A Comprehensive Analysis of the Dual Roles of BMPs in Regulating Adipogenic and Osteogenic Differentiation of Mesenchymal Progenitor Cells

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Pluripotent mesenchymal stem cells (MSCs) are bone marrow stromal progenitor cells that can differentiate into osteogenic, chondrogenic, adipogenic, and myogenic lineages. Several signaling pathways have been shown to regulate the lineage commitment and terminal differentiation of MSCs. Here, we conducted a comprehensive analysis of the 14 types of bone morphogenetic protein (BMPs) for their abilities to regulate multilineage specific differentiation of MSCs. We found that most BMPs exhibited distinct abilities to regulate the expression of Runx2, Sox9, MyoD, and PPAR γ 2. Further analysis indicated that BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 effectively induced both adipogenic and osteogenic differentiation in vitro and in vivo. BMP-induced commitment to osteogenic or adipogenic lineage was shown to be mutually exclusive. Overexpression of Runx2 enhanced BMP-induced osteogenic differentiation, whereas knockdown of Runx2 expression diminished BMP-induced bone formation with a decrease in adipocyte accumulation in vivo. Interestingly, overexpression of PPAR γ 2 not only promoted adipogenic differentiation, but also enhanced osteogenic differentiation upon BMP-2, BMP-6, and BMP-9 stimulation. Conversely, MSCs with PPAR γ 2 knockdown or mouse embryonic fibroblasts derived from PPAR γ 2^{-/-} mice exhibited a marked decrease in adipogenic differentiation, coupled with reduced osteogenic differentiation and diminished mineralization upon BMP-9 stimulation, suggesting that PPAR γ 2 may play a role in BMP-induced osteogenic and adipogenic differentiation. Thus, it is important to understand the molecular mechanism behind BMP-regulated lineage divergence during MSC differentiation, as this knowledge could help us to understand the pathogenesis of skeletal diseases and may lead to the development of strategies for regenerative medicine.

Introduction

MESENCHYMAL STEM CELLS (MSCs) hold great promise for tissue bioengineering and regenerative medicine. MSCs are adherent marrow stromal cells that can self-renew and differentiate into osteogenic, chondrogenic, adipogenic, and myogenic lineages [1–5]. Although

primarily residing in within the bone marrow compartment [5–7], MSCs have been isolated from periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, and deciduous teeth [8]. MSCs represent a very small fraction (e.g., 0.001–0.01%) of the total population of nucleated cells in the marrow [3]. Currently, the biologic properties

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of MSCs, specifically with respect to their existence in the adult organism and postulation of their biological niche, are not well understood. It is essential to understand the molecular mechanisms that govern the stem cell potential and lineage-specific differentiation of MSCs because MSCs without directed differentiation may become tumorigenic in vivo [9,10].

Several signaling pathways have been implicated in regulating stem cell self-renewal and lineage commitment [11–16]. Bone morphogenetic proteins (BMPs) play an important role in regulating cell proliferation and differentiation during development [17,18] and have been shown to play an important role in stem cell biology [19,20]. BMPs belong to the TGF- β superfamily and consist of at least 15 members in humans [17,18,21,22]. Genetic disruptions of BMPs have resulted in various skeletal and extraskeletal abnormalities during development [21,23]. BMPs fulfill their signaling activity by interacting with the heterodimeric complex of two transmembrane serine/threonine kinase receptors, BMPR type I and BMPR type II [17]. The activated receptor kinases phosphorylate the transcription factors Smads 1, 5, or 8, which in turn form a heterodimeric complex with Smad4 in the nucleus and activate the expression of target genes in concert with other coactivators [17].

Although not well understood, BMPs play an important role in regulating osteoblast differentiation and subsequent bone formation [22,24–27]. We have demonstrated that several BMPs are potent inducers of osteogenesis of MSCs [27–32], and that osteogenic BMPs (e.g., BMP-2, BMP-6, and BMP-9) regulate a distinct set of downstream targets that may play a role in regulating BMP-induced osteoblast differentiation [27,30–33]. BMP-2, BMP-4, and BMP-7, in coordination with other signaling molecules or supplementary cofactors, have been shown to promote preadipocyte differentiation [34–46]. However, the biological effects of BMPs on multiple lineage commitment and terminal differentiation of MSCs have not comprehensively analyzed.

In this study, using a well-characterized mesenchymal stem cell line C3H10T1/2 [47] and primary mesenchymal progenitor cells, we conducted a comprehensive analysis of 14 types of BMPs for their ability to induce lineage-specific differentiation of MSCs. BMPs were shown to exhibit distinct abilities to regulate expression of the master regulators of four different lineages. When the osteogenic and adipogenic lineages were further analyzed, BMP-2, -4, -6, -7, and -9 were shown to effectively induce both adipogenic and osteogenic differentiation of MSCs in vitro and in vivo, although the commitment of MSCs to osteoblast or adipogenic lineage was shown to be mutually exclusive. Overexpression of Runx2 synergizes with BMP-induced osteogenic differentiation, while exerting not detectable effects on adipogenic differentiation. However, overexpression of PPAR γ 2 promotes both osteogenic and adipogenic differentiation in vitro and in vivo. Conversely, knockdown or deletion of PPAR γ 2 not only inhibited adipogenic differentiation, but also diminished BMP-induced ossification, suggesting that PPAR γ 2 may also play an important role in osteogenic differentiation. Taken together, findings from our in vitro and in vivo studies suggest that BMPs may play an important role in regulating multilineage commitment and terminal differentiation of MSCs. Understanding the regulatory mechanisms

behind BMP-regulated lineage divergence of MSC differentiation may lead to the development of novel therapeutic strategies for human disorders (such as osteoporosis and obesity) and for regenerative medicine.

Materials and Methods

Cell culture and chemicals

HEK293 and C3H10T1/2 cell lines were obtained from the ATCC (Manassas, VA), and were maintained in complete DMEM and Basal Medium Eagle, respectively. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

Adenoviral vectors expressing BMPs, PPAR γ 2, and Runx2

Recombinant adenoviruses expressing the 14 types of human BMPs (i.e., AdBMPs) were generated as previously described [28,29,48]. The coding regions for mouse PPAR γ 2 and Runx2 were amplified by PCR and subcloned into a shuttle vector for recombinant adenovirus generation using the AdEasy system [48,49]. The resultant vectors are designated as AdPPAR γ 2 and AdRunx2. An analogous adenovirus expressing GFP only (i.e., AdGFP) was used as a control [49–51].

Quantitative real-time PCR (qPCR) analysis

The qPCR was carried out as described [31–33]. Ten micrograms of total RNA were used to generate cDNA templates by reverse transcription with hexamer and Superscript II reverse transcriptase (Invitrogen). The first strand cDNA products were further diluted 5- to 10-fold and used as qPCR templates. The qPCR primers (Supplementary Table 1; Supplementary Table 1 is available online at <http://www.liebertpub.com/scd>) were 18-mers, designed by using the Primer3 program, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, to amplify the 3'-end (approximately 120 bps) of the mouse Runx2, Sox9, MyoD, or PPAR γ 2 genes. SYBR Green-based qPCR analysis was carried out by using the Opticon DNA Engine (MJ Research). The specificity of each qPCR reaction was verified by melting curve analysis and further confirmed by resolving the PCR products on 1.5% agarose gels. Ten-fold serially diluted pUC19 was used as a standard. Triplicate reactions were carried out for each sample. All samples were normalized by the expression level of GAPDH.

Alkaline phosphatase activity

Alkaline phosphatase activity was assessed by the colorimetric assay and/or histochemical staining assay as previously described [28–33].

Construction and verification of Runx2 and PPAR γ 2 knockdown vectors

We used our recently developed pSOS system to select and validate efficacious short interfering double-stranded RNA (siRNA) target sites of mouse Runx2 and PPAR γ 2 [52]. Briefly, we designed four pairs of oligonucleotides

containing siRNA target sites for mouse Runx2 or PPAR γ 2 using Dharmacon's *siDESIGN* program (Supplementary Table 1). The oligo pairs were annealed and subcloned into the *Sfi* I site of pSOS, resulting in pSOS-simRunx2 or pSOS-simPPAR γ 2. To assess the knockdown efficiency, we further subcloned the coding regions of mouse Runx2 and PPAR γ 2 into the pSOS-simRunx2 and pSOS-simPPAR γ 2, resulting in pSOS-simRunx2-WT and pSOS-simPPAR γ 2-WT, respectively. The pSOS-simRunx2-WT and pSOS-simPPAR γ 2-WT were transfected into HEK-293 cells, and GFP signal levels were used to assess the silencing efficiency of different siRNA target sites [52]. Authenticity of PCR amplified sequences and the oligonucleotide cassettes were verified by DNA sequencing. Cloning and construction details are available upon request.

Establishment of stable C3H10-Runx2-kd and C3H10-PPAR γ 2-kd lines

As the pSOS is a retrovirus-based vector, we pooled the three pSOS-simRunx2 or pSOS-simPPAR γ 2 plasmids and packaged retroviral viruses by transfecting HEK-293 cells with the packaging plasmid pAmpho [52]. The produced retroviral supernatants were used to infect subconfluent C3H10T1/2 cells (e.g., usually 3–4 rounds of infection within 24–36 h). The infected cells were selected against blasticidin S (Invitrogen) for 5–7 days. The empty pSOS vector was used to establish a control stable line. The stable lines were designated as C3H10-Runx-kd, C3H10-PPAR γ 2, and C3H10-Control.

PPAR γ 2 null mouse embryonic fibroblasts (MEFs)

MEFs derived from animals with the homozygous and heterozygous deletion of PPAR γ 2 (i.e., MEF-PPAR γ 2^{-/-} and MEF-PPAR γ 2^{+/-}) were obtained from Bruce Spiegelman of Dana Farber Cancer Institute.

Immunofluorescence staining

Immunofluorescence staining was carried out as described [31,32]. Briefly, subconfluent C3H10-Runx-kd and C3H10-Control cells were fixed with methanol at -20°C for 15 min and washed with PBS. The fixed cells were permeabilized, blocked with 10% goat serum, and followed by incubation with anti-Runx2 antibody. After washing, the cells were incubated with anti-mouse IgG secondary antibody labeled with Alexa Fluor 594 (Molecular Probes). The presence of Runx2 protein was examined under a fluorescence microscope. Stains without the primary antibody, or with control IgG, were used as negative controls.

Stem cell implantation

The use and care of animals were approved by the Institutional Animal Care and Use Committee. Subconfluent C3H10T1/2, its derivative lines, or MEFs were infected with AdBMPs, AdGFP, and/or AdPPAR γ 2 or AdRunx2 for 15 h, and collected for subcutaneous injection (5×10^6 cells per injection) into the flanks of athymic nude (nu/nu) mice (five animals per group, 4 to 6-week-old, male, Harlan Sprague

Dawley). Five weeks after implantation, animals were sacrificed and the implantation sites were retrieved for microCT analysis and histologic evaluation and other stains.

Hematoxylin and Eosin, Masson's Trichrome, and Alcian Blue staining

Retrieved tissues were decalcified, fixed in 10% formalin overnight, and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H&E). Masson's Trichrome stain was carried out as described [29]. Deparaffinized and rehydrated sections were stained with 1% Alcian Blue pH 2.5.

Oil Red-O staining

Decalcified and paraformaldehyde fixed tissues were subjected to frozen sectioning. The sections were rinsed with PBS, water and ethanol, and then stained with freshly prepared Oil Red-O solution (six parts saturated Oil Red-O dye in isopropanol plus four parts water) at 37°C for 15 min, followed by washing with 70% ethanol and PBS.

Transmission electron microscopy analysis

For transmission electron microscopy, MSCs were infected with AdGFP or AdBMPs for 7 or 14 days, and fixed with 0.5% glutaraldehyde (in 0.1 M sodium phosphate buffer, pH 7.4) at 4°C for 30 min. The fixed cells were collected by using cell scrapers followed by a brief centrifugation. The cell pellets were washed with 0.1 M sodium barbital buffer, pH 9.4, 2–3 times, and incubated in alkaline phosphatase (ALP) substrate solution (0.1 M barbital buffer, pH 9.4, 0.1 M β -glycerophosphate, 0.5 M MgCl₂, and 0.2 M CaCl₂) at room temperature for 60 min, followed by washing with 0.1 M sodium barbital buffer, pH 9.4 or PBS. The cell pellets were then incubated in precold 0.05 M lead nitrate for 4 min at room temperature and washed extensively with PBS. The cell pellets were fixed for additional 2 h in 2.5% glutaraldehyde, and postfixed for 2 h with 1% osmium tetroxide. The cell pellets were dehydrated in an ascending ethanol series washes and embedded in Epon 812. Serial ultrathin sections were stained with uranyl acetate and lead citrate, and then examined using a Zeiss 900 electron microscope. Magnifications, 7,000 $\times$ to 12,000 $\times$.

MicroCT analysis

The cone-beam micro-CT system used for acquiring mouse data consists of a microfocal X-ray source, an orthogonally-mounted rotary stage with object holder, and a CsI-coupled CMOS detector. The microfocal X-ray source (MX-20, Faxitron, USA) comprises of a tungsten anode with beryllium exit window, and it has a focal spot of 20 μ m and was operated at 28 KeV. The 14-bit digital camera (Bioptics, USA) includes a CsI scintillator plate and a 2048 $\times$ 1024 array of CMOS thin-film transistors and photodiodes with a pixel size of 50 μ m. The new developed back projection-filtration (BPF) algorithm with a unit appodization function was used for reconstructing volumetric mouse images from the acquired cone-beam data. Volume-rendered views were generated with opacity-weighted compositing

and gradient-based shading (VolView 2.0: Kitware, Inc) techniques.

Results

BMPs exert distinctive abilities to induce lineage-specific regulators of mesenchymal progenitor cells

We previously demonstrated that BMP-2, -6, and -9 are the most potent inducers of osteoblast differentiation [22,27–32]. Here, we sought to further determine how the 14 BMPs regulate multilineage commitment and differentiation of MSCs. We focused on four commonly known lineages derived from MSCs, and analyzed the expression of the known respective lineage-specific regulators: Runx2 (osteogenic), Sox9 (chondrogenic), PPAR γ 2 (adipogenic), and MyoD (myogenic). Most BMPs, except BMP-3 and BMP-8, exhibit a modest ability to up-regulate Runx2 expression (Fig. 1A). These results are intriguing because, although they are consistent with the recent observation that BMP-3 has been shown to inhibit osteogenesis [53], we and others have demonstrated that *only* BMP-2, -4, -6, -7, and 9 can induce *de novo* bone formation [29,54]. One possibility is that Runx2 is not only associated with osteogenesis. When the expression of chondrogenic regulator Sox9 was examined, we found that osteogenic BMP-2, -4, and -7 significantly induced Sox9 expression, whereas BMP-11, -10, -13, and -14 also up-regulated Sox9 expression (Fig. 1B). However, most BMPs, except BMP-11 and 15, exhibited a weak ability to induce the expression of the myogenic regulator MyoD under the same conditions (Fig. 1C). Lastly, we analyzed the expression of the adipogenic regulator PPAR γ 2 and found that BMP-2, -4, -5, -6, -7, -9, and -10 effectively induced PPAR γ 2 expression in MSCs (Fig. 1D).

BMPs regulate both osteogenic and adipogenic differentiation

The overlapping osteogenic and chondrogenic activities of several BMPs may not be surprising, as chondrogenesis and osteogenesis are closely related and well-coordinated processes during skeletal development. In fact, endochondral ossification is the major bone formation process for long bone growth [22]. However, we were intrigued by the strong adipogenic activity of several osteogenic BMPs. Although not well defined, it has long been speculated that the disrupted balance between osteogenesis and adipogenesis may be responsible for certain diseases, such as age-related osteoporosis [55–61]. We sought to further dissect the osteogenic versus adipogenic activity of the BMPs in MSCs. Consistent with our earlier findings, only BMP-2, -4, -6, -7, and -9 of the 14 BMPs were shown to induce the early osteogenic marker alkaline phosphatase in MSCs (Fig. 2A). Under the same conditions, the above five osteogenic BMPs were shown to effectively induce adipogenic differentiation, as assessed with Oil Red-O staining (Fig. 2B). We also analyzed the expression of the late marker of adipogenic differentiation lipoprotein lipase (LPL) upon BMP stimulation, and found that the five osteogenic BMPs exhibited the greatest ability to induce LPL expression in C3H10T1/2 cells (data not shown). Interestingly, BMP-12, -13, and -14 also exhibited a modest ability to induce terminal adipogenic differentiation, while BMP-5 and BMP-10, although able to induce PPAR γ 2 expression (Fig. 1D), did not induce significant Oil Red-O staining under the same assay conditions (Fig. 2B). Similar results were also obtained using primary MEFs and primary marrow stromal cells, although these progenitor cells exhibited a detectable level of spontaneous differentiation (data not shown).

FIG. 1. Quantitative analysis of the four lineage-specific regulators affected by the 14 types of BMPs in C3H10T1/2 MSCs. BMP-regulated expression of the lineage-specific regulators in MSCs was assessed with quantitative real-time PCR. The MSCs were infected with AdBMPs or AdGFP for 5 days prior to RNA isolation for real time-PCR analysis. (A) Expression of osteoblastic lineage regulator Runx2. (B) Expression of chondrogenic lineage regulator Sox9. (C) Expression of myogenic lineage regulator MyoD. (D) Expression of adipogenic lineage regulator PPAR γ 2. The dotted lines indicate 50% upregulation of gene expression.

FIG. 2. BMP-induced osteogenic and adipogenic differentiation in MSCs. (A) Osteogenic differentiation. C3H10T1/2 cells were infected with AdBMPs or AdGFP for 7 days, followed by histochemical staining of early osteogenic marker ALP activity. (B) Adipogenic differentiation. C3H10T1/2 cells were infected with AdBMPs or AdGFP for 20 days, followed by Oil Red-O staining. PPAR γ agonist Troglitazone (TRO, 10 μ M) was used as a positive control.

BMP-regulated osteogenic and adipogenic lineage commitment of MSCs is mutually exclusive

We next examined whether MSCs committed to adipogenesis or osteogenesis were derived from distinct cell populations. Briefly, C3H10T1/2 cells were infected with adenovirus vectors expressing BMP-2, -4, -6, -7, or

-9, or GFP control [22,28,29,31,32,48,50,51]. At day 14, cells were first stained for alkaline phosphatase activity, followed by Oil Red-O staining. As shown in Figure 3A, as an early osteogenic marker, alkaline phosphatase staining was still readily detectable in the cells stimulated with all five osteogenic BMPs at day 14. The osteogenic BMP-stimulated C3H10T1/2 cells also exhibited positive

Oil Red-O staining. These double staining experiments suggest that the BMP-induced osteogenic and adipogenic lineage commitments of mesenchymal progenitor cells may be mutually exclusive, although the progenitor cells undergoing osteogenic or adipogenic differentiation are closely located and are usually intertwined. These findings were also confirmed by electron microscope analysis. As shown in Fig. 3B, BMP-2 and BMP-9 effectively induced the cytoplasm membrane-bound ALP activity in certain cells, and lipid accumulation in other cells. It was also noted that the ALP positive cells were metabolically active and highly rich in organelles, such as mitochondria and ER apparatuses. Under this assay condition, we did not observe any cells that exhibited both high ALP activity and lipid accumulation, suggesting that BMP-induced osteogenic and adipogenic lineage commitment is mutually exclusive.

BMPs regulate both osteogenic and adipogenic differentiation of mesenchymal progenitor cells in vivo

We then determined whether BMPs could induce both osteogenic and adipogenic differentiation in vivo. We conducted a series of stem cell transplantation experiments, in which MSCs transduced with AdBMP-2, AdBMP-9, or AdGFP, were injected subcutaneously into athymic nude mice. After 5 weeks, the animals were sacrificed for microCT imaging and the implanted cell masses were retrieved for histologic evaluation. While no palpable mass was formed in the animals injected with AdGFP-infected cells, the AdBMP-2 or AdBMP-9-infected cells formed solid cell/tissue masses, and larger masses were found in the animals injected with BMP-9-expressing MSCs (Fig. 4A). These results suggest

that these BMPs may not only promote lineage specific differentiation but also may be important for the early proliferation and repopulation of the MSCs. Consistent with the in vitro findings, BMP-2 and -9 induced both osteogenesis and adipogenesis, while chondrogenesis was readily detectable in some of these samples, most notably BMP-2-transduced MSC masses. Representative histologic findings from AdBMP-2 and AdBMP-9 infected cell implants are shown in Fig. 4B. Consistent with our earlier findings [29], these findings indicate that BMP-2 induces predominantly endochondral ossification, while BMP-9 primarily induces intramembraneous ossification. The results were confirmed when the retrieved MSCs masses were stained with Alcian Blue. A significant number of Alcian Blue-stained positive cells were found in BMP-2-treated MSC implants, whereas a scattered few positive cells were detected in BMP-9-transduced MSC implants (Fig. 4C). Nevertheless, mature mineralized bone-like matrix was readily found in both BMP-2 and BMP-9-transduced MSC implants, as demonstrated by Masson's Trichrome staining (Fig. 4D). Oil Red-O staining revealed that both mature adipocytes and preadipocytes were readily found in BMP-2 or BMP-9-transduced MSC implants (Fig. 4E). Overall, our results confirm the in vitro findings, and suggest that some of these BMPs may regulate both osteogenic and adipogenic lineage commitment and terminal differentiation of MSCs.

Overexpression of Runx2 synergizes BMP-induced ALP activity without affecting adipogenic differentiation

We next examined the role of Runx2, a well-established regulator of osteoblast differentiation, in BMP-9-induced MSC differentiation. Using an adenoviral vector expressing

FIG. 3. BMP-induced osteogenic and adipogenic differentiation in MSCs is mutually exclusive. (A) ALP and Oil Red-O costaining. C3H10T1/2 cells were infected with AdBMP-2, -4, -6, -7, -9, or AdGFP. At Day 14, cells were fixed and first stained for ALP activity, followed by Oil Red-O staining. (B) Transmission electron microscope analysis of BMP-induced MSC osteogenic and adipogenic differentiation. C3H10T1/2 cells were infected with the indicated AdBMP-2, AdBMP-9, or AdGFP. At Day 7 and Day 14, cells were processed for transmission electron microscopy (TEM) analysis. Magnification, 12,000 $\times$. Abbreviations: AP, membrane-bound ALP activity; LP, accumulated lipid droplets.

FIG. 4. BMP-2 and BMP-9 induce both osteogenic and adipogenic differentiation in vivo. (A) MicroCT analysis of the tissue masses formed by GFP, BMP-2, or BMP-9-transduced MSCs. Transduced cells were implanted subcutaneously (5×10^6 cells per injection) into the flanks of athymic nude mice. After 5 weeks, animals were sacrificed and subjected to microCT analysis and histologic evaluation. Arrows indicate injection sites. (B) Histology of the tissue masses formed by BMP2- or BMP-9-transduced MSCs. Serial sections of decalcified tissues were stained with H&E. Dotted lines indicate the margin of masses. Boxed regions indicate the subsequently magnified areas. (C) Cartilage formation assessed with Alcian Blue staining. Serial sections were stained with Alcian Blue. Cartilage matrix was stained blue. (D) Mineralized bone matrix assessed with Masson's Trichrome staining. Serial sections of the decalcified tissues were stained with Masson's Trichrome. Fully mineralized bone matrix was stained red, while unmineralized osteoid matrix was stained light or dark blue. (E) Adipocyte formation assessed with Oil Red-O staining. Decalcified tissues were frozen sectioned and stained with Oil Red-O. Lipid-containing/producing cells, including preadipocytes, were stained red. Abbreviations: Ac, adipocyte; Ch, chondrocyte; MB, mineralized bone matrix; OM, osteoid matrix; Pac, preadipocyte; PC, progenitor cells.

Runx2, we found that overexpression of Runx2 in C3H10T1/2 cells resulted in approximately 140%, 190%, and 170% increase in ALP activity upon BMP-2, BMP-6, and BMP-9 stimulation, respectively (Fig. 5A). Similar results were obtained for ALP histochemical staining assays (Supplementary Fig. 3A) (Supplementary Fig. 3 is available online at <http://www.liebertpub.com/scd>). However, overexpression of Runx2 did not inhibit, if not increase the adipogenic differentiation of MSCs stimulated by BMP-2, -6, and -9 (Fig. 5B). These findings suggest that Runx2 may primarily regulate osteogenic differentiation of MSCs without affecting adipogenic differentiation.

Overexpression of PPAR γ 2 promotes both osteogenic and adipogenic differentiation

The role of PPAR γ 2 in BMP-induced differentiation of MSCs is rather intriguing. PPAR γ 2 has been shown to be significantly up-regulated by several BMPs, especially osteogenic BMPs (Fig. 1D) [30]. To examine the role of PPAR γ 2 in BMP-induced differentiation of MSCs, we used an adenoviral vector to express mouse PPAR γ 2. As shown in Fig. 5C, overexpression of PPAR γ 2 enhanced ALP activity by 79%, 63%, and 64% for BMP-2, BMP-6, and BMP-9 stimulated MSCs, respectively. These results were also confirmed

FIG. 5. Effect of overexpression of Runx2 or PPAR γ 2 on BMP-induced osteogenic and adipogenic differentiation. (A) Synergistic effect of Runx2 on BMP-induced ALP activity. C3H10T1/2 cells were coinfecting with BMP-2, -6, -9, GFP, and/or Runx2 adenoviral vectors. ALP activity was assayed at Day 7. (B) Runx2 exhibits no significant effect on BMP-induced adipogenic differentiation. C3H10T1/2 cells were coinfecting with BMP-2, -6, -9, GFP, and/or Runx2 adenoviral vectors. Oil Red-O stain was carried out at Day 14. (C) PPAR γ 2 enhances BMP-induced ALP activity. C3H10T1/2 cells were coinfecting with BMP-2, -6, -9, GFP, and/or PPAR γ 2 adenoviral vectors. ALP activity was analyzed at Day 7. (D) PPAR γ 2 augments BMP-induced adipogenic differentiation. C3H10T1/2 cells were coinfecting with BMP-2, -6, -9, GFP, and/or PPAR γ 2 adenoviral vectors. Oil Red-O stain was conducted at Day 14. (E) PPAR γ 2 overexpression enhances both adipogenic and osteogenic differentiation in vivo. C3H10T1/2 cells were transduced with BMP-9, GFP, and/or PPAR γ 2 adenoviral vectors and implanted subcutaneously (5×10^6 cells per injection) into the flanks of athymic nude mice. After 5 weeks, animals were sacrificed and subjected to microCT analysis (see Fig. 7B) and histologic evaluation. Abbreviations: Ac, adipocyte; MB, mineralized bone matrix.

by histochemical staining (Supplementary Fig. 3B). As expected, overexpression of PPAR γ 2 promoted BMP-induced adipogenic differentiation (Fig. 5D). The *in vivo* stem cell implantation experiments confirmed that overexpression of PPAR γ 2 promoted both osteogenic and adipogenic differentiation (Fig. 5E and Fig. 7A panels a and b). These findings strongly suggest that PPAR γ 2 may play an important role in regulating BMP-mediated osteogenic and adipogenic lineage commitment of MSCs.

Knockdown of Runx2 or PPAR γ 2 expression leads to a decrease in BMP-mediated osteogenic differentiation of MSCs

To complement the above overexpression experiments, we sought to further investigate the effect of silencing Runx2 and PPAR γ 2 on BMP-induced osteogenic and adipogenic differentiation of MSCs. We used our recently developed pSOS system to select and validate the siRNA target sites of mouse Runx2 and PPAR γ 2 [52]. We chose four candidate siRNA sites that targeted the coding region of mouse Runx2 or PPAR γ 2 (Supplementary Table 1). We demonstrated that three of the four tested siRNA sites effectively silenced GFP-Runx2 chimeric transcript in transient transfection assays (Supplementary Fig. 1A and B) (Supplementary Fig. 1 is available online at <http://www.liebertpub.com/scd>). We then pooled the pSOS vectors expressing the three effective siRNA sites and established a stable pool of C3H10T1/2 cells, designated as C3H10-Runx2-kd. The effective silencing of Runx2 expression in this stable line was confirmed by immunofluorescence staining (Supplementary Fig. 1C). The siRNA sites targeting the coding region of mouse PPAR γ 2 were selected and validated in a similar fashion (Supplementary Fig. 2A and B) (Supplementary Fig. 2 is available online at <http://www.liebertpub.com/scd>). The pooled three PPAR γ 2 siRNA sites were used to establish a stable pool of C3H10T1/2 cells, designated as C3H10-PPAR γ 2-kd. The knockdown efficiency (>50%) of C3H10-Runx2-kd and C3H10-PPAR γ 2-kd was further confirmed by quantitative real-time PCR assays (Fig. 6A and C). Our results demonstrated that silencing either Runx2 or PPAR γ 2 expression resulted in a decrease in BMP-induced ALP activity. Specifically, the ALP activity decreased to approximately 16%, 7%, and 13% for BMP-2, BMP-6, and BMP-9-stimulated C3H10-Runx2-kd cells, respectively, compared to that of the empty vector control line C3H10-Control (Fig. 6B). Similarly, compared to that of the empty vector control line C3H10-Control, the ALP activity decreased to approximately 21%, 25%, and 19% for BMP-2, BMP-6, and BMP-9-stimulated C3H10-PPAR γ 2-kd cells, respectively (Fig. 6D). Similar results were obtained using the histochemical staining assays of ALP activity (Supplementary Fig. 3C and D). On the contrary, the knockdown of either Runx2 or PPAR γ 2 expression in MSC C3H10T1/2 cells did not significantly affect adipogenic differentiation *in vitro*, as judged by Oil Red-O staining (Fig. 6E and F).

We next performed stem cell implantation experiments to investigate the *in vivo* effect of silencing Runx2 or PPAR γ 2 expression on BMP-9-induced bone and fat formation in MSCs. The microCT imaging demonstrated that knockdown of either Runx2 or PPAR γ 2 expression in MSCs resulted in the formation of smaller heterotopically ossified masses (Fig. 7A, panel a, vs. panels c and d). Histologic

evaluation revealed that osteoid matrix and not fully mineralized bone matrix were readily detected in the samples retrieved from either Runx2 or PPAR γ 2 knockdown MSC implantation (Fig. 7B). These results were reproducible in three batches of independent experiments. In the Runx2 knockdown samples, there was also a decrease in adipocyte accumulation. Noticeable decrease in adipocyte accumulation was obtained in PPAR γ 2 knockdown samples. These *in vivo* results were in general consistent with that of the *in vitro* findings shown in Figs. 5 and 6. Our findings strongly suggest that PPAR γ 2, in addition to its critical role in adipogenic differentiation, may play a role in regulating BMP-9-induced terminal differentiation of osteogenic progenitor cells.

Deletion of PPAR γ 2 results in decreased ossification of BMP-9-stimulated PPAR γ 2^{-/-} MEFs

Considering a possible partial phenotype of RNAi knockdown of PPAR γ 2 in C3H10T1/2 cells, we used the MEFs derived from animals in which the PPAR γ 2 was genetically deleted and investigated that the loss of PPAR γ 2 would affect BMP-9-induced bone formation. The relative PPAR γ 2 expression was analyzed in PPAR γ 2 heterozygous and homozygous MEFs, and confirmed that PPAR γ 2 level was low in PPAR γ 2 homozygous MEFs (Fig. 7C). The ALP activity was slightly decreased in PPAR γ 2 null MEFs upon BMP-9 stimulation, while as expected the adipogenic differentiation was notably decreased in PPAR γ 2 null MEFs upon BMP-9 stimulation (Fig. 7D). Stem cell implantation experiments revealed that PPAR γ 2 null MEFs formed smaller palpable masses than that of the wildtype or heterozygous MEFs (data not shown), whereas much low or no detectable ossified masses were observed with microCT imaging analysis in PPAR γ 2 MEFs injected the animals (Fig. 7E). Histologically, the masses retrieved from PPAR γ 2 null MEF implantation displayed a feature of more immature or non-mineralized osteoid matrix containing chondrocytes (Fig. 7F). As expected, no significant adipogenic differentiation was observed in PPAR γ 2 null MEFs derived masses (Fig. 7F). Thus, the results obtained from PPAR γ 2^{-/-} MEFs are consistent with that from the C3H10-PPAR γ 2-kd cell. Taken together, these findings suggest that PPAR γ 2 may play an important role in regulating both osteogenic and adipogenic differentiation of MSCs, although the exact mechanism through which PPAR γ 2 regulates osteocyte and adipocyte lineage commitment and terminal differentiation remains to be elucidated.

Discussion

Using a well-characterized mesenchymal progenitor cell line C3H10T1/2 [47], we conducted a comprehensive analysis of the 14 types of BMPs for their abilities to regulate multilineage specific differentiation of MSCs. Most BMPs exhibited distinct abilities to regulate expression of the master regulators of four common lineages derived from MSCs. When the osteogenic and adipogenic lineages were analyzed, BMP-2, -4, -6, -7, and -9 were shown to effectively induce both adipogenic and osteogenic differentiation of MSCs *in vitro* and *in vivo*. However, the BMP-induced commitment to osteogenic or adipogenic lineage was mutually exclusive. Overexpression of Runx2 enhanced

FIG. 6. Effect of RNAi-mediated silencing of Runx2 and PPAR γ 2 on BMP-induced osteogenic and adipogenic differentiation. (A) Verification of silencing Runx2 expression in C3H10-Runx2-kd and empty vector stable lines by qPCR. (B) Runx2 knock-down inhibits BMP-induced ALP activity. C3H10-Runx2-kd and empty vector stable lines were infected with AdBMP-2, AdBMP-6, AdBMP-9, or AdGFP. ALP assays were carried out 7 days after infection. (C) Verification of silencing PPAR γ 2 expression in C3H10-PPAR γ 2-kd and empty vector stable lines by qPCR. (D) PPAR γ 2 knock-down inhibits BMP-induced ALP activity. C3H10-PPAR γ 2-kd and empty vector stable lines were infected with AdBMP-2, AdBMP-6, AdBMP-9, or AdGFP. ALP assays were carried out 7 days after infection. (E) Runx2 knock-down exerts no significant effect on BMP-induced adipogenic differentiation. C3H10-Runx2-kd and empty vector stable lines were infected with AdBMP-2, AdBMP-6, AdBMP-9, or AdGFP. Oil Red-O stain was carried out 14 days after infection. (F) PPAR γ 2 knock-down exerts no significant effect on BMP-induced adipogenic differentiation. C3H10-PPAR γ 2-kd and empty vector stable lines were infected with AdBMP-2, AdBMP-6, AdBMP-9, or AdGFP. Oil Red-O stain was carried out 14 days after infection.

BMP-induced osteogenic differentiation, whereas knock-down of Runx2 expression diminished BMP-induced bone formation. Changes in the Runx2 expression status did not significantly affect BMP-induced adipogenic differentiation in vitro, although there was a slight decrease in adipocyte accumulation in Runx2 knockdown MSC-formed ectopic bone masses. However, overexpression of PPAR γ 2 promoted both osteogenic and adipogenic differentiation upon BMP-2, -6 and -9 stimulation. Conversely, both knockdown and deletion of PPAR γ 2 not only reduced adipogenic differentiation but also significantly inhibited osteogenic differentiation, especially the full mineralization of osteoid matrix.

The ability of PPAR γ 2 to enhance BMP-induced osteogenic differentiation is rather intriguing. It has long been

hypothesized that osteoblast and marrow adipocyte lineages may have a close but reciprocal relationship [61]. Changes in the balance between osteogenesis and adipogenesis have been postulated as contributing factors to physiologic and pathologic conditions, such as ageing and osteoporosis [55–62]. A recent study indicated that aging activates adipogenic and suppresses osteogenic programs in marrow stem cells [62]. A decrease in bone volume in age-related osteoporosis is usually accompanied by an increase in marrow adipose tissue [55–59,60]. PPAR γ insufficiency was shown to enhance osteogenesis [63], whereas the PPAR γ agonist Rosiglitazone causes bone loss in mice [64,65]. Conversely, patients with progressive osseous hyperplasia have heterotopic bone formation within adipose tissue [66]. However, *Runx2* null mice

FIG. 7. The in vivo effect of Runx2 and PPAR γ 2 depletion on BMP-9-induced osteogenic and adipogenic differentiation. (A) MicroCT analysis of BMP-9-induced bone formation affected by PPAR γ 2 overexpression, PPAR γ 2 knock-down, and Runx2 knockdown. Cell lines were transduced with AdBMP-9, AdGFP, and AdBMP-9+AdPPAR γ 2 for 15 h and subjected to subcutaneous implantation (5×10^6 cells per injection) into the flanks of athymic nude mice. After 5 weeks, animals were sacrificed and subjected to microCT analysis and histologic evaluation. Dotted lines indicate the margin of the ossified masses. (B) Histologic evaluation of the ossified masses retrieved from (A). (C) qPCR analysis of PPAR γ 2 expression in MEFs derived from PPAR γ 2 $^{+/+}$ and PPAR γ 2 $^{-/-}$ animals. (D) BMP-9-induced ALP activity and Oil Red-O stain in PPAR γ 2 $^{+/+}$ and PPAR γ 2 $^{-/-}$ MEFs. The MEFs were infected with AdBMP-9 and stained for ALP activity and Red Oil-O at Day 7 and Day 14, respectively. (E) Genetic deletion of PPAR γ 2 significantly inhibits BMP-9-induced ossification. BMP-9-transduced MEFs were subcutaneously implanted in athymic nude mice. MicroCT analysis was carried out 5 weeks after implantation. Dotted lines indicate the margin of the palpable masses. (F) Histologic evaluation of the samples retrieved from (E). Abbreviations: Ac, adipocyte; Ch, chondrocyte; MB, mineralized bone matrix; OM, osteoid matrix.

exhibited impaired bone formation and reduced adipogenesis [67], suggesting that osteogenic and adipogenic differentiation is closely related. Thus, the possible role of PPAR γ 2 in BMP-induced osteogenic and adipogenic differentiation

of MSCs remains to be defined. Our earlier studies indicate that PPAR γ 2 is readily up-regulated by osteogenic BMPs in preosteoblast progenitor cells [30], as well as in this study (Fig. 1D). We also demonstrated that PPAR γ 2 agonist

Troglitazone inhibited osteogenic BMP-induced ALP activity in MSCs (Supplementary Fig. 3E and F). While we do not have any satisfactory explanations for the different outcomes of our PPAR γ 2 overexpression/knockdown/deletion versus Troglitazone treatment and other in vivo observations, it is conceivable that: (1) Troglitazone as a small molecule agonist may exert off-target effect; (2) the action of PPAR γ 2 may be context-dependent, while we used a rather homogenous population of MSCs; (3) PPAR γ 2 may interplay with other factors during skeletal development, which is difficult to replicate in our stem cell implantation experiments; and (4) there may be a mechanistic difference between BMP-induced bone formation and developmental osteogenesis. Nonetheless, our findings clearly demonstrated that PPAR γ 2 may play an important role in regulating BMP-induced osteogenic and adipogenic differentiation, although it remains to be understood what exact role PPAR γ 2 may play in BMP-induced osteogenesis and adipogenesis of MSCs.

While the roles of BMPs in bone and skeletal development are well recognized [16,22,23,27], little is known about the role of BMP signaling in adipose development. A recent study reported that mice lacking the BMP-2 regulated zinc finger protein Schnurri-2 exhibited a significant reduction of in white fat mass [68]. Although several transcription factors have been identified as the “master regulators” of muscle, adipose, bone and cartilage differentiation [4,5], the molecular mechanisms which control the early stages of MSC lineage commitment and possible lineage plasticity remain to be understood.

MSC precursors have been shown to be highly heterogeneous in terms of their multilineage differentiation potential or “plasticity” [3,69]. In fact, only about 30% of the initial adherent bone marrow-derived MSC clones are pluripotent (mostly tri-lineage, osteo/chondo/adipogenic) [3,69], and the remainder displays a bi-lineage (osteo/chondrogenic) or uni-lineage (osteogenic) potential [69,70]. Nevertheless, MSCs can be rather easily isolated from bone marrow and expanded in vitro, and they have generated a great deal of interest and become a prime target for researchers of tissue regeneration and replacement of damaged tissues, such as bone, cartilage, tendon, and ligament [3,7,8].

Future investigations should be directed toward understanding how lineage commitment and divergence of MSCs is regulated [15,27,71]. Due to their low frequency and the lack of knowledge on cell surface markers and their location of origin, most information concerning MSCs is derived from in vitro studies. The cell populations could also represent different points of a hierarchy or a continuum of MSC differentiation. These issues reinforce the urgent need for a more comprehensive analysis of the MSC identity and characteristics, which should help to understand the regulatory mechanisms behind lineage determination and terminal differentiation of MSCs. Ultimately, understanding the regulatory mechanisms behind BMP-regulated lineage divergence during MSC differentiation should aid us in understanding the causes of skeletal diseases (such as osteoporosis and obesity) and may lead to the development of novel therapeutic strategies for regenerative medicine.

Acknowledgments

We thank Bruce M. Spiegelman of Dana-Farber Cancer Institute, Boston, MA, for providing the PPAR γ 2 null mouse embryo fibroblasts (MEFs). We also thank Parke-Davis/

Pfizer for generously providing Troglitazone. The reported work was supported, in part, by research grants from the American Cancer Society (H.H.L. and T.C.H.), The Brinson Foundation (T.C.H.), the Natural Science Foundation of China and the Chinese Ministry of Science and Technology (QL, #30500602; QK, #30600625; Z.L.D. and T.C.H.), the 863 Project Grant from the Chinese Ministry of Science and Technology (Z.L.D., T.C.H., Q.K., and Q.L.), the Orthopaedic Research and Education Foundation (R.C.H. and H.H.L.), and the National Institutes of Health (Q.K., R.C.H., T.C.H., and H.H.L.).

Author Disclosure Statement

The authors declare no competing financial interests.

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Received for publication May 18, 2008

Accepted after revision July 10, 2008

Prepublished on Liebert Instant Online July 10, 2008