VEGF-C and TGF-β reciprocally regulate mesenchymal stem cell commitment to differentiation into lymphatic endothelial or osteoblastic phenotypes

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Abstract. The direction of mesenchymal stem cell (MSC) differentiation is regulated by stimulation with various growth factors and cytokines. We recently established MSC lines, [transforming growth factor-β (TGF-β)-responsive SG-2 cells, bone morphogenetic protein (BMP)-responsive SG-3 cells, and TGF-β/BMP-non-responsive SG-5 cells], derived from the bone marrow of green fluorescent protein-transgenic mice. In this study, to compare gene expression profiles in these MSC lines, we used DNA microarray analysis to characterize the specific gene expression profiles observed in the TGF-β-responsive SG-2 cells. Among the genes that were highly expressed in the SG-2 cells, we focused on vascular endothelial growth factor (VEGF) receptor 3 (VEGFR3), the gene product of FMS-like tyrosine kinase 4 (*Flt4*). We found that VEGF-C, a specific ligand of VEGFR3, significantly induced the cell proliferative activity, migratory ability (as shown by Transwell migration assay), as well as the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 in the SG-2 cells. Additionally, VEGF-C significantly increased the expression of prospero homeobox 1 (*Prox1*) and lymphatic vessel endothelial hyaluronan receptor 1 (*Lyve1*), which are lymphatic endothelial cell markers, and decreased the expression of osteogenic differentiation marker genes in these cells. By contrast, TGF-β significantly increased the expression of early-phase osteogenic differentiation marker genes in the SG-2 cells and markedly decreased the expression of lymphatic endothelial cell markers. The findings of our study strongly suggest the following: i) that VEGF-C promotes the proliferative activity and migratory ability of MSCs; and ii) VEGF-C and TGF-β reciprocally regulate MSC commitment to differentiation into lymphatic endothelial or osteoblastic phenotypes, respectively. Our findings provide new insight into the molecular mechanisms underlying the regenerative ability of MSCs.

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Introduction

Mesenchymal stem cells (MSCs) were first derived from bone marrow and are characterized by their self-renewal ability and their capacity to develop into various mesenchymal tissue cells (1-3). Much of this differentiation process depends on the ability of the MSCs to proliferate and differentiate under the influence of various growth factors and cytokines (4-7). For example, the role of growth factors in bone repair is widely recognized, particularly with regard to bone morphogenetic protein (BMP), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-I (IGF-I) and transforming growth factor-β (TGF-β) (8,9). In a recent study of ours, we demonstrated that PDGF-induced phosphoinositide 3-kinase (PI3K)-mediated signaling promoted the TGF-β-induced osteogenic differentiation of MSCs in a TGF-β-activated extracellular signal-regulated kinase (ERK) kinase-dependent manner (10). In a another recent study, we demonstrated that MSCs-secreted protein, scrapie responsive gene-1 (SCRG1), and its receptor bone marrow stromal cell antigen-1 (BST1), which played important roles in the maintenance of stemness and in the suppression of the osteogenic differentiation of MSCs (11).

VEGF, an important growth factor for bone repair, regulates numerous cellular events associated with angiogenesis and vasculogenesis, such as tissue remodeling during embryonic development and in adults (12). The mammalian VEGF signaling pathway consists of 5 glycoprotein ligands from the VEGF family (VEGF-A, -B, -C, -D and placental growth factor), 3 transmembrane receptors [VEGF receptor (VEGFR)1, VEGFR2 and VEGFR3] and 2 co-receptors (neuropilin-1 and -2) (13-22). VEGF-A binding to VEGFR2 is believed to be the key signaling pathway mediating angiogenesis (14,23). VEGF-A enhances proliferation and survival, promotes cell migration, increases vascular permeability, and alters gene expression in endothelial cells (13,14). VEGF-B binding to VEGFR1 promotes the survival of endothelial cells, pericytes, and smooth muscle cells and upregulates the expression of prosurvival genes (24). VEGF-C and VEGF-D bind to the receptors, VEGFR2 and VEGFR3 (22). VEGF-C expression has been shown to be associated with advanced metastasis in colorectal cancer (25) and to play a role in lymphangiogenesis and/or metastasis to lymph nodes in multiple types of cancer, including colorectal (26) and breast cancer (27,28). VEGF-D is also involved in lymphangiogenesis and lymphatic metastasis (29,30). On the other hand, in contrast to the well-studied VEGF signaling in endothelial cells, the VEGF signaling pathways in cells involved in bone repair, such as MSCs and osteoblasts, remains less well known (31). Osteoblasts express VEGFR1, VEGFR2 and the co-receptor, neuropilin (32). The expression of VEGF and its receptors in differentiating osteoblasts has been detected in cultured cells (32,33), and an *in vitro* cell culture study suggested a role for VEGFR2 in both osteoblast differentiation and survival (34).

In a recent study of ours, we established 3 MSC lines (SG-2, SG-3, and SG-5) derived from the bone marrow of green fluorescent protein (GFP)-transgenic mice (35). These cell lines clearly expressed the mouse MSC markers, stem cells antigen-1 (Sca-1) and CD44, and the SG-2 and SG-5 cells retained their potential for osteogenic and adipogenic differentiation. In addition, we examined the reactions of the TGF-β superfamily in these MSC lines. The analysis of cytokine and cytokine receptor expression in these MSC lines revealed that BMP receptor 1B was most strongly expressed in the SG-3 cells, which underwent osteogenesis in response to BMP. TGF-β receptor II was more strongly expressed in the SG-3 and SG-5 cells. However, we unexpectedly noted that the phosphorylation of Smad2, a major transcription factor, was induced by TGF-β1 in the SG-2 cells, but not in the SG-3 or SG-5 cells. These findings demonstrated the establishment of TGF-β-responsive SG-2 MSCs, BMP-responsive SG-3 MSCs, and TGF-β/BMP-non-responsive SG-5 MSCs.

In the present study, we focused on membrane proteins that are expressed specifically in SG-2 cells in order to facilitate the sorting and identification of the MSCs. VEGFR3, the gene product of FMS-like tyrosine kinase 4 (*Flt4*), was strongly expressed only in the SG-2 cells, but not in the SG-3 and SG-5 cells. Our findings demonstrate the role of VEGF-C, a specific ligand of VEGFR3, in the regenerative ability of the mouse MSC line, TGF-β-responsive SG-2 cells.

Materials and methods

Mouse MSC lines. In a recent study of ours, we described the establishment process and culture method for all MSC lines derived from the bone marrow of GFP-transgenic mice: TGF-β-responsive SG-2, BMP-responsive SG-3 and TGF-β/BMP-non-responsive SG-5 cells (35). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, GE Healthcare Life Sciences, Logan, UT, USA, Logan, UT, USA) at 37˚C under hypoxic conditions (5% O_2, 5% CO_2 and 90% N_2).

DNA microarray analysis. Whole genome expression was analyzed for the bone-marrow derived SG-2, SG-3 and SG-5 MSC lines. Total RNA was extracted using ISOGEN reagent (Nippon Gene Co., Ltd., Tokyo, Japan). Filgen, Inc. (Nagoya, Japan) performed the DNA microarray analyses, including reverse transcription labeling, microarray hybridization, scanning and raw data analyses. For hybridization, 3 GeneChip Mouse Gene 2.0 ST arrays (Affymetrix, Santa Clara, CA, USA) were used. These analyses were conducted by the Research Institute of Bio-System Informatics (Tohoku Chemical Co., Ltd., Morioka, Japan).

Flow cytometry. Almost confluent SG-2, SG-3 and SG-5 cells (1.0×10^5) were suspended in ice-cold phosphate-buffered saline (PBS) containing 0.5% FBS and 2 mM EDTA. The cells were incubated with phycoerythrin (PE)-conjugated anti-mouse VEGFR3 (CD310) antibody (1:10, clone AFL4, #130-102-216; Miltenyi Biotec, Bergisch Gladbach, Germany) for 1 h at 4˚C in the dark. Acquisition was performed with an EPICS XL ADC system (Beckman Coulter, Inc., Brea, CA, USA).

Western blot analysis. The SG-2, SG-3 and SG-5 cells were serum-starved overnight and stimulated with 10 ng/ml VEGF-C (R&D Systems, Inc., Minneapolis, MN, USA) for 1 h at 37˚C under hypoxic conditions. The cells were washed twice with ice-cold PBS and then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein content was measured using BCA reagent (Pierce/Thermo Fisher Scientific, Waltham, MA, USA). Samples containing equal amounts of protein were separated using 12.5% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany). After blocking with 5% non-fat dry milk in T-TBS (50 mM Tris-HCl, pH 7.2, 150 mM NaCl and 0.1% Tween-20), the membrane was incubated with primary anti-Akt (#9272), anti-phospho-Akt (Ser473) [phosphorylated (p-)Akt; #9271], anti-p44/42 mitogen-activated protein kinase (MAPK; ERK1/2; #9102), anti-phospho-p44/42 MAPK (Thr202/Tyr204) (p-ERK1/2; #9101), anti-p38 MAPK (p38; #9212), anti-phospho-p38 MAPK (T180/Y182) (p-p38; #9211), anti-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (JNK; #9252) and anti-phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK; #9251) antibodies (all from Cell Signaling Technology, Danvers, MA, USA), and anti-β-actin (clone C4; Santa Cruz Biotechnology, Dallas, TX, USA) antibody as a loading control for normalization. The blots were incubated with an alkaline phosphatase-conjugated secondary antibody and developed using the BCIP/NBT membrane phosphatase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA).

Table I. Primer sequences.

Gene name	Symbol	Primer sequence (5'→3')
Prospero homeobox 1	*Prox1*	Forward: CGCTTAGCATTGCTGTTGCTG Reverse: GAGCCATTCCTGGGTGATGTC
Lymphatic vessel endothelial hyaluronan receptor 1	*Lyve1*	Forward: GAGCCATTCAAAGTACCAGGTCCTAA Reverse: ACATGTGCCTGGTTCCAAAG
Runt-related transcription factor 2	*Runx2*	Forward: GACGTGCCCAGGCGTATTTC Reverse: AAGGTGGCTGGGTAGTGCATTC
Alkaline phosphatase, liver/bone/kidney	*Alpl*	Forward: ACACCTTGACTGTGGTTACTGCTGA Reverse: CCTTGTAGCCAGGCCCGTTA
Integrin-binding sialoprotein	*Ibsp*	Forward: AGAACAATCCGTGCCACTCACTC Reverse: AGTAGCGTGGCCGGTACTTAAAGA
Bone gamma-carboxyglutamate (gla) protein	*Bglap*	Forward: CGGCCCTGAGTCTGACAAA Reverse: TCTGTAGGCGGTCTTTAAGCCATA
Glyceraldehyde 3-phosphate dehydrogenase	*Gapdh*	Forward: TGTGTCCGTCGTGGATCTGA Reverse: TTGCTGTTGAAGTCGCAGGAG

Cell proliferation assay. Cell proliferation was analyzed using a colorimetric assay for cleavage of the tetrazolium salt WST-1 (Roche Diagnostics, Basel, Switzerland) by mitochondrial dehydrogenases in viable cells. The measured absorbance of the dye directly correlates with the number of metabolically active cells in the culture. The cells were cultured in 96-well plates (Nunc; Thermo Fisher Scientific) in growth medium with/without 10 ng/ml VEGF-C under hypoxic conditions. After 5 days, the cells were incubated for a further 1 h at 37˚C with 100 μl medium containing 10 μl WST-1 reagent. The samples were shaken for 1 min, and absorbance was measured at 450 nm using an MPR-A4i microplate reader (Tosoh Corp., Tokyo, Japan).

Transwell migration assay. The migration assay was performed as reported previously using Transwell cell culture inserts (BD Biosciences, Franklin Lakes, NJ, USA) that were 6.5 mm in diameter with 8-μm pore filters (11). The cells ($5.0\text{x}10^4$) were suspended in 350 μl serum-free DMEM containing 0.1% BSA (Sigma-Aldrich) and seeded into the upper well; 600 μl normal growth medium with/without 10 ng/ml VEGF-C was placed in the lower well of the Transwell plate. Following incubation for 15 h under hypoxic conditions, the cells that had not migrated from the upper side of the filter were scraped off with a cotton swab, and the filters were stained with the three-step stain set (Diff-Quik; Sysmex, Kobe, Japan). The number of cells that had migrated to the lower side of the filter was counted under a light microscope in 5 high-power fields (x400 magnification; Olympus IX70; Olympus Corp., Tokyo, Japan). The experiment was performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The SG-2, SG-3 and SG-5 cells were stimulated with 10 ng/ml VEGF-C (R&D Systems) or 5.0 ng/ml TGF-β (Calbiochem, Merck Millipore). After 48 h, total RNA from each cell was isolated using ISOGEN reagent (Nippon Gene Co., Ltd.) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using the PrimeScript RT Reagent kit (Takara Bio, Otsu, Japan). RT-qPCR was performed on a Thermal Cycler Dice Real-Time system with SYBR Premix Ex Taq II (both from Takara Bio) and specific oligonucleotide primers (Table I) using a two-step cycle procedure (denaturation at 95˚C for 5 sec, annealing and extension at 60˚C for 30 sec) for 40 cycles. For each test run, cDNA derived from 50 ng total RNA as a template and 0.4 μM primer pair was used. mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), and the relative amounts of each mRNA in each sample were calculated using the ΔΔCq method. The relative mRNA expression levels are expressed as fold increase or decrease relative to the control.

Statistical analysis. All experiments were repeated at least 3 times. Representative images or data are shown. The numerical data are presented as the means ± standard deviation (SD). Differences between averages and percentages between control and tests were statistically analyzed using paired two-tailed Student's t-tests. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Higher expression of VEGFR3 in SG-2 cells. To identify the genes that modulate the regenerative ability of MSCs, we used DNA microarrays to characterize the specific gene expression profiles observed in the TGF-β-responsive SG-2 cells. We identified 105 genes that were ≥10-fold more strongly expressed in the SG-2 cells compared to the SG-3 and SG-5 cells (Table II). Among these genes, we focused on VEGFR3, the *Flt4* gene product, since, as a cell surface antigen, it is useful for identifying and sorting MSCs from various tissues. The gene expression level of *Flt4* in the SG-2 cells was more than 16.5- and 32.0-fold higher than that in the SG-3 and SG-5 cells, respectively. These results were further confirmed by flow cytometry (Fig. 1) and indicated that the SG-2 cells expressed higher levels of VEGFR3 on the cell surface than the SG-3 and SG-5 cells.

Table II. Genes expressed ≥10-fold stronger in the SG-2 cells compared to the SG-3 and SG-5 cells.

Symbol	Gene name	Fold change in SG-2 vs. SG-3	Fold change in SG-2 vs. SG-5
Thrb	Thyroid hormone receptor beta	75.7	58.4
Grhl2	Grainyhead-like 2 (*Drosophila*)	72.9	56.5
Olfr1497	Olfactory receptor 1497	69.6	53.1
Arhgap15	Rho GTPase activating protein 15	66.5	51.4
Hoxd11	Homeobox D11	57.5	44.1
Cfc1	Cripto, FRL-1, cryptic family 1	56.2	43.8
Kcnq5	Potassium voltage-gated channel, subfamily Q, member 5	53.5	41.5
Scgb2b7	Secretoglobin, family 2B, member 7	50.6	39.0
Fn3krp	Fructosamine 3 kinase related protein	55.4	36.2
Tcp10a	T-complex protein 10a	43.5	41.6
Serpina6	Serine (or cysteine) peptidase inhibitor, clade A, member 6	46.0	35.0
Cym	Chymosin	40.4	30.3
Ppp1r3fos	Protein phosphatase 1, regulatory subunit 3F, opposite strand	39.2	30.6
Syn3	Synapsin III	69.7	22.7
Lypd6	LY6/PLAUR domain containing 6	38.3	30.4
Olfr772	Olfactory receptor 772	38.6	29.7
Olfr2	Olfactory receptor 2	38.4	29.4
Zfp92	Zinc finger protein 92	26.5	43.1
Fam81b	Family with sequence similarity 81, member B	38.1	28.8
Ssxb1	Synovial sarcoma, X member B, breakpoint 1	37.4	28.6
Vmn2r25	Vomeronasal 2, receptor 25	36.7	28.7
Olfr368	Olfactory receptor 368	35.9	28.2
Arhgef4	Rho guanine nucleotide exchange factor (GEF) 4	31.8	30.9
Il21	Interleukin 21	35.7	27.8
Fpr-rs3	Formyl peptide receptor, related sequence 3	34.8	27.1
Cldn13	Claudin 13	34.4	27.0
Trim43c	Tripartite motif-containing 43C	35.1	26.6
Caskin1	CASK interacting protein 1	34.4	26.3
Commd7	COMM domain containing 7	34.1	25.3
Prom2	Prominin 2	33.5	25.4
St6galnac3	ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetyl-galactosaminide α-2,6-sialyltransferase 3	32.6	25.8
Slco6c1	Solute carrier organic anion transporter family, member 6c1	30.4	23.6
Nhlrc4	NHL repeat containing 4	29.8	23.7
Chrd	Chordin	21.6	33.8
Olfr117	Olfactory receptor 117	31.0	22.5
Igkv4-53	Immunoglobulin κ variable 4-53	26.3	25.3
Ctag2	Cancer/testis antigen 2	28.8	22.9
F2rl3	Coagulation factor II (thrombin) receptor-like 3	28.9	22.6
Kynu	Kynureninase (L-kynurenine hydrolase)	28.4	21.2
Spata22	Spermatogenesis associated 22	27.2	21.2
Vmn1r191	Vomeronasal 1 receptor 191	27.1	21.2
Ctnnd1	Catenin (cadherin associated protein), delta 1	26.0	20.5
Vmn1r234	Vomeronasal 1 receptor 234	26.2	19.8
Zfp174	Zinc finger protein 174	26.4	19.5
Dgat2l6	Diacylglycerol O-acyltransferase 2-like 6	25.7	19.7
Nudt12os	Nudix (nucleoside diphosphate linked moiety X)-type motif 12, opposite strand	25.3	19.6
Zap70	Zeta-chain (TCR) associated protein kinase	25.3	19.1
Flt4	FMS-like tyrosine kinase 4	16.5	32.0
Hes3	Hairy and enhancer of split 3 (*Drosophila*)	24.8	19.3
Sema6d	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	15.8	33.9
Slc17a4	Solute carrier family 17 (sodium phosphate), member 4	16.9	29.5
Tbx2	T-box 2	24.0	18.1
Wdr95	WD40 repeat domain 95	22.7	17.6
Fer1l5	Fer-1-like 5 (*C. elegans*)	22.4	17.8

Table II. Continued.

		Fold change in SG-2	
Symbol	Gene name	vs. SG-3	vs. SG-5
Gbx2	Gastrulation brain homeobox 2	15.0	29.1
Cd33	CD33 antigen	22.4	17.6
Tdrd5	Tudor domain containing 5	22.8	17.0
Lix1	Limb expression 1 homolog (chicken)	21.4	16.7
Lgals4	Lectin, galactose binding, soluble 4	21.5	16.2
Pip5k1b	Phosphatidylinositol-4-phosphate 5-kinase, type 1β	21.4	15.9
Gsdma2	Gasdermin A2	21.3	15.9
Ifi27l2b	Interferon, α-inducible protein 27 like 2 beta	16.0	19.8
Zcchc18	Zinc finger, CCHC domain containing 18	17.2	17.8
Olfr384	Olfactory receptor 384	19.5	15.5
Olfr1123	Olfactory receptor 1123	11.4	33.8
Myo18b	Myosin XVIIIb	11.8	29.6
Ss18	Synovial sarcoma translocation, Chromosome 18	23.4	13.0
Plxna4os1	Plexin A4, opposite strand 1	19.5	14.4
Fam115e	Family with sequence similarity 115, member E	19.1	14.5
Fbxl5	F-box and leucine-rich repeat protein 5	13.7	20.1
Megf10	Multiple EGF-like-domains 10	18.5	14.3
Robo3	Roundabout homolog 3 (*Drosophila*)	18.3	14.3
Igkv4-91	Immunoglobulin kappa chain variable 4-91	18.3	14.2
Elavl3	ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like 3 (Hu antigen C)	15.7	16.1
Slc17a8	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8	11.7	24.4
Gpr39	G protein-coupled receptor 39	15.7	15.8
Catsper1	Cation channel, sperm associated 1	18.3	13.6
Zscan4a	Zinc finger and SCAN domain containing 4A	17.4	13.8
Ceacam14	Carcinoembryonic antigen-related cell adhesion molecule 14	17.8	13.3
Acnat1	Acyl-coenzyme A amino acid N-acyltransferase 1	12.4	17.8
Arhgap26	Rho GTPase activating protein 26	17.4	12.6
Olfr1270	Olfactory receptor 1270	16.6	13.0
Lrrc25	Leucine rich repeat containing 25	11.4	19.1
Capsl	Calcyphosine-like	16.5	12.5
Gnl2	Guanine nucleotide binding protein-like 2 (nucleolar)	16.1	12.8
Ctnna3	Catenin (cadherin associated protein), alpha 3	16.4	12.6
Itm2a	Integral membrane protein 2A	16.4	12.4
Sema5b	Sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B	20.5	10.6
Il17c	Interleukin 17C	15.7	12.2
Chrna9	Cholinergic receptor, nicotinic, alpha polypeptide 9	15.4	12.1
Acap1	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1	10.8	17.0
Spint5	Serine protease inhibitor, Kunitz type 5	15.1	11.4
Rab5b	RAB5B, member RAS oncogene family	12.5	13.3
Olfr1122	Olfactory receptor 1122	10.5	16.4
Grm8	Glutamate receptor, metabotropic 8	17.6	10.1
Igh-VJ558	Immunoglobulin heavy chain (J558 family)	14.4	11.2
Ptprz1	Protein tyrosine phosphatase, receptor type Z, polypeptide 1	14.4	11.1
Cd300lh	CD300 antigen-like family member H	15.2	10.4
Mcoln1	Mucolipin 1	11.7	12.8
Olfr649	Olfactory receptor 649	13.9	10.9
Tlr8	Toll-like receptor 8	13.9	10.7
Mb21d1	Mab-21 domain containing 1	10.8	13.2
Itgb1bp2	Integrin beta 1 binding protein 2	13.3	10.5
Defb28	Defensin beta 28	13.3	10.4
Cypt2	Cysteine-rich perinuclear theca 2	13.4	10.3

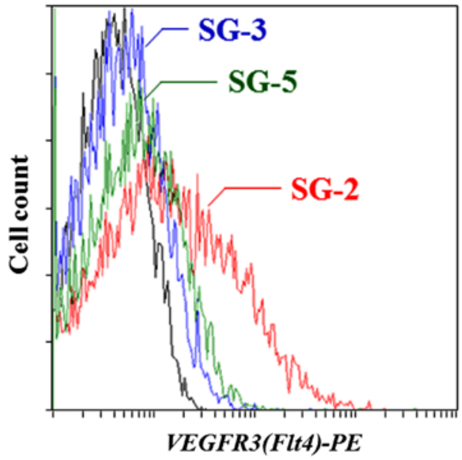

Figure 1. Vascular endothelial growth factor receptor 3 (VEGFR3) expression on the cell surface was detected in SG-2 cells. Cell surface expression of VEGFR3 was analyzed with a VEGFR3-specific antibody in SG-2 (red), SG-3 (blue) and SG-5 (green) cells and an isotype control IgG (black) using flow cytometry.

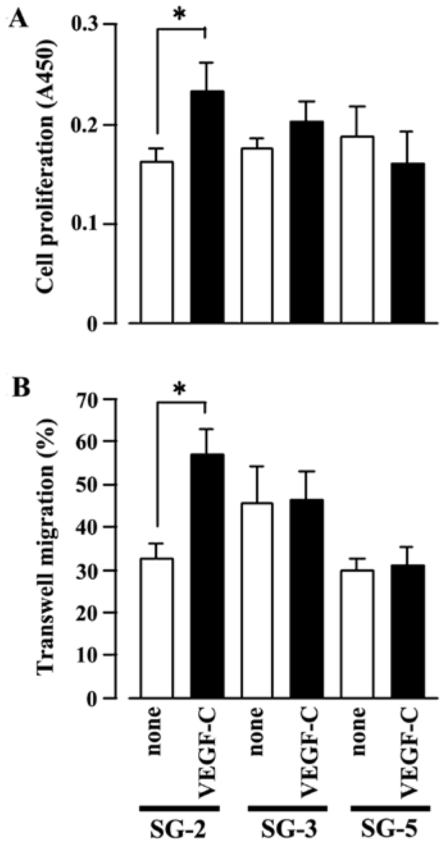

Figure 2. Cell proliferative activity and migratory ability increases with vascular endothelial growth factor-C (VEGF-C) stimulation in FMS-like tyrosine kinase 4 (*Flt4*)-positive SG-2 cells. (A) Cell proliferation assay for SG-2, SG-3 and SG-5 cells stimulated with (VEGF-C) or without (none) 10 ng/ml VEGF-C measured as the absorbance at 450 nm (A450). (B) Transwell migration assay for SG-2, SG-3 and SG-5 cells. Cell numbers were counted in 5 fields under a light microscope. In (A) and (B), data are presented as the means ± SD. *p<0.05 vs. unstimulated control (none) within each cell line.

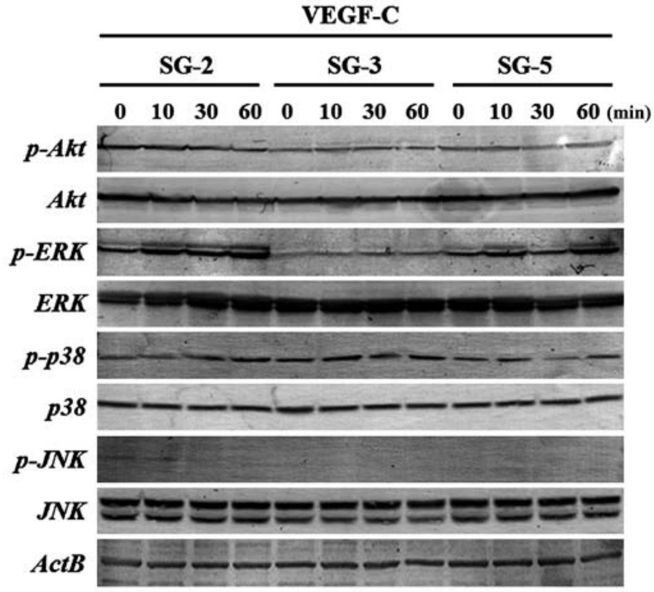

Figure 3. Extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation is induced by vascular endothelial growth factor-C (VEGF-C) stimulation in FMS-like tyrosine kinase 4 (*Flt4*)-positive SG-2 cells. Phosphorylation levels were measured using western blot analysis in SG-2, SG-3 and SG-5 cells stimulated with 10 ng/ml VEGF-C.

Promotion of the migratory ability and proliferative activity of SG-2 cells by VEGF-C. Subsequently, we examined the effects of VEGF-C, a specific ligand of VEGFR3, on the MSC lines (SG-2, SG-3 and SG-5). Indeed, VEGF-C significantly stimulated SG-2 cell proliferation (Fig. 2A) and cell migration (Fig. 2B), but had no effect on the SG-3 or SG-5 cells. These results strongly suggest that VEGF-C specifically promotes the proliferative activity and migratory ability of the SG-2 cells through VEGFR3.

Phosphorylation of ERK1/2 in SG-2 cells by stimulation with VEGF-C. To clarify the signaling pathways activated by VEGF-C in SG-2 cells, we evaluated the phosphorylation status of molecules in the PI3K/Akt- and MAPK-mediated pathways. ERK1/2 phosphorylation was markedly upregulated in the SG-2 cells upon VEGF-C stimulation, whereas that in the SG-3 and SG-5 cells was unaffected (Fig. 3). These results suggest that VEGF-C enhances the proliferative activity and migratory ability of the MSCs through the ERK1/2 pathway in *Flt4*-positive SG-2 cells.

Increase in the expression of lymphatic endothelial differentiation marker genes following stimulation of SG-2 cells with VEGF-C. The VEGF-C gene encodes a ligand for VEGFR3 that is expressed mainly in lymphatic endothelia (18). Furthermore, the VEGF-C/VEGFR3 pathway was the first critical pathway described for the development of the lymphatic vascular tree (36). As the SG-2 cells respond to both TGF-β and VEGF-C, we examined the effects of TGF-β or VEGF-C stimulation on their multi-differentiation potential. VEGF-C clearly and significantly increased the mRNA expression levels of the lymphatic endothelial cell markers, prospero homeobox 1 (*Prox1*) and lymphatic vessel endothelial hyaluronan receptor 1 (*Lyve1*) (p<0.01), in the SG-2 cells, whereas the levels were clearly and significantly decreased following stimulation of the cells with TGF-β (p<0.05; Fig. 4A and B). We previously reported that TGF-β promotes the osteogenic

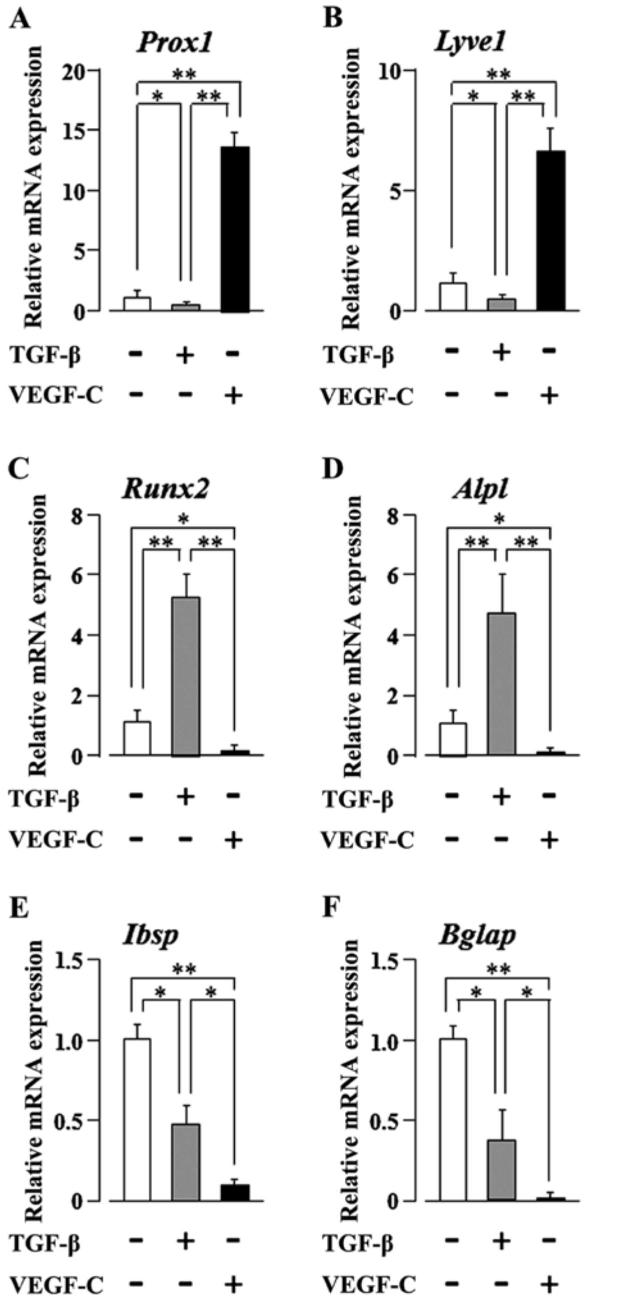

Figure 4. Vascular endothelial growth factor-C (VEGF-C) and transforming growth factor-β (TGF-β) reciprocally regulate the commitment of mesenchymal stem cells (MSCs) to differentiation into lymphatic endothelial or osteoblastic phenotypes, respectively. RT-qPCR of (A) *Prox1*, (B) *Lyve1*, (C) *Runx2*, (D) *Alpl*, (E) *Ibsp* and (F) *Bglap* in SG-2 cells stimulated with 10 ng/ml VEGF-C or 5.0 ng/ml TGF-β. Reported values are normalized to *Gapdh* expression. The results are expressed as the fold change relative to the respective control. Data are presented as the means ± SD. *$p<0.05$, **$p<0.01$.

differentiation of bone marrow-derived MSCs (10). Therefore, in this study, we further investigated the mRNA expression of osteogenic differentiation markers in the SG-2 cells following TGF-β or VEGF-C stimulation. TGF-β clearly and significantly increased the expression of the early-stage osteogenic differentiation marker genes, Runt-related transcription factor 2 (*Runx2*) and alkaline phosphatase, liver/bone/kidney (*Alpl*) ($p<0.01$), in the SG-2 cells; by contrast, VEGF-C clearly and significantly decreased the expression of these early-stage osteogenic differentiation markers ($p<0.05$; Fig. 4C and D). Of note, TGF-β unexpectedly decreased the expression of the late-stage osteogenic differentiation markers, integrin-binding sialoprotein (*Ibsp*) and bone gamma-carboxyglutamate (Gla) protein (*Bglap*) ($p<0.05$; Fig. 4E and F). On the other hand, as expected, VEGF-C suppressed the expression of these late-stage differentiation markers ($p<0.01$; Fig. 4E and F). These results suggest that VEGF-C and TGF-β reciprocally regulate the commitment of MSCs to differentiate into lymphatic endothelial or osteoblastic phenotypes, respectively. On the other hand, both TGF-β and VEGF-C appear to suppress the final maturation of osteoblastic MSC differentiation during late-stage osteogenesis.

Discussion

As demonstrated in our previous study, the TGF-β-responsive, *Flt4*-positive SG-2 MSC line retained both osteogenic and adipogenic differentiation potentials (35). Herein, we focused on SG-2-specific membrane protein expression and identified high expression levels of VEGFR3, the *Flt4* gene product (Table II and Fig. 1). Furthermore, we found that the VEGFR3-specific ligand, VEGF-C, significantly increased the proliferative activity and migratory ability of the SG-2 cells (Fig. 2). VEGF potently promotes angiogenesis and is indispensable for vascular development (37,38), and the tyrosine kinase receptor, VEGFR2, is the primary transmitter of VEGF signals in endothelial cells (39,40). The binding of VEGF-A to VEGFR2 activates downstream signaling, including the MAPK pathways (41,42). Other VEGF family members and other signaling mediators affect and overlap with the function of VEGF-A (22,43,44). VEGFR3 is activated by the VEGF homologues, VEGF-C and VEGF-D, which, when fully proteolytically processed, also stimulate VEGFR2 and induce the formation and activation of VEGFR2-VEGFR3 heterodimers (36,45,46). Since in this study VEGF-C stimulation induced ERK1/2 phosphorylation in the SG-2 cells, the promotion of the migratory ability and proliferative activity of *Flt4*-positive MSCs appears to depend on the activation of the MAPK cascade (Fig. 3).

The VEGF-C/VEGFR3 pathway was the first critical pathway described for the development of the lymphatic vascular tree (36). It has been demonstrated that VEGFR3 expression starts during mouse embryonic day 8.5 in developing blood vessels, and VEGFR3-deficient embryos die at midgestation from defects in the remodeling of primary vascular networks (47). In adult tissues, VEGFR3 expression occurs mainly in lymphatic endothelial cells (47-50), and VEGFR3-positive lymphatic vessels appear concurrently with blood vessels during wound healing, but regress rapidly (51). However, VEGFR3-expressing endothelial cells may also be found in the fenestrated capillaries of several adult organs, including the bone marrow, splenic and hepatic sinusoids, kidney glomeruli and endocrine glands (50). Notably, in human cancer, VEGFR3-expressing vascular endothelial cells are detected in angiogenic capillaries (52,53), and the inactivation of VEGFR3 signaling with blocking antibodies in nude mice has been shown to suppress tumor growth by inhibiting angiogenesis (54).

MSCs have been reported to home towards hypoxic microenvironments *in vivo*, and hypoxic tumor cells specifically recruit MSCs by activating survival pathways that facilitate tumor progression (55). Based on these findings, the results of our study suggest that *Flt4*-positive MSCs play an important role in tumor angiogenesis and lymphatic vessel formation. Previous studies have demonstrated the VEGF-mediated differentiation of lymphatic endothelial cells from bone marrow-derived MSCs (56,57).

In this study, our results indicated that stimulation with VEGF-C increased the expression of lymphatic endothelial cell marker genes in *Flt4*-positive SG-2 cells (Fig. 4A and B). More interestingly, VEGF-C suppressed the expression of osteogenic differentiation marker genes (Fig. 4C and D). On the other hand, TGF-β suppressed the lymphatic endothelial commitment of SC-2 cells (Fig. 4A and B). Thus, we concluded that VEGF-C and TGF-β reciprocally regulate MSC commitment to differentiation into lymphatic endothelial or osteoblastic phenotypes, respectively. However, TGF-β and VEGF-C both seem to suppress the maturation of osteoblastic MSC differentiation at the late stage of the osteogenic process, suggesting that additional cellular signals must be necessary for the progression of osteoblastic differentiation of some types of MSCs. In addition, VEGF-C positively regulated the migration and proliferation of the *Flt4*-positive SG-2 cells (Fig. 2). The migratory ability and the proliferative activity are necessary conditions of MSCs, suggesting the novel possibility that VEGF-C plays an important role in determining MSC characteristics.

Our findings provide new insight into the molecular mechanisms underlying the regenerative activity of MSCs. In future studies, we aim to determine whether these results are reproducible *in vivo* by transplanting GFP-expressing SG-2 cells into suitable animal experimental models to facilitate their discrimination from the surrounding donor cells.

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