phase contrast images (A-F) were taken immediately after scraping (A and D), after 5 h (B and E) and after 10 h (C and F). At one-hour intervals after scraping, the relative cell-free area in the absence (G, control) or presence of GDF-5 (G, GDF-5) was calculated as a percentage of the cell-free area compared to that at 0 h. After 5 h, the cell-free area shown in red was reduced in the presence of GDF-5 (E) compared to the control (B). The cell-free area disappeared after 10 h of culturing in the presence of GDF-5 (F). Scale bar in (F) : 100μm. *p $<$0.05 compared to the control.

intensity of Alcian blue staining increased in a dose-dependent manner (Fig. 2U, V). We observed no difference compared with the control (Fig. 2A) in the diameter of the cartilaginous nodule formation area in micromass cultures at 2.0×10^7 cells/mL in the presence of GDF-5 (Fig. 2F, K, P). At cell densities of 0.5×10^7 (Fig. 2R) and 0.25×10^7 cells/mL (Fig. 2S), a reduction in the cartilaginous formation area in the presence of GDF-5 compared with the control was observed (Fig. 2C, D). Nonetheless, enhanced Alcian blue staining was observed in the center zone of the nodule formation area. The diameter of the nodule formation areas was reduced significantly in the presence of 500 ng/mL GDF-5 compared to that in the controls using 0.5×10^7 (Fig. 2W) and 0.25×10^7 cells/mL (Fig. 2X). These results suggested that GDF-5 increases cell density because of cell migration or aggregation, which promotes differentiation to cartilaginous nodules in the center zone of micromass cultures.

Cell migration assays (cell scratch assays) were performed to clarify how the cartilaginous nodule formation area was reduced. We hypothesized that GDF-5 promotes cell migration around the cartilaginous nodule formation area. Micromass culturing of mouse limb mesenchymal cells at 0.5×10^7 cells/mL was performed in the absence or presence of GDF-5 after scratching the attached cells. After washing, the cell-free area in each culture caused by the scratch was measured. In the absence of GDF-5, the cell-free area 5 h (Fig. 3B) after scratching was smaller when compared to the initial size of this area (Fig. 3A). After 10 h, a cell-free area was still present in the micromass culture (Fig. 3C). In the presence of GDF-5, the cell-free area was also reduced in size after 5 h culturing (Fig. 3E) and no apparent cell-free area was present in the micromass culture 10 h after scratching (Fig. 3F). A significant difference in the cell-free area was observed in the presence of GDF-5 (Fig. 3G, GDF-5) 2 h after scratching compared to that in the culture without GDF-5 (Fig. 3G, Control). This difference persisted for up to 5 h (Fig. 3G).

We examined the effect of GDF-5 on cell migration using the scratch assay and micromass cultures of C3H10T1/2 mouse pluripotent mesenchymal cells or ATDC5 prechondrogenic cells. In the presence of GDF-5, the cell-free area in the C3H10T1/2 culture (Fig. 4E, GDF-5) was significantly smaller than in the absence of GDF-5 after 2 h culturing (Fig. 4E, Control). After 5 h culturing in the presence of GDF-5, the cell-free area was essentially absent (Fig. 4D), whereas a cell-free area was still present in cultures not exposed to GDF-5 (Fig. 4B). In contrast, the cell-free area in ATDC5 cells cultured in the presence of GDF-5 (Fig. 4J,

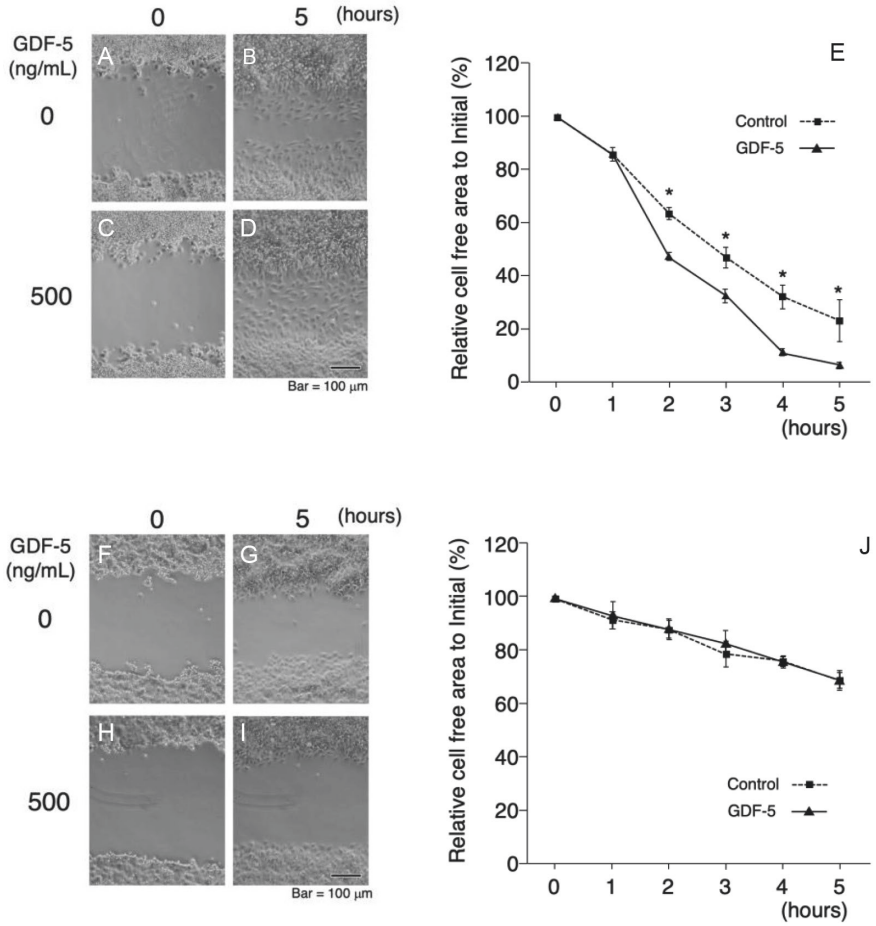

Fig. 4 GDF-5promotes cell migration in C3H10T1/2 mouse mesenchymal cells but not in ATDC5 prechondrogenic cells. After plating cells at 1.5×10^7 cells/mL, cultures were scraped and cultured in the absence or presence of 500 ng/mL GDF-5. Phase contrast images of each cell line, C3H10T1/2 (A-D) and ATDC5 (F-I), were captured immediately after scraping (A, C, F, H) and after 5 h (B, D, G, I). At one-hour intervals after scraping, the relative cell-free area of C3H10T1/2 cells in the absence (E, Control) or presence of GDF-5 (E, GDF-5) was calculated. The cell-free area of ATDC5 cells was also calculated in the absence (J, Control) or presence of GDF-5 (J, GDF-5). After 2 h, the cell-free area of C3H 10T1/2 cells was significantly reduced by GDF-5 (E, GDF-5) compared to that in the control (E, Control) but not for ATDC5 cells (J, Control, GDF-5). The scale bar in (D, I) : 100μm. *$p<0.05$ compared to the control.

GDF-5) was not significantly reduced when compared to cells cultured in the absence of GDF-5 (Fig. 4J, Control) for 5 h. The reduced cell-free area in ATDC5 cell cultures (Fig. 4J) was moderate compared to that in C3H10T1/2 cell cultures (Fig. 4E) in the presence or absence of GDF-5. These results suggested that GDF-5 promotes pluripotent mesenchymal cell migration but minimally affects prechondrogenic cell migration in micromass cultures.

The scratch assay was then performed in the presence of a BMP receptor inhibitor or p38 MAP kinase inhibitor to investigate the role of the GDF-5 signaling pathway in cell migration of C3H10T1/2 cell micromass cultures (Fig. 5). After 3 h, the relative cell-free area in cells treated with GDF-5 (Fig. 5E, GDF-5) was significantly reduced compared to that in the control (Fig. 5E, Control). However, in the presence of the BMP receptor inhibitor and GDF-5 (Fig. 5E, GDF-5+K02288), the relative cell-free area increased significantly compared to that in cells cultured with

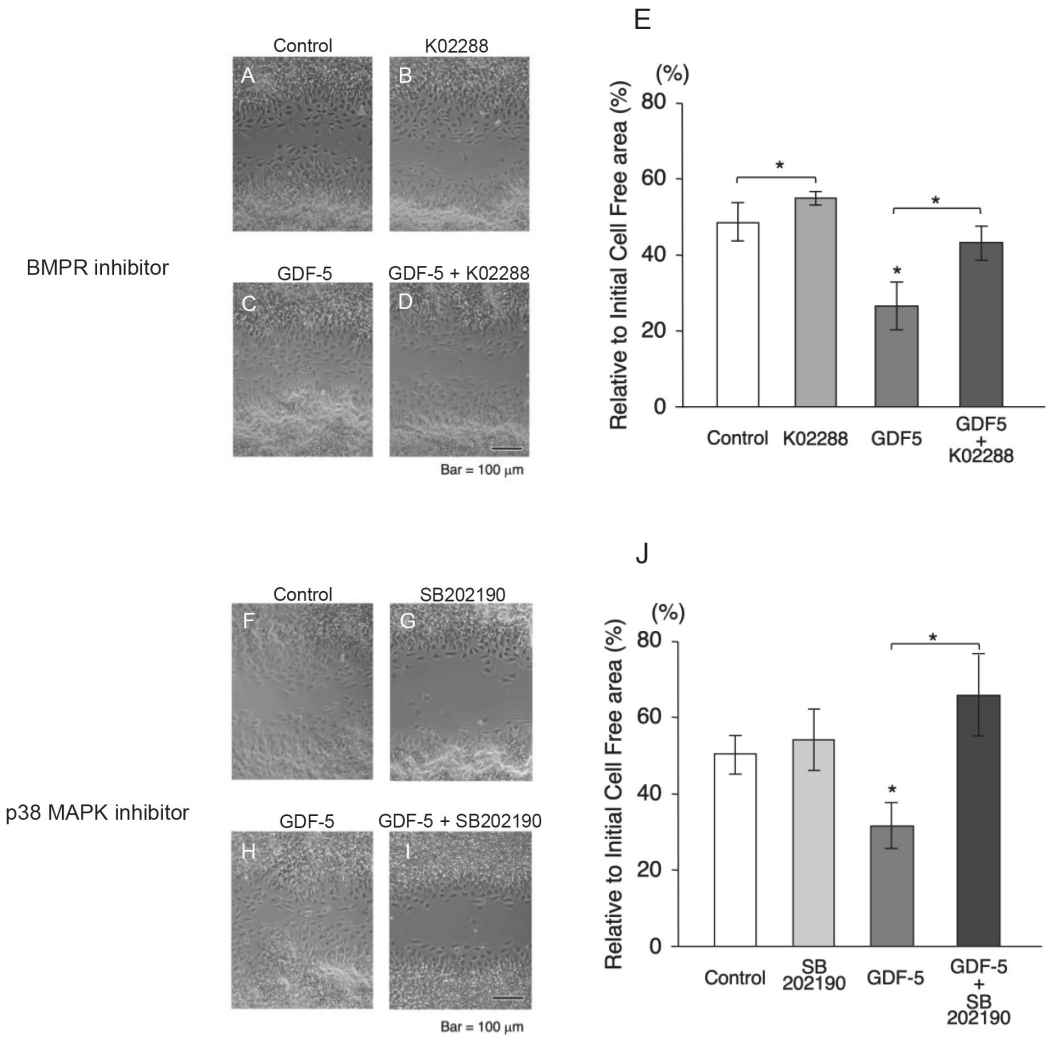

Fig. 5 Both BMPR and p38 MAPK inhibitors inhibit GDF-5-mediated C3H10T1/2 cell migration. After plating cells, cultures were scraped and cultured in the absence or presence of 500 ng/mL GDF-5 with or without the BMPR inhibitor K02288 (A-D) or the p38 MAPK inhibitor SB202190 (F-I). After 3 h, the relative cell-free area in the presence of GDF-5 (E, GDF5) was significantly reduced compared to that in the control (E, Control). However, co-treatment with BMPR inhibitor and GDF-5 (E, GDF + K02288) or p38 MAP kinase inhibitor SB202190 and GDF-5 (J, GDF5 + SB202190) increased the relative cell-free area compared to that in cells treated with only GDF-5 (E, GDF5). The scale bar in (D, I) : 100μm. *$p < 0.05$ compared to the control or GDF-5 only treatment.

only GDF-5 (Fig. 5E, GDF-5). Treatment with only K02288 also significantly increased the relative cell-free area (Fig. 5E, K02288) compared to the control. 1, the cell scratch assay in the presence of the MAPK inhibitor SB202190 was performed. After culturing for 3 h, the relative cell-free area increased in the presence of SB202190 and GDF-5 (Fig. 5J, GDF-5 + SB202190) compared to the cell-free area in the presence of GDF-5 only (Fig. 5J, GDF-5). Microscopic observation after 5 h of culturing showed that the cell-free area in the presence of GDF-5 (Fig. 5C and 5H) almost disappeared, but in the presence of GDF-5 and either K02288 (B) or SB202190 (G), the cell-free areas were similar to that of the control (Fig. 5A and F). These results indicated that the BMPR/p38 MAP kinase signaling pathway is involved in mesenchymal cell migration promoted by GDF-5 in micromass cultures.

Gene expression in C3H10T1/2 cells in the presence

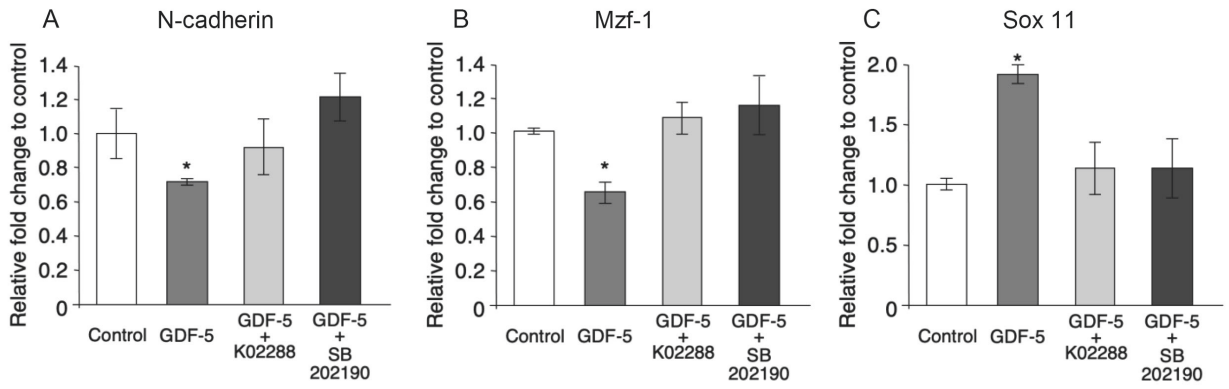

Fig. 6 GDF-5 suppresses N-cadherin expression and promotes Sox-11 expression. After plating cells at 1.5×10^7 cells/mL, cultures were incubated with or without 500 ng/mL GDF-5, K02288 or SB202190. RT-PCR was performed on cDNA generated from total RNA, which was isolated from four samples. The expression of N-cadherin (A) and Mzf-1 (B) was significantly suppressed in the presence of GDF-5 compared to the expression in the absence of GDF-5 (Control). K02288 and SB202190 rescued N-cadherin (A) and Mzf-1 (B) expressions. Sox-11 expression was promoted by GDF-5 and was suppressed by K02288 or SB202190 (C). $*p<0.05$ compared to the control.

or absence of GDF-5 was examined to investigate the molecular mechanism promoting mesenchymal cell migration via GDF-5. After 3 h of culturing in the presence of GDF-5, the expression of N-cadherin decreased significantly compared to that in the absence of GDF-5 (Fig. 6A). Mzf-1 gene expression was tested because Mzf-1 is an N-cadherin transcription factor[36]. In the presence of GDF-5, Mzf-1 expression was significantly suppressed compared to its expression in control cells (Fig. 6B). Expression of N-cadherin and Mzf-1 were rescued by treatment with K02288 or SB202190 and GDF-5 (Fig. 6A, B). The expression of Sox-11 in cells, which is related to mesenchymal cell migration[37], increased significantly in the presence of GDF-5 compared to that in the control (Fig. 6C) and was suppressed in the presence of K02288 and SB202190 (Fig. 6C). These data indicated that Mzf-1 regulates N-cadherin gene expression and that Sox-11 and N-cadherin may be associated with cell migration promoted by GDF-5 in mesenchymal micromass cultures.

Discussion

Cartilage development is a multistep process involving uncommitted mesenchymal cells undergoing cell fate determination to become chondroprogenitors that differentiate into chondrocytes. An initial cell condensation stage is critical for skeletal development during the formation of the cartilage primordium. Previous studies reported that GDF-5 is involved in cell condensation[16,28,29] and that cell migration directly modulates cell condensation[30,31]. This study attempted to clarify the effect of GDF-5 on cell condensation using micromass cultures.

In serially diluted mouse mesenchymal cell micromass cultures, the cartilaginous nodule formation area in the presence of GDF-5 was dose-dependently reduced in low cell density cultures. As a result of cell condensation in center of micromass culture, the intensity of Alcian blue staining increased in the presence of GDF-5 (Fig. 2). These data confirmed our previous study showing that GDF-5 promotes mesenchymal condensation in micromass cultures[29]. Because cell migration directly modulates the cell condensation of mesenchymal cells[30,31], cell migration in micromass cultures using a cell scratch assay was examined. Mouse primary mesenchymal cell migration was significantly promoted in the presence of GDF-5 compared to that in the control (Fig. 3). GDF-5 also promoted the migration of bone marrow-derived stem cells, C3H10T1/2 mouse mesenchymal cells, but did not promote ATDC5 prechondrogenic cell migration (Fig. 4). In a comparative study of cell migration in different stages of human mesenchymal progenitor

cells derived from bone marrow, both BMP-2 and BMP-4 stimulated mesenchymal progenitor cell migration compared to differentiated progenitor cells or primary osteoblasts[38]. C3H10T1/2 cells differentiate into chondrogenic cells in the presence of BMPs, including GDF-5[29,39-43]. The results herein indicated that GDF-5 promotes cell migration of undifferentiated mesenchymal cells but not differentiated cells into chondrocytes.

A previous study showed that squamous cancer cell migration is enhanced by BMP-6/-7 but not by BMP-2/-4[44]. In contrast, BMP-4 inhibits endothelial cell migration[45]. In C2C12 mouse myoblast or human chondrosarcoma cells, cell migration is promoted by BMP-2[46] or BMP-7[47]. In chicken primary fibroblasts, BMP-2 promotes cell migration in a three-dimensional culture using collagen gel[48]. In addition to BMP-2, GDF-5 promotes the migration of bone marrow-derived stem cells[49]. A recent study reported that GDF-5 induces epidermal stem cell migration[50], suggesting that GDF-5 promotes both epithelial and mesenchymal cell migration.

C3H10T1/2 cell migration promoted by GDF-5 was inhibited by a BMPR inhibitor (Fig. 5E). The migration of squamous cancer cells induced by BMP-6 or BMP-7 is enhanced through ALK-2 or ActR-II[44]. In mesenchymal stem cells derived from the bone marrow, BMP-7 induces a cell signaling cascade through the ActR-Ia homodimer, ActR-Ia/BMPR-IA heterodimer or BMPR-IA homodimer[51]. The gene expression pattern of BMPRs in mesenchymal stem cells derived from the bone marrow differed from that in squamous cancer cells, with BMPR-IA and BMPR-II expression higher than BMPR-IB expression[52]. Similar to mesenchymal stem cells, C3H10T1/2 cells express BMPR-IA, BMPR-II[53] and ActR-II[54] but not BMPR-IB[53] and ActR-IIB[54]. Notably, ATDC5 prechondrogenic cells express transcripts for BMPR-II but not BMPR-IB[55]. Although GDF-5 binds BMPR-IB and BMPR-IA, its affinity for BMPR-IB is higher than that for BMPR-IA[18,19]. Thus, undifferentiated mesenchymal cell migration in micromass cultures would be promoted through GDF-5 binding to the BMPR-IA.

In this study, GDF-5-mediated cell migration was suppressed in the presence of the p38 MAPK inhibitor SB202190 (Fig. 5J). There are canonical and non-canonical BMPR signaling pathways downstream of GDF-5 and other BMP family members[3]. The Smad canonical pathway is induced by GDF-5[17,26,28,56,57]. The GDF-5 non-canonical pathway involving p38 MAPK plays an important role in chondrogenic differentiation[16,27,58]. Regarding the involvement of p38 MAP kinase in GDF-5-activated cell migration, two opposing hypotheses have been proposed[16,28]. However, these previous studies only observed cartilage nodule formation using Alcian blue or peanut agglutinin lectin staining, a chondrogenic mesenchymal cell aggregation marker. Thus, our study indicated that GDF-5 promotes cartilage nodule formation, which results from cell migration via the p38 MAP kinase signaling pathway.

In our study, N-cadherin (Fig. 6A) and Mzf-1 (Fig. 6B) gene expression were suppressed, and Sox11 (Fig. 6C) expression increased in the presence of GDF-5. Because the overexpression of Sox11 in mesenchymal stem cells increases cell migration[37], increased Sox11 expression during short-term culturing indicates that Sox11 may initially promote cell migration via GDF-5. Previous studies showed that BMP-2 stimulates N-cadherin gene expression in C3H10T1/2 micromass cultures after 24 h[59], and GDF-5 increases N-cadherin gene expression in rat tendon fibroblasts after 6 days[60]. In contrast, in micromass cultures of chicken limb mesenchymal cells, no significant alteration was observed in the N-cadherin protein level compared to that in the control after 3 days[28]. Although there are no studies reporting that GDF5 regulates N-cadherin gene expression in mesenchymal cells, our study suggests that GDF-5 suppresses the expression of the Mzf-1 gene, causing a decrease in N-cadherin gene expression in mesenchymal micromass cultures because Mzf-1 is a transcriptional activator of the N-cadherin gene[36]. Notably, gradient expression of the extracellular or cytoplasmic N-cadherin domain regulates different cell migration phenotypes, i.e., single-cell migration without forming cell clusters or cell cluster rigid movement[61]. This observation suggested that BMP

signaling regulates the formation of mesenchymal cell clusters and coordinates their movement as a single unit to associate and form mesenchymal condensations[62]. Furthermore, Sox11 regulates β-catenin expression transcriptionally in the chondrogenic differentiation of mesenchymal stem cells[37),63]. Because the interaction of N-cadherin and β-catenin regulates the chondrogenic differentiation of bone marrow mesenchymal stem cells[64], further studies should clarify the mechanism of regulation of N-cadherin expression by GDF-5 to cell cluster movement in high-density micromass cultures of mesenchymal cells.

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原著論文

高密度間葉細胞培養における GDF-5 の細胞遊走に与える影響

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GDF-5 は肢芽の細胞凝集領域に発現しているが，試験管内における軟骨形成モデルとなる未分化間葉細胞高密度培養（マイクロマスカルチャー）における GDF-5 の役割は不明である。そこで，本研究は未分化間葉細胞の遊走能に着目して検討した。マウス肢芽間葉細胞を用いたマイクロマスカルチャーにおいてピペットチップにより無細胞領域を作成した。細胞培養培地に GDF-5 を添加した群では，無細胞領域が対照群と比較して有意に減少していた。また未分化細胞株 C3H10T1/2 では無細胞領域が減少していたが，軟骨細胞株 ATDC5 では減少がみられなかった。さらに C3H10T1/2 細胞における BMP 受容体阻害剤および p38MAK キナーゼ阻害剤添加群では対照群と有意な差が認められなかった。C3H10T1/2 細胞を用いたマイクロマスカルチャーでは，GDF-5 添加群において N-cadherin および Sox11 遺伝子発現の変化がみられた。これらのことから，GDF-5 は BMP 受容体および p38MAP キナーゼ経路によりマイクロマスカルチャーにおける細胞遊走を促進している可能性が示唆された。