Predominant control of PDGF/PDGF receptor signaling in the migration and proliferation of human adipose-derived stem cells under culture conditions with a combination of growth factors

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Abstract. Human adipose-derived stem cells (hASCs) play important roles in regenerative medicine and tissue engineering. However, their clinical applications are limited because of their instability during cell culture. Platelet lysates (PLTs) contain large amounts of growth factors that are useful for manufacturing cellular products. Platelet-derived growth factor (PDGF) is a major growth factor in PLTs and a potent mitogen in hASCs. To optimize growth conditions, the effects of a combination of growth factors on the promotion of hASC proliferation were investigated. Moreover, PDGF-BB combined with vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) markedly enhanced the viability of hASCs compared with the effects of PDGF-BB alone. Neither VEGF nor HGF had any effect alone. All growth factor receptor inhibitors inhibited cell proliferation. Wound healing assays revealed that VEGF and HGF stimulated PDGF-dependent cell migration. The effects of these growth factors on the activation of their cognate receptors and signaling enzymes were assessed using immunoblotting. Phosphorylation of PDGF receptor (PDGFR)β, VEGF receptor (VEGFR)2 and MET proto-oncogene and receptor tyrosine kinase was induced by PDGF-BB treatment, and was further increased by treatment with PDGF-BB/VEGF and PDGF-BB/HGF. The levels of phospho-ERK1/2 and phospho-p38MAPK were increased by these treatments in parallel. Furthermore, the expression levels of SRY-box transcription factor 2 and peroxisome proliferator-activated receptor g were increased in PDGF-BB-treated cells, and PDGF-BB played a dominant role in spheroid formation. The findings of the present study highlighted that PDGF/PDGFR signaling played a predominant role in the proliferation and migration of hASCs, and suggested that PDGF was responsible for the efficacy of other growth factors when hASCs were cultured with PLTs.

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Introduction

Mesenchymal stem cells (MSCs) represent a promising source for cell-based clinical applications (1,2). MSCs are adult pluripotent stem cells present in almost every tissue and organ and the bone marrow is an important site for MSC isolation (1,2). Adipose tissue is a promising source of MSCs with a comprehensive impact on various clinical applications such as the treatment of osteoarthritis (2,3). Adipose tissue is relatively abundant in the body and can be collected in large amounts with minimally invasive procedures, resulting in low rates of morbidity (3). Human adipose-derived stem cells (hASCs) can differentiate into multiple lineages, including osteogenic (4), chondrogenic (5), adipogenic (6) and neurogenic (7) lineages. Based on their differentiation abilities, hASCs have been used in regenerative medicine to promote bone regeneration (8), wound healing and age suppression (9), and to facilitate nerve regeneration (8). Additionally, their high proliferation rate as well as their immune-privileged and -tolerant properties make hASCs effective and a potential novel therapy in the field of regenerative medicine (8,9).

Human platelet lysates (PLTs) contain several mitogenic growth factors, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and transforming growth factor (TGF)-β (10,11). PLT can be used in MSC cultures without adversely affecting the immunophenotype or metabolism of MSCs, and can replace fetal bovine serum (FBS) for cell proliferation (12,13). The superiority of PLT over human AB serum (14), human plasma (15) and human autologous serum (15) for *ex vivo* expansion of MSCs is due to its high growth factor content, low cost and ease of large-scale production (12-15). Therefore, this method can be applied in clinical studies concerning hASCs using PLTs. PLTMax, a derivative of platelet-rich plasma (PRP), is a commercialized PLT product created from the whole blood of American donors, which has been tested

for infections. Alonso-Camino and Mirsch (16) demonstrated that PLTMax exhibited proliferative effects similar to those of PRP. Furthermore, our group previously reported that culturing hASCs with PLTMax resulted in a superior effect on cell proliferation compared to culturing with FBS (17).

PDGF is a major growth factor in PLT that stimulates the proliferation, survival and motility of MSCs (18). PDGF belongs to the family of cationic homo- and disulfide-linked dimers of A- and B-polypeptide chains (19). PDGF isoforms bind to α- and β-tyrosine kinase receptors, and promote autophosphorylation and kinase activity (19). Previous studies on the PDGF signaling pathway and target genes have demonstrated that the activation of PDGF receptor (PDGFR) downstream molecules contributes to PDGF-promoted cell proliferation and migration (19,20). However, PDGF does not account for the mitogenic ability of PLT (20). In addition, PLTMax (or PLT) contains proliferation and differentiation factors released by platelets, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and EGF (16). These factors act as mitogens and motogens for diverse cell types and angiogenesis promoters (18). Previous *in vitro* studies have demonstrated that hASCs cultured with individual or combinations of growth factors differentiate into endothelial, chondrogenic, myogenic, osteogenic and neural cells (21,22). However, the cooperative role of the individual factors present in PLTMax in the proliferation and differentiation of hASCs remains unknown, and the precise mechanisms involved in the recruitment of PDGF and its receptor to other signaling pathways involved in the proliferation and migration of hASCs are unclear.

In the present study, the contribution of proliferative factors to hASC potency was systematically investigated. Our group previously reported the positive effects of PLT (or PRP), PDGF-BB and FGF-2 on the proliferation and migration of hASCs (20,23,24). The present study aimed to optimize hASC proliferation in 2-dimensional (D) and 3-D cultures by supplementing hASCs with growth factors. PDGF-BB combined with VEGF, HGF or EGF had a synergistic effect on the PDGF-BB-dependent migration and proliferation of hASCs. PDGF-BB/PDGFR signaling predominantly controlled different signaling pathways to activate the ERK1/2 and p38 MAPK mitogenic enzymes, followed by cell proliferation and migration.

Materials and methods

Ethics approval. The present study was approved by The Ethics Review Board of Kansai Medical University (Hirakata, Japan; approval no. 2017094) in accordance with the ethical guidelines of the Declaration of Helsinki of 1975.

Reagents. Human platelet lysate (PLTMax) was purchased from MilliporeSigma (cat. no. SCM141). PDGF-BB was purchased from PeproTech EC Ltd. VEGF-A (VEGF) and HGF were obtained from Takara Biotechnology Co., Ltd. FBS was purchased from HyClone (Cytiva). Trypsin, trypsin inhibitor and imatinib (a PDGFR inhibitor) were purchased from FUJIFILM Wako Pure Chemical Corporation. VEGFR tyrosine kinase inhibitor II (a VEGFR inhibitor) and tivantinib [a MET proto-oncogene, receptor tyrosine kinase (c-Met) inhibitor] were purchased from Cayman Chemical Company. All chemicals used in the present study were of analytical grade.

Isolation of hASCs. The present study complies with the International Society for Stem Cell Research guidelines (https://www.isscr.org/guidelines). Adipose tissue was obtained from a 42-year-old male patient, who had provided informed oral and written consent, whilst undergoing plastic surgery at Kansai Medical University in 2017. hASCs were isolated as previously described (20,24). Briefly, adipose tissue was cut into small pieces and digested with collagenase (MilliporeSigma). After the addition of Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Ltd.; cat. no. 05915) containing 10% FBS (Hyclone; Cytiva) and 2% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.), the tissue was centrifuged at 400 x g for 3 min at room temperature. The obtained supernatant was passed through a 100-μm nylon mesh (Falcon; Corning Life Sciences) to remove cellular debris. The resulting primary hASCs were cultured for 4-5 days until the cells reached 90% confluency. These cells were defined as passage '0'. Cells from passages 7-9 were used for all the experiments. hASCs were identified through the presence (CD73, CD90 and CD105) or absence (CD14 and CD31) of cell surface markers by flow cytometric analysis (6,8,20).

Cell proliferation assay. hASCs were seeded into 96-well cell culture plates at a density of $3.0x10^3$ cells/well and incubated in complete medium overnight at 37˚C. The cell culture medium was then replaced with serum-free DMEM (control medium). After incubation for 18 h, hASCs were cultured in control medium supplemented with PLTMax (1-5%), PDGF-BB (20 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml), VEGF (1 ng/ml) and PDGF-BB (20 ng/ml)/HGF (1 ng/ml) for 48 h at 37˚C. For pharmacological inhibition assays, imatinib (Cayman Chemical Company; cat. no. 13139), VEGFR inhibitor II (Cayman Chemical Company; cat no. 17654) and tivantinib (Cayman Chemical Company; cat. no. 17135) were added to the control medium 1 h before incubation at 37˚C with medium containing growth factors. Cell proliferation was examined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions as described previously (17,20,24). The optical absorbance was measured at 450 nm using a multi-well plate reader (EnSpire 2300 Multilabel Reader; PerkinElmer, Inc.).

Immunofluorescent analysis. hASCs ($1x10^4$) were treated with PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) and PDGF-BB (20 ng/ml)/HGF (1 ng/ml) for 24 h. The cells were fixed in 4% formaldehyde solution for 20 min at room temperature and permeabilized with 0.3% Triton X-100 for 15 min (17). After blocking with phosphate-buffered saline (PBS) containing 3% FBS (Hyclone; Cytiva) for 2 h at room temperature, cells were incubated with a monoclonal antibody against Ki-67 (1:800; cat. no. #9449; Cell Signaling Technology, Inc.) for 2 h at room temperature, followed by incubation with FITC-conjugated anti-rabbit immunoglobulin (IgG; 1:100; cat. no. SA00003-1;

Table I. Primer sequences used for quantitative PCR analysis.

Gene	Forward primer, 5'-3'	Reverse primer, 5'-3'
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
COL10A1	CGCTGAACGATACCAAATGCCC	TGGACCAGGAGTACCTTGCTCT
RUNX2	CCCAGTATGAGAGTAGGTGTCC	GGGTAAGACTGGTCATAGGACC
PPARG	AGCCTGCGAAAGCCTTTTGGTG	GGCTTCACATTCAGCAAACCTGG
ACTA1	AGGTCATCACCATCGGCAACGA	GCTGTTGTAGGTGGTCTCGTGA
SOX2	GCTACAGCATGATGCAGGACCA	TCTGCGAGCTGGTCATGGAGTT
CD73	AGTCCACTGGAGAGTTCCTGCA	TGAGAGGGTCATAACTGGGCAC

PPARG, peroxisome proliferator-activated receptor γ; RUNX2, RUNX family transcription factor 2; COL10A1, collagen type X α1 chain; ACTA, actin α1; SOX2, SRY-box transcription factor 2.

Proteintech Group, Inc.) and DAPI (1:1,000; cat. no. D523; Dojindo Molecular Technologies, Inc.) for 1 h at room temperature. Images of the antigens were captured using a fluorescence microscope (BZ9000; Keyence Corporation).

Western blot analysis. hASCs ($5x10^5$) were treated without or with PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) and PDGF-BB (20 ng/ml)/HGF (1 ng/ml) for 20 min. Cells were lysed in M-PER solution (cat. no. 78501; Thermo Fisher Scientific, Inc.) supplemented with a protease and phosphatase inhibitor cocktail (cat. no. 78440; Thermo Fisher Scientific, Inc.) (20). Protein concentration was estimated using a BCA assay kit (cat. no. A53225; Thermo Fisher Scientific, Inc.). Cellular proteins (20 μg/lane) were separated by SDS-PAGE on 4-15% gradient gels (cat. no. NP0321BOX; Thermo Fisher Scientific, Inc.) and electroblotted onto polyvinylidene difluoride membranes (cat. no. IPVH00010; Thermo Fisher Scientific, Inc.). After blocking with Blocking One-P reagent (cat. no. 05999-84; Nacalai Tesque, Inc.) for 60 min at room temperature, the membranes were incubated overnight at 4˚C with the following primary antibodies: Anti-phospho-Erk1/2 (1:1,000; cat. no. #4370; Cell Signaling Technology, Inc.), anti-Erk1/2 (1:1,000; cat. no. #4695; Cell Signaling Technology, Inc.), anti-phospho-PDGFRb (1:1,000; cat. no. GTX133525; GeneTex, Inc.), anti-PDGFRb (1:1,000; cat. no. 134491AP; Proteintech Group, Inc.), anti-phospho-c-Met (1:1,000; cat. no. 600401989S; Rockland Immunochemicals Inc.), anti-c-Met (1:1,000; cat. no. GTX631992; GeneTex, Inc.), anti-phospho-VEGFR2 (1:1,000; cat. no. CSBPA000703; Cusabio Technology, LLC), anti-VEGFR2 (1:1,000; cat. no. CSBPA008334; Cusabio Technology, LLC), anti-phospho-p38 MAPK (1:1,000; cat. no. #4511; Cell Signaling Technology, Inc.), anti-p38 MAPK (1:1,000; cat. no. #8690; Cell Signaling Technology, Inc.) and anti-β-actin (1:1,000; cat. no. #4970; Cell Signaling Technology, Inc.). Next, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; cat. no. SC2357; Santa Cruz Biotechnology, Inc.) or rabbit anti-mouse IgG (1:10,000; cat. no. SC2031; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence reagent (cat. no. 296-69901; FUJIFILM Wako Pure Chemical Corporation), and signals were quantified using ImageJ software (version 1.53t; National Institutes of Health) with β-actin as the loading control for normalization.

Wound healing assay. hASCs were seeded into 24-well cell culture plates at $6.0x10^4$ cells/well and incubated in complete medium at 37˚C overnight, followed by incubation with serum-free medium for 4 h at 37˚C. The cell monolayer was then scratched using a sterilized 200-μl disposable pipette tip. Subsequently, the cells were washed twice with PBS and cultured in DMEM containing PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml), and PDGF-BB (20 ng/ml)/HGF (1 ng/ml). Images of the scratched areas from three independent experiments were compared at 0, 12 and 24 h using an optical microscope (Primovert; Zeiss, AG), and the area between the two edges of the wound was analyzed using ImageJ software (National Institutes of Health; version 1.53t).

Gene expression. Total RNA was isolated from the hASCs using a Maxwell RSC kit (cat. no. AS1340; Promega Corporation) (25). Reverse transcription-quantitative PCR (RT-qPCR) was performed using SYBR Green RT-qPCR Master Mix (cat. no. 204243; Qiagen GmbH), according to the manufacturer's protocol, on a Rotor-Gene Q HRM Real-Time PCR System (Qiagen GmbH). The PCR thermocycling conditions were 40 cycles of 10 sec at 95˚C and 20 sec at 60˚C. Relative gene expression changes were calculated using the $2^{-\Delta\Delta Cq}$ method (26) with GAPDH as the internal reference gene. The PCR primers used in the present study are listed in Table I.

Spheroid formation assays. hASCs were suspended in DMEM containing PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) and PDGF-BB (20 ng/ml)/HGF (1 ng/ml) at a density of $1x10^4$ cells/100 μl and seeded in a Corning ultra-low-attachment 96-well plate (Corning, Inc.). The cells were cultured in the indicated medium at 37˚C for 6 days, and 50 μl DMEM containing PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) and PDGF-BB (20 ng/ml)/HGF (1 ng/ml) was added every

2 days. Phase-contrast images were acquired using an optical microscope (Primovert; Zeiss GmbH). Quantification of spheroid diameters was performed using ImageJ software (version 1.53t; National Institutes of Health).

Statistical analysis. Data are expressed as the mean ± standard deviation (n=3/4). All statistical analyses were performed using JMP® Pro v.16.2 (JMP Statistical Discovery LLC). Data homogeneity was examined using the Shapiro-Wilk test. Significant differences were evaluated using one-way ANOVA followed by Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

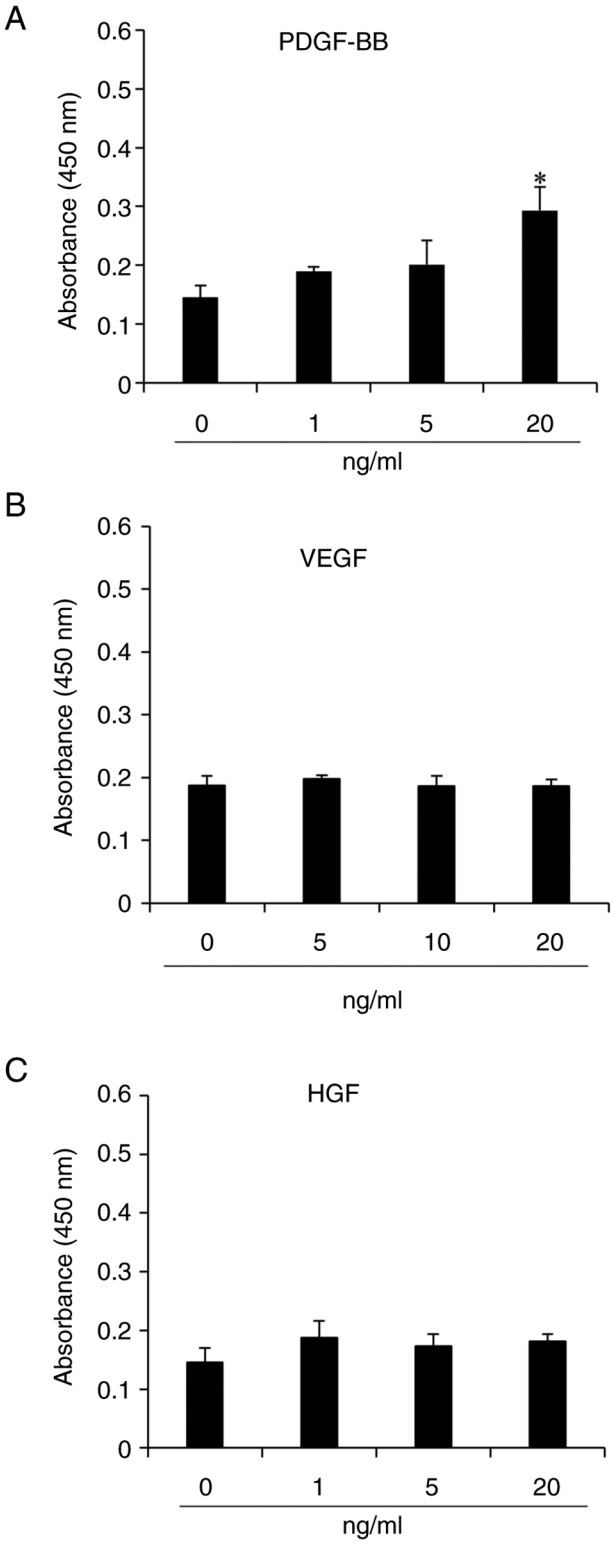
Results

Proliferative effects of PLTMax, PDGF-BB, VEGF, HGF and EGF on hASCs. When cells were cultured with PLTMax, hASC viability increased gradually with 1-3% PLTMax in a dose-dependent manner, and the greatest stimulation was observed at 3% PLTMax with 3% PLTMax showing higher viability of the cells than 10% FBS (Fig. S1A). Trypsin treatment, but not trypsin inhibitor (TI) treatment, significantly reduced hASC proliferation, indicating that growth factors and other proteins in PLTMax played essential roles in cell proliferation (Fig. S1B).

Next, the proliferative effects of PDGF-BB, a major growth factor in PLTMax, were examined. PDGF-BB caused a dose-dependent stimulation of hASC proliferation at 0-20 ng/ml (Fig. 1A). At >20 ng/ml PDGF-BB concentrations, the extent of the proliferation curve decreased (Fig. S2). Conversely, other growth factors such as VEGF and HGF did not exhibit any significant proliferative effects on hASCs (Fig. 1B and C). When cells were treated with VEGF and PDGF-BB, the addition of 1 ng/ml VEGF to 20 ng/ml PDGF-BB-containing medium further enhanced PDGF-dependent cell proliferation. Cells treated with PDGF-BB and higher concentrations of VEGF showed a decrease in cell viability, compared with the lower concentration. The potency of stimulation with PDGF-BB and VEGF appeared to be stronger than stimulation with PDGF-BB alone (Fig. 1D). Treatment with 20 ng/ml PDGF-BB and 1 ng/ml HGF resulted in the greatest cell proliferation, similar to that observed with the combination of PDGF and VEGF (Fig. 1E).

Furthermore, EGF alone did not enhance cell viability but the enhancement of the proliferation with PDGF-BB (20 ng/ml) and EGF (1 ng/ml) in combination was ~2.1-fold (Fig. S3A). The extent of enhancement by EGF combined with PDGF-BB was lower than that induced by PDGF-BB combined with either VEGF or HGF. Thus, in the presence of PDGF-BB, growth factors such as VEGF, HGF and EGF showed a synergistic effect on hASC proliferation. The combined use of growth factors resulted in maximal hASC proliferation, similar to that achieved by PLTMax.

Effects of PDGF-BB, VEGF and HGF on the number of Ki-67⁺ cells. To examine proliferative markers of cell proliferation, fluorescent immunostaining of hASCs with anti-Ki-67 antibody was performed (Fig. 2A). The percentage of Ki-67⁺ cells in the control was low (8%). However, the percentage of Ki-67⁺ cells in the VEGF and HGF groups were 12 and 26%, respectively. The addition of PDGF-BB increased the number of Ki-67-positive cells by 52%, which was similar to that observed after PDGF-BB/HGF treatment. The highest expression level was observed in the PDGF-BB/VEGF group (Fig. 2B). These results confirmed that PDGF-BB combined with VEGF resulted in an improved proliferative ability of hASCs.



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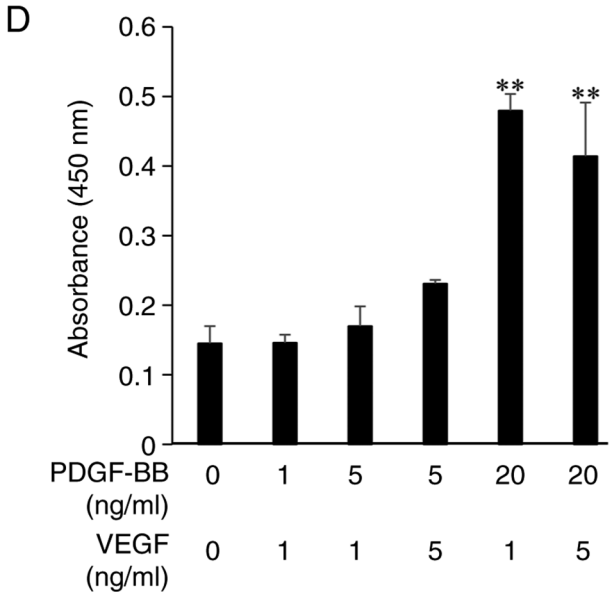


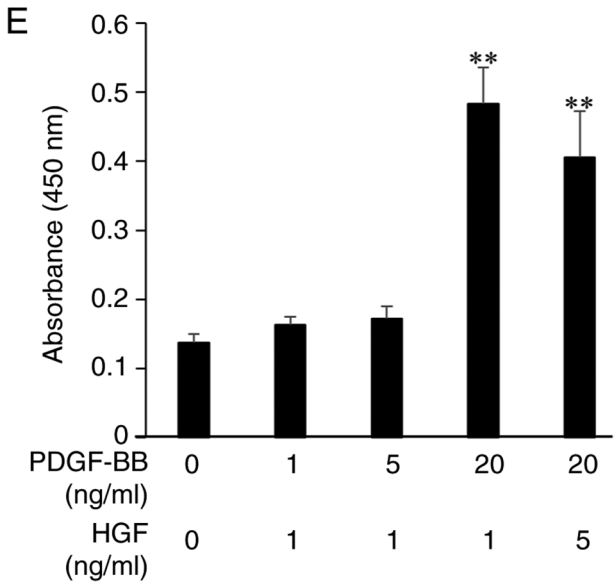


Figure 1. Growth factor-dependent proliferation of hASCs. The cells were cultured in DMEM with or without the indicated concentrations of (A) PDGF-BB, (B) VEGF, (C) HGF, (D) PDGF-BB/VEGF and (E) PDGF-BB/HGF for 48 h. Cell viability was measured by Cell Counting Kit-8 assay. Data are shown as the mean ± SD (n=4). *P<0.05; **P<0.01 vs. control. PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; hASCs, human adipose-derived stem cells.

Pharmacological effects on hASC viability. The effects of pharmacological inhibitors of PDGF, VEGF and HGF receptors on cell viability were investigated. Proliferation of hASCs induced by PDGF-BB was significantly decreased when cells were treated with imatinib (a PDGFR inhibitor), tivantinib (a c-Met inhibitor) and VEGFR inhibitor II (Fig. 3A).

Imatinib significantly inhibited the proliferation of PDGF-BB/VEGF-treated hASCs (Fig. 3B), and further inhibition was observed with a combination of imatinib and the aforementioned VEGFR inhibitor. Furthermore, inhibition by imatinib and/or tivantinib was observed after treatment with PDGF-BB/HGF (Fig. 3C).

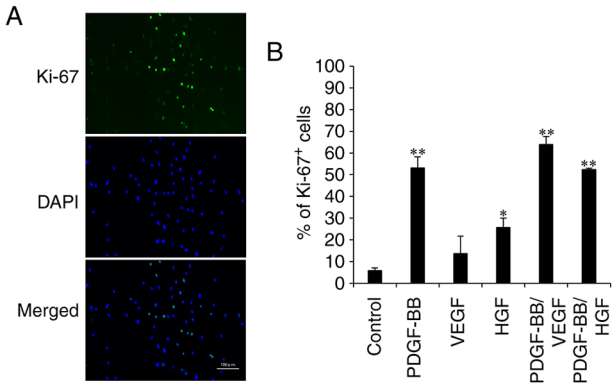


Figure 2. Effect of growth factors on the proliferative ability of hASCs. (A) Representative microscopy images of immunochemical staining for Ki-67 (green)/DAPI (blue) in hASCs cultured with PDGF-BB (20 ng/ml) plus VEGF (1 ng/ml) for 24 h (scale bar, 100 μm). (B) Quantification of Ki67^{+} cells. A total of 300 cells were counted after the indicated treatment and data are expressed as the mean ± SD (n=3). *P<0.05; **P<0.01 vs. control. hASCs, human adipose-derived stem cells; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor.

PD153035, an EGFR inhibitor, inhibited the proliferation induced by PDGF-BB and EGF (Fig. S3B). These results indicated that all growth factor receptor inhibitors investigated in the present study exhibited a similar potent effect on the inhibition of hASC proliferation.

Activation of receptors for growth factors and signaling enzymes by treatment with PDGF-BB, PDGF-BB/VEGF and PDGF-BB/HGF. To clarify the involvement of receptors in PDGF-, VEGF- and HGF-mediated signaling pathways, immunoblotting was performed with hASCs. Phospho-PDGFR levels increased after treatment with PDGF-BB (Fig. 4). Treatment with a combination of PDGF-BB and VEGF resulted in significantly higher phosphorylation level of PDGFRβ compared with other treatment groups. Furthermore, PDGF-BB stimulated the phosphorylation of VEGFR2 but VEGF did not significantly affect phosphorylated protein levels. A marked increase in phospho-VEGFR2 levels was observed in PDGF-BB/VEGF-treated cells. The increased phosphorylation of c-Met was not observed in HGF-treated cells, but it was increased by PDGF-BB/HGF, compared with HGF-only treatment. In response to the increase in phospho-PDGFRβ, phospho-VEGFR2 and phospho-c-Met, the levels of phospho-ERK1/2 and phospho-p38 MAPK increased in PDGF-BB, PDGF-BB/VEGF and PDGF-BB/HGF groups. These results indicated that the activation of PDGF/PDGFR signaling played a predominant role in the stimulation of VEGFR and c-Met, subsequently triggering the phosphorylation of ERK and p38, followed by the enhancement of hASC proliferation.

Effects of PDGF-BB, VEGF and HGF on hASC migration. To investigate the effects of growth factors on hASC migration, wound closure was measured after 12 h of incubation. PDGF-BB, PDGF-BB/VEGF and PDGF-BB/HGF markedly increased cell migration compared with the control (Fig. 5A and B). PDGF-BB/VEGF and PDGF-BB/HGF treatments showed 100% wound closure by 24 h of incubation.

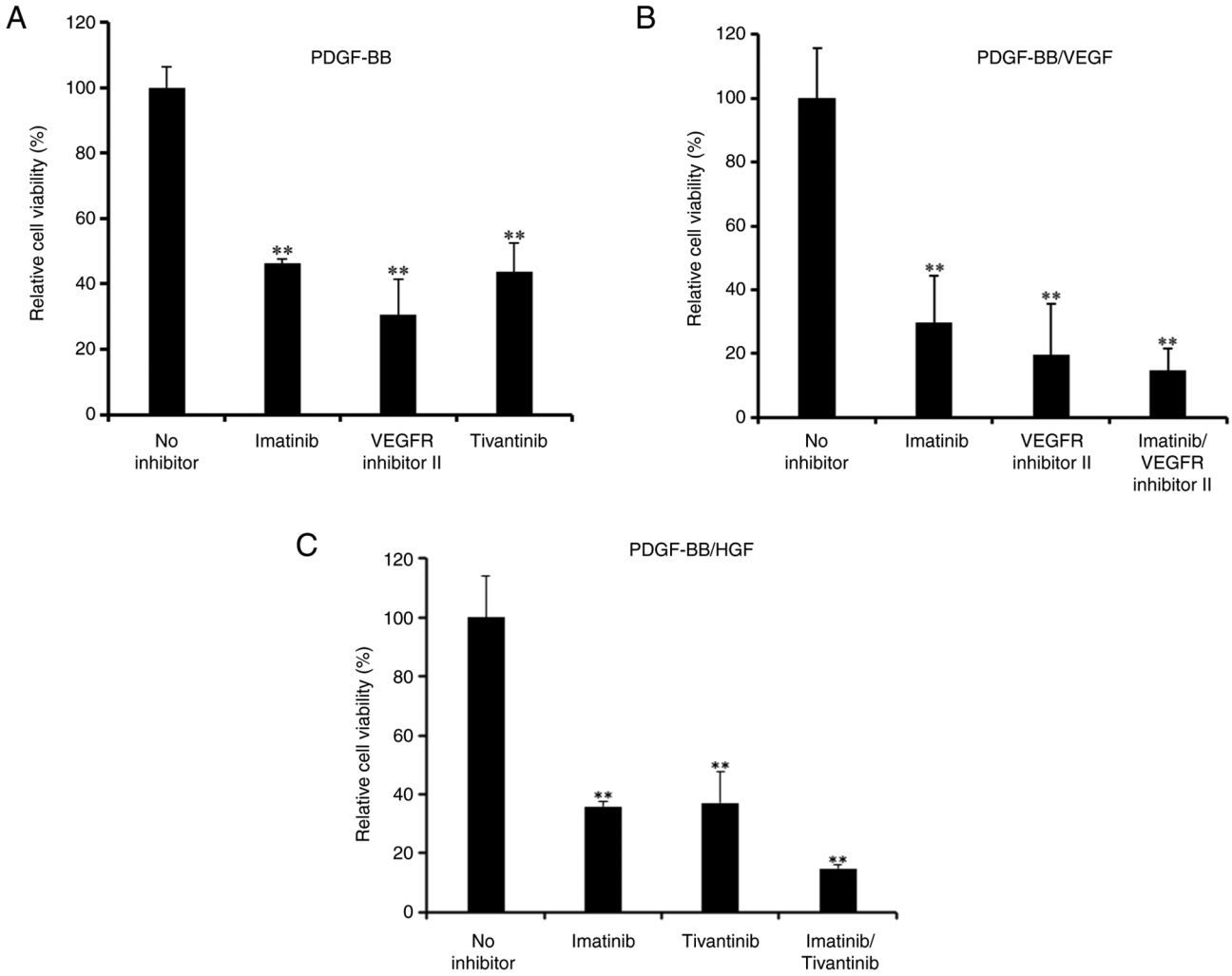
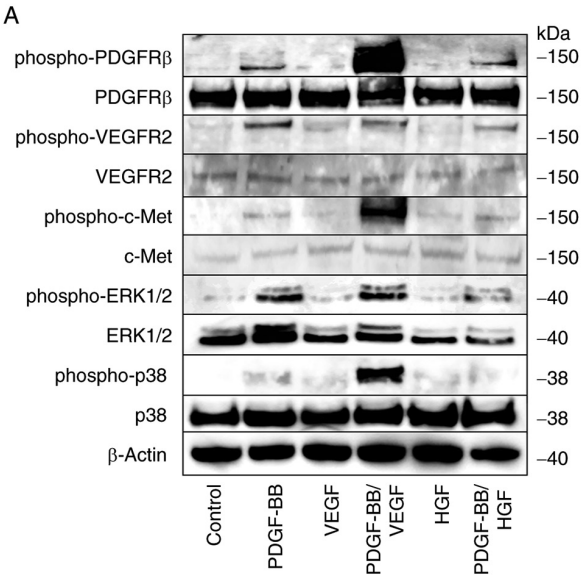


Figure 3. Pharmacological inhibition of hASC viability cultured with inhibitors of growth factor receptors. The cells were cultured in DMEM containing (A) PDGF-BB (20 ng/ml), (B) PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) and (C) PDGF-BB (20 ng/ml)/HGF (1 ng/ml), without or with the indicated receptor inhibitors for 48 h. Cell viability was examined by Cell Counting Kit-8 assays. The inhibitors used were imatinib (5 μM), VEGFR tyrosine kinase inhibitor II (10 μM) and tivantinib (0.5 nM). Data are expressed as a percentage of the control value (no inhibitor) and as the mean ± SD (n=4). **P<0.01 vs. no inhibitor. hASCs, human adipose-derived stem cells; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; VEGFR, VEGF receptor.

PDGF-BB/VEGF and PDGF-BB/HGF appeared to be the most effective combination to induce cell migration. Treatment with VEGF or HGF alone had no effects on cell migration.

Gene expression in PDGF-BB-, VEGF- and HGF-treated cells. To examine the effects of PDGF-BB, VEGF and HGF on the gene expression of stem cell and differentiation markers, RT-qPCR was performed on growth factor-treated cells for 6 days. As shown in Fig. 6, PDGF-BB, PDGF-BB/VEGF and PDGF-BB/HGF increased the mRNA levels of peroxisome proliferator-activated receptor γ (PPARG), an adipogenic marker, indicating that these growth factors could be involved in adipocyte differentiation. Conversely, the levels of RUNX family transcription factor 2 (RUNX2), an osteogenic marker, and actin α1 (ACTA), a myogenic marker, were decreased in PDGF-BB-treated cells. Additionally, the expression level of collagen type X α1 chain (COL10A1), a chondrogenesis-related gene, was decreased by treatment with PDGF-BB, but was restored in PDGF-BB/VEGF- and PDGF-BB/HGF-treated



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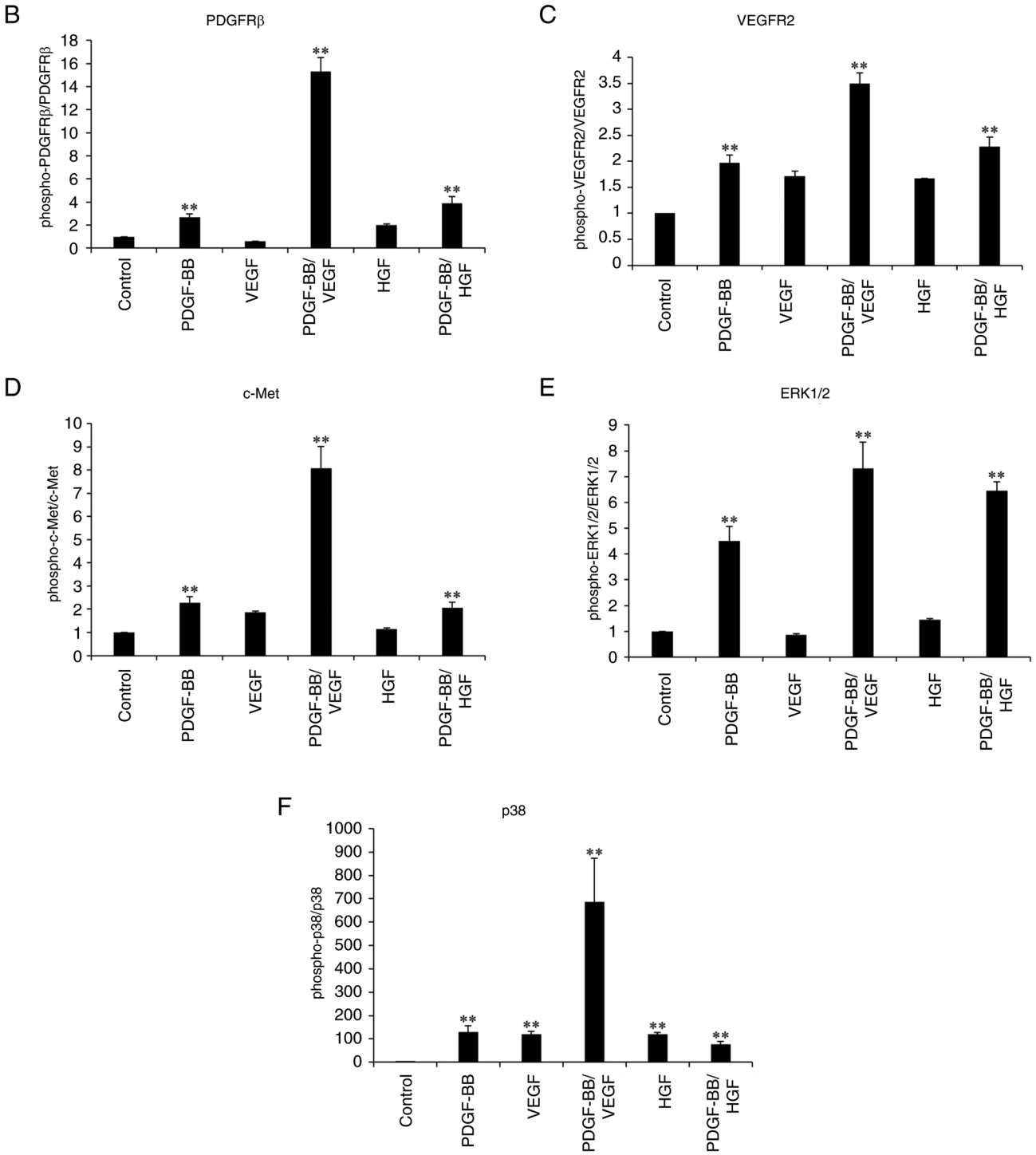

Figure 4. Activation of growth factor receptors and signal enzymes in growth factor-treated hASCs. hASCs were cultured in DMEM containing 10% FBS, followed by starvation for 16 h. The cells were then incubated in DMEM containing PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) or PDGF-BB (20 ng/ml)/HGF (1 ng/ml) for 20 min. The cells then were washed, collected and lysed. Next, cellular proteins were analyzed by SDS-PAGE using 4-15% gels, followed by (A) immunoblotting with the indicated primary antibodies. (B) Ratio of phospho-PDGFRb versus total PDGFRb, (C) ratio of phospho-VEGFR2 versus total VEGFR2, (D) ratio of phospho-c-Met versus total c-Met, (E) ratio of phospho-ERK1/2 versus total ERK1/2 and (F) ratio of phospho-p38 versus total p38 were calculated. Data are presented as the mean ± SD (n=3). **P<0.01 vs. control. hASCs, human adipose-derived stem cells; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor.

cells. An increase in the levels of SRY-box transcription factor 2 (SOX2), a stem cell marker, was observed in the PDGF-BB/VEGF and PDGF-BB/HGF groups, but not in the PDGF-BB group. The levels of CD73, a mesenchymal stem cell marker, were decreased by treatment with PDGF-BB and PDGF-BB/VEGF.

Effects of PDGF-BB, VEGF and HGF on hASC spheroid formation. Cell aggregates were observed when suspended cells were cultured in a low-attachment culture plate with notably few adherent cells on day 2 (Fig. 7A). The cells formed spheroids of various sizes and the number of suspended cells decreased after further incubation.

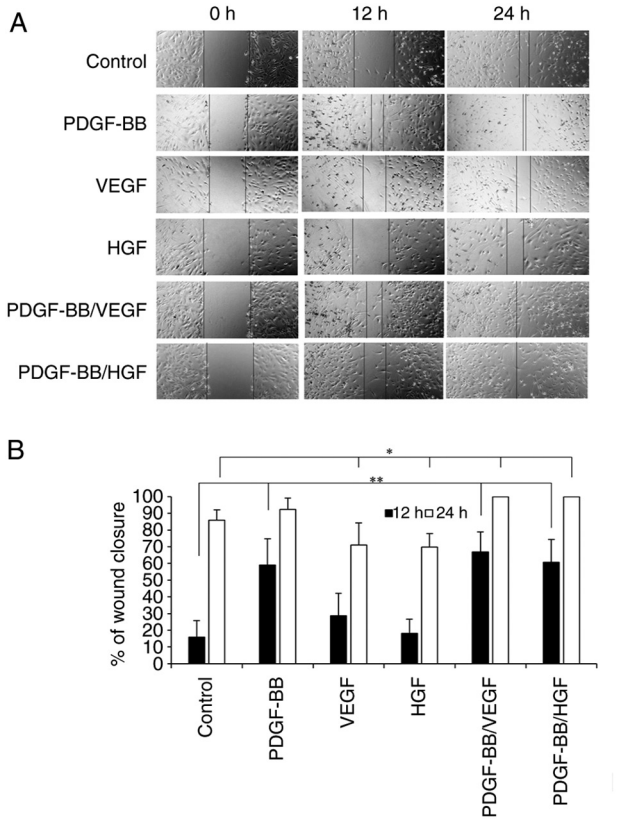

Figure 5. *In vitro* hASC wound healing assay. (A) Wounds were created on hASCs seeded in 24-well plates at 90% confluence using a pipette tip, and the cells were then incubated in DMEM without or with PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) and PDGF-BB (20 ng/ml)/HGF (1 ng/ml) for 12 and 24 h. Wounds were observed under an optical microscope (magnification, x40). (B) Quantitative analysis of wound closure after 12 and 24 h incubation. Wound healing closure rates were calculated using ImageJ software (version 1.53t). Values are presented as the percentage of wound closure ± SD (n=4). *P<0.05; **P<0.01 vs. control. hASCs, human adipose-derived stem cells; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor.

Treatment of cells with PDGF-BB, PDGF-BB/VEGF and PDGF-BB/HGF increased the number of spheroids (diameter >100 μm) with incubation time. Since the spheroid number in all groups was zero at the start of the experiment, following incubation there was a significant formation of spheroids in all groups. At day 6, the number of spheroids 100-200 μm was high after treatment with PDGF-BB or PDGF-BB/VEGF, and spheroids >200 μm were observed in PDGF-BB-, PDGF-BB/VEGF- and PDGF-BB/HGF-treated cells (Fig. 7B). Thus, PDGF plays a dominant role in spheroid formation.

Gene expression in hASCs from day 6 spheroids was examined. RT-qPCR data showed that COL10A1 and RUNX2 mRNA levels were increased by treatment with PDGF-BB/VEGF compared with the control group, and PDGF-BB/HGF treatment also increased COL10A1 mRNA levels. Furthermore, treatment with PDGF-BB md PDGF-BB/HGF led to an increase in PPARG mRNA, but ACTA mRNA levels were decreased in PDGF-BB, PDGF-BB/VEGF, and PDGF-BB/HGF groups (Fig. 8). SOX2 mRNA levels were increased in PDGF-BB/VEGF-treated cells, and CD73 mRNA levels increased with all treatments. These results indicated that the expression of stem cell markers in the spheroids was high.

Discussion

To the best of our knowledge, the present study is the first to demonstrate that PDGF/PDGFR signaling predominantly stimulates hASC migration and proliferation (Fig. 9). PDGF-BB is a major growth factor present in both PLTMax and PRP (17,20). Proteolytic treatment with PLTMax reduced the enhancement of hASCs proliferation, suggesting that proteins, including growth factors and adhesion molecules, were involved in such stimulation. Our group previously reported that PDGF treatment induced the proliferation of hASCs (20). However, the effect of PDGF on cell proliferation was lower than that of PRP (or PLTMax) (20). In the present study, although it was examined whether growth factors such as VEGF, HGF and EGF stimulated hASC proliferation, treatment with VEGF, HGF or EGF alone did not exert any proliferative effect. When hASCs were incubated with PLTMax for 48 h, the highest proliferative effect was observed with 3-5% PLTMax. A stimulatory effect similar to that of PLTMax was observed with the combined use of PDGF-BB, VEGF and HGF. Furthermore, the number of Ki-67^{+} cells increased significantly after treatment with PDGF-BB, PDGF-BB/VEGF and PDGF-BB/HGF, but not with VEGF or HGF alone. EGF or insulin-like growth factor (IGF) combined with PDGF was less effective for hASC proliferation than PRP alone (20). Thus, VEGF and HGF synergistically enhanced the PDGF-dependent proliferation of hASCs. In addition, as shown in the present study PDGF is a primary factor responsible for cell proliferation and can draw on the potential of other growth factors including VEGF, HGF and EGF in PLTMax when hASCs are cultured with PLTMax (or PRP).

The addition of 20 ng/ml PDGF-BB resulted in significantly higher stimulation of hASC proliferation (Fig. 1). Kang *et al* (27) also found that PDGF induced the chemotactic migration of human adipose tissue-derived MSCs in a dose-dependent manner (5-25 ng/ml) and increased the number of cells after incubation for 48 h. Additionally, PDGF isoforms stimulate the migration and proliferation of equine epithelial cells, keratinocytes (28), and mesoderm-derived epithelial and glial cells (29) to different extents, indicating that the extent of the PDGF proliferative effect is dependent on different cell types owing to different quantities of receptors. Thus, the distribution of PDGFR and the concentration of PDGF isoforms are important determinants of the fate of different cell types.

Accompanied by the stimulation of cell proliferation, the wound-healing assays revealed that the migration of hASCs was enhanced by treatment with PDGF and VEGF. Growth factors released after activation could stimulate the chemotaxis of fibroblasts into wound tissues (30). Among these growth factors, PDGF is a potent chemotactic factor that activates cell adhesion molecules, including integrins (31). HGF, EGF and TGF-β positively regulate focal adhesion molecules and integrins (32-34). During inflammation, PDGF induces the migration and proliferation of monocytes, fibroblasts and

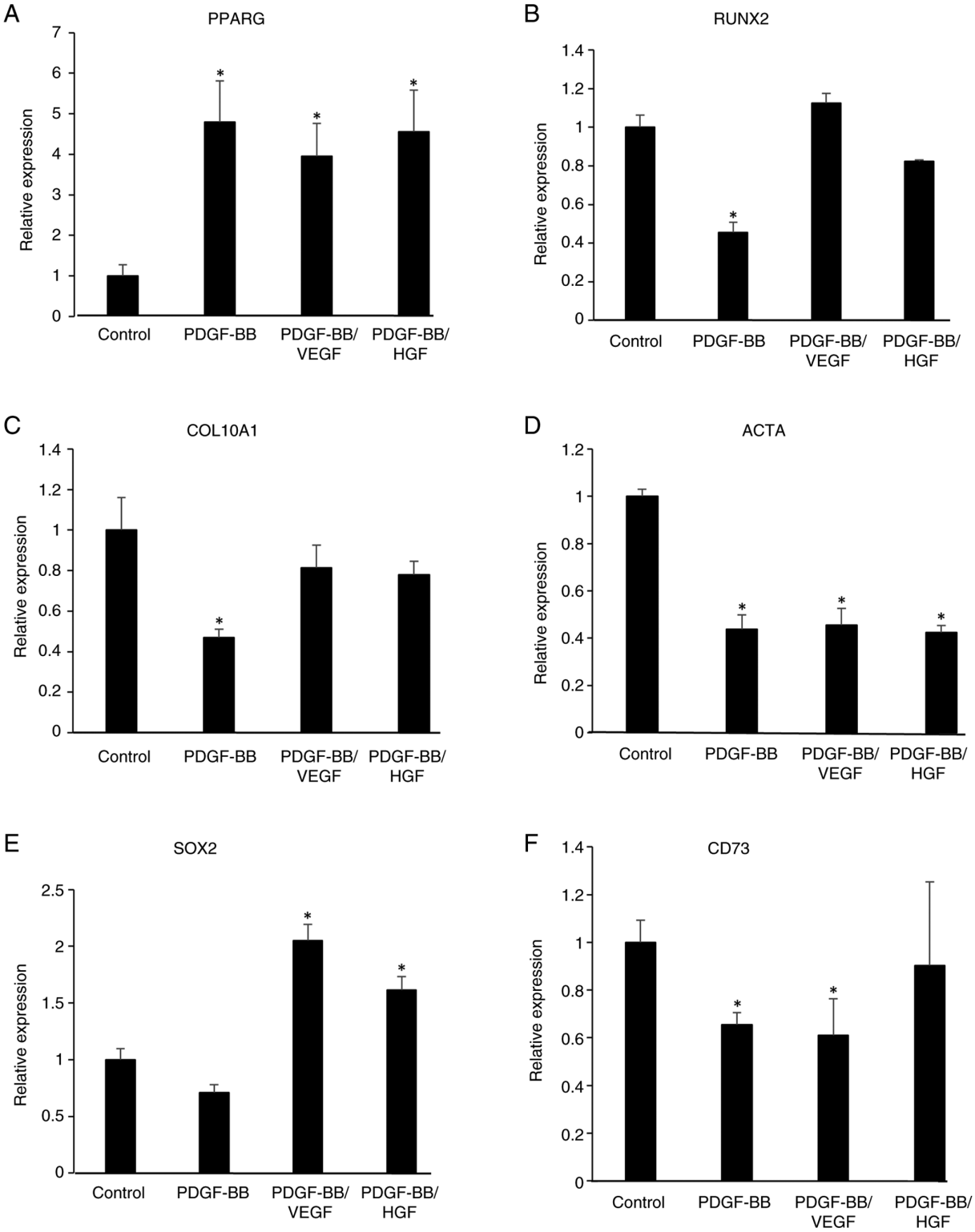

Figure 6. Gene expression of differentiation-related genes, including (A) PPARG, (B) RUNX2, (C) COL10A1 and (D) ACTA, and stem cell-related genes, including (E) SOX2 and (F) CD73 in growth factor-treated hASCs. hASCs were incubated in DMEM without or with PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) and PDGF-BB (20 ng/ml)/HGF (1 ng/ml), and the cells were cultured for 6 days. The individual medium was changed every 2 days. Total RNA in the cells was isolated and reverse transcription-quantitative PCR was performed. mRNA levels were normalized to GAPDH mRNA expression. Data are presented as the mean ± SD (n=3). *P<0.05 vs. control. hASCs, human adipose-derived stem cells; PPARG, peroxisome proliferator-activated receptor γ; RUNX2, RUNX family transcription factor 2; COL10A1, collagen type X α1 chain; ACTA, actin α1; SOX2, SRY-box transcription factor 2.

vascular smooth muscle cells; attracts monocytes to sites of vascular injury; and limits proinflammatory events through autocrine feedback inhibition of platelet aggregation (35). Furthermore, PDGF is a strong pro-angiogenic factor that stimulates the migration and proliferation of endothelial cells (36).

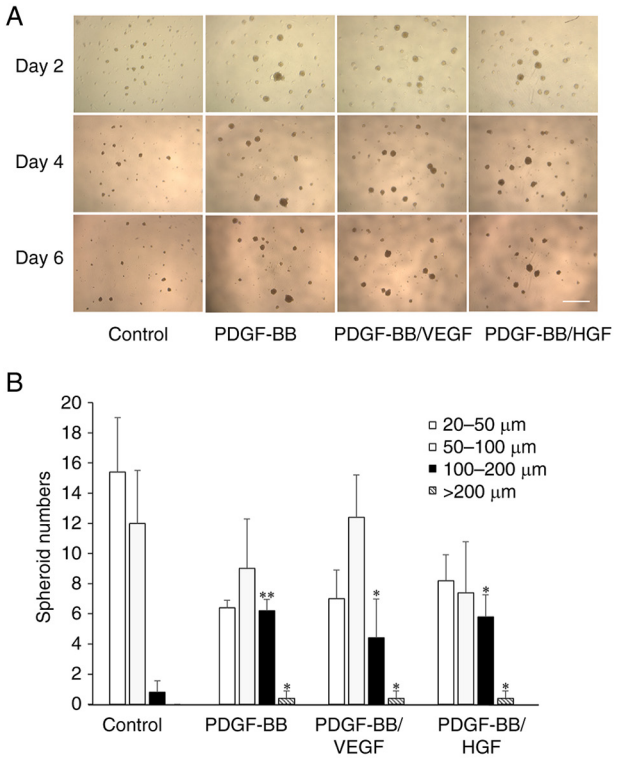

Figure 7. Spheroid formation of hASCs with PDGF-BB, PDGF-BB/VEGF and PDGF-BB/HGF. (A) Representative microscopy images of spheroids under the indicated conditions. Cells were seeded at 1,000 cells/well in low-attachment plates (96-wells), and cultured with DMEM containing PDGF-BB (20 ng/ml), VEGF (1 ng/ml), HGF (1 ng/ml), PDGF-BB (20 ng/ml)/VEGF (1 ng/ml) and PDGF-BB (20 ng/ml)/HGF (1 ng/ml) for 6 days. The cells were also cultured with serum-free DMEM as a control (scale bar, 1 mm). (B) Quantification of total spheroids distributed by size. The shapes of the spheroids were analyzed by ImageJ software, and spheres <20 μm were excluded. The diameter of spheroids was divided into 20-50, 50-100, 100-200 and >200 μm, and the number of spheroid in each group was shown. Data are presented as the mean ± SD (n=3). *P<0.05; **P<0.01 vs. control. hASCs, human adipose-derived stem cells; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor.

PDGFR consists of α and β subunits (37). PDGFR-α can bind to either PDGF-A or PDGF-B, while PDGFR-β can bind only to PDGF-B. PDGF-BB binds to the two subunits of PDGFR and is ~10-fold more mitogenic than PDGF-AA (37). Since hASCs express high levels of PDGFR-β, which is fundamental in cell proliferation, adhesion and migration at the commencement of angiogenesis (38,39), it is possible that the PDGF-B/PDGFR-β signaling may play an important role in cell stimulation. On the other hand, PDGF-B is not expressed by hASCs, whereas PDGF-D and PDGFR-β are, and PDGF-D also upregulates growth factor expression and exerts a strong mitogenic effect on hASCs (40). PDGF-D is a minor component of platelets (41). Therefore, it mainly acts as a mitogenic factor in response to inflammation and tissue injury regeneration.

The present study demonstrated that rapid phosphorylation of VEGFR2 and c-Met occurred upon treatment of hASCs with PDGF-BB. This indicated that transactivation of these receptors by PDGF-BB induced the phosphorylation of the mitogenic enzymes ERK1/2 and p38MAPK, leading to cell migration and proliferation. Conversely, neither VEGF nor HGF activated any receptor for PDGF, HGF or VEGF in hASCs, which was consistent with the lack of stimulation on cell proliferation. VEGFR and c-Met may be located intracellularly in hASCs under resting conditions. By contrast, phosphorylation of VEGFR2 and c-Met occurs without binding of the corresponding ligands once PDGFR-β activation occurs. Interactions between PDGFR and EGFR have also been reported (42). Increased PDGFR-β kinase activity leads to upregulation of VEGF and VEGFR2 mRNAs in bone marrow MSCs, acting directly on endothelial cells and leading to increased vessel formation (43). To the best of our knowledge, no previous studies have examined the transactivation of PDGFR to VEGFR and c-Met in any cell type. Although the mechanisms underlying VEGFR2 and c-Met activation by PDGF are unclear, it is possible that the activation signal of PDGFR-β transactivates other receptors in a manner mediated by intracellular molecules. G protein-coupled receptors and sphingosine 1-phosphate receptor can transfer activating signals to cell surface receptors (44). Thus, the predominant control of PDGF/PDGFR signaling to activate other growth factor receptors is essential for promoting hASC migration and proliferation.

In the presence of PDGF-BB, VEGF further increased the levels of phospho-VEGFR and phospho-c-Met, and subsequently markedly activated ERK1/2 and p38 MAPK. These events were ascribed to the synergistic effect of VEGF and c-Met on PDGF-dependent enhancement of hASC migration and proliferation. Considering that all inhibitors of PDGFR, VEGFR and c-Met reduced the proliferation of hASCs in the presence of PDGF-BB, the activation of these receptors by PDGF appears to be required for cellular activation. Among the growth factors present in PLTs, PDGF, IGF and FGF promote proliferation and cell cycle transition in human MSCs (27,41). However, the effects of TGF-β and EGF on MSCs remain unknown. Treatment of hASCs with EGF and basic FGF promotes the cell proliferation and differentiation of neural lineage (45). When hASCs were treated with a combination of FGF2 and VEGF, the promotion of cell proliferation and endothelial differentiation was accompanied by an increase in the expression of the endothelial markers CD31, von Willebrand factor and CD144 (46). The present data demonstrated that VEGF and HGF promoted the PDGF-dependent proliferation of hASCs, with high mRNA expression of the stem cell markers SOX2 and CD73. Similarly, PDGF-BB combined with VEGF or HGF enhanced the formation of spheroids in which hASCs abundantly expressed SOX2 and CD73 mRNAs. These results indicated that PDGF may be an essential growth factor that promoted maintenance of hASC stemness.

Several studies have shown that MSCs do not express VEGFR (37) and that VEGF can bind to PDGFR (47), suggesting that VEGF induces MSC proliferation by activating the PDGF/PDGFR axis. By contrast, the present data clearly demonstrated that no PDGFR-β activation occurred by treating hASCs with VEGF alone. The reason for this discrepancy is not clear, but it is possible that PDGFR is localized on the cell surface of MSCs. However, growth hormone receptors are generally translocated to the plasma membrane upon phosphorylation (48). As shown in the present study,

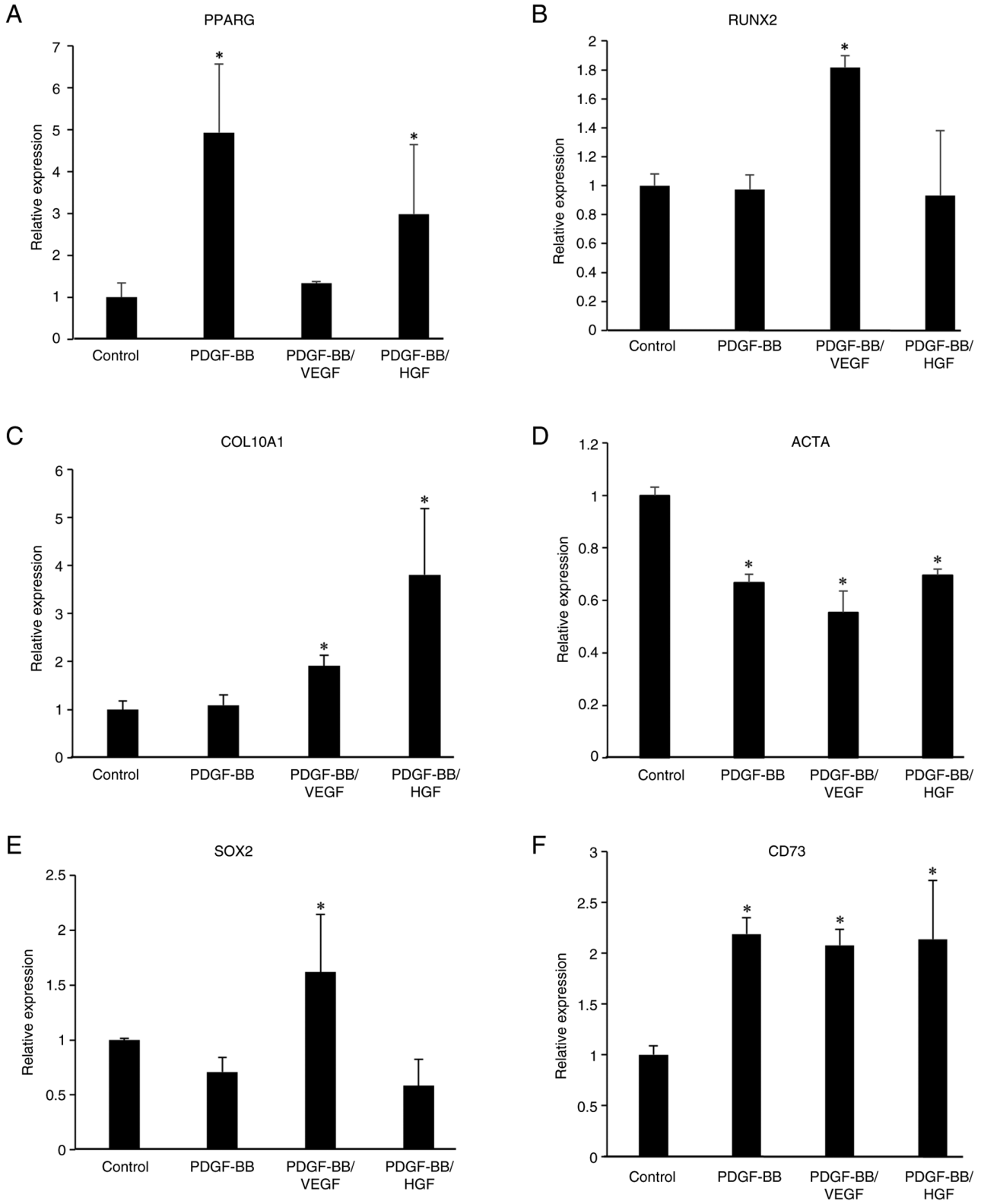

Figure. 8. Gene expression of differentiation-related genes, including (A) PPARG, (B) RUNX2, (C) COL10A1 and (D) ACTA, and stem cell-related genes, including (E) SOX2 and (F) CD73 in growth factor-treated hASC spheroids. Spheroids treated with the indicated growth factors for 6 days were collected by centrifugation at 1,000 x g for 5 min at room temperature. Total RNA in the cells was isolated and reverse transcription-quantitative PCR was performed. Data are presented as the mean ± SD (n=3). *P<0.05 vs. control. hASCs, human adipose-derived stem cells; PPARG, peroxisome proliferator-activated receptor γ; RUNX2, RUNX family transcription factor 2; COL10A1, collagen type X α1 chain; ACTA, actin α1; SOX2, SRY-box transcription factor 2.

once PDGFR-β and PDGF-BB phosphorylate VEGFR2 and c-Met in hASCs, the activated receptors are translocated to the cell surface and bind to their cognate ligands. Thus, the activation of PDGF/PDGFR signaling is indispensable for the stimulation of other signaling pathways in hASCs, improving the wound healing properties of hASCs.

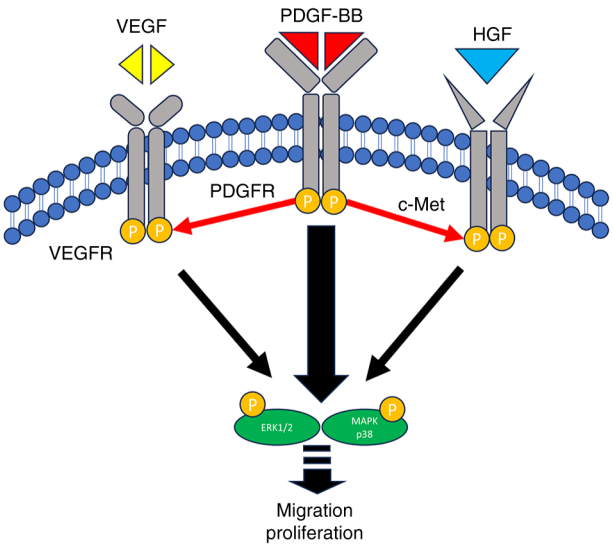

Figure 9. PDGF-BB/PDGFR signal dominates the stimulation of the migration and proliferation of hASCs via activation of multiple growth factor receptors. VEGF and HGF synergistically enhanced such stimulation. hASCs, human adipose-derived stem cells; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; PDGFR, PDGF receptor; VEGFR, VEGF receptor; c-Met, HGF receptor; hASCs, human adipose-derived stem cells.

PDGF has multiple effects on the differentiation of hASCs. PDGF-B enhances vascular network stability and osteogenic differentiation, resulting in the development of vascularized bone tissues by hASCs (49). PDGF promotes the tenogenic differentiation of hASCs (49,50). RT-qPCR analysis showed that mRNA expression of the osteogenic marker RUNX2 was slightly increased upon treatment with PDGF-BB and VEGF, and this effect was potent in spheroids. The mRNA levels of the chondrogenic marker COL10A1 increased in spheroids treated with all the evaluated growth factors. Although PDGF treatment of hASCs reduced the expression of adipogenesis-related genes in a previous study (51), the present data showed an increase in the mRNA expression of the adipogenic marker PPARG by PDGF-BB, PDGF-BB/VEGF and PDGF-BB/HGF treatment compared to the control. The reasons for such differences in gene expression between previous and present data are unclear. hASCs produce various paracrine factors that are dependent on different culture conditions (52,53) and therefore the cells may develop along different lineages. In the present study, SOX2 and CD73 were selected as stem cell markers to examine the maintenance of stem cell self-renewal. SOX2 mRNA was increased by treatment with PDGF-BB/VEGF under monolayer and spheroid culture conditions, and was virtually unchanged with PDGF-BB exposure. CD73 mRNA levels increased in all spheroid treatment groups, but remained unchanged in monolayer cells. Thus, the supplementation of hASC spheroids with PDGF-BB, VEGF and HGF positively affected stem cell pluripotency. Furthermore, VEGF and HGF synergistically enhanced the PDGF-dependent migration and proliferation of hASCs. However, other additional effects of VEGF and HGF on the fate of hASCs were not observed. The proliferation and differentiation potential of hASC spheroids differs depending on the culture conditions, including supplementation with growth factors, scaffold environment and cell density, and therefore further systematic gene expression studies are required to elucidate the roles of VEGF, HGF and PDGF in the differentiation potential of hASCs.

Several studies have reported the proliferation-promoting effects of PLTs on bone marrow-derived MSCs (12,54). Additionally, Huang *et al* (55) reported that PLTs enhanced neuronal proliferation and differentiation to a greater extent than FBS. Additionally, PLTs contain higher concentrations of various growth factors other than FBS (17). Collectively, PLTs are a promising xenogeneic-free substitute for FBS in hASC cultures, with an underlying mechanism of growth factor-induced proliferation. The present study demonstrated that the combination of the growth factors PDGF-BB, VEGF and HGF promoted the migration and proliferation of hASCs. It is unclear whether the cells could acquire multiple functions for tissue regeneration or senescence, although this may be induced upon long-term culture (53). This may be further explained by the fact that PLTs (or PRP), which also contain adhesion molecules, chemokines and various plasma proteins, share similar importance with platelets and leukocytes during wound healing (56). The combination of other factors, including chemokines and adhesion molecules, with VEGF and HGF could further enhance the proliferative effect on hASCs through PDGF/PDGFR signaling. Improving the viability and stability of hASCs by preserving their homogeneity may contribute to the development of stem cell therapeutics using biofunctional materials.

In conclusion, the present study demonstrated that VEGF and HGF treatment synergistically enhanced the PDGF-BB-dependent proliferation of hASCs. hASC migration after PDGF-BB/VEGF and PDGF-BB/HGF treatment was greater than that after PDGF-BB treatment alone. These enhancements were accompanied by the phosphorylation of PDGFR, VEGFR2 and c-Met. RT-qPCR analysis revealed high expression of stem cell markers in growth factor-treated cells. During hASC spheroid formation, PDGF-BB played a predominant role in the synergistic effects of VEGF and HGF. These observations provide new insights for future investigations surrounding the beneficial effects of supplementing cultured hASCs with PDGF-BB and VEGF to repair injured tissues.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZS conducted all the laboratory work, acquired data and drafted the manuscript. NK and ST designed this study and revised the manuscript. MF, AK and SK acquired the RT-qPCR data. The first draft of the manuscript was written by ZS, MF, ST and NK. ZS, MF, ST and NK confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Review Board of Kansai Medical University in accordance with the ethical guidelines of the Helsinki Declaration of 1975 (approval no. 2017094; Hirakata, Japan). All specimens were collected from one donor and informed oral and written consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR: Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147, 1999.
2. Pittenger MF, Discher DE, Péault BM, Phinney DG, Hare JM and Caplan AI: Mesenchymal stem cell perspective: Cell biology to clinical progress. NPJ Regen Med 4: 22, 2019.
3. Koźlik M and Wójcicki P: The use of stem cells in plastic and reconstructive surgery. Adv Clin Exp Med 23: 1011-1017, 2014.
4. Zhang M, Zhang P, Liu Y and Zhou Y: GSK3 inhibitor AR-A014418 promotes osteogenic differentiation of human adipose-derived stem cells via ERK and mTORC2/Akt signaling pathway. Biochem Biophys Res Commun 490: 182-188, 2017.
5. Galeano-Garces C, Camilleri ET, Riester SM, Dudakovic A, Larson DR, Qu W, Smith J, Dietz AB, Im HJ, Krych AJ, *et al*: Molecular validation of chondrogenic differentiation and hypoxia responsiveness of platelet-lysate expanded adipose tissue-derived human mesenchymal stromal cells. Cartilage 8: 283-299, 2017.
6. Paul NE, Denecke B, Kim BS, Dreser A, Bernhagen J and Pallua N: The effect of mechanical stress on the proliferation, adipogenic differentiation and gene expression of human adipose-derived stem cells. J Tissue Eng Regen Med 12: 276-284, 2018.
7. Jahromi M, Razavi S, Amirpour N and Khosravizadeh Z: Paroxetine can enhance neurogenesis during neurogenic differentiation of human adipose-derived stem cells. Avicenna J Med Biotechnol 8: 152-158, 2016.
8. Gaur M, Dobke M and Lunyak VV: Mesenchymal stem cells from adipose tissue in clinical applications for dermatological indications and skin aging. Int J Mol Sci 18: 208, 2017.
9. Takahashi H, Ishikawa H and Tanaka A: Regenerative medicine for Parkinson's disease using differentiated nerve cells derived from human buccal fat pad stem cells. Hum Cell 30: 60-71, 2017.
10. Iudicone P, Fioravanti D, Bonanno G, Miceli M, Lavorino C, Totta P, Frati L, Nuti M and Pierelli L: Pathogen-free, plasma-poor platelet lysate and expansion of human mesenchymal stem cells. J Transl Med 12: 28, 2014.
11. Huang CJ, Sun YC, Christopher K, Pai AS, Lu CJ, Hu FR, Lin SY and Chen WL: Comparison of corneal epitheliotrophic capacities among human platelet lysates and other blood derivatives. PLoS One 12: e0171008, 2017.
12. Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X and Lataillade JJ: Platelet lysates promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications. J Cell Physiol 205: 228-236, 2005.
13. Crespo-Diaz R, Behfar A, Butler GW, Padley DJ, Sarr MG, Bartunek J, Dietz AB and Terzic A: Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability. Cell Transplant 20: 797-811, 2011.
14. Díez JM, Bauman E, Gajardo R and Jorquera JI: Culture of human mesenchymal stem cells using a candidate pharmaceutical grade xeno-free cell culture supplement derived from industrial human plasma pools. Stem Cell Res Ther 6: 28, 2015.
15. Stute N, Holtz K, Bubenheim M, Lange C, Blake F and Zander AR: Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol 32: 1212-1225, 2004.
16. Alonso-Camino V and Mirsch B: Rapid expansion of mesenchymal stem/stromal cells using optimized media supplemented with human platelet lysate PLTMax® or PLTGold®, suitable for cGMP expansion at large scale. Cytotherapy 21 (Suppl): S85, 2019.
17. Kakudo N, Morimoto N, Ma Y and Kusumoto K: Differences between the proliferative effects of human platelet lysate and fetal bovine serum on human adipose-derived stem cells. Cells 8: 1218, 2019.
18. Zhang JM, Feng FE, Wang QM, Zhu XL, Fu HX, Xu LP, Liu KY, Huang XJ and Zhang XH: Platelet-derived growth factor-bb protects mesenchymal stem cells (MSCs) derived from immune thrombocytopenia patients against apoptosis and senescence and maintains MSC-mediated immunosuppression. Stem Cells Transl Med 5: 1631-1643, 2016.
19. Papadopoulos N and Lennartsson J: The PDGF/PDGFR pathway as a drug target. Mol Aspects Med 62: 75-88, 2018.
20. Lai F, Kakudo N, Morimoto N, Taketani S, Hara T, Ogawa T and Kusumoto K: Platelet-rich plasma enhances the proliferation of human adipose stem cells through multiple signaling pathways. Stem Cell Res Ther 9: 107, 2018.
21. Imam SS, Al-Abbasi FA, Hosawi S, Afzal M, Nadeem MS, Ghoneim MM, Alshehri S, Alzarea SI, Alquraini A, Gupta G and Kazmi I: Role of platelet rich plasma mediated repair and regeneration of cell in early stage of cardiac injury. Regen Ther 19: 144-153, 2022.
22. Chun SY, Lim JO, Lee EH, Han MH, Ha YS, Lee JN, Kim BS, Park MJ, Yeo M, Jung B and Kwon TG: Preparation and characterization of human adipose tissue-derived extracellular matrix, growth factors, and stem cells: A concise review. Tissue Eng Regen Med 16: 385-393, 2019.
23. Ma Y, Kakudo N, Morimoto N, Lai F, Taketani S and Kusumoto K: Fibroblast growth factor-2 stimulates proliferation of human adipose-derived stem cells via Src activation. Stem Cell Res Ther 10: 350, 2019.
24. Kakudo N, Minakata T, Mitsui T, Kushida S, Notodihardjo FZ and Kusumoto K: Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. Plast Reconstr Surg 122: 1352-1360, 2008.
25. Fukui M, Matsuoka Y, Taketani S, Higasa K, Hihara M, Kuro A and Kakudo N: Accelerated angiogenesis of human umbilical vein endothelial cells under negative pressure was associated with the regulation of gene expression involved in the proliferation and migration. Ann Plast Surg 89: e51-e59, 2022.
26. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
27. Kang YJ, Jeon ES, Song HY, Woo JS, Jung JS, Kim YK and Kim JH: Role of c-Jun N-terminal kinase in the PDGF-induced proliferation and migration of human adipose tissue-derived mesenchymal stem cells. J Cell Biochem 95: 1135-1145, 2005.
28. Haber M, Cao Z, Panjwani N, Bedenice D, Li WW and Provost PJ: Effects of growth factors (EGF, PDGF-BB and TGF-beta 1) on cultured equine epithelial cells and keratocytes: Implications for wound healing. Vet Ophthalmol 6: 211-217, 2003.
29. Knorr M, Völker M, Denk PO, Wunderlich K and Thiel HJ: Proliferative response of cultured human tenon's capsule fibroblasts to platelet-derived growth factor isoforms. Graefes Arch Clin Exp Ophthalmol 235: 667-671, 1997.
30. Seppä H, Grotendorst G, Seppä S, Schiffmann E and Martin GR: Platelet-derived growth factor in chemotactic for fibroblasts. J Cell Biol 92: 584-588, 1982.

31. Donovan J, Abraham D and Norman J: Platelet-derived growth factor signaling in mesenchymal cells. Front Biosci (Landmark Ed) 18: 106-119, 2013.
32. Bellas RE, Bendori R and Farmer SR: Epidermal growth factor activation of vinculin and beta 1-integrin gene transcription in quiescent Swiss 3T3 cells. Regulation through a protein kinase C-independent pathway. J Biol Chem 266: 12008-12014, 1991.
33. Celotti F, Colciago A, Negri-Cesi P, Pravettoni A, Zaninetti R and Sacchi MC: Effect of platelet-rich plasma on migration and proliferation of SaOS-2 osteoblasts: Role of platelet-derived growth factor and transforming growth factor-beta. Wound Repair Regen 14: 195-202, 2006.
34. Li JF, Yin HL, Shuboy A, Duan HF, Lou JY, Li J, Wang HW and Wang YL: Differentiation of hUC-MSC into dopaminergic-like cells after transduction with hepatocyte growth factor. Mol Cell Biochem 381: 183-190, 2013.
35. Lopatina T, Favaro E, Grange C, Cedrino M, Ranghino A, Occhipinti S, Fallo S, Buffolo F, Gaykalova DA, Zanone MM, *et al*: PDGF enhances the protective effect of adipose stem cell-derived extracellular vesicles in a model of acute hindlimb ischemia. Sci Rep 8: 17458, 2018.
36. Lopatina T, Bruno S, Tetta C, Kalinina N, Porta M and Camussi G: Platelet-derived growth factor regulates the secretion of extracellular vesicles by adipose mesenchymal stem cells and enhances their angiogenic potential. Cell Commun Signal 12: 26, 2014. Veevers-Lowe J, Ball SG, Shuttleworth A and Kielty CM: Mesenchymal stem cell migration is regulated by fibronectin through α5β1-integrin-mediated activation of PDGFR-β and potentiation of growth factor signals. J Cell Sci 124: 1288-1300, 2011.
37. Ball SG, Shuttleworth CA and Kielty CM: Mesenchymal stem cells and neovascularization: Role of platelet-derived growth factor receptors. J Cell Mol Med 11: 1012-1030, 2007.
38. Veevers-Lowe J, Ball SG, Shuttleworth A and Kielty CM: Mesenchymal stem cell migration is regulated by fibronectin through α5β1-integrin-mediated activation of PDGFR-β and potentiation of growth factor signals. J Cell Sci 124: 1288-1300, 2011.
39. Jung KH, Chu K, Lee ST, Bahn JJ, Jeon D, Kim JH, Kim S, Won CH, Kim M, Lee SK and Roh JK: Multipotent PDGFRβ-expressing cells in the circulation of stroke patients. Neurobiol Dis 41: 489-497, 2011.
40. Gao Z, Daquinag AC, Su F, Snyder B and Kolonin MG: PDGFRα/PDGFRβ signaling balance modulates progenitor cell differentiation into white and beige adipocytes. Development 145: dev155861, 2018.
41. Yan L, Zhou L, Yan B, Zhang L, Du W, Liu F, Yuan Q, Tong P, Shan L and Efferth T: Growth factors-based beneficial effects of platelet lysate on umbilical cord-derived stem cells and their synergistic use in osteoarthritis treatment. Cell Death Dis 11: 857, 2020.
42. Brizzi MF, Tarone G and Defilippi P: Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche. Curr Opin Cell Biol 24: 645-651, 2012.
43. Magnusson PU, Looman C, Ahgren A, Wu Y, Claesson-Welsh L and Heuchel RL: Platelet-derived growth factor receptor-beta constitutive activity promotes angiogenesis in vivo and in vitro. Arterioscler Thromb Vasc Biol 27: 2142-2149, 2007.
44. Kilpatrick LE and Hill SJ: Transactivation of G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs): Recent insights using luminescence and fluorescence technologies. Curr Opin Endocr Metab Res 16: 102-112, 2021.
45. Hu F, Wang X, Liang G, Lv L, Zhu Y, Sun B and Xiao Z: Effects of epidermal growth factor and basic fibroblast growth factor on the proliferation and osteogenic and neural differentiation of adipose-derived stem cells. Cell Reprogram 15: 224-232, 2013.
46. Khan S, Villalobos MA, Choron RL, Chang S, Brown SA, Carpenter JP, Tulenko TN and Zhang P: Fibroblast growth factor and vascular endothelial growth factor play a critical role in endotheliogenesis from human adipose-derived stem cells. J Vasc Surg 65: 1483-1492, 2017.
47. Mamer SB, Chen S, Weddell JC, Palasz A, Wittenkeller A, Kumar M and Imoukhuede PI: Discovery of high-affinity pdgf-vegfr interactions: Redefining RTK dynamics. Sci Rep 7: 16439, 2017.
48. Bergeron JJ, Di Guglielmo GM, Dahan S, Dominguez M and Posner BI: Spatial and temporal regulation of receptor tyrosine kinase activation and intracellular signal transduction. Annu Rev Biochem 85: 573-597, 2016.
49. Kim WS, Park HS and Sung JH: The pivotal role of PDGF and its receptor isoforms in adipose-derived stem cells. Histol Histopathol 30: 793-799, 2015.
50. Younesi Soltani F, Javanshir S, Dowlati G, Parham A and Naderi-Meshkin H: Differentiation of human adipose-derived mesenchymal stem cells toward tenocyte by platelet-derived growth factor-BB and growth differentiation factor-6. Cell Tissue Bank 23: 237-246, 2022.
51. Artemenko Y, Gagnon A, Aubin D and Sorisky A: Anti-adipogenic effect of PDGF is reversed by PKC inhibition. J Cell Physiol 204: 646-653, 2005.
52. Hye Kim J, Gyu Park S, Kim WK, Song SU and Sung JH: Functional regulation of adipose-derived stem cells by PDGF-D. Stem Cells 33: 542-556, 2015.
53. Lee CS, Nicolini AM, Watkins EA, Burnsed OA, Boyan BD and Schwartz Z: Adipose stem cell microbeads as production sources for chondrogenic growth factors. J Stem Cells Regen Med 10: 38-48, 2014.
54. Carrancio S, López-Holgado N, Sánchez-Guijo FM, Villarón E, Barbado V, Tabera S, Díez-Campelo M, Blanco J, San Miguel JF and Del Cañizo MC: Optimization of mesenchymal stem cell expansion procedures by cell separation and culture conditions modification. Exp Hematol 36: 1014-1021, 2008.
55. Huang CT, Chu HS, Hung KC, Chen LW, Chen MY, Hu FR and Chen WL: The effect of human platelet lysate on corneal nerve regeneration. Br J Ophthalmol 105: 884-890, 2021.
56. Verma R, Kumar S, Garg P and Verma YK: Platelet-rich plasma: A comparative and economical therapy for wound healing and tissue regeneration. Cell Tissue Bank 24: 285-306, 2023.