A Novel Regulatory Role for Stromal-derived Factor-1 Signaling in Bone Morphogenic Protein-2 Osteogenic Differentiation of Mesenchymal C2C12 Cells*

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Stromal-derived factor-1 (SDF-1) is a chemokine with important functions in development and postnatal tissue homeostasis. SDF-1 signaling via the G-protein-coupled receptor CXCR4 regulates the recruitment of stem and precursor cells to support tissue-specific repair or regeneration. In this study we examined the contribution of SDF-1 signaling to osteogenic differentiation of mesenchymal C2C12 cells induced by bone morphogenic protein 2 (BMP2). Blocking SDF-1 signaling before BMP2 stimulation by treatment with siRNA, antibodies against SDF-1 or CXCR4, or the G-protein-coupled receptor inhibitor pertussis toxin strongly suppressed BMP2 induction of osteogenic differentiation in C2C12 cells, as evidenced by an early decrease in the expression of the myogenesis inhibitor Id1, the osteogenic master regulators Runx2 and Oxs, the osteoblast- associated transcription factors JunB, Pflz, Mxs2, and Dlx5, and later of the bone marker proteins osteocalcin and alkaline phosphatase. Similarly, blocking SDF-1/CXCR4 signaling strongly inhibited BMP2-induced osteogenic differentiation of ST2 bone marrow stromal cells. Moreover, we found that the interaction between SDF-1 and BMP2 signaling was mediated via intracellular Smads and MAPK activation. Our data provide the first evidence for a co-requirement of the SDF-1/CXCR4 signaling axis in BMP2-induced osteogenic differentiation of C2C12 and ST2 cells and, thus, uncover a new potential target for modulation of osteogenesis.

Stromal-derived factor-1 (SDF-1), which is also referred to as CXC group of chemokine ligand 12 (CXCL12) or pre-B cell stimulating factor (1, 2), functions via activation of the G-protein-coupled receptor (GPCR), CXC chemokine receptor 4 (CXCR4) (3–5). The interaction between SDF-1 and CXCR4 leads to cytoskeletal rearrangement in cells and integrin-mediated cell-cell or cell-matrix adhesion (6), which in turn results in directional migration of CXCR4-expressing cells toward a gradient of SDF-1 (6, 7). The SDF-1/CXCR4 signaling axis is essential for normal physiological functions, including the inflammatory response (8), blood homeostasis (9), maintenance of the immune system (10, 11), and bone remodeling (12). Moreover, the expression of CXCR4 by different types of tumor cells is thought to be responsible for metastatic spread of tumors to organs where high levels of SDF-1 are present (7, 13–16). CXCR4 also serves as a co-receptor for the entry of human immunodeficiency type I virus (7, 17). During development, CXCR4 expression is a prerequisite for germ cell migration to SDF-1-expressing gonads (18). Mice lacking SDF-1 or CXCR4 die perinatally and display multiple defects in the developing brain, heart, and intestine as well as in the vasculature and hematopoietic tissues (19–21).

Expression of SDF-1 and CXCR4 has also been detected in stem cells and various precursor cells, such as in human bone marrow-derived mesenchymal stem cells (22) and in the pluripotent mesenchymal C2C12 cell line (23). SDF-1 and CXCR4 are predominantly expressed by these stem and precursor cells at a primitive stage, but their expression rapidly declines once cells commit to a differentiation pathway (22, 23), suggesting that SDF-1 signaling has a role in the initiation of their differentiation. Moreover, SDF-1 expression is up-regulated at sites of injury such as in massive liver injury and in ischemic stroke, where it serves as a potent chemoattractant to recruit circulating or resident CXCR4-expressing stem cells in support of tissue-specific repair or regeneration (24–30). At such sites, SDF-1 is thought to promote growth and survival of stem and precursor cells and protect against cytokine-induced apoptosis.
until the differentiation of these cells is triggered in response to environmental cues (24–30). Although a complex array of local paracrine and autocrine signaling molecules as well as physiochemical and mechanical stimuli is likely to be required for tissue repair and regeneration, these observations suggest that SDF-1 signaling contributes to the differentiation of stem and precursor cells.

Among the local stimuli that are known to induce the osteogenic differentiation of stem and precursor cells are bone morphogenic proteins (BMPs), which are growth factors originally identified from bovine bone based on their ability to induce de novo ectopic bone formation (31, 32). At the cellular level, BMPs bind to type I and II serine/threonine kinase receptors, and both the Smad and the mitogen-activated protein kinase (MAPK) signaling machinery relay the BMP signal from the cell surface to the nucleus to regulate target gene expression (31, 32). Although it is well established that several members of the BMP family such as BMP-2, -4, -6, and -7 induce the differentiation of stem or precursor cells into osteoblastic lineages (33–39), much remains to be learned about the molecular mechanisms that underlie BMP-dependent osteoinduction. In this study we provide the first evidence for a critical role of SDF-1/CXCR4 in the differentiation of mesenchymal C2C12 and ST2 marrow stromal cells into osteoblastic cells after their treatment with BMP2.

**MATERIALS AND METHODS**

**Reagents and Antibodies**—Recombinant (r) BMP2 protein, rSDF-1 protein, anti-SDF-1, anti-CXCR4 neutralizing antibodies, and isotype-matched control antibodies were purchased from R&D Systems (Minneapolis, MN). 12G5 anti-CXCR4 antibody was purchased from BD Biosciences (San Jose, CA). The CXCR4 antagonist AMD3100 and Trizol reagent were obtained from Sigma. Reagents for reverse transcription were from Bio-Rad. Primary antibody was purchased from Santa Cruz (Santa Cruz, CA). Enhanced chemiluminescence (ECL) detection reagent was purchased from Amersham Biosciences.

**Cell Culture**—C2C12 myoblastic cells were acquired from American Type Culture Collection (ATCC, Manassas, VA). ST2 marrow stromal cells were obtained from the RIKEN cell bank (Tsukuba, Japan). For maintenance of stock plates, C2C12 cells or ST2 cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium or α-minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Identical culture conditions were used in experiments examining the role of SDF-1 signaling during BMP2-stimulated osteogenic differentiation, except that the serum concentration was lowered to 2% to reduce cell proliferation. rBMP2 protein or rSDF-1 protein was added at 300 ng/ml, anti-SDF-1 or anti-CXCR4 neutralizing antibody was added at 100 μg/ml, 12G5 antibody was added at 10 μg/ml, and AMD3100 was added at 630 μM unless indicated otherwise. The doses were selected for optimal signal transduction or inhibition based on the manufacturer’s recommendations or previous publications (22, 23, 40, 41).

**Quantitative Reverse Transcription-PCR**—Total RNA was extracted from C2C12 cells using Trizol reagent, 1 μg of total RNA from each sample was reverse-transcribed (Applied Biosystems), and levels of target gene expression were quantified in real time PCR by using a MyiQ Single Color Real Time PCR detection system (Bio-Rad). Relative expression of target genes was calculated based on ΔCT values, which are differences in the number of threshold cycles between the target gene and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as described previously (42). The primer sequences of target genes that were analyzed in this study are listed in Table 1.

**Small Interfering RNA (siRNA) Knockdown of Gene Expression**—SDF-1 siRNA and non-targeting control siRNA (Dharmacon, Chicago, IL) were used to transfect C2C12 cells at 100 nM. 24 and 48 h after transfection, the effectiveness of siRNA knockdown was determined by quantifying the level of SDF-1 gene expression by real time PCR as described above and by measuring the amount of SDF-1 protein released into the cell supernatant by commercially available ELISA (R&D Systems). Subsequently, SDF-1 siRNA or control siRNA- transfected cells were stimulated with rBMP2 protein.

**GPCR Inhibition**—C2C12 or ST2 cells were seeded overnight in 6-well plates at a density of 2 × 10⁵ cells per well. Pertussis toxin (PTX, Calbiochem), which blocks signaling via G₁-protein-coupled GPCRs (7, 43, 44), was used to treat cells at 200 ng/ml in serum-free medium for 4 h at 37 °C. Subsequently, PTX-treated cells were incubated with rBMP2 or rSDF-1 protein using the concentrations indicated above.

**Transient Transfection and Luciferase Assay**—The SBE-Luc reporter construct, in which BMP-specific Smad binding ele-
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ments (SBE) are repeated 12 times (GCCG_{12}) in an osteocalcin minimal promoter that drives the expression of the luciferase (Luc) gene, was obtained from Drs. Ming Zhao and Stephen Harris (University of Texas Health Science Center, San Antonio, TX). The day before transfection, C2C12 cells were plated in 100-mm diameter culture dishes at 3 × 10^7 cells per dish and then co-transfected with SBE-Luc and a pCMV-βGal plasmid (Stratagene, Cedar Creek, TX) at 5 μg each per dish by using a modified bovine serum albumin transfection kit following the manufacturer’s protocol (Stratagene). After transfection, cells were divided into 12-well plates at a density of 1 × 10^7 cells per well and subsequently treated with rBMP2, rSDF-1, anti-SDF-1, anti-CXCR4, or in combination. Luciferase activities in cells were assessed 2 and 18 h after stimulation using a LMaxII luminometer (Molecular Devices, Sunnyvale, CA) and normalized to the activity of β-galactosidase.

Western Blotting—C2C12 cells were kept in low serum medium with 0.5% bovine serum albumin overnight to render the cells quiescent. Subsequently, cells were stimulated with rBMP2-, anti-SDF-1-, or anti-CXCR4-neutralizing antibodies in serum-free medium. Cell lysates were collected in lysis buffer (5 mM sodium fluoride, 5 mM sodium vanadate, 1% Triton X-100, and 5 mM EDTA in 50 mM Tris, pH 7.5) after 15 min of stimulation, and total cellular proteins were measured by the bicinchoninic acid assay (Pierce). Equal loading of 10-μg aliquots of total protein from each sample was performed for Western blotting (Nupage Bis-Tris gel systems, Invitrogen) as described previously (42, 45). The phosphorylation of intracellular Smad1/5/8 and MAPK components Erk1/2 and p38 was detected by using the appropriate primary antibodies followed by secondary goat anti-rabbit IgG. Subsequently, the blots of phosphorylated Smads and MAPKs were stripped in 0.1 M glycine solution (pH 2.0) and re-probed for the detection of respective total protein kinase. Bound antibodies were visualized with ECL reagents and exposure of premium autoradiography film (Denville, Metuchen, NJ). The intensity of immunoreactive bands was quantified by gel image analysis software (ImageJ_1.32, NIH).

Determination of Osteogenic Differentiation—Osteogenic differentiation of cells was evaluated by the synthesis of bone matrix proteins found in mature osteoblasts during matrix deposition and mineralization and the expression of several key transcription factors that regulate the expression of bone-specific genes. The amount of osteocalcin (OCN) was detected in cell supernatants by a commercially available ELISA kit (Biomedical Technologies, Stoughton, MA). The activity of alkaline phosphatase (ALP) was determined in cell lysates by colorimetric assay (Sigma) and normalized to total cellular protein (Pierce) as described previously (42, 45).

Real time PCR was also performed to quantify the expression of runt-related transcription factor 2 (Runx2) and osterix (Osx), which are two “master” regulators of the BMP2-osteogenic pathway (46, 47). Additionally, the expression of several osteoblast-associated early transcription factors, such as JunB (a proto-oncogene member of the activator protein-1 transcription factor family) and Plzf (promyelocytic zinc finger protein) (48, 49); Msx2 (msh homeobox homolog 2) and Dlx5 (distalless homeobox 5) (50, 51) was further quantified by real time PCR. All relative expression of target genes was calculated based on ΔCT values and normalized to GAPDH as described above.

Statistical Analysis—All experiments were repeated two to three times independently, with three to six samples included in each treatment group. Measurements in each experiment were run in triplicate. Results were reported as the mean ± S.D. Differences among treatment groups were compared by analysis of variance followed by the Tukey-Kramer post-hoc test, with values of p < 0.05 considered to be statistically significant.

RESULTS

SDF-1 and CXCR4 Expression during BMP2-induced Osteogenic Differentiation—As a first step toward evaluating a potential role of SDF-1 and CXCR4 in BMP2-induced osteogenic differentiation of C2C12 cells, we analyzed the expression of both genes by quantitative real time PCR (Fig. 1). We found that stimulation with BMP2 for 4 days at 100 ng/ml reduced the expression of CXCR4 (Fig. 1A) and SDF-1 (Fig. 1B) by 19 and 33%, respectively, when compared with base-line levels in control cells stimulated with medium only. Furthermore, CXCR4 expression was reduced 35% by BMP2 stimulation at 300 ng/ml and 49% by BMP2 stimulation at 500 and 1000 ng/ml (Fig. 1A). Similarly, SDF-1 gene expression was reduced by 78% upon stimulation with BMP2 at 300 ng/ml and 75 and 90% by BMP2 stimulation at 500 and 1000 ng/ml, respectively, in comparison to control cells (Fig. 1B).

When we analyzed the time course of SDF-1 protein released from C2C12 cells after stimulation with 300 ng/ml BMP2, we observed a time-dependent decrease in SDF-1 protein levels (Fig. 1C). Although stimulation with BMP2 for 1 day did not significantly alter the level of SDF-1 protein when compared with control cells, SDF-1 synthesis declined 59% upon BMP2 stimulation for 4 days and continuously decreased to 88 and 94% at 7 and 10 days of BMP2 stimulation, respectively (Fig. 1C).

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Role of SDF-1 Expression in BMP2-induced OCN Synthesis in C2C12 Cells—To evaluate whether SDF-1 is required for BMP-induced osteogenic differentiation, we tested how inhibition of SDF-1 expression by siRNA affects synthesis of OCN, a well-established marker for osteoblast differentiation. OCN synthesis was strongly increased in C2C12 cells in response to BMP2 stimulation (Fig. 2A). However, pretreatment of cells with SDF-1 siRNA for 2 days led to a 57% lower OCN protein concentration when compared with cells treated with BMP2, whereas the addition of non-targeting control siRNA did not significantly affect OCN synthesis (Fig. 2A). The inhibitory effect of SDF-1 siRNA was largely reversed by the addition of an excess of rSDF-1 (300 ng/ml), which produced an OCN level comparable with that in cells stimulated with BMP2 alone (Fig. 2A). Moreover, the addition of SDF-1 before BMP2 stimulation further enhanced BMP2-induction of OCN synthesis by 33% (Fig. 2A).

The 59% reduction of SDF-1 mRNA expression in cells after 1 day of treatment with SDF-1 siRNA compared with cells treated with control siRNA was confirmed by quantitative real-time PCR (Fig. 2B). After 2 days of incubation with SDF-1 siRNA versus control siRNA, the level of SDF-1 protein was reduced by 50% (Fig. 2C). There were no significant differences in SDF-1 levels between cells treated with control siRNA and medium only (Fig. 2, B and C).

GPCR Inhibition Abrogates BMP2-induced OCN Synthesis in C2C12 Cells—SDF-1 is a ligand for the GPCR CXCR4 (22, 23). Therefore, we tested how pertussis toxin (PTX), a broad spectrum inhibitor that uncouples Gi proteins from GPCRs and, thus, blocks CXCR4 signaling, affects BMP2-induced osteogenesis. Treatment with PTX strongly reduced BMP2-induced OCN synthesis (93% reduction) to a level comparable with control cells treated with medium only (Fig. 3). In addition, the inhibitory effect of PTX on BMP2-induced OCN synthesis could not be rescued by the addition of SDF-1 (Fig. 3), corroborating that SDF-1 signaling in C2C12 cells is G-protein-dependent, and that is required for BMP2-induced OCN synthesis.

To further confirm the involvement of CXCR4 in BMP2-induced OCN synthesis, we tested how a CXCR4 antagonist, AMD3100 (41), and a monoclonal antibody directly against an extracellular domain of CXCR4, 12G5 (40), affect BMP2-induced OCN expression in these cells. In the presence of 12G5 and AMD3100, a 41 and 54% reduction in BMP2-induced OCN synthesis, respectively, was observed (Fig. 3), which further corroborates the requirement of CXCR4 in BMP2 induction of OCN synthesis. Treatment with PTX, 12G5, or AMD3100 alone did not result in significant changes in OCN levels when compared with control cells maintained in medium only (data not shown).

The SDF-1/CXCR4 Signaling Axis Is a Prerequisite for BMP2-induced OCN Synthesis and ALP Activity in C2C12 Cells—To provide additional lines of evidence for a role of SDF-1/CXCR4 signaling in BMP2-induced osteogenesis, we tested whether function blocking monoclonal antibodies against SDF-1 or its receptor affect synthesis of OCN and ALP, marker proteins for later stages of osteogenic differentiation. Pretreatment of C2C12 cells with anti-SDF-1 or anti-CXCR4 antibodies for 1 h reduced BMP2-stimulated OCN synthesis by 44%, and simultaneous addition of both antibodies further decreased OCN synthesis by 63% (Fig. 4A). The inhibitory effect of anti-SDF-1 neutralizing antibodies (at 100 μg/ml) could be rescued in the presence of an excess of SDF-1 at 300 ng/ml (Fig. 4A). Treatment with PTX, 12G5, or AMD3100 was less effective than treatment with SDF-1 antibodies alone at reducing OCN synthesis, and combined treatment with anti-SDF-1 and anti-CXCR4 antibodies reduced OCN synthesis by 64% (Fig. 4A).
Role of SDF-1 in BMP-2-stimulated expression of bone matrix proteins. C2C12 cells were treated with rBMP2 alone at 300 ng/ml for 4 days. In parallel, cells were pretreated with anti-SDF-1 and anti-CXCR4 at 100 μg/ml each, a combination of anti-SDF-1 and anti-CXCR4 for 1 h at 37°C, or pre-treated with anti-SDF-1 for 1 h followed by the addition of 300 ng/ml rSDF-1 for another hour before BMP2 stimulation. As controls, cells were maintained in medium only. A, synthesis of OCN protein under different treatment conditions. B, enzymatic activity of ALP normalized to total protein. * and #, p < 0.05 versus medium only control and rBMP2 stimulation, respectively. To address the temporal requirement of SDF-1, C2C12 cells were pretreated with PTX at 200 ng/ml for 4 h at 37°C followed by rBMP2 stimulation for 4 days. In comparison, cells were stimulated with rBMP2 for 1 h at 37°C followed with treatment of PTX. C, percentage of OCN synthesis of that in cells stimulated with BMP2 alone. *, p < 0.05 versus rBMP2 stimulation.

anti-SDF-1 and anti-CXCR4 lowered the ALP activity by 76% (Fig. 4B). The effects of anti-SDF-1 could be rescues by an excess of SDF-1, resulting in ALP levels that were comparable with those seen in cells treated only with BMP2 (Fig. 4B). Identical doses of isotype-matched control antibodies for anti-SDF-1 and anti-CXCR4 had no significant effect on OCN synthesis or ALP activity compared with control cells maintained in medium only (data not shown).

In the experiments described above C2C12 cells were pre-treated with antibodies against SDF-1 or CXCR4 before the addition of BMP2. To further address the temporal requirement of SDF-1/CXCR4 signaling with respect to BMP2-induced osteogenic differentiation, we added anti-SDF-1 or anti-CXCR4 1 h after the addition of BMP2 but did not observe significant effects on the BMP2-dependent induction of OCN synthesis under these conditions (data not shown). Moreover, when the GPCR inhibitor PTX was added 1 h after BMP2 stimulation, the level of BMP2-induced OCN synthesis was affected to a much lesser extent (30% reduction) than when cells were pretreated with PTX before the addition of BMP2 (93% reduction, Fig. 4C).

Blocking SDF-1/CXCR4 Signaling Also Inhibits BMP2-induced OCN Synthesis in ST2 Cells—To address whether SDF-1 plays a role in osteogenic differentiation of a different cell line, we tested how blocking SDF-1/CXCR4 affects BMP2-induced OCN synthesis in ST2 cells, a mouse bone marrow-derived stromal cell line, which also undergoes osteogenic differentiation upon BMP2 stimulation (52). In ST2 cells a 78% reduction in BMP2-induced OCN synthesis by anti-SDF-1 treatment was observed, whereas anti-CXCR4 or PTX inhibition reduced OCN synthesis to levels that were comparable with those in untreated control cells (Fig. 5).

SDF-1/CXCR4 Signaling Is Involved in BMP2-dependent Activation of Smad and MAPK—Upon BMP2 binding, the activated BMP type I receptors phosphorylate Smad proteins and MAPK components (31, 32). To further delineate the pathway by which SDF-1 contributes to BMP2-induced osteogenic differentiation, we examined how blocking SDF-1 signaling affects the activation of Smad and MAPK by BMP2.

Western blot analysis showed that treatment of C2C12 cells with BMP2 for 15 min led to a 4-fold increased phosphorylation of Smad1/5/8 when compared with control cells (Fig. 6A). This BMP2-stimulated Smad1/5/8 phosphorylation was reduced by 50% after pretreatment with anti-SDF-1 and by 54% after pretreatment with anti-CXCR4 (Fig. 6A). There were no changes in the levels of total Smad1/5 proteins regardless of treatment (Fig. 6A). In addition, isotype-matched control antibodies for anti-SDF-1 or anti-CXCR4 did not affect Smad phosphorylation when compared with control cells maintained in medium only (data not shown).

To further corroborate that SDF-1 affects Smad signaling, C2C12 cells were transfected with SBE-Luc, a luciferase reporter construct for Smad-mediated OCN promoter activity. SBE-Luc-transfected C2C12 cells were then either stimulated with BMP2 alone or pretreated with anti-SDF-1 or anti-CXCR4 before the addition of BMP2. Two hours after treatment with BMP2 alone the luciferase activity was 5-fold greater than in control cells stimulated with medium only (data not shown), and after 18 h of BMP2 stimulation the luciferase activity was 8-fold greater than that in control cells (Fig. 6B). Treatment with anti-SDF-1 or anti-CXCR4 antibodies strongly decreased the level of BMP2-induced luciferase activity at both time points (anti-SDF1 by 96% at 2 h and 78% at 18 h, anti-CXCR4 by 84% at 2 h and 78% at 18 h; Fig. 6B, the 2-h time point data are not shown). The effects of anti-SDF-1 antibodies could be largely reversed by the addition of SDF-1 protein (Fig. 6B and data not shown for the 2-h time point).

Western blot analysis of the phosphorylation of two components of the MAPK signaling pathway, p38 and Erk1/2, was used to determine whether anti-SDF-1 or anti-CXCR4 antibodies also affected BMP2 signaling through this pathway (Fig. 7). BMP2 treatment for 15 min strongly stimulated the phos-
Blocking SDF-1/CXCR4 Increases Expression of MyoD and Reduces Expression of Inhibitor of Differentiation-1 (Id1) in BMP2-treated C2C12 Cells—BMP2 is known to promote differentiation of C2C12 cells along the osteogenic pathway while preventing myogenic differentiation, particularly in low serum culture conditions, which strongly favor myogenesis in the absence of BMP2 (33–39). We, therefore, tested whether blocking SDF-1/CXCR4 affects the expression of MyoD, an early marker for myogenesis (53), and the expression of Id1, a BMP-induced inhibitor for myogenesis (54), within 6 h of BMP2 stimulation. Although there was no significant difference in MyoD expression between cells treated with BMP2 and control cells maintained in medium only, pretreatment with anti-SDF-1 increased MyoD expression by 3.4-fold over controls after 1 and 6 h of BMP2 stimulation (Fig. 8A). Similarly, anti-CXCR4 increased MyoD expression by 2- and 2.6-fold over controls at 1 and 6 h of BMP2 stimulation, respectively (Fig. 8A).

Moreover, BMP2 stimulation increased Id1 gene expression by 9.2-fold over control cells at 1 h of stimulation and 6-fold over control after 6 h of stimulation (Fig. 8B). However, pretreatment with anti-SDF-1 resulted in a 51% decrease in BMP2-induced Id1 expression at 1 and 6 h of BMP2 stimulation (Fig. 8B). Treatment with anti-CXCR4 reduced BMP2-stimulated Id1 expression by 43% after 1 h and 47% after 6 h of BMP2 stimulation (Fig. 8B). Taken together, these data suggest that blocking SDF-1/CXCR4 signaling promotes myogenic differentiation of C2C12 cells at the expense of osteogenic differentiation. Treatment with isotype-matched control antibodies for anti-SDF-1 and anti-CXCR4 did not affect the expression of MyoD and Id1 when compared with control cells maintained in medium only (data not shown).

Blocking SDF-1/CXCR4 Inhibits the Temporal Expression of Osteoblast-associated Transcription Factors—Downstream of either the BMP2-Smad or BMP-MAPK pathways is a cascade of transcription factors that are involved in the regulation of osteoblast gene expression. These transcription factors include the master regulators Runx2 and Osx, which increase the syn-
thesis of the bone matrix proteins described above (46, 47), JunB and Plzf, two early transcription factors upstream of Runx2 (48, 49), and Msx2 and Dlx5, two early transcription factors upstream of Osx (50, 51). Dlx5 is also thought to mediate Runx2 expression (55). We, therefore, tested whether blocking SDF-1/CXCR4 affects the expression of these factors at different time points after the addition of BMP2.

As shown in Fig. 9A, Runx2 expression was induced by BMP2 at 1 h of stimulation (4.1-fold) and reached peak expression at 6 h (11.2-fold). Osx expression (Fig. 9B) was increased by BMP2 at 6 h (78-fold), suggesting a slightly delayed expression pattern. Both BMP2-induced Runx2 and Osx expression declined at 24 h and lasted for 4 days (Fig. 9, A and B). However, in both cases treatment with anti-SDF-1 or anti-CXCR4 strongly reduced BMP2-stimulated Runx2 and Osx expression at all time points (Fig. 9, A and B). BMP2- induced JunB expression (Fig. 9C) was highest after 1 h of stimulation (16-fold) and then declined at 6 h and continued to decline until day 4. Similarly, Plzf expression (Fig. 9D) was increased by BMP2 at 1 h (11-fold), reached a peak at 6 h (65-fold), and declined afterward. After 1 h JunB expression was strongly reduced by anti-SDF-1 and anti-CXCR4 treatment when compared with BMP2-stimulated cells (Fig. 9C). Similarly, BMP2-induced Plzf expression was also significantly inhibited by treatment with anti-SDF-1 or anti-CXCR4 during the entire time course, particularly after 1 and 24 h of stimulation, when its expression was reduced to levels comparable with that in control cells (Fig. 9D).

Finally, BMP2-dependent Msx2 expression (Fig. 9E) was slightly increased at 1 h, reached a peak between 6 h (12.5-fold) and 24 h, and then declined at day 4. Dlx5 expression (Fig. 9F) showed a slightly different pattern as it was increased 6 h after BMP2 stimulation (45-fold), was highest at 24 h (164-fold), and declined at day 4. Both BMP2-induced Msx2 and Dlx5 expression were also effectively blocked by treatment with anti-SDF-1 or anti-CXCR4 during the time course and were at comparable levels to control cells at day 4 (Fig. 9, E and F).

**DISCUSSION**

The chemokine SDF-1 and its receptor CXCR4 are known regulators of chemotactic migration of various types of cells, including stem and precursor cells (8–16, 22, 23). The results presented here provide the first evidence to our knowledge that SDF-1/CXCR4 signaling plays an important role in BMP2-induced osteogenic differentiation of C2C12 cells and ST2 cells and, thus, uncover a novel role for these two molecules in the regulation of osteogenic differentiation of mesenchymal precursor cells.

Consistent with the results of a previous study, which reported that the CXCR4 receptor was predominantly expressed by undifferentiated muscle satellite cells (C2C12 and G7 cells) before myotube formation (23), we also detected an initially high expression of SDF-1 and CXCR4 in C2C12 cells. However, after the addition of BMP2, the levels of SDF-1 and CXCR4 dropped over time. These findings are similar to those
of a study with human mesenchymal stem cells, in which a drop in SDF-1 expression was observed when these cells were cultured in osteogenic medium containing dexamethasone (22). Moreover, in vivo studies have identified high expression of SDF-1 in immature mesenchymal stem cells surrounding blood capillaries in bone marrow but not in mature osteoblasts lining bone surfaces and osteocytes within the bone matrix (13, 25, 56). The drop in SDF-1/CXCR4 expression after treatment with BMP2 is consistent with two models of how SDF-1/CXCR4 signaling could affect osteogenic differentiation. The first is that expression of SDF-1 and CXCR4 must be reduced for osteogenic differentiation to proceed. The second model is that SDF-1 and CXCR4 are required for initiation of BMP2-dependent osteogenesis but are then no longer needed, and therefore, their expression decreases during osteogenic differentiation. To distinguish between these two models, we examined the role of SDF-1/CXCR4 signaling in osteogenic differentiation of mesenchymal C2C12 cells in more detail.

When blocking antibodies against SDF-1 or CXCR4 were added to C2C12 cells before the addition of BMP2, the phosphorylation of Smad proteins and MAPK components was reduced, suggesting that SDF-1 is involved in the early phases of BMP2-dependent osteoinduction. Moreover, blocking SDF-1/CXCR4 before the addition of BMP2 also resulted in decreased expression of several osteoblast genes, which serve as markers for early stages of osteogenic differentiation. These included the myogenesis inhibitor Id1 and the transcription factors JunB and Plzf, and their downstream target Runx2. Furthermore, in both C2C12 and ST2 cells, blocking SDF-1/CXCR4 reduced expression of markers for later stages of osteogenic differentiation, including OCN, a bone matrix protein abundantly expressed by mature osteoblasts during mineralization of bone (57). Consistent with this observation, we found that other steps in the intracellular MAPK p38 and Erk pathways were also affected, such as the expression of early osteogenic markers Msx2 and Dlx5 and of the downstream master regulator Otx as well as the synthesis of bioactive ALP, which regulates the initiation of mineralization (58). The broad effects of SDF-1/CXCR4 on these distinct steps of the BMP2-osteogenic pathway suggest that there is a previously unrecognized co-requirement for SDF-1 and BMP2 signaling for osteogenic induction in C2C12 cells. Moreover, these findings suggest that SDF-1 is provided in sufficient amounts by C2C12 cells to fulfill the co-requirement, such that only BMP2 must be added to induce osteogenic differentiation. Finally, because blocking SDF-1/CXCR4 signaling in C2C12 cells also increased expression of the early myogenesis marker, MyoD, this suggests that SDF-1/CXCR4 is also involved in preventing myogenic differentiation of C2C12 cells.

In this context it should be noted that, whereas C2C12 cells are commonly employed as a model system to study both myogenic and osteogenic differentiation, the temporal expression of genes associated with osteogenesis in these cells, including Runx2, Otx, Dlx5, and Msx2, differs from that in other osteogenic cell lines, such as ST2 (59, 60), C3H10T1/2 (59), and ROB-C26 cells (61). Moreover, OCN, the most abundant non-collagenous protein of the bone matrix, serves as a late marker of osteoblast differentiation in several osteogenic lines, yet in C2C12 cells and ST2 cells significantly increased OCN mRNA can be detected as early as 24 h after BMP2 stimulation (59, 60, 62). Future studies will, therefore, focus on determining how SDF-1/CXCR4 signaling affects differentiation of other osteogenic cell lines as well as primary osteoblasts derived from bone marrow. Finally, three OCN-related genes have been identified in mice (osteocalcin-1, osteocalcin-2, and osteocalcin-related gene) (63). Of these, osteocalcin-1 is the predominant form expressed in C2C12 cells in response to BMP2 stimulation (64), so it will be interesting to determine whether the expression of osteocalcin-2 and osteocalcin-related gene is also differentially regulated by SDF-1/CXCR4 in other osteogenic cultures.

The timing of blocking SDF-1/CXCR4 signaling to elicit a block of BMP2-induced osteogenesis in C2C12 cells appears to be crucial. The most pronounced effect of antibody blockage or PTX treatment was seen if these inhibitors were added before the addition of BMP2. If the antibodies or PTX were added afterward, we found that they were less effective or ineffective in inhibiting BMP2-induced OCN synthesis. This strongly suggests that SDF-1-dependent stimulation of GPCR signaling via CXCR4 is a prerequisite for BMP2 osteoinduction. The results of siRNA treatment are also consistent with this notion, because siRNA treatment for 2 days before the addition of BMP2 lowered the amount of SDF-1 produced by C2C12 cells that would be available for osteoinduction. Finally, the observation that treatment with antibodies had the strongest effect even though no new antibodies were added together with BMP2 suggests that the antibodies block SDF-1 that is associated with cells or the extracellular matrix as opposed to soluble SDF-1. This is consistent with the previously reported observation that SDF-1 exists in a soluble form released into the cell supernatant and in an insoluble form which is associated with the extracellular matrix (65). Taken together, these results suggest that the expression of SDF-1 and CXCR4 may decrease after osteogenic induction with BMP2 because SDF-1/CXCR4 are no longer required once the osteogenic differentiation pathway has been set in motion.

Previous studies showed that BMP2 failed to stimulate ALP activity and OCN mRNA expression in calvarial cells derived from mice deficient in cyclo-oxgenase (Cox)-2, an enzyme that controls the production of the GPCR ligand prostaglandin E2, whereas the expression of ALP and OCN was rescued by the addition of PGE2 (66). Because treatment with PTX completely blocked BMP2-stimulated osteogenesis in C2C12 cells, whereas inhibition of SDF-1/CXCR4 with antibodies or siRNA reduced but did not abolish BMP2-dependent osteogenesis, it appears likely that another GPCR besides CXCR4 has a role in SDF-1 signaling or that GPCR ligands other than SDF-1 might be crucial. The most pronounced effect of antibody blockage or PTX treatment was seen if these inhibitors were added before the addition of BMP2. If the antibodies or PTX were added afterward, we found that they were less effective or ineffective in inhibiting BMP2-induced OCN synthesis. This strongly suggests that SDF-1-dependent stimulation of GPCR signaling via CXCR4 is a prerequisite for BMP2 osteoinduction. The results of siRNA treatment are also consistent with this notion, because siRNA treatment for 2 days before the addition of BMP2 lowered the amount of SDF-1 produced by C2C12 cells that would be available for osteoinduction. Finally, the observation that treatment with antibodies had the strongest effect even though no new antibodies were added together with BMP2 suggests that the antibodies block SDF-1 that is associated with cells or the extracellular matrix as opposed to soluble SDF-1. This is consistent with the previously reported observation that SDF-1 exists in a soluble form released into the cell supernatant and in an insoluble form which is associated with the extracellular matrix (65). Taken together, these results suggest that the expression of SDF-1 and CXCR4 may decrease after osteogenic induction with BMP2 because SDF-1/CXCR4 are no longer required once the osteogenic differentiation pathway has been set in motion.

In summary, our results uncovered a novel regulatory role for SDF-1 and CXCR4 in BMP2-stimulated osteogenic differentiation of mesenchymal C2C12 and ST2 cells. SDF-1/CXCR4 sig-
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...naling appears to be a prerequisite for BMP2-dependent osteogenic signaling. Because only BMP2 is needed to induce osteogenesis in C2C12 cells, the SDF-1/CXCR4 signaling axis must be fully functional in these cells. These observations provide critical new insights into the mechanisms underlying the osteogenic differentiation of mesenchymal precursor cells and may potentially lead to better control of the differentiation of these cells to support tissue repair and regeneration. Finally, it will now be interesting to examine the role of SDF-1/CXCR4 signaling in non-osteogenic differentiation, such as in chondrogenesis or adipogenesis.

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