J Med Dent Sci 2018; 65: 73–82

Original Article

PDGF-induced proliferation and differentiation of synovial mesenchymal stem cells is mediated by the PI3K-PKB/Akt pathway

Mari Uomizu[1)], Takeshi Muneta[1)], Miyoko Ojima[1)], Ichiro Sekiya[2)], Hideyuki Koga[1)] and Kunikazu Tsuji[3)]

1) Department of Joint Surgery and Sports Medicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University
2) Center for Stem Cell and Regenerative Medicine, Tokyo Medical and Dental University
3) Department of Cartilage Regeneration, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University

Abstract

Background
Platelet-derived growth factors (PDGFs) have been reported to enhance proliferation of synovial mesenchymal stem cells (MSCs) without reducing their multi-lineage differentiation potential in vitro. This study was aimed to elucidate the intracellular molecular pathways activated by PDGFs.

Methods
Synovial MSCs were isolated from patients who underwent total knee arthroplasty with institutional approval and patients' permission. Cell proliferation and differentiation assays were performed in the presence of small inhibitor molecules specific for intracellular kinases.

Results
Both PDGF-AA and -BB enhanced cell proliferation in medium containing reduced serum. These effects were significantly reduced by a phosphatidylinositol-3 kinase (PI3K) inhibitor, LY290042. During chondrogenic spheroid formation in vitro, LY290042 significantly reduced the size of spheroids enhanced by PDGF-AA. LY290042 also significantly inhibited in vitro chondrogenic and osteoblastic differentiation of synovial MSCs.

Conclusion
Our data indicated that activation of the PI3K-PKB/Akt pathway by PDGFs plays an important role in both proliferation and differentiation of synovial MSCs. Hence, these data could be beneficial for optimizing the in vitro culture conditions of synovial MSCs for clinical use.

Key words: synovial MSC, PDGF, PI3K, proliferation, differentiation

Corresponding Author: Kunikazu Tsuji, Ph. D.
Department of Cartilage Regeneration, Tokyo Medical and Dental University 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan.
Tel: +81-3-5803-4080 Fax: +81-3-5803-4675
E-mail: tsuji.orj@tmd.ac.jp
Received November 7, 2017; Accepted March 9, 2018

Introduction

The synovial membrane is a thin layer of membranous tissue, which lines the inner surface of the joint capsule and tendon sheath[1]. Fibroblast-like cells isolated from the synovial membrane in the knee joint are multipotent with an ability to differentiate into osteoblasts, chondrocytes, and adipocytes, and express various surface antigens, such as CD73, CD90, and CD105[2, 3], suggesting that cultured synovial fibroblasts have the characteristics of mesenchymal stem cells (MSCs)[4]. The cartilage-inductive potential of these cells in vivo was demonstrated by the transplantation of synovial MSCs into the knee joint in various animal experimental osteoarthritis models[5-13]and thus the autologous MSC transplantation therapy for the articular cartilage and/or meniscus regeneration is now going on[14]. In our protocol, we transplant the cells that are created by 2-weeks of two-dimensional culture of the autologous synovial cells obtained by synovial biopsy[14]. The trial was basically successful. However, in some cases, we experienced somehow impaired growth rate of MSCs by unknown reasons. In such cases, we

regard the optimization of the culture conditions is of great importance, since our current protocol requires at least 2 x 10^7 cells for transplantation. To make it possible, we now aimed to understand the intracellular signaling that control the proliferation and differentiation of synovial MSCs.

Several cytokines and growth factors, including PDGFs (platelet-derived growth factors), IL1*β* (interleukin 1 beta), and TNF*α* (tumor necrosis factor alpha) have been reported to enhance proliferation and/or differentiation of synovial MSCs in vitro[3, 15-17]. However, the physiological roles of IL1*β* and TNF*α* are still questionable, due to the ambiguous optimal doses in previous reports in comparison to their physiological serum concentrations. In contrast, the roles of PDGF seem to be reasonable. PDGFs are multifunctional secreted proteins that are involved in various physiological processes[18]. Previous studies have shown that MSCs isolated from the synovial membrane express two types of PDGF receptors (PGDFRs), PDGFR*α*/CD140a and PDGFR*β*/CD140b[3, 19], and have greater potential of proliferation with respect to the amounts of PDGFs in the serum compared to bone marrow-derived MSCs[3, 15]. In bone marrow-derived MSCs, inhibition of PDGF signaling enhances the expression of stem cell-related transcription factors (Oct4 and Nanog) and promotes pluripotency and differentiation into endodermal and ectodermal lineages[19]. This suggests that PDGF signaling is involved in both proliferation and differentiation of synovial MSCs. However, the molecular mechanisms by which PDGF promotes synovial MSC proliferation and differentiation are yet to be elucidated.

Therefore, in this study, we focused on answering to two major experimental questions with regard to the use of synovial MSCs for cell transplantation therapy. The first aim of this study was to elucidate the intracellular molecular pathways involved in PDGF-dependent synovial MSC growth. The second was to evaluate the physiological roles of these pathways in the process of osteoblastic, chondrogenic, and adipogenic differentiation of synovial MSCs in vitro. For this purpose, we inhibited the canonical PDGF signaling pathway by small inhibitor molecules for specific kinases and found that both phosphatidylinositol-3 kinase (PI3K) and extracellular signal-regulated kinase (Erk) activities play some roles in proliferation of synovial MSCs. In addition, we demonstrated that PI3K activity also play roles in the differentiation process of MSCs.

Methods

Growth factors and small inhibitor molecules for kinases downstream of PDGFR

Recombinant human PDGF-AA (#221-AA) and PDGF-BB (#220-BB) were purchased from R&D Systems (MN). A PI3K inhibitor, LY290042 (#129-04861), and a PKC (protein kinase C) inhibitor, GF10923X (#079-03811), were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). A p38/MAPK inhibitor, SB203580 (#19-135), was purchased from Upstate Biotechnology (NY). An Erk1/Erk2 inhibitor, PD98059 (#9900), was purchased from Cell Signaling Technology (MA).

Isolation and expansion of synovial MSCs

This study was approved by the Ethics Committee of Tokyo Medical and Dental University (approved No. 2121). All patients gave their full written and informed consent for participation in this study. Primary human synovial cells were isolated from the synovial tissues obtained from patients who underwent total knee arthroplasty, as described previously [2]. All experiments were performed using cells within passage 6.

Cell proliferation assay

Synovial MSCs at passage 4–5 were seeded at 5.7 x 10^2 cells/cm^2 on 10-cm-diameter dishes in MEM-alpha supplemented with 10% FBS and antibiotics. On the next day, the culture medium was replaced with fresh MEM-alpha supplemented with 0.1% FBS and/or PDGFs. Concentrations of PDGF-AA and -BB were determined as 3-fold of ED50 (effective dose 50%) based on the technical data sheets provided by the manufacturer (ED50 of PDGF-AA was 200 ng/mL and of PDGF-BB was 6 ng/mL). Cells were detached on days 0, 2, 5, and 8 by trypsin (Gibco, MA) treatment and total cell numbers were measured by an automated cell counter (TC20; BioRad, Hercules, CA). Experiments were performed using cells isolated from 4 unrelated patients. Statistical analysis was performed using paired t-test (Figure 2A).

Inhibition of cell proliferation by small inhibitor molecules for kinases downstream of PDGF signals

Synovial MSCs at passage 4–5 were seeded at 5.0 x 10^2 cells/well in 96-well plates. On the next day, the culture medium was replaced with fresh MEM-alpha supplemented with 0.1% FBS, PDGFs (3-fold of ED50), and/or inhibitor molecules (3-fold of IC50). Cell proliferation rate was determined by the MTT assay [20]. Experiments were performed in quadruplicate using

cells isolated from 1 donor for LY290042, SB203580, and GF10923X. For PD98059, sample size was 13~16. Statistical analysis was performed using unpaired t-test (Figure 3).

Expression analysis of surface antigens

Fluorochrome-conjugated antibodies for the analysis of cell surface markers, including CD73, CD90, CD105, CD44, CD45, CD140a, and CD140b, were purchased from BD Biosciences (NJ). Cells were detached with TrypLE (Thermo Fisher Scientific, MA) for 5 min and stained with antibodies as described previously[2]. The surface antigen-positive cell fraction was measured using FACSVerse flow cytometer (BD Biosciences).

Intracellular phosphoprotein analysis

Ligand binding activation of PDGFRβ/CD140b was detected by phosphorylation of tyrosine residues at 857, 1009, and 1021 in the amino acid sequence. Phosphotyrosine (PY)-specific antibodies (PY857, PY1009, and PY1021) for PDGFRβ/CD140b were purchased from BD Biosciences. Cells were starved in MEM-alpha supplemented with 0.5% FBS and antibiotics for 24 h to reduce the phosphorylation of tyrosine residues, and then treated with PDGF-BB (3-fold of ED50) for up to 20 min. Cell fixation and permeabilization were performed using Phosflow buffer system (BD Biosciences). The population of cells with tyrosine phosphorylation was measured using FACSVerse flow cytometer (BD Biosciences).

Western blotting

Cells were starved in MEM-alpha supplemented with 0.5% FBS and antibiotics for 24 h to reduce phosphorylation of tyrosine residues, and then treated with PDGF-AA or -BB (3-fold of ED50) for up to 30 min. Total cell lysate was prepared using cell lysis buffer (#9803S; Cell Signaling Technology, MA). Western blotting was performed as described previously[21]. Antibodies against phospho-p44/42 (#4370), p44/42 (#9102), phospho-Akt (#9271), and Akt (#9272) were purchased from Cell Signaling Technology.

In vitro differentiation assay

In vitro differentiation assay was performed according to the method described by Colter[22].

For chondrogenic spheroid formation, 2.5 x 10^5 cells were placed in a 15 mL polypropylene tube (BD Biosciences), centrifuged at 450 x *g* for 10 min, and cultured in chondrogenic medium containing 1000 ng/mL rhBMP-2 (Infuse Bone Graft; Medtronic, TN), 10 ng/mL transforming growth factor-β3 (R&D Systems, MN), and 100 nM dexamethasone (Sigma-Aldrich, MO) for 14 days. Experiments were performed using cells isolated from 4 unrelated patients. Statistical analysis was performed using paired t-test (Figure 4A).

For calcification, cells were cultured in normal growth medium supplemented with 1 nM dexamethasone, 20 mM beta-glycerol phosphate, and 50 μg/mL ascorbate-2-phosphate (Sigma-Aldrich) for 21 days. Calcified nodule formation was visualized by alizarin red staining (Sigma-Aldrich). Experiments were performed using cells isolated from 4 unrelated patients. Statistical analysis was performed using paired t-test (Figure 4E).

For adipogenesis, cells were cultured in MEM-alpha supplemented with 10% FBS, 100 nM dexamethasone (Sigma-Aldrich), 0.5 mM IBMX (isobutyl-methylxanthine; Sigma-Aldrich), and 50 μM indomethacin (Wako, Japan) for 21 days. The adipogenic cultures were fixed and stained with fresh Oil red-O solution (Sigma-Aldrich). Experiments were performed using cells isolated from 4 unrelated patients. Representative images were indicated in Figure 4F.

Glycosaminoglycan (GAG) assay

Amounts of GAG in each chondrogenic spheroid were measured using the Blyscan Assay Kit (Biocolor Ltd, Newtonabbay, Ireland) according to the manufacturer's instructions. By using this kit, the total amount of soluble sulfated proteoglycans and sulfated glycosaminoglycans was quantitatively evaluated by colorimetric method using a dye specifically binding to sulfated sugar chain. Experiments were performed in sextuplicate from 1 donor. Statistical analysis was performed using unpaired t-test (Figure 4B).

Histochemical and immunohistochemical analysis

Sections of the chondrogenic spheroids were deparaffinized in xylene, rehydrated in graded alcohol, and saturated with phosphate buffered saline (PBS). Proteoglycan deposition in the extracellular matrix was visualized by Safranin O staining (Sigma-Aldrich). Type II collagen staining was performed as described previously[2].

Statistical analysis

All statistical analyses were performed with EZR[23]. Paired t-test and unpaired t-test (Student's t-test) were employed for statistical analyses. P values less than 0.05 were considered statistically significant.

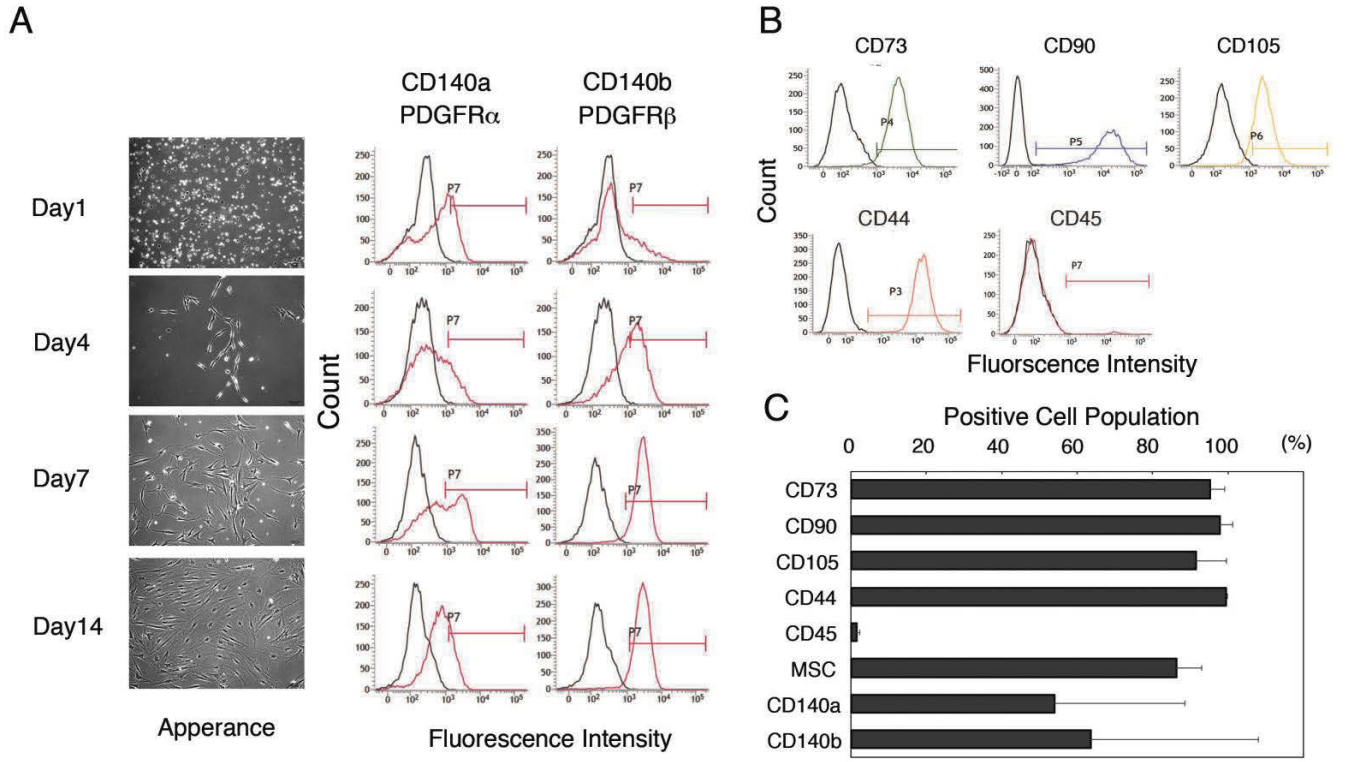

Fig. 1. Expanded fibroblastic cells isolated from the synovial membrane express PDGFRs and exhibit characteristics of MSCs.

A. Primary cultures of adherent cells isolated from the human synovial membrane. Left column; Microscopic appearance of the cells during primary culture (10X). Middle and right columns; Flow cytometric analysis of PDGFRs. Histograms in black indicate the distribution of the fluorescence intensity of the cells stained with isotype control antibodies. Histograms in red indicate the distribution of the fluorescence intensity of the cells stained with antibodies against CD140a/PDGFα (left) or CD140b/PDGFRβ (middle). Marker P7 designates positive events.

B. Flow cytemetric analysis of MSC-related surface antigens expressed on the cells isolated from the synovial membrane. Primary cells were detached from dishes after 14 days of culture and stained with MSC-specific antibodies (CD73, CD90, CD105, and CD44). CD45 is a negative marker for MSCs. Histograms in black indicate the distribution of the fluorescence intensity of the cells stained with isotype control antibodies. Histograms in color indicate the distribution of the fluorescence intensity of the cells stained with antibodies against surface markers indicated. Markers (P3-P7) designate positive events.

C. Surface antigen expression profiles of cells after 14 days in culture. MSC: population of $CD73^{+}CD90^{+}CD105^{+}CD44^{+}CD45^{-}$ MSCs. Representative data from 6 independent experiments are indicated in this figure.

Results

Cultured fibroblastic cells isolated from the synovial membrane have characteristics of MSCs and express PDGFRs

The synovial tissue is reported to be composed of two major cell types: synovial macrophages and synovial fibroblasts. At day 1 in culture, the appearance of the adherent cells isolated from the synovial tissue was quite heterogeneous and most of the cells had a round shape and did not express PDGFRs (Figure 1A). This heterogeneity disappeared with time, and only the cells with fibroblastic appearance were expanded on day 14 (Figure 1A). Surface antigen expression analyses indicated that most of these expanded cells were negative for CD45, but positive for CD73, CD90, CD105, and CD44 (Figure 1B, 1C). Multicolor flow cytometric analyses showed that more than 80% of these cells expressed CD73, CD90, CD105, and CD44 on the same cell surface, but lacked CD45 (Figure 1C, MSC). Flow cytometric analyses further indicated that more than half of the cells expressed CD140a/PDGFR*α* (54.1+/-34.6%) and CD140b/PDGFR*β* (63.6+/-44.4%, Figure 1C), suggesting that PDGF signaling is involved in the growth of synovial MSCs.

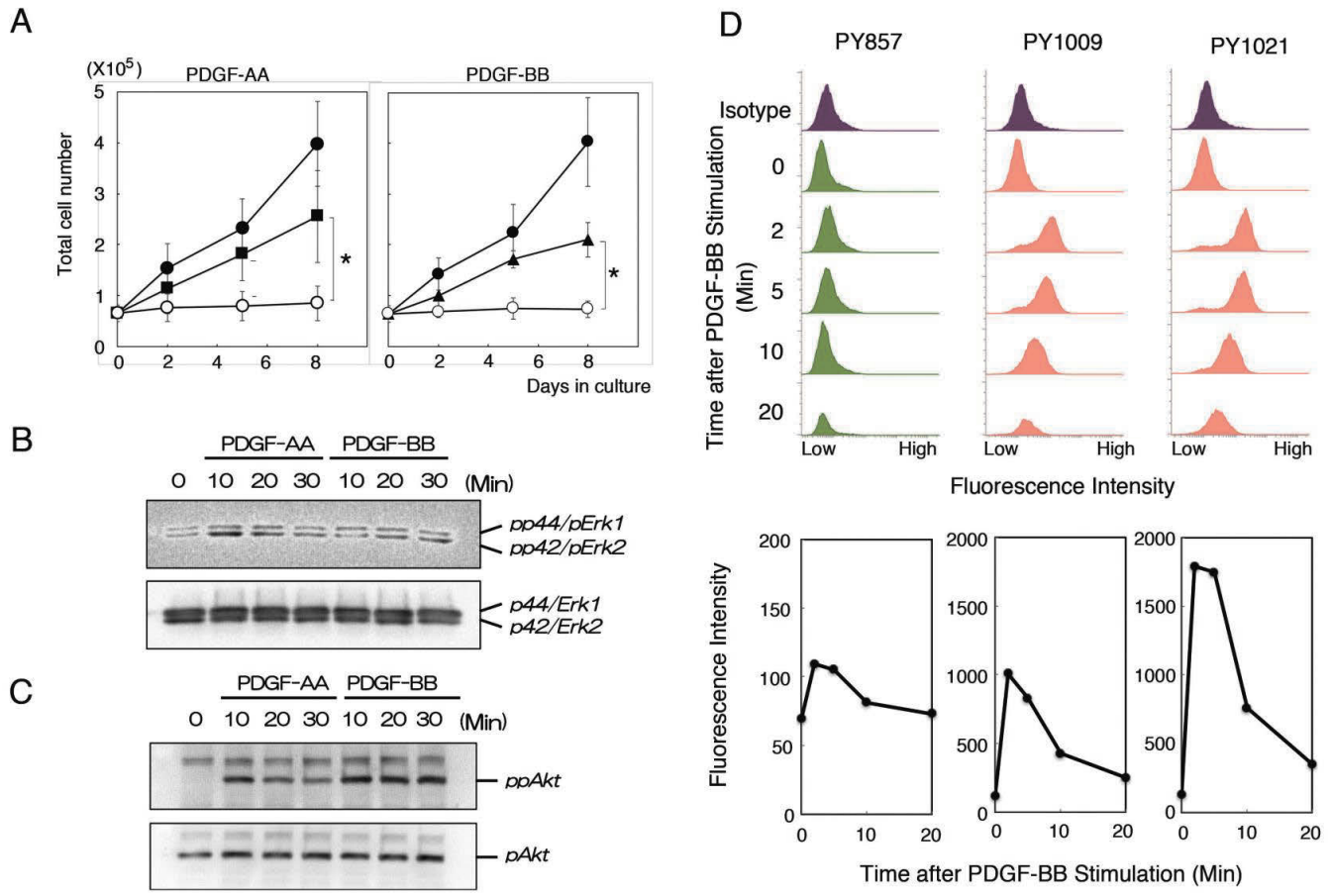

Fig. 2. Analysis of intracellular signaling molecules activated by PDGF-AA and -BB.

A. Both PDGF-AA and -BB enhance proliferation of synovial MSCs in medium containing reduced serum. Synovial MSCs were cultured in the presence or absence of PDGFs and the numbers of cells were counted on the days indicated. Data are expressed as mean ± standard deviation (SD) of 4 independent experiments. Open circle; 0.1%FBS, Closed circle; 10%FBS, Closed rectangle; 0.1%FBS+PDGF-AA, Closed triangle; 0.1%FBS+PDGF-BB. Asterisk (*) indicates that the difference is statistically significant ($p<0.05$, Paired T-test). Exact p values were as follows. Open circle vs closed rectangle; 0.013 (day8). Open circle vs closed triangle; 0.004 (day8).

B. Immunoblot analysis of phospho-Erk1/2.

C. Immunoblot analysis of phospho-Akt.
Representative images from 3 independent experiments are shown.

D. Transient phosphorylation of tyrosine residues in the intracellular domain of CD140b/PDGFRβ that are involved in the PLC signaling pathway. (Upper panels) Time course changes of fluorescence intensity of tyrosine residues after PDGF-BB stimulation. phosphorylation of tyrosine 857, 1009, and 1021 quickly occurs after stimulation. Histograms in Blue indicate the distribution of the fluorescence intensity of the cells stained with isotype control antibodies. (Lower panels) Peak fluorescence intensity at each time point is measured and plotted. Representative images from 2 independent experiments are shown.

Activation of PI3K and Erk1/Erk2 is important for PDGF-dependent proliferation of synovial MSCs

To examine if PDGF signal play roles in MSC growth, cell growth assay was performed. As shown in Figure 2A, synovial MSCs were increased almost fourfold at day 8 in culture in the normal growth medium (10% FBS, closed circle). In the medium with reduced serum (0.1%), cell proliferation was not observed (open circle). Supplementation of PDGFs in the medium with reduced serum significantly increased cell numbers after day 8 in culture. Since the effect of PDGFs on cell proliferation was observed in medium containing reduced concentration of FBS (0.1%), the intracellular signals activated by PDGFRs seemed to play some roles in the proliferation of synovial MSCs.

Canonical PDGF signals include 3 different pathways, Erk1/2, PI3K, and PLC (phospholipase C). Among them, to identify the signaling pathways involved in the proliferation of synovial MSCs, ligand binding-dependent activation of intracellular signals downstream of PDGFs

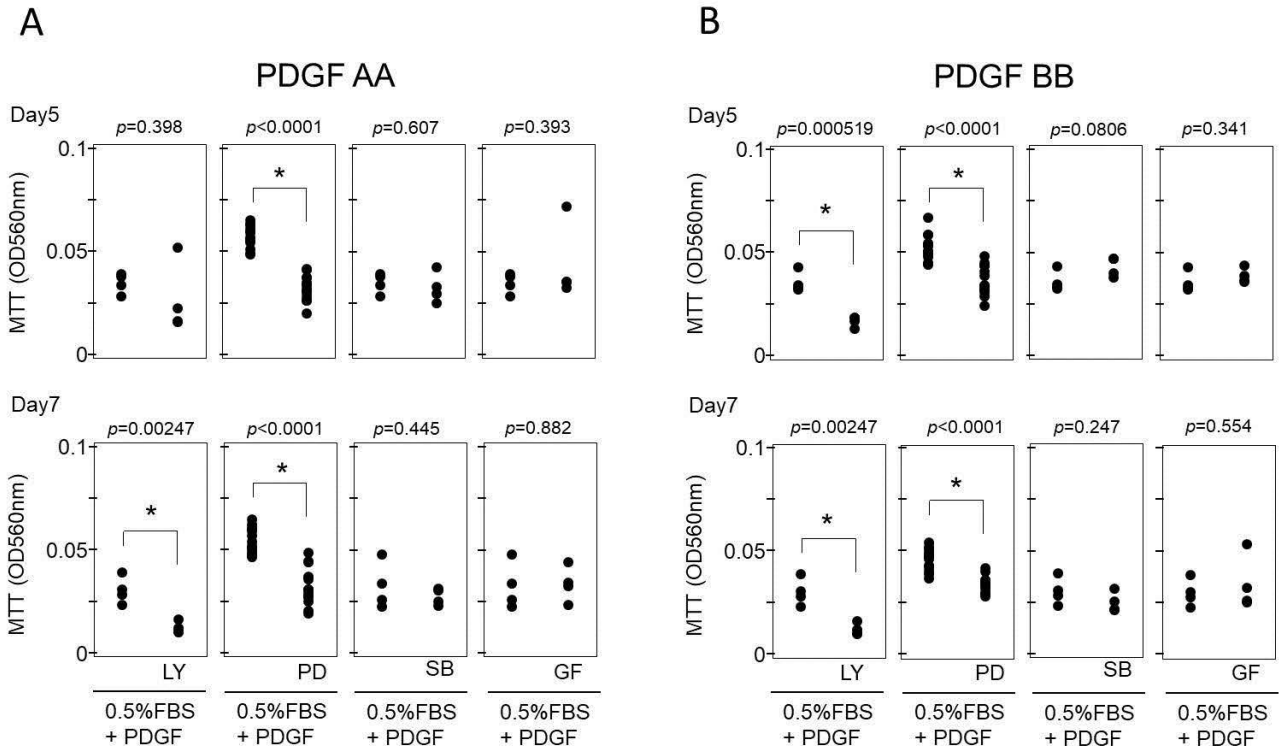

Fig. 3. The PI3K and Erk pathways play important roles in the proliferation of synovial MSCs.

MTT assay was performed to examine the inhibitory effect of small inhibitor molecules on the proliferation of synovial MSCs induced by PDGF-AA (A) and -BB (B). Inhibitor molecules were supplemented at 3-fold of IC50, as indicated in the instruction manual provided by each manufacturer. LY:LY290042, PD:PD98059, SB:SB203580, and GF:GF10923X. Asterisk (*) indicates that the difference is statistically significant (Unpaired t-test, $P<0.05$). Data are expressed as mean ± standard deviation (SD). Number of samples were 4 for LY, SB, and GF and 13~16 for PD. Exact p values are indicated in the figure. Of note that due to the larger sample size, much smaller p values were observed in PD.

was evaluated by phosphorylation of p44/Erk1, p42/Erk2, and PKB/Akt (western blot analysis), and tyrosine residues (857Y, 1009Y, and 1021Y) of CD140b/PDGFRβ those are important in PLC activation (flow cytometry). As shown in figures 2B and 2C, PDGF-AA rapidly enhanced the phosphorylation levels of p44/Erk1, p42/Erk2, and PKB/Akt in synovial MSCs within 10 min. PDGF-BB strongly enhanced phosphorylation of PKB/Akt within 10 min after stimulation. Effects of PDGF-BB on PKB/Akt phosphorylation lasted longer than 30 min in synovial MSCs (Figure 2C). However, Phosphorylation levels of p44/Erk1 and p42/Erk2 did not seem to change significantly up to 30 min after PDGF-BB stimulation. Flow cytometric analyses suggested that PDGF-BB also activates the PLC signaling pathway, since a rapid increase in the phosphorylation of tyrosine residues 857, 1009, and 1021 (PY857, PY1009, and PY1021) in PDGFRβ/CD140b was observed within 2 min after PDGF-BB stimulation in synovial MSCs (Figure 2D).

Next, to elucidate which signaling pathways are functionally involved in the process of proliferation of synovial MSCs, cell growth assay was performed in the presence of various types of pathway-specific protein kinase inhibitors. As shown in Figure 3, the PI3K (LY290042) and Erk1/Erk2 (PD98059) inhibitors significantly inhibited MSC proliferation induced by PDGF-AA and PDGF-BB, while the MAPK (SB203580) had subtle effects on it. Of note that much smaller p values were observed in PD98059 (Figure 3). These seemed due to the larger sample size only for this experiment in technical issue. Flow-cytometric analyses indicated the transient phosphorylation of tyrosine residues in the intra-cellular domain of PDGFRβ (Figure2D), which is involved in the activation of PLC-PKC pathway, however, PKC inhibitor, GF109203X, did not inhibit proliferation of synovial MSCs, suggesting that PLC-PKC signal pathway is dispensable in the proliferation of synovial MSCs (Figure 3). Taken together, these data suggested that both PI3K-PKB/Akt and Erk1/Erk2 signaling pathways play important roles in PDGF-mediated MSC proliferation.

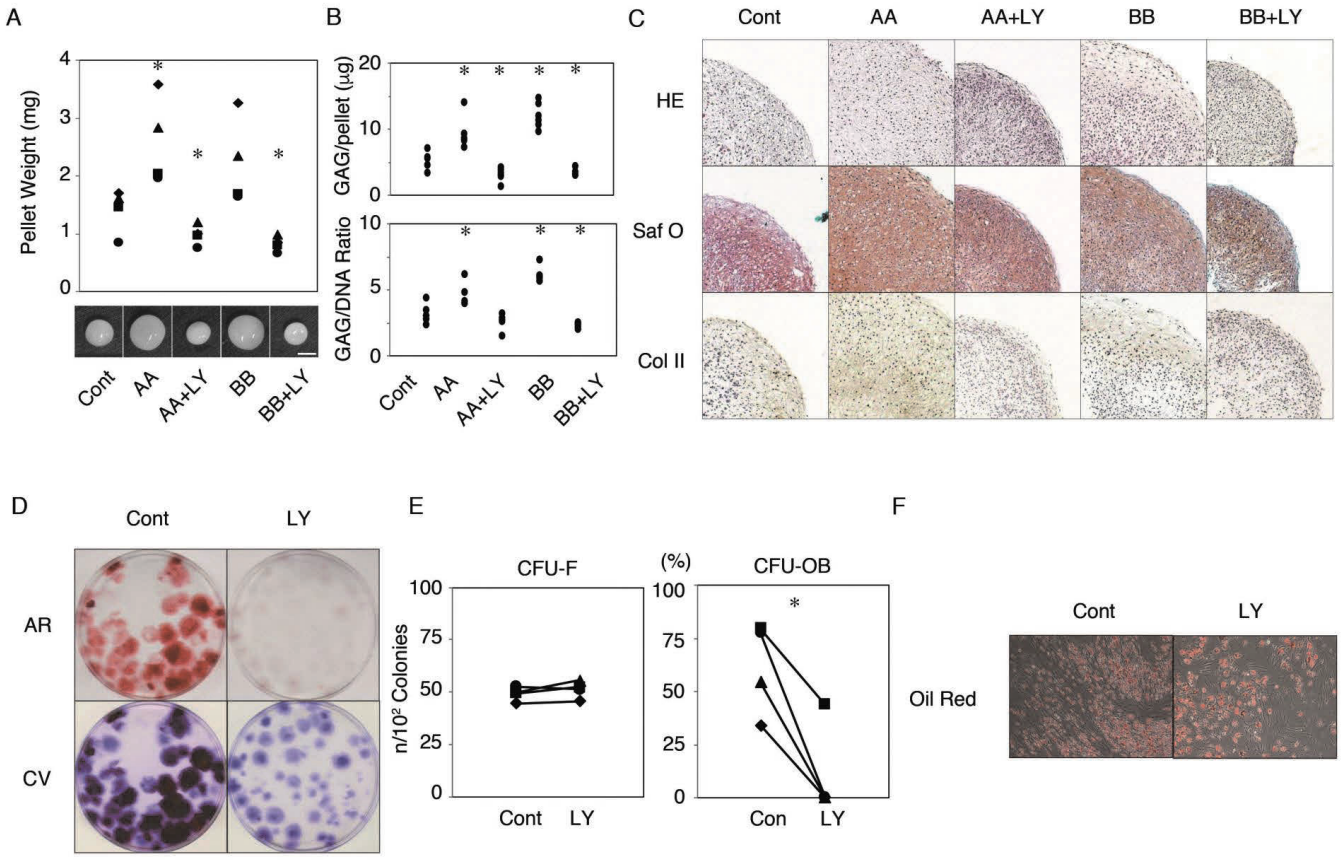

Fig. 4. PI3K activity plays a pivotal role during chondrogenic and osteogenic differentiation of synovial MSCs.

A. In vitro chondrogenic spheroid formation. Pellet weights of 4 independent experiments were indicated (data from same patient indicated in each marker). Asterisk (*) indicates that the difference is statistically significant if compared with that of control (Paired T-test, P<0.05). Exact p values were as follows. AA:0.021, AA+LY:0.043, BB:0.058, BB+LY:0.026

B. Upper panel indicates the GAG amounts in each spheroid. GAG amounts are normalized to DNA amounts in each spheroid and indicated in the lower panel. Measured values from sextuplicate from 1 donor were indicated. Due to the experimental difficulties, there are several missing values in each data. Asterisks (*) indicates that the difference is statistically significant if compared with that of control (Paired T-test, P<0.05). Exact p values and numbers of samples were as follows. Upper panel; AA:0.006 (n=5), AA+LY:0.029 (n=6), BB:<0.0001(n=6), BB+LY:0.043 (n=6). Lower panel; AA:0.031 (n=4), AA+LY:0.202 (n=5), BB:0.0001 (n=5), BB+LY:0.035 (n=5)

C. Histological analysis of the chondrogenic spheroids.

D. Osteogenic differentiation of synovial MSCs in the presence or absence of LY290042. Representative images from 4 independent experiments are shown. AR: Alizarin Red S, CV: Crystal Violet

E. CFU-F (colony forming unit-fibroblast: number of colonies which is visualized by CV staining) and CFU-OB (colony forming unit-osteoblast: number of colonies with calcified extra-cellular matrix, which is visualized by AR staining/CFU-F (%)) were counted. Data of 4 independent experiments were indicated (data from same patient indicated in each marker). Asterisk (*) indicates that the difference is statistically significant (paired T-test, CFU-OB: P<0.0001).

F. Adipogenic differentiation of synovial MSCs. Representative images from 4 independent experiments are indicated.

PDGF enhanced chondrogenic spheroid formation via the PI3K pathway

It is reported that the expression of stem cell phenotype-related transcription regulators, Oct4 and Nanog, is negatively regulated by PI3K activity in bone marrow derived MSCs[19]. To evaluate the effects of PDGF-PI3K-Akt/PKB axis on the chondrogenic differentiation of synovial MSCs, spheroid culture was performed in the presence or absence of either PDGF-AA or -BB. As indicated in Figure 4A, PDGF-AA significantly increased the wet weight of chondrogenic spheroids. PDGF-BB also increased the wet weight although the difference was not statistically significant. GAG amounts in each pellet were significantly increased in the presence of either PDGF-AA or -BB (Figure 4B, upper panel). The ratio of GAG to DNA amounts was also increased by PGDF supplementation (Figure 4B, lower panel). Histologically, the inter-nuclear distances seemed to be increased in

the presence of PDGF-AA or -BB. (Hematoxylin and Eosin staining, the top row). Extracellular matrices were more abundant in type II collagen in the presence of PDGFs than in the presence of LY290042 (Figure 4C, the bottom row). In contrast, the levels of proteoglycan deposition seemed comparable regardless of LY290042 treatment (Figure 4C, the second row). These data suggest that supportive function of PDGF-AA or –BB on chondrogenic spheroid culture that may be combination of the effects on cell proliferation and differentiation was dependent on the PI3K activity.

Inhibition of the PI3K pathway strongly diminished calcified extracellular matrix deposition by synovial MSCs with slight effects on adipogenic differentiation

To further analyze the importance of PI3K activity during osteoblastic and adipogenic differentiation of synovial MSCs, cells were maintained for two weeks in differentiation medium. As shown in Figure 4D and 4E, inhibition of PI3K activity strongly reduced the size of the colonies and calcium deposition (colony forming unit osteoblast, CFU-OB). In contrast, PI3K activity seemed to be dispensable during adipogenic differentiation of synovial MSCs (Figure 4F).

Discussion

In previous studies, we reported that serum PDGFs have great potential to enhance the proliferation of synovial MSCs without affecting multipotency in two-dimensional culture conditions[15]. In this study, we showed that fibroblastic cells positive for PDGFRs were expanded during the primary culture of synovial MSCs (Figure 1). This suggests that PDGF signaling plays an important role in the proliferation of synovial MSCs. Two types of receptor molecules, CD140a/PDGFRα and CD140b/PDGFRβ are found in the PDGF system, and their specific ligands, PDGF-AA and PDGF-BB, respectively, have already been identified. Distinctive phosphorylation patterns of PKB/Akt and Erk1/Erk2 between the responses to the PDGF-AA and –BB (Figure 2B and C) may suggest separate roles of these factors on the regulation of synovial MSCs. However, since our observation also indicated that physiological functions of these factors and receptors on synovial MSC proliferation are comparable, the distinctive phosphorylation patterns may have only a subtle influence on proliferation. Distinctive roles on other physiological phenomena are still possible, but are not unveiled yet. Further investigation may be required.

Important roles of the PI3K-PKB/Akt axis on proliferation have been reported in MSCs from other types of tissues. Gharibi et al reported that inhibition of PI3K by LY290042 significantly reduced PDGF-BB-induced DNA synthesis in human bone marrow-derived MSCs[24]. They showed that PDGF-BB enhanced the expression of cyclin D1 in these cells, and this effect was suppressed by LY290042. Qiu et al also reported similar findings using human cord blood-derived MSCs[25]. In our study, we did not analyze the expression of cyclin D1 in synovial MSCs. However, it is feasible to assume that similar molecular mechanisms exist. This is the first study reporting the intracellular signaling pathway responsible for cell cycle progression in synovial MSCs.

In addition to the roles of PI3K in proliferation, we showed that PI3K activity is also involved in the chondrocytic- and osteoblastic-differentiation of synovial MSCs. PDGF-AA significantly enhanced chondrogenic spheroid formation, while the PI3K inhibitor, LY290042, completely reversed it (Figure 4A and 4B upper panel). Biochemical and immunohistochemical analyses indicated that PDGF treatment during chondrogenic differentiation enhanced the expression of cartilaginous extracellular matrix proteins, such as type II collagen (Figure 4C). These pro-chondrogenic effects of PI3K may be through both the increase in cell numbers and cartilage matrix production in each spheroid (Figure 4B).

Inhibitory effect of LY290042 on extracellular matrix mineralization was more striking. In the presence of LY290042, CFU-OB was almost completely abolished (Figure 4D); colony number was not altered, however, the size of each colony appeared reduced (Figure 4D and E). Although the underlying molecular mechanisms were not elucidated, PI3K activity seemed to be a prerequisite for osteogenic differentiation of synovial MSCs. In contrast to our results, Gharibi et al reported that PDGF-BB inhibited osteogenic differentiation of bone marrow-derived MSCs and indicated that this was partly due to the inhibited expression of osteoblast-related markers, such as type I collagen and alkaline phosphatase[24]. The reason for this discrepancy remains unclear, and further analyses are required to determine the characteristics of MSCs derived from different adult tissues.

In summary, we analyzed the intracellular signaling pathways activated by PDGFs in synovial MSCs and identified that PI3K signaling is important in both in vitro proliferation and differentiation. In this study, we also identified that Erk activity is also play some roles in the proliferation of synovial MSCs. The findings presented in this study may be useful in the optimization of in vitro culture conditions of synovial MSCs, facilitating their

application in cell transplantation therapy. Furthermore, these results may also be useful to develop a new serum-free culture medium for clinical usage.

Acknowledgments

This study was supported by the Japan Society for the Promotion of Science (JSPS) to KT (25293317, 25460716, 26462287). This study was also supported by "the Project for Realization of Regenerative Medicine" from the Japan Agency for Medical Research and Development to IS (122011A182). We thank Ms. Masako Akiyama for great support in statistical analyses.

References

1. E.B. de Sousa, P.L. Casado, V. Moura Neto, et al. Synovial fluid and synovial membrane mesenchymal stem cells: latest discoveries and therapeutic perspectives. Stem cell research & therapy. 2014; 5: 112.
2. K. Tsuji, M. Ojima, K. Otabe, et al. Effects of Different Cell-Detaching Methods on the Viability and Cell Surface Antigen Expression of Synovial Mesenchymal Stem Cells. Cell transplantation. 2017; 26: 1089-1102.
3. A. Nimura, T. Muneta, H. Koga, et al. Increased proliferation of human synovial mesenchymal stem cells with autologous human serum: comparisons with bone marrow mesenchymal stem cells and with fetal bovine serum. Arthritis Rheum. 2008; 58: 501-10.
4. M. Dominici, K. Le Blanc, I. Mueller, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006; 8: 315-7.
5. S. Kondo, T. Muneta, Y. Nakagawa, et al. Transplantation of autologous synovial mesenchymal stem cells promotes meniscus regeneration in aged primates. J Orthop Res. 2017; 35: 1274-82.
6. N. Ozeki, T. Muneta, H. Koga, et al. Not single but periodic injections of synovial mesenchymal stem cells maintain viable cells in knees and inhibit osteoarthritis progression in rats. Osteoarthritis Cartilage. 2016; 24: 1061-70.
7. N. Ozeki, T. Muneta, S. Matsuta, et al. Synovial mesenchymal stem cells promote meniscus regeneration augmented by an autologous Achilles tendon graft in a rat partial meniscus defect model. Stem Cells. 2015; 33: 1927-38.
8. D. Hatsushika, T. Muneta, M. Horie, et al. Intraarticular injection of synovial stem cells promotes meniscal regeneration in a rabbit massive meniscal defect model. J Orthop Res. 2013; 31: 1354-9.
9. D. Hatsushika, T. Muneta, T. Nakamura, et al. Repetitive allogeneic intraarticular injections of synovial mesenchymal stem cells promote meniscus regeneration in a porcine massive meniscus defect model. Osteoarthritis Cartilage. 2014; 22: 941-50.
10. H. Katagiri, T. Muneta, K. Tsuji, et al. Transplantation of aggregates of synovial mesenchymal stem cells regenerates meniscus more effectively in a rat massive meniscal defect. Biochem Biophys Res Commun. 2013; 435: 603-9.
11. Y. Nakagawa, T. Muneta, S. Kondo, et al. Synovial mesenchymal stem cells promote healing after meniscal repair in microminipigs. Osteoarthritis Cartilage. 2015; 23: 1007-17.
12. M. Okuno, T. Muneta, H. Koga, et al. Meniscus regeneration by syngeneic, minor mismatched, and major mismatched transplantation of synovial mesenchymal stem cells in a rat model. J Orthop Res. 2014; 32: 928-36.
13. S. Suzuki, T. Muneta, K. Tsuji, et al. Properties and usefulness of aggregates of synovial mesenchymal stem cells as a source for cartilage regeneration. Arthritis Res Ther. 2012; 14: R136.
14. I. Sekiya, T. Muneta, M. Horie, et al. Arthroscopic Transplantation of Synovial Stem Cells Improves Clinical Outcomes in Knees With Cartilage Defects. Clin Orthop Relat Res. 2015; 473: 2316-26.
15. M. Mizuno, H. Katano, K. Otabe, et al. Platelet-derived growth factor (PDGF)-AA/AB in human serum are potential indicators of the proliferative capacity of human synovial mesenchymal stem cells. Stem cell research & therapy. 2015; 6: 243.
16. E. Matsumura, K. Tsuji, K. Komori, et al. Pretreatment with IL-1beta enhances proliferation and chondrogenic potential of synovium-derived mesenchymal stem cells. Cytotherapy. 2017; 19: 181-93.
17. M. Shioda, T. Muneta, K. Tsuji, et al. TNFalpha promotes proliferation of human synovial MSCs while maintaining chondrogenic potential. PLoS One. 2017; 12: e0177771.
18. J. Andrae, R. Gallini, C. Betsholtz. Role of platelet-derived growth factors in physiology and medicine. Genes Dev. 2008; 22: 1276-312.
19. S.G. Ball, A. Shuttleworth, C.M. Kielty. Inhibition of platelet-derived growth factor receptor signaling regulates Oct4 and Nanog expression, cell shape, and mesenchymal stem cell potency. Stem Cells. 2012; 30: 548-60.
20. K. Tsuji, M. Noda. Transient suppression of core-binding factor alpha 1 expression by basic fibroblast growth factor in rat osteoblast-like osteosarcoma ROS17/2.8 cells. J Bone Miner Metab. 2001; 19: 213-9.
21. K. Tsuji, N. Kraut, M. Groudine, et al. Vitamin D (3) enhances the expression of I-mfa, an inhibitor of the MyoD family, in osteoblasts. Biochim Biophys Acta. 2001; 1539: 122-30.
22. D.C. Colter, I. Sekiya, D.J. Prockop. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci U S A. 2001; 98: 7841-45.
23. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013;48(3):452-8.
24. B. Gharibi, M.S. Ghuman, F.J. Hughes. Akt- and Erk-mediated regulation of proliferation and differentiation during PDGFRbeta-induced MSC self-renewal. Journal of cellular and molecular medicine. 2012; 16: 2789-801.

25. P. Qiu, W. Song, Z. Niu, et al. Platelet-derived growth factor promotes the proliferation of human umbilical cord-derived mesenchymal stem cells. Cell biochemistry and function. 2013; 31: 159-65.