Stromal Cell-Derived Factor-1 β Potentiates Bone Morphogenetic Protein-2-Stimulated Osteoinduction of Genetically Engineered Bone Marrow-Derived Mesenchymal Stem Cells *In Vitro*

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Skeletal injuries are among the most prevalent clinical problems and bone marrow-derived mesenchymal stem/stromal cells (BMSCs) have successfully been used for the treatment thereof. Stromal cell-derived factor-1 (SDF-1; CXCL12) is a member of the CXC chemokine family with multiple splice variants. The two most abundant variants, SDF-1 α and SDF-1 β , share identical amino acid sequences, except for four additional amino acids at the C-terminus of SDF-1 β , which may mediate surface stabilization via glycosaminoglycans and protect SDF-1 β from proteolytic cleavage, rendering it twice as potent as SDF-1 α . Increasing evidence suggests that SDF-1 is involved in bone formation through regulation of recruitment, engraftment, proliferation, and differentiation of stem/progenitor cells. The underlying molecular mechanisms, however, have not yet been fully elucidated. In this study, we tested the hypothesis that SDF-1 β can potentiate bone morphogenetic protein-2 (BMP-2)-stimulated osteogenic differentiation and chemotaxis of BMSCs *in vitro*. Utilizing retrovirus-mediated gene transfer to generate novel Tet-Off-SDF-1 β BMSCs, we found that conditional SDF-1 β expression is tightly regulated by doxycycline in a dose-dependent and temporal fashion, leading to significantly increased SDF-1 β mRNA and protein levels. In addition, SDF-1 β was found to enhance BMP-2-stimulated mineralization, mRNA and protein expression of key osteogenic markers, and regulate BMP-2 signal transduction via extracellular signal-regulated kinases 1/2 (Erk1/2) phosphorylation in genetically engineered BMSCs *in vitro*. We also showed that SDF-1 β promotes the migratory response of CXC chemokine receptor 4 (CXCR4)-expressing BMSCs *in vitro*. Taken together, these data support that SDF-1 β can play an important role in BMP-2-stimulated osteogenic differentiation of BMSCs and may exert its biological activity in both an autocrine and paracrine fashion.

Introduction

SKELETAL INJURIES AND their complications continue to be major causes of morbidity and mortality, and this problem is accentuated in patients with osteoporosis and osteoporosis-related fractures.¹ Over the last decade, bone marrow-derived mesenchymal stem/stromal cells (BMSCs) have successfully been used to repair or regenerate bone in experimental and clinical studies, as BMSCs can give rise to cells of the osteoblast lineage and form bone.^{2,3} Thus, stem cell-based therapy has become one of the most promising

modalities for bone repair in challenging cases, such as nonunions and large bone defects.¹ In addition, accumulating evidence suggests that aging-associated osteoporosis is a stem cell disease and that regenerative medicine approaches may be clinically useful for its treatment.⁴

Stromal cell-derived factor-1 (SDF-1) was originally identified as a secreted product of a BMSC line,⁵ and subsequently as a pre-B-cell growth-stimulating factor.⁶ SDF-1 (CXCL12) is a member of the proinflammatory CXC chemokine family and a potent chemoattractant for T cells, monocytes, and lymphohematopoietic progenitor cells. In

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contrast to the expression of most chemokines, which is induced by cytokines, SDF-1 is produced constitutively.^{7,8} In fact, both SDF-1 and its cognate G-protein-coupled transmembrane CXC chemokine receptor 4 (CXCR4) are widely expressed in many tissue types and have been shown to be fundamental for embryonic development. Mice lacking either of them die *in utero* or perinatally due to severe defects in developing nervous, hematopoietic, and cardiovascular systems. After birth, SDF-1 signaling continues to be important in regulating physiological tissue homeostasis, hematopoiesis, and angiogenesis.^{9–11} Binding of SDF-1 to CXCR4 mediates the migration of CXCR4-expressing cells toward gradients of SDF-1 and this chemotactic property regulates the homing and retention of hematopoietic stem cells and MSCs within the bone marrow.^{12–14} Furthermore, this also accounts for reactive endochondral bone formation postinjury during the acute phase of bone repair.^{15–17} Of note, a second receptor, CXCR7/RDC1, has recently been identified in several cell types and although CXCR7 binds SDF-1 with high affinity, typical chemokine signaling has not been demonstrated.¹⁸

SDF-1, which is highly conserved among species, has six identified splice variants (SDF-1 α , - β , - γ , - δ , - ϵ , - ϕ) derived from the same gene^{7,8,19} and its N-terminal residues 1–8 are required to form the binding domain for CXCR4.^{20,21} The two most abundant splice variants, SDF-1 α and SDF-1 β , share identical amino acid sequences except for the presence of four additional amino acids at the C-terminus of SDF-1 β . SDF-1 α is subject to proteolytic degradation on both the N-terminus and C-terminus, whereas processing of SDF-1 β primarily occurs on the N-terminus due to its four additional C-terminal amino acids, which render the C-terminus more protected from proteolytic cleavage in the peripheral circulation. These additional amino acids also mediate glycosaminoglycan-dependent stabilization on cell and extracellular surfaces, which increases its stability in highly vascularized tissues, such as the bone marrow, and ultimately doubles its potency compared to SDF-1 α .^{22–24} Thus, SDF-1 β may be of significant interest in developing regenerative medicine treatment protocols.

Previous experimental studies investigating the role of SDF-1 in bone formation are limited and have only focused on SDF-1 α ; however, no studies to date have explored the potential of SDF-1 β as an osteogenic mediator in normal bone formation and bone injury. Researchers have utilized transient adenoviral-mediated overexpression of SDF-1 α in BMSCs and *in vitro* preconditioning of BMSCs with SDF-1 α before transplantation, which resulted in enhanced cell engraftment and new tissue formation in different animal models.^{25–27} However, none of these approaches allowed for direct modulation of SDF-1 levels *in vivo*. Tetracycline (Tet)-dependent regulatory systems, such as Tet-Off, allow for tight control of transgene expression *in vitro* and *in vivo*, and these features provided the basis for using the Tet-Off regulatory system in the present study.

Given the dearth of scientific literature examining the contribution of SDF-1 β to bone growth, we investigated the normal distribution of SDF-1 β in whole bone marrow, enriched BMSCs, and peripheral circulation in mature animals and the specific contribution of SDF-1 β to the regulation of bone morphogenetic protein-2 (BMP-2)-stimulated bone formation in an *in vitro* setting, as a prelude to *in vivo* ap-

plications. Specifically, we utilized the Tet-Off regulatory system to conditionally overexpress SDF-1 β in BMSCs and tested the hypothesis that SDF-1 β enhances BMP-2-stimulated osteogenic differentiation and chemotaxis of genetically engineered BMSCs *in vitro*.

Materials and Methods

Animals

C57BL/6J male mice were purchased from Jackson Laboratories (Bar Harbor, ME) or the aged rodent colony at the National Institute on Aging (Bethesda, MD). Animals were maintained at the Animal Research Facility of the Veterans Affairs Medical Center and used at the age of 3 and 18 months. All aspects of the animal research were conducted in accordance with the guidelines set by the Institutional Animal Care and Use Committee of the Department of Veterans Affairs Medical Center in Augusta.

Isolation of whole BM cells

Ten 3-month-old male C57BL/6J mice were euthanized by isoflurane overdose followed by thoracotomy. Blood samples were drawn by cardiac puncture. The femora and tibiae were dissected free of soft tissues and kept on ice. A 15-mL conical tube was filled with 2 mL of phosphate-buffered saline (PBS) before cutting the bones into small pieces to release their BM cells. Following vigorous mixing by inverting the tube, the BM cells were separated from the bone chips using a 100- μ L pipette and divided in two aliquots. BM cells were subsequently centrifuged at 1500 rpm for 5 min and cell pellets were transferred to -80°C for later use.

Isolation and culture of BMSCs

BMSCs derived from 3-month-old and 18-month-old male C57BL/6J mice were generously provided by Dr. Xingming Shi. The isolation process, negative immunodepletion (CD-11b, CD45R/B220, CD11c, plasmacytoid dendritic cell antigen-1 [PDCA-1]), positive immunoselection (stem cell antigen-1 [Sca-1]), and retroviral infection (GFP) have been described previously.^{28,29} First, six mice per age group were euthanized by CO₂ overdose followed by thoracotomy. The femora and tibiae were dissected free of soft tissues and kept in cold PBS on ice. The bones were cut open at both ends and flushed with complete isolation media (CIM) (RPMI-1640; Cellgro, Mediatech, Manassas, VA), 9% heat-inactivated fetal bovine serum (FBS), 9% horse serum (both from Atlanta Biologicals, Lawrenceville, GA), and 12 μ M L-glutamine (Gibco, Invitrogen, Carlsbad, CA) using a 22-gauge syringe followed by filtration through a 70- μ m nylon mesh filter. The combined whole bone marrow aspirate was dispersed with a 25-gauge syringe to produce a single-cell suspension. Next, BMSCs were isolated using a modified protocol^{30–32} by plating the single-cell suspension in 175-cm² flasks at a density of 2×10^7 cells/flask. After a 3-h incubation at 37°C in 5% CO₂, the nonadherent cells were removed and the adherent cells washed two times gently with PBS to reduce the degree of hematopoietic lineage cell contamination. The cells were cultured in CIM for 3–4 weeks with media change every 3–4 days. At 70%–80% confluence, the cells were lifted with trypsin/EDTA, washed, and resuspended at a density

of 5×10^6 cells/mL in PBS containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA followed by negative immunodepletion using magnetic microbeads conjugated to anti-mouse CD11b, CD45R/B220 (BD Biosciences Pharmingen, San Diego, CA), CD11c, and PDCA-1 (Miltenyi Biotec, Bergisch Gladbach, Germany) monoclonal antibodies according to the manufacturer's instructions. Resulting CD11b, CD45R/B220, CD11c, PDCA-1-negative cells were subjected to positive immunoselection using anti-Sca-1 microbeads (Miltenyi Biotec) following the manufacturer's recommendations. Enriched BMSCs (3 months: 1.35% CD45, 1.45% CD11b, 74.46% Sca-1; 18 months: 0.33% CD45, 0.13% CD11b, 83.18% Sca-1 by FACS analysis²⁸), which are depleted of monocytes, granulocytes, macrophages, myeloid-derived dendritic cells (DCs), natural killer cells, B-1 cells, B lymphocytes, T lymphocytes, classical DCs, plasmacytoid DCs, and macrophage progenitors, were maintained in the Dulbecco's modified Eagle's medium (DMEM; Cellgro) with 10% heat-inactivated FBS (Atlanta Biologicals), and shown of possessing multilineage potential.²⁸ Of note, BMSCs derived from 3-month-old animals at this stage were used for comparisons with whole BM cell isolates (see above) derived from a second set of 3-month-old mice. BMSCs derived from 18-month-old mice were subjected to retroviral-mediated transduction with Δ U3-GFP plasmid DNA, constructed in the replication defective Δ U3nlsLacZ vector by inserting the full-length coding region of *Gfp* cDNA.²⁹ BMSCs were then retransplanted in tibiae of 2-month-old C57BL/6J mice and their osteogenic potential was confirmed *in vivo* (unpublished data). After 4 weeks, animals were euthanized, the marrow cells harvested, and seeded at low density for clonal selection. Well-isolated, GFP-positive BMSCs (clone 2) were maintained in DMEM supplemented with 10% heat-inactivated FBS and used as parental cells for further genetic modification with the Tet-Off system at 70%–80% confluence.

Culture of retroviral packaging cells

293GPG cells³³ were maintained in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate (Gibco, Invitrogen), 2 mM L-glutamine, 1 μ g/mL Tet (Sigma-Aldrich, St. Louis, MO), 300 μ g/mL G418 (MP Biomedicals, Solon, OH), and 2 μ g/mL puromycin (Sigma-Aldrich), and used at 70%–80% confluence.

Retroviral constructs

Retroviral Tet-Off expression vectors from Clontech Laboratories (Mountain View, CA) were propagated in DH5 α (Invitrogen). The murine *Sdf-1 β* full-length coding sequence (NM_013655) was amplified by reverse transcription–polymerase chain reaction (RT-PCR) using total RNA isolated from parental BMSCs with TRIZOL[®] reagent (Invitrogen). Two micrograms of RNA was reverse transcribed using the iScript[™] cDNA Synthesis Kit (Bio-Rad, Hercules, CA). *NotI* and *EcoRI* restriction sites were incorporated into the 5'- and 3'-end of the PCR product, respectively, when *Sdf-1 β* cDNA was amplified by PCR. The RT-PCR primers were (Thermo Fisher Scientific, Waltham, MA) (5'-3'): forward, GCGCGGCCGCGCCATGGACGCCAAGGTC and reverse, GCGCGAATTCCTCACATCTTGAGCCTCT.

Virus production and infection

293GPG cells were transfected at passage 8 with retroviral constructs using the CalPhos[™] Mammalian Transfection Kit (Clontech Laboratories). Retrovirus production was induced by withdrawing Tet from the culture medium. Supernatants were collected 24 h post-transfection and passed through 0.45- μ m cellulose filter units. Retroviral titers were determined using a quantitative RT-PCR (qRT-PCR) Titration Kit (Clontech Laboratories). The day before infection, cells were counted using a NucleoCounter[™] system (New Brunswick Scientific, Edison, NJ) and 2.0×10^5 BMSCs/well were plated in 6-well plates. Multipotent BMSCs (clone 2) (differentiation along osteogenic, adipogenic, and chondrogenic pathways at passage 14; data not shown) derived from 18-month-old mice were infected at passage 10 with 2 mL of retroviral supernatant containing 4 μ g/mL polybrene (Sigma-Aldrich) and 100 ng/mL doxycycline (Dox; Sigma-Aldrich) for 24 h at a multiplicity of infection of 100. The next day, cells were split 1:4 using fresh medium before initiating the selection process by supplementing 400 μ g/mL G418 and 2.5 μ g/mL puromycin. Healthy small colonies (~ 5 –10 cells) of transduced cells were picked using cloning cylinders. Genetically engineered BMSCs were maintained in DMEM supplemented with 10% Tet System Approved FBS (Tet-FBS; Clontech Laboratories), 400 μ g/mL G418, and 2.5 μ g/mL puromycin. For *in vitro* experiments, cells at passage 16 were plated at 2.5×10^3 cells/cm², and then treated with Dox starting the next day. The medium was exchanged daily.

Quantitative reverse transcription–polymerase chain reaction

Cells were harvested in TRIZOL reagent (Invitrogen) for RNA isolation and subsequent cDNA synthesis (Bio-Rad). Fifty to one-hundred nanograms of cDNA was amplified in duplicates in each 40-cycle reaction using an iCycler[™] (Bio-Rad) with annealing temperature set at 60°C, ABsolute[™] QPCR SYBR[®] Green Fluorescein Mix (ABgene, Thermo Fisher Scientific), and custom-designed qRT-PCR primers (Table 1) (Thermo Fisher Scientific). A melt curve was used to assess the purity of amplification products. mRNA levels were normalized to β -actin, and gene expression was calculated as fold change using the comparative C_T method. If not otherwise indicated, experimental groups were compared to control groups (Dox-suppressed, Tet-Off-EV empty vector).

SDF-1 α and SDF-1 β ELISA

Whole-cell lysates were prepared in lysis buffer containing protease inhibitors (Complete Lysis-M EDTA-free buffer, Roche Diagnostics, Indianapolis, IN). The anti-SDF-1 capture antibody (R&D Systems, Minneapolis, MN) in the sodium bicarbonate buffer pH 9.4 was bound to MaxiSorp[™] 96-well plates (Nunc, Thermo Fisher Scientific) overnight. Plates were blocked for 2 h with 1% bovine serum albumin (BSA) in PBS. Murine SDF-1 α or SDF-1 β (PeproTech, Rocky Hill, NJ) standards and samples were incubated for 2 h before incubating with the biotinylated anti-SDF-1 α and anti-SDF-1 β detection antibody (R&D Systems), respectively. Streptavidin-horseradish peroxidase (HRP) (R&D Systems) was incubated for 20 min followed by the substrate reagent (R&D Systems) for 40 min. 2 N sulfuric acid was added to stop the enzymatic

TABLE 1. LIST OF OLIGONUCLEOTIDE PRIMER SEQUENCES FOR QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Gene		Sequence (5'-3')	Product size	Accession number
SDF-1 α	Fwd	GTGAGAACATGCCTAGATTTACCC	105	NM_021704
	Rev	ATAGGACTCAGGGACAATTACCAA		
SDF-1 β	Fwd	GCTGAAGAACAACAACAGACAAGT	98	NM_013655
	Rev	CTCACATCTTGAGCCTCTTGTTTA		
Runx2	Fwd	GGAAAGGCACTGACTGACCTA	103	NM_009820
	Rev	ACAAATTCTAAGCTTGGGAGGA		
BMP-2	Fwd	TGTTTGGCCTGAAGCAGAGA	83	NM_007553
	Rev	TGAGTGCCTGCGGTACAGAT		
OCN	Fwd	ATTAGGACCTGTGCTGCCCTA	120	NM_007541
	Rev	GGAGCTGCTGTGACATCCATAC		
Collal	Fwd	GCCCATTAGCCGGTATGTTATTA	112	MMU50767
	Rev	TCCCTGGTACCTATGGAGACTGT		
β -actin	Fwd	TGACAGACTACCTCATGAAGATCC	103	NM_007393
	Rev	ACATAGCACAGCTTCTCTTTGATG		

SDF-1 α / β , stromal cell-derived factor-1 α / β ; Runx2, runt-related transcription factor 2; BMP-2, bone morphogenetic protein-2; OCN, osteocalcin; Collal, collagen alpha 1 type 1.

color reaction and absorbance was read at 450 nm. SDF-1 α and SDF-1 β protein expression was calculated using standard curves and normalized to total protein, which was quantified using the EZQ[®] Protein Quantitation Kit (Invitrogen).

Osteogenic differentiation

Genetically engineered BMSCs were treated with DMEM supplemented with 5% Tet-FBS, 0.25 mM ascorbic acid (Sigma-Aldrich), 0.1 μ M dexamethasone (Sigma-Aldrich), and 10 mM β -glycerophosphate (Sigma-Aldrich) $\pm$ 100 ng/mL Dox for 21 days with daily medium exchange. The medium was supplemented with 100 ng/mL rhBMP-2 (R&D Systems), or vehicle control, for the first 24 h in culture to further stimulate osteogenic differentiation.

Detection and quantification of calcium mineral content

Alizarin Red S (ARS) staining was performed as described previously.³⁴ In brief, monolayers of genetically engineered BMSCs were washed with PBS and fixed in 3% paraformaldehyde (Sigma-Aldrich) for 30 min. Cells were stained with 40 mM ARS pH 4.1 (Sigma-Aldrich) for 15 min followed by washing with excess dH₂O. Stained monolayers were visualized by phase-contrast microscopy using an inverted microscope (Nikon, Melville, NY). For quantitative destaining, 10% acetic acid was added for 30 min. Samples were transferred to a 1.5-mL microcentrifuge tube, overlaid with mineral oil (Sigma-Aldrich), heated to 85°C for 10 min, and transferred to ice for 5 min. Following centrifugation at 14,000 rpm for 15 min, supernatants were removed and neutralized with 10% ammonium hydroxide. Aliquots were transferred to a 96-well plate and absorbance was read at 405 nm.

Quantification of alkaline phosphatase activity and osteocalcin production

Whole-cell lysates were prepared in lysis buffer containing protease inhibitors (Roche Diagnostics). Alkaline phosphatase (ALP) activity in genetically engineered BMSCs was determined after 3 days using a colorimetric assay kit (Cell Biolabs, San Diego, CA). Osteocalcin (OCN) production was analyzed after 21 days using an ELISA kit

(Biomedical Technologies, Stoughton, MA). Data were calculated using respective standard curves and normalized to total protein.

Western blotting

Genetically engineered BMSCs were kept in DMEM supplemented with 1% Tet-FBS $\pm$ 100 ng/mL Dox overnight to render cells quiescent. The next day, cells were incubated with the highly selective CXCR4 antagonist AMD3100 (Tocris Bioscience, Ellisville, MO) at 600 μ M, the specific MEK 1/2 inhibitor U0126 (Cell Signaling Technology, Danvers, MA) at 50 μ M, or vehicle control, for 4 h before stimulation with 300 ng/mL rhBMP-2 (R&D Systems) for 30 min. Whole-cell lysates were prepared in lysis buffer containing protease and phosphatase inhibitors (Roche Diagnostics). Equal amounts (15 μ g) of protein lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% Nu-PAGE[®] Bis-Tris gels (Invitrogen) and transferred to 0.2 μ m PVDF membranes (Millipore, Billerica, MA). Membranes were blocked with 5% BSA in TBST. The phosphorylation of intracellular extracellular signal-regulated kinases 1/2 (Erk1/2) and Smad1/5/8 was detected using specific primary antibodies (anti-phospho-Erk1/2, anti-Erk1/2, anti-phospho-Smad1/5/8: Cell Signaling Technology; anti-Smad1/5/8: Santa Cruz Biotechnology, Santa Cruz, CA; anti- β -actin: Sigma-Aldrich) followed by HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Subsequently, the blots of phosphorylated Erks and Smads were stripped using stripping buffer (Western Blot Stripping Buffer, Pierce, Thermo Fisher Scientific) and reprobed for the detection of the respective total protein. Bound antibodies were visualized with the ECL detection system (Pierce, Thermo Fisher Scientific) on autoradiography film (Denville Scientific, Metuchen, NJ). The intensity of immunoreactive bands was quantified using Photoshop CS4 v11.0 (Adobe Systems, San Jose, CA).

Transwell migration assay

Conditioned media of genetically engineered BMSCs were collected after 24 h and used freshly. Parental BMSCs were

transferred to DMEM supplemented with 1% FBS overnight. The conditioned media were added to the lower wells of a Fluorometric CytoSelect 96-well Transwell Migration Assay Kit plate (8- μ m pore size) (Cell Biolabs). Parental cells were lifted with trypsin/EDTA, resuspended at a density of 8.0×10^5 cells/mL in a low serum medium, added to the upper wells of the transwell plate, and exposed to conditioned media for 8 h to allow migration across the porous membrane. Migratory cells were visualized by fluorescence microscopy using an inverted microscope (Carl Zeiss, Jena, Germany) equipped with an Exfo X-Cite 120 fluorescence lamp (Lumen Dynamics, Mississauga, Ontario, Canada), detached from the lower surface of the transwell membrane, and lysed. Total DNA in the cell lysates was stained with CyQuant[®] GR Dye and fluorescence was read at 485/535 nm.

Statistical analysis

Experiments were performed three independent times ($n=3-10$). All data are expressed as means $\pm$ SD. The Student's *t*-test and analysis of variance (ANOVA) followed by

the Tukey's *post hoc* test were used to determine mean differences between groups. Null hypotheses were rejected at the 0.05 level. Data were analyzed using SigmaPlot 11.0 software (Systat Software, Inc., Chicago, IL).

Results

Abundance of SDF-1 splice variants

SDF-1 α and SDF-1 β are splice variants derived from the same gene, and only differ by 4 C-terminal amino acids (Fig. 1A). We analyzed the relative abundance of these splice variants in whole BM cells, enriched BMSCs, and sera from 3-month-old animals (Fig. 1B-E). SDF-1 α mRNA expression was significantly greater than SDF-1 β in whole BM cells (22.3 ± 10.0 -fold, $p < 0.0001$) and BMSCs (10.7 ± 4.4 -fold, $p < 0.0001$). Additionally, the relative abundance of both splice variants was significantly higher in whole BM cells compared to enriched BMSCs (SDF-1 α : 74.4 ± 30.8 -fold; SDF-1 β : 155.4 ± 74.0 -fold; $p < 0.005$) (Fig. 1C). A similar trend was found for SDF-1 α and SDF-1 β protein expression in the BM cell and BMSC lysates (SDF-1 α : 14.6 ± 6.6 -fold, $p < 0.001$; SDF-1 β : 2.0 ± 0.4 -fold, $p < 0.05$) (Fig. 1D). Interestingly, SDF-1 α

FIG. 1. SDF-1 α and SDF-1 β splice variants are differentially expressed in whole BM cells and BMSCs, and can be found in the peripheral circulation. **(A)** Schematic of the murine SDF-1 gene map, alternatively spliced SDF-1 α and SDF-1 β mRNAs, and amino acid sequences with accession numbers. The white boxes depict 5'- and 3'-untranslated regions of the respective exon. The italicized amino acids in gray show the differences compared to the human SDF-1 α and SDF-1 β protein sequences, respectively. The additional four C-terminal amino acids of SDF-1 β are underlined. **(B)** Schematic of sample preparation from 3-month-old C57BL/6J male mice. **(C)** Normalized SDF-1 α mRNA expression was significantly greater than SDF-1 β in whole BM cells and BMSCs ($###, $$$, p < 0.0001$). Both SDF-1 α and SDF-1 β mRNA expression was significantly greater in whole BM cells compared to BMSCs ($***p < 0.005$). **(D)** Intracellular SDF-1 α and SDF-1 β protein levels by ELISA confirmed this trend ($*p < 0.05$, $***p < 0.001$). In contrast, SDF-1 α protein levels were significantly lower than SDF-1 β in whole BM cells and BMSCs ($^{\#}, \$p < 0.05$). **(E)** Serum ELISA revealed circulating levels of both splice variants; however, SDF-1 α was significantly lower than SDF-1 β ($*p < 0.05$). SDF-1, stromal cell-derived factor-1, BMSC, bone marrow-derived mesenchymal stem/stromal cells.

protein levels were significantly lower than SDF-1 β in whole BM cells (3.2 ± 0.9 -fold, $p < 0.05$) and BMSCs (22.5 ± 10.0 -fold, $p < 0.05$), despite the overall higher mRNA levels (Fig. 1D). The presence of SDF-1 β protein in systemic circulation has not been reported to date. Serum ELISA analysis revealed substantial amounts of SDF-1 α and SDF-1 β in 3-month-old C57BL/6J mice. Circulating levels of SDF-1 α protein were significantly lower than SDF-1 β (1.7 ± 0.3 -fold, $p < 0.05$), confirming previous results (Fig. 1E). These studies show, for the first time, that detectable quantities of SDF-1 β exist in peripheral circulation and, together with its expression in whole BM and BMSCs, provide the rationale for studying the effect of this isoform on BMSC osteogenic differentiation.

Regulation of SDF-1 β transgene expression

Since basal SDF-1 β mRNA is not relatively highly expressed in BMSCs, but has a potential greater bioavailability than the alpha isoform, we sought to develop and characterize a transgenic *in vitro* system to overexpress SDF-1 β in BMSCs. Initially, we confirmed the sequence accuracy of the retroviral constructs following SDF-1 β gene transfer and selected for the highest possible expression of Tet-components (data not shown). We then characterized the expression of genetically engineered SDF-1 β in Tet-Off-SDF-1 β BMSCs (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Cells were cultured for 24 h with increasing concentrations of Dox (0.001–1000 ng/mL) and SDF-1 β mRNA levels were analyzed relative to baseline without Dox. A sharp drop in SDF-1 β mRNA expression to $53.8\% \pm 6.1\%$ of the baseline level was observed with as little as 0.01 ng/mL Dox. This trend continued and SDF-1 β transgene expression was undetectable starting at 10 ng/mL Dox (Supplementary Fig. S1A). For time course studies, Tet-Off-SDF-1 β BMSCs were supplemented with 100 ng/mL Dox over the course of 1 week (0.5–168 h). After 3 h, the SDF-1 β mRNA expression was reduced to $34.9\% \pm 8.8\%$ of the baseline level. This trend continued and SDF-1 β transgene expression was undetectable starting at approximately 18 h (Supplementary Fig. S1B). Subsequently, 24 h was selected due to complete suppression of SDF-1 β transgene expression in Tet-Off-SDF-1 β BMSCs.

SDF-1 β transcript and protein analysis

Using the optimized *in vitro* experimental conditions, we quantified the total SDF-1 β mRNA and protein expression in genetically engineered BMSCs (Fig. 2). SDF-1 β mRNA levels in Tet-Off-SDF-1 β BMSCs were significantly increased compared to Dox-suppressed and Tet-Off-EV controls (Tet-Off-SDF-1 β : –Dox, 29.7 ± 8.3 -fold, +Dox, 1.0 ± 0.3 -fold; Tet-Off-EV: –Dox, 1.0 ± 0.3 -fold, +Dox, 1.1 ± 0.4 -fold; $p < 0.0005$); no differences in SDF-1 α mRNA levels were found between any of the groups (Fig. 2A).

Genetically engineered BMSCs were then characterized with regard to intracellular and secreted/extracellular SDF-1 α and SDF-1 β levels normalized to total protein. We utilized custom-designed ELISAs with splice variant-specific detection antibodies. In accordance with mRNA expression results, intracellular SDF-1 β protein levels in Tet-Off-SDF-1 β BMSCs were significantly increased compared to Dox-suppressed and Tet-Off-EV controls (Tet-Off-SDF-1 β : –Dox, 1828.4 ± 429.0 pg/mg, +Dox, 390.5 ± 57.7 pg/mg; Tet-Off-EV: –Dox, 351.7 ± 84.1 pg/mg, +Dox, 306.1 ± 73.0 pg/mg; $p < 0.0005$); SDF-1 α protein levels were comparable among all groups (Fig. 2B). Secreted extracellular SDF-1 β levels in the culture media mirrored those seen in the BMSC lysates (Tet-Off-SDF-1 β : –Dox, 1012.4 ± 165.0 pg/mg, +Dox, 178.5 ± 80.9 pg/mg; Tet-Off-EV: –Dox, 205.9 ± 43.4 pg/mg, +Dox, 141.3 ± 77.3 pg/mg; $p < 0.0005$); again, no differences were found for secreted SDF-1 α levels between groups (Fig. 2C).

In vitro osteogenic differentiation

To investigate the role of SDF-1 β in osteogenic differentiation *in vitro*, we cultured genetically engineered BMSCs for 21 days in the osteogenic induction medium. In the groups treated with BMP-2, it was supplemented only for the first 24 h, as continuous stimulation was found to result in saturated levels of mineralization by ~12 days (data not shown). Calcium mineral content in monolayers was detected and quantified by standard ARS staining. SDF-1 β in Tet-Off-SDF-1 β BMSCs significantly accelerated (by 14 days vs. 21 days; data not shown) and enhanced osteogenic differentiation relative to Dox-suppressed controls ($A_{405 \text{ nm}}$: –Dox, 1.8 ± 0.2 , +Dox, 1.4 ± 0.1 ; $p < 0.005$) (Fig. 3A, B) and potentiated the known osteoinductive properties of BMP-2 ($A_{405 \text{ nm}}$: –Dox, 2.9 ± 0.2 , +Dox, 1.5 ± 0.5 ; $p < 0.005$) (Fig. 3A, B). ARS

FIG. 2. SDF-1 β mRNA and protein levels are significantly increased relative to controls. (A) Normalized SDF-1 β mRNA and (B, C) intra- and extracellular SDF-1 β protein levels in tetracycline (Tet)-Off-SDF-1 β BMSCs were significantly increased compared to Dox-suppressed and Tet-Off-EV controls (24 h, ± 100 ng/mL Dox, *** $p < 0.0005$); SDF-1 α mRNA and protein levels were comparable among groups.

FIG. 3. SDF-1 β significantly enhances osteogenic differentiation and potentiates osteoinductive characteristics of bone morphogenetic protein-2 (BMP-2) *in vitro*. (**A**, **C**) Representative Alizarin Red S (ARS)-stained BMSC monolayers (21 days, $\pm$ 100 ng/mL Dox, $\pm$ 100 ng/mL BMP-2 for the first 24 h in an osteoinductive medium, 20 $\times$, scale bar 100 μ m). (**B**, **D**) Colorimetric quantification of extracted ARS showed significantly increased mineralization in Tet-Off-SDF-1 β with, or without, BMP-2 stimulation relative to Dox-suppressed and Tet-Off-EV controls (** p < 0.005).

staining of Tet-Off-EV control cells confirmed this finding, as no differences were found among groups, all expressing basal levels of SDF-1 (A_{405 nm}: -Dox, -BMP-2, 1.1 ± 0.1 , +Dox, -BMP-2, 1.1 ± 0.1 ; -Dox, +BMP-2, 1.4 ± 0.2 , +Dox, +BMP-2, 1.4 ± 0.2) (Fig. 3C, D).

qRT-PCR analysis of key osteogenic markers

Next, we investigated mRNA expression levels of key osteogenic markers in Tet-Off-SDF-1 β BMSCs and Tet-Off-EV control cells after 21 days of osteogenic differentiation with, or without, BMP-2 stimulation (Fig. 4). Runt-related transcription factor (Runx) 2, BMP-2, OCN, and collagen alpha 1 type 1 (Col1a1) transcript levels were 1.1–1.7-fold greater in Tet-Off-SDF-1 β BMSCs compared to Dox-suppressed controls, which were not statistically significant for Runx2 ($p=0.454$) and BMP-2 ($p=0.460$), but close to significance for OCN ($p=0.051$) and Col1a1 ($p=0.056$) (Fig. 4A–D). In contrast, SDF-1 β significantly potentiated gene expression levels in BMP-2-stimulated Tet-Off-SDF-1 β BMSCs relative to Dox-suppressed controls (Runx2: -Dox, 2.5 ± 0.5 -fold, +Dox, 1.0 ± 0.2 -fold, $p < 0.005$; BMP-2: -Dox, 3.6 ± 0.7 -fold, +Dox, 1.0 ± 0.4 -fold, $p < 0.005$; OCN: -Dox, 7.6 ± 0.7 -fold, +Dox, 1.0 ± 0.4 -fold, $p < 0.0001$; Col1a1: -Dox, 4.3 ± 0.9 -fold, +Dox, 1.0 ± 0.2 -fold, $p < 0.005$) (Fig. 4A–D). Transcript levels of osteogenic markers in Tet-Off-EV control cells were comparable among groups, verifying our previous findings (Fig. 4E–H).

ALP activity and OCN production in vitro

To further characterize the role of SDF-1 β in osteogenic differentiation *in vitro*, genetically engineered BMSCs were harvested after 3 and 21 days of osteogenic differentiation, respectively, to look at early and late markers for osteogenesis. ALP activity and OCN production were normalized to

total protein and quantified (Supplementary Fig. S2). ALP activity in Tet-Off-SDF-1 β BMSCs was significantly increased compared to Dox-suppressed controls (-Dox, -BMP-2, 21.1 ± 0.3 U/mg, +Dox, -BMP-2, 13.8 ± 1.9 U/mg; $p < 0.05$). In addition, SDF-1 β significantly potentiated ALP activity in BMP-2-stimulated Tet-Off-SDF-1 β BMSCs relative to Dox-suppressed controls (-Dox, +BMP-2, 60.0 ± 0.6 U/mg, +Dox, +BMP-2, 49.2 ± 3.4 U/mg; $p < 0.05$) (Supplementary Fig. S2A). ALP activity levels were comparable among Tet-Off-EV controls (Supplementary Fig. S2B). These findings were in agreement with transcript analyses, which showed significantly enhanced ALP mRNA levels in Tet-Off-SDF-1 β BMSCs relative to Dox-suppressed controls at 3 days, while other markers were not yet elevated (data not shown).

ELISA analysis revealed that OCN production in Tet-Off-SDF-1 β BMSCs without BMP-2 stimulation was 1.6-fold greater relative to Dox-suppressed controls, which was not quite significant ($p=0.053$). However, SDF-1 β significantly enhanced OCN protein levels in BMP-2-stimulated Tet-Off-SDF-1 β BMSCs compared to Dox-suppressed controls (-Dox, +BMP-2, 5.3 ± 0.8 ng/mg, +Dox, +BMP-2, 1.4 ± 0.5 ng/mg, $p < 0.05$); no differences were found among Tet-Off-EV control groups (Supplementary Fig. S2C, D).

Intracellular signal transduction

Next, we investigated the potential role of SDF-1 β in modulating BMP-2 signaling utilizing Tet-Off-SDF-1 β BMSCs and Tet-Off-EV control cells. The selective CXCR4 antagonist AMD3100 and the specific MEK 1/2 inhibitor U0126 were used to block total SDF-1-mediated G-protein-coupled receptor signaling. We examined the phosphorylation of Erk1/2 and Smad1/5/8, two key players in SDF-1 and BMP-2 signaling, respectively (Fig. 5). Western blot analysis showed that the ratios of phosphorylated (p) to

FIG. 4. SDF-1 β significantly upregulates key osteogenic markers during BMP-2-stimulated osteogenic differentiation *in vitro*. SDF-1 β significantly potentiated (A, E) runt-related transcription factor (Runx2), (B, F) BMP-2, (C, G) osteocalcin (OCN), and (D, H) collagen alpha 1 type 1 (Col1a1) transcript levels in BMP-2-stimulated Tet-Off-SDF-1 β BMSCs compared to Dox-suppressed and Tet-Off-EV controls (21 days, ± 100 ng/mL Dox, ± 100 ng/mL BMP-2 for the first 24 h in an osteoinductive medium, ** $p < 0.005$, *** $p < 0.0001$).

total Erk1/2 and Smad1/5/8 in Tet-Off-SDF-1 β BMSCs were comparable to Dox-suppressed controls and Tet-Off-EV control cells in normal control media (Fig. 5A–F). In contrast, SDF-1 β significantly potentiated phosphorylation of Erk1/2 and Smad1/5/8 in BMP-2-stimulated Tet-Off-SDF-1 β BMSCs relative to Dox-suppressed normal controls (pErk1/2/Erk1/2: –Dox, +BMP-2, 6.1 ± 0.2 -fold, +Dox, +BMP-2, 2.5 ± 0.2 -fold; pSmad 1/5/8/Smad 1/5/8: –Dox, +BMP-2, 90.5 ± 2.1 -fold, +Dox, +BMP-2, 39.6 ± 1.8 -fold; $p < 0.0001$)

(Fig. 5A–C). No differences between groups were found in BMP-2-stimulated Tet-Off-EV controls (pErk1/2/Erk1/2: –Dox, +BMP-2, 2.5 ± 0.1 -fold, +Dox, +BMP-2, 2.5 ± 0.1 -fold; pSmad 1/5/8/Smad 1/5/8: –Dox, +BMP-2, 35.9 ± 1.8 -fold, +Dox, +BMP-2, 39.1 ± 1.8 -fold) (Fig. 5D–F). Pretreatment with AMD3100 or U0126 completely abolished the BMP-2-induced Erk1/2 and Smad1/5/8 phosphorylation in Tet-Off-SDF-1 β BMSCs; a similar trend was found in Tet-Off-EV control cells.

FIG. 5. SDF-1 β significantly potentiates phosphorylation of extracellular signal-regulated kinases 1/2 (Erk1/2) and Smad1/5/8 *in vitro*. **(A, D)** Representative Western blots of key players involved in SDF-1/CXC chemokine receptor 4 (CXCR4) and BMP-2/BMPRI/II signal transduction pathways. Densitometry quantification of immunoreactive bands revealed that relative phosphorylation of **(B, E)** intracellular Erk1/2 and **(C, F)** Smad1/5/8 in BMP-2-stimulated Tet-Off-SDF-1 β BMSCs was significantly increased relative to Dox-suppressed and Tet-Off-EV controls (± 100 ng/mL Dox, ± 300 ng/mL rhBMP-2 for 30 min, *** $p < 0.0001$). Pretreatment with AMD3100, or U0126, abolished these effects (± 600 μ M AMD3100, ± 50 μ M U0126 for 4 h).

BMSC migration *in vitro*

One key feature of SDF-1 is its ability to mediate the migration of CXCR4-expressing cells. This chemotactic property is involved in both mobilization and homing of stem cell populations in the bone marrow. Chemotactic migration of parental BMSCs in response to conditioned media, derived from Tet-Off-SDF-1 β BMSCs, was significantly greater compared to Dox-suppressed and Tet-Off-EV controls (Tet-Off-SDF-1 β : -Dox, 5522 ± 453 RFU, +Dox, 4183 ± 94 RFU; Tet-Off-EV: -Dox, 4071 ± 469 RFU, +Dox, 4185 ± 313 RFU; $p < 0.05$) (Fig. 6A, B). As expected, the migratory response of parental BMSCs was comparable across all control groups, since the conditioned media from these cells contain similar basal levels of both SDF-1 α and SDF-1 β . Parental BMSCs migrated toward normal culture media supplemented with exogenous SDF-1 α or SDF-1 β in a comparable fashion, and this chemotactic effect was blocked when the CXCR4 antagonist AMD3100 was added to the media (data not shown).

Discussion

In the present study, our aim was to use genetically engineered Tet-Off-SDF-1 β BMSCs, which conditionally overexpress SDF-1 β , to investigate whether SDF-1 β could enhance BMP-2-stimulated osteogenic differentiation and chemotaxis of BMSCs *in vitro*. SDF-1 β was chosen over SDF-

1 α due to its greater resistance to proteolytic cleavage by carboxypeptidases N and M, which is mediated directly through its C-terminal extension, and indirectly through protecting the N-terminus via enhanced binding to cell- and extracellular matrix molecules, suggesting that SDF-1 β may be convenient for local applications directly into the BM microenvironment.²²⁻²⁴ We showed that both SDF-1 α and SDF-1 β were highly expressed in cells from whole BM as well as isolated BMSCs of 3-month-old C57BL/6J mice; SDF-1 α showed an expected greater abundance compared to SDF-1 β . In contrast, SDF-1 α protein expression was found to be significantly lower than SDF-1 β in both whole BM cells and BMSCs, despite the overall higher transcript levels. This suggests less proteolytic clearance of SDF-1 β and longer bioavailability in the highly vascularized BM microenvironment as a result of its additional C-terminal amino acids. These results are in agreement with data suggesting that SDF-1 is not only expressed by endothelial cells, stromal cells, and osteoblasts in the BM, but that it can also be found in the endosteal region as well as throughout the entire thickness of bone.³⁵ Similar levels of SDF-1 α and SDF-1 β mRNA levels were found in bone extracts, indicating that bone tissues could serve as a reservoir of both major SDF-1 splice variants.³⁵ In our studies, serum analysis, using custom-designed ELISAs, revealed the presence of both splice variants. Despite the more than 20-fold lower SDF-1 β mRNA expression compared to SDF-1 α , levels of SDF-1 β protein in

FIG. 6. SDF-1 β significantly promotes chemotaxis of CXCR4-expressing BMSCs. **(A)** Representative fluorescence micrographs of migratory BMSCs in response to conditioned media from genetically engineered BMSCs (8 h, $\pm$ 100 ng/mL Dox, 20 $\times$, scale bar 100 μ m). **(B)** Fluorometric quantification of migratory BMSCs at 8 h showed significantly increased chemotactic response to media derived from Tet-Off-SDF-1 β compared to Dox-suppressed and Tet-Off-EV controls ($*p < 0.05$). Color images available online at www.liebertpub.com/tea

circulation remained detectable, again suggesting a longer half-life of the less abundant splice variant. This is the first report of immunoreactive circulating levels of SDF-1 β *in vivo*, and is consistent with the beta isoform of SDF-1 possessing significant resistance to proteolytic cleavage by serum proteases, a slower turnover, and consequently an increased bioavailability. Taken together, our findings suggest the unexpected possibility that SDF-1 β , independent of the more labile SDF-1 α , may also act as a modulator of BMSC homing and mobilization to distant bone injury sites as well as a potential key player in normal and pathological bone remodeling.

These initial studies prompted the development of the Tet-Off-SDF-1 β BMSCs to specifically examine the contribution of SDF-1 β to BMSC osteogenic differentiation *in vitro*. Administration of 100 ng/mL Dox for 24 h was sufficient to completely and stably suppress (without leaking) SDF-1 β transgene expression. The Tet-Off technology for overexpression of SDF-1 β in BMSCs was selected over previously attempted protocols, that is, preconditioning²⁶ and adenoviral-mediated gene transfer,²⁷ because of its advantage to tightly regulate transgene expression *in vitro* and *in vivo*.³⁶ In the Tet-Off system, doxycycline prevents binding of the Tet-controlled transactivator to the Tet-promoter, and thus abolishes transcription of the downstream target gene.³⁶ We demonstrated that SDF-1 β mRNA expression by Tet-Off-SDF-1 β BMSCs was 30-fold increased compared to controls and this increase was accompanied by a similar augmentation in immunoreactive SDF-1 β protein levels, validating our model.

Previous studies have suggested a critical role of the SDF-1/CXCR4 signaling axis in regulating BMP-2-induced osteogenic differentiation of progenitor cells and ectopic

bone formation.^{37–39} Among osteoinductive growth factors, BMPs are well known to drive bone formation *in vitro* and *in vivo*⁴⁰ by enhancing the recruitment of osteoblast progenitor cells and angiogenesis as well as promoting the osteogenic differentiation of MSCs.^{41,42} We asked whether it was possible for SDF-1 β to potentiate BMP-2-regulated osteogenic differentiation. Using our novel Tet-Off-SDF-1 β BMSCs, we demonstrated for the first time that SDF-1 β significantly accelerated and enhanced calcium mineral deposition compared to controls, independent of BMP-2 costimulation. Second, using qRT-PCR, we showed that key osteogenic markers Runx2, BMP-2, OCN, and Col1a1 were significantly upregulated during BMP-2-stimulated osteogenic differentiation relative to controls. Runx2, the bone-related product of the *Cbfa1* gene, is a critical transcription factor for osteoblast and hypertrophic chondrocyte differentiation. It also regulates the osteoblast-specific expression of OCN, the most specific osteoblast gene.⁴³ In addition, BMPs are known to directly upregulate Runx2, and subsequently activate downstream genes necessary for the osteoblast phenotype, such as Col1a and OCN. Hence, our studies suggest that SDF-1 β , independent of SDF-1 α , can potentiate the osteoinductive properties of BMP-2, and thus may be involved in the mesenchymal-to-osteoblast transition of BMSCs. Furthermore, we showed that SDF-1 β significantly enhanced ALP activity, which is essential for both mineralization and the production of bone matrix proteins, and OCN protein production following brief costimulation with BMP-2.

These findings led to the investigation of the interaction between the SDF-1/CXCR4 and BMP-2/BMPRI/II signal transduction pathways. Binding of SDF-1 to CXCR4 initiates divergent signal transduction pathways. Among those, the

mitogen-activated protein kinase (MAPK) pathway plays a major role in regulating embryogenesis, cell differentiation, proliferation, and death. The MAPK cascades ultimately effect cellular function through the activation of Erk1/2.¹⁸ Signaling by BMP-2, through a functional complex of type I (BMPRI) and type II (BMPRII) receptors, results in phosphorylation of receptor-regulated Smad1/5/8. These Smad proteins then complex with the common partner protein Smad4 and translocate into the nucleus where they act to regulate gene expression. In addition, BMP receptors can initiate other signaling pathways, distinct from the Smad pathway, resulting in the activation of p38 MAPK and JNK.⁴⁴

We show here that phosphorylation of intracellular Erk1/2 and Smad1/5/8 was significantly enhanced in Tet-Off-SDF-1 β BMSCs compared to controls, suggesting that SDF-1 β can potentiate BMP-2 signal transduction during osteogenic differentiation. Given that the MAPK pathway is a predominant mechanism of CXCR4-mediated signaling, it is possible that this pathway is directly involved in the regulation of Smad1/5/8 signaling. Blocking the SDF-1/CXCR4 signaling axis at the extracellular receptor level with AMD3100 or the intracellular MAPK pathway signaling level with U0126 resulted in complete inhibition of BMP-2-stimulated activation of Erk1/2 and Smad1/5/8, further supporting the finding of SDF-1 β potentiation of BMP-2 signaling. Additional studies from our laboratory have shown that systemic application of AMD3100 for 4 weeks significantly decreased the BM cell mRNA levels of osteogenic markers, such as Runx2, BMP-2, OCN, and Col1a1 in C57BL/6j mice, suggesting impaired bone formation (unpublished data). Previous studies have reported that SDF-1-dependent stimulation of CXCR4 signaling is a prerequisite for BMP-2-mediated osteoinduction^{37,38}; however, the exact mechanisms have not yet been elucidated. Our studies reveal the specific contribution of SDF-1 β to the regulation of BMP-2 signaling *in vitro*. Future studies aim to investigate the detailed kinase-mediated temporal activation mechanisms and elucidate the identity of the molecular players involved in regulating this cross talk between the SDF-1- and BMP-2-mediated signal transduction pathways.

In recent years, evidence has accumulated suggesting that SDF-1 is upregulated at sites of injury. This chemokine serves as a potent chemoattractant to recruit circulating or resident CXCR4-expressing stem and progenitor cells, which subsequently undergo tissue-specific differentiation and aid in tissue regeneration.^{6,12–14} In fracture healing, the contribution of circulating MSCs to new bone formation during the acute phase of the bone repair has been shown to depend largely on the involvement of the SDF-1/CXCR4 axis.^{15–17} A shortcoming of these studies, however, is that no clear distinction between involved SDF-1 splice variants was made, and thus the specific contribution of SDF-1 β remains unknown. Given the recent report that similar levels of SDF-1 α and SDF-1 β mRNA levels can be found in bone extracts, suggesting that bone tissues may serve as a reservoir for both major SDF-1 splice variants,³⁵ we asked whether it was possible for secreted SDF-1 β to stimulate a chemotactic response of distant BMSCs *in vitro*. We demonstrated for the first time that, independent of SDF-1 α , SDF-1 β significantly promoted the migratory response of BMSCs compared to control groups, suggesting that genetically engineered Tet-Off-SDF-1 β BMSCs secrete bioavailable levels of chemoat-

tractant. Stimulation with either exogenous SDF-1 α or SDF-1 β resulted in a comparable chemotactic response of BMSCs, and this effect was abolished with AMD3100. These findings suggest that SDF-1 β may exert its biological activity in both an autocrine and paracrine fashion *in vivo*. SDF-1 α , which lacks the C-terminal lysine, displays a significantly reduced ability to stimulate pre-B-cell proliferation and chemotaxis compared to SDF-1 β , which is protected against carboxypeptidases N and M-mediated cleavage due to its additional four C-terminal amino acids compared to SDF-1 α .^{22,24} Hence, this differential processing could provide a mechanism for fine regulation of the local and distant functional activity of SDF-1, favoring SDF-1 β over SDF-1 α . It appears possible that SDF-1 α with a shorter active half-life may stimulate more acute CXCR4 signaling in the BM, whereas the more stable SDF-1 β may be critical in mediating chronic effects on BMSC osteoinduction. Future studies aim to investigate the *in vivo* role of SDF-1 in bone homeostasis to specifically elucidate the contribution of SDF-1 β to new bone formation, both locally and systemically, in bone development, and disorders, such as fracture repair and osteoporosis.

Conclusion

In the present study, we provide novel evidence that SDF-1 β , a more potent splice variant compared to SDF-1 α , is unexpectedly abundant and plays a critical role in regulating BMP-2-stimulated osteogenic differentiation of CXCR4-expressing BMSCs *in vitro*. Furthermore, our studies shed new light on how differential processing of SDF-1 splice variants may provide a mechanism for controlling its autocrine and paracrine chemokine activity. Using our novel Tet-Off-SDF-1 β BMSCs, we demonstrate the potential of SDF-1 β , independent of the more studied SDF-1 α , to serve as a modulator of BMSC migration, suggesting it may also have a greater role in homing and mobilization to distant bone injury sites than expected. Our studies may provide new opportunities to support BMSC-based therapies for improving regenerative medicine approaches to treat acute and chronic bone injuries.

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Disclosure Statement

The authors have no conflict of interests.

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