BMP-2 promotes chondrogenesis of rat adipose-derived stem cells by using a lentiviral system

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ABSTRACT. Osteoporosis poses a major public health threat in aging societies. Adipose-derived stem cells (ADSCs) are multipotent adult stem cells that have the ability to yield mesenchymal stem cells, and have the potential to undergo osteogenesis and bone regeneration. Bone morphogenetic proteins (BMPs) have been demonstrated to upregulate bone gene expression after mechanical injury and to improve bone injury repair. This study aimed to produce BMP-2 expression in ADSCs by using lentiviral vectors. Subcutaneous adipose tissue from 4-week-old male Sprague-Dawley rats was used. Oil red O staining was used to detect adipocyte formation from ADSCs. Induction of ADSC osteogenesis was confirmed with Alizarin red S staining. The recombinant lenti-hBMP-2/
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neo was constructed to infect ADSCs, BMP-2 expression was measured by immunoblotting analysis, and cellular alkaline phosphatase levels were examined. We found that >70% of ADSC cells could be induced to differentiate into osteocytes or adipocytes. Under osteogenic induction, ADSCs showed increased intracellular calcium deposition, the formation of calcium tubercles, and the disappearance of cellular structures in calcium tubercles. After infection of ADSCs by lenti-hBMP-2/neo, BMP-2 was expressed after doxycycline induction. We, thus, conclude that ADSCs maintain vigorous growth ex vivo and possess stem cell-like properties. When infected with lenti-hBMP-2/neo, ADSCs can be induced to promote BMP-2 expression.

Key words: Bone morphogenetic protein 2; Adipose-derived stem cells; Osteogenic differentiation; Osteoporosis; Bone defect

INTRODUCTION

In China, almost 70 million people over the age of 50 have osteoporosis, and the disease results in approximately 687,000 hip fractures each year (China CHPFWP, 2008). Over the last decade, estrogen replacement therapy has been a common treatment for osteoporosis in women; however, prolonged estrogen therapy has been found to increase the risk of breast and endometrial cancers, coronary heart disease and strokes, and venous thromboembolic diseases. Autologous and homologous bone grafts have been used to treat fractures and traumatic bone defects, which is associated with compromised vascularity and osteoporosis, and has yielded only modest outcomes (Colterjohn and Bednar, 1997; Gamradt and Lieberman, 2003).

Tissue-engineering strategies for bone defect repair have been widely studied using resorbable biomaterial scaffolds, stem cells of various origins, and biologically active molecules such as cytokines or growth factors (Hubbell, 2003). In particular, adult stem cells such as bone marrow-derived mesenchymal stem cells (BMSCs) have been studied for this purpose. The proliferation and osteogenic differentiation of BMSCs are associated with bone healing in osteoporotic bone. Osteoblasts originate from BMSCs, which have the potential to differentiate into several different lineages including osteoblasts, chondroblasts, adipocytes, and myoblasts. Of these lineages, the osteogenic and adipogenic lineages are closely related.

A successful cell-based therapy for bone defect repair requires an appropriate cell source that is easily accessible, abundant, non-immunogenic, and which possesses osteogenic potential. Compared with osteocytes, BMSCs have higher yields and are more readily accessible, and have therefore been studied as a source of osteogenic cells for bone defect repair, and as an alternative to autologous primary osteocytes. However, BMSCs are difficult to isolate, and expansion of BMSCs ex vivo is a time-consuming process that is prone to contamination. In addition, BMSCs may yield inadequate numbers of stem cells from bone marrow, and their differentiation potential decreases with increasing age of the donor (Banfi et al., 2002; Stenderup et al., 2003).

On the other hand, adipose-derived stem cells (ADSCs) are multipotent adult stem cells that are capable of angiogenesis and osteogenesis (Philippe et al., 2010). They contain stem cells similar to BMSCs (Zuk et al., 2002; Guilak et al., 2006) and have the ability to yield...
mesenchymal stem cells with the potential to undergo osteogenesis and bone regeneration. Additionally, ADSCs are easily accessible, abundant, easily expandable, non-immunogenic, and harvesting is minimally invasive (Zuk et al., 2002; Guilak et al., 2006). For example, 6 x 10^6 ADSCs can easily be obtained from adipose tissue and rapidly expanded \textit{ex vivo}; by contrast, only approximately one BMSC can be obtained per 10^6 bone marrow stromal cells. Additionally, ADSCs are among the fastest growing stem cells and do not require immortalization. Halvorsen et al. (2001) showed that ADSCs growing under osteogenic induction expressed osteoblast-related genes and proteins such as alkaline phosphatase, type I collagen, osteocalcin, connexin 43, RunX-1, receptors for bone morphogenetic protein (BMP) I and II, and receptors for parathyroid hormone. ADSCs can also be induced to produce mineralized substrates when cultured under osteogenic conditions \textit{in vitro} (Leong et al., 2008). It has also been shown that human ADSCs inoculated into mice with severe combined immunodeficiency could form bones \textit{in vivo} (Hong et al., 2006).

Lentiviruses, which are based on the human immunodeficiency virus, have been used as delivery systems to insert genetic information into the DNA of host cells. BMSCs expressing keratinocyte growth factor via an inducible lentivirus have been shown to protect against bleomycin-induced pulmonary fibrosis (Aguilar et al., 2009). In the present study, we engineered BMP-2 expression in rat ADSCs \textit{in vitro} using lentiviral vectors expressing BMP-2, and investigated the osteogenic properties of ADSCs infected with BMP-2-expressing lentiviruses growing under osteogenic conditions.

**MATERIAL AND METHODS**

The study protocol was approved by the local Institution Review Board and all animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Harbin Medical University. All of the procedures were conducted in accordance with the Declaration of Helsinki and relevant policies in China.

**Isolation and characterization of ADSCs**

Subcutaneous adipose tissues (5 cm³) were removed from the nape of the neck of 4-week-old male Sprague-Dawley rats, which were purchased from the Experimental Animal Center of the Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China. The animals were housed in environmentally controlled conditions (22°C in a 12-h light/dark cycle with the light cycle from 6:00 to 18:00) with \textit{ad libitum} access to standard laboratory rat chow.

The isolated adipose tissue was minced and digested with 0.1% type II collagenase (Invitrogen, Carlsbad, CA, USA) at 37°C for 40 min, and resuspended in a red blood cell lysis buffer at room temperature for 15 min. The digest was then filtered through 200 x 50-mm mesh and centrifuged at 600 g for 5 min to produce a pellet. The cells were then resuspended, and 2 x 10^6 cells were cultured in Dulbecco’s modified Eagle’s medium with 10 mM glucose (DMEM-HG) (Gibco BRL, Grand Island, NY, USA) containing 5% fetal bovine serum at 37°C in a humidified 5% CO₂ incubator. Isolated ADSCs were characterized for cell surface markers by flow cytometry as described previously (Donnenberg et al., 2007). CD34 (stem cell marker) and CD90 (progenitor cell marker) (Beckman Coulter, San Jose, CA, USA) were
used to examine the ADSCs for stem cell-like features, CD44 for mesenchymal cell origin (CD44 is expressed by most mesenchymal cells and other cell types including adipocytes, fibroblasts, macrophages, and ADSCs), CD45 (hematopoietic marker; BD Biosciences, San Jose, CA, USA), and CD11b (T-lymphocyte marker; BD Biosciences).

**Induction of ADSC differentiation**

To explore the potential of isolated ADSCs for osteogenic differentiation, ADSCs were seeded at $1 \times 10^4$ cells/cm$^2$ on 10-cm culture dishes, and were grown under osteogenic induction conditions in DMEM supplemented with 0.1 mM dexamethasone, 50 μM ascorbate, and 10 mM β-glycerophosphate sodium. Mineralization of ADSCs was determined by Alizarin red S staining at day 16 or 21 post-drug treatment as previously described (Gregory et al., 2004). Alizarin red S concentrations were calculated by comparison with an Alizarin red S dye standard curve, and are reported as nmol/mL after normalization against the total cellular protein and as nmol/mg protein.

For induction of adipocyte differentiation, post-confluent cells, designated at day zero, were treated with 100 nM insulin, 500 μM 3-isobutyl-1-methylxanthine (cAMP phosphodiesterase inhibitor), and 250 nM dexamethasone for 48 h. Cells were subsequently incubated in DMEM supplemented with 10% calf serum and 100 nM insulin for 8 days. Oil red O staining was performed as previously described (Schwarz et al., 1997).

**Lentiviral preparation and infection of ADSCs**

Recombinant lentiviruses (lenti-rtTA/puro, lenti-emGFP/hygro, or lenti-hBMP-2/neo) were prepared by transfecting the plasmid along with packaging plasmids (Cyagen Gwen Zhou, China) into human Hek293T cells used to facilitate optimal lentivirus production. The cells were inoculated at a multiplicity of infection of 25, and incubated at 37°C in a humidified incubator containing 5% CO$_2$. Following incubation for 16 h, the medium was aspirated and the cells were washed with phosphate-buffered saline (PBS) twice and were then incubated as indicated. Lentiviruses were concentrated by centrifuging the lentiviral supernatant at 20,000 g for 2 h. The pellet was resuspended in PBS with 0.1% bovine serum albumin, aliquoted, and stored at -80°C. ADSCs were plated at $1 \times 10^5$ cells per well onto 12-well plates, and after an overnight incubation at 37°C, were infected with lenti-emGFP/hygro or lenti-hBMP-2/neo with or without osteogenic conditions. The ADSC-emGFP cell line was a lentiviral vector carrying a green-fluorescent protein, which served an infected control of ADSCs vs lenti-hBMP-2/neo.

**Immunoblotting studies and enzyme-linked immunosorbent assays (ELISA)**

Induced BMP-2 levels from ADSCs after infection with lenti-emGFP/hygro or lenti-hBMP-2/neo under osteogenic conditions was determined by immunoblotting and ELISA. Cells were rinsed with ice-cold PBS and lysed using RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The cell lysates were then sonicated for 30 s. Cell debris was removed by centrifugation at 12,000 g for 10 min. The cell lysates were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene
fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The blots were incubated in 5% PBS for 5 min, and then the PVDF membranes were blocked with 5% nonfat dry milk in Tris borate saline containing 1% Tween-20 (TBST) for 1 h and incubated overnight with anti-BMP-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody at 4°C with gentle shaking. The blots were extensively washed with TBST, and were then incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 1 h at room temperature. The blots were then washed, and the signals were visualized by chemiluminescence and quantified and analyzed with the Quantity-One package (Bio-Rad). α-GADPH was used as a loading control.

The content of BMP-2 in the cell culture medium and cell lysates was measured using commercially available ELISA kits, following manufacturer instructions (Boster Biological Technology). The BMP-2 content was normalized against the total cellular protein content and is reported as ng/mg protein. These experiments were performed at least three times independently.

Assay for lentiviral-infected ADSC proliferation

To determine the growth status of ADSCs after infection with lentiviral lenti-emGFP/hygro or lenti-hBMP-2/neo under osteogenic conditions, cells were seeded at 1 x 10^5 per well on 6-well plates and cell viability was assessed by the tetrazolium-based semi-automated colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Absorbance was read at 490 nm using a microtiter plate reader (KHB Labsystems Wellscan K3, Finland).

Alkaline phosphatase activity and mineralization of lentiviral-infected ADSCs

Alkaline phosphatase activity of ADSCs after infection with lentiviral lenti-emGFP/hygro or lenti-hBMP-2/neo under osteogenic conditions was determined by measuring p-nitrophenyl phosphate using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as previously described (Li et al., 2003), and was normalized against total cellular protein content. The experiment was performed at least three times independently. Mineralization of ADSCs was determined by Alizarin red S staining at day 21 post-drug treatment as described.

Statistical analysis

Data are reported as means ± standard deviation of three or more independent experiments. Statistical significance was estimated by one-way analysis of variance (ANOVA) with Bonferroni’s correction for multiple comparisons, and the Student Newman-Keuls post hoc ANOVA (comparisons between two groups) was used where appropriate. A value of P < 0.05 was considered to indicate statistical significance.

RESULTS

ADSCs show stem cell-like properties

We grew the ADSCs under osteogenic or adipogenic conditions. Flow cytometric
analysis showed that these cells were 99.81% CD44 positive, 99.89% CD90 positive, 0.64% CD34 positive, 0.36% CD11b positive, and 0.08% CD45 positive (Figure 1A). We further demonstrated that >70% of the cells could be induced to differentiate into osteocytes or adipocytes. Oil red O staining revealed fat droplets in the induced ADSCs, indicating the formation of adipocytes (Figure 1B). Furthermore, Alizarin red S assays revealed that ADSCs under osteogenic induction (Figure 1C) exhibited increased intracellular calcium deposits, the formation of calcium tubercles, and the disappearance of cellular structures in calcium tubercles compared with non-osteogenic induced ADSCs (Figure 1D).

Figure 1. Characteristics of isolated adipose-derived stem cells (ADSCs). A. Flow cytometric analysis of ADSCs with antibodies against cell surface markers CD34 and CD90 (stem/progenitor marker), CD44 (mesenchymal marker), CD45 (hematopoietic marker), and CD11b (T-lymphocyte marker). B. Adipose formation of ADSCs after adipogenic induction for 12 days (Oil red O, 100X). C. Osteogenic differentiation of ADSCs after 16 days of induction (Alizarin red S, 100X). D. ADSCs without osteogenic induction (Alizarin red S, 100X).
Inducible BMP-2 was detected in ADSCs infected with lenti-hBMP-2/neo

We investigated whether lenti-hBMP-2/neo-infected ADSCs expressed higher levels of BMP-2. There were 10 lentiviral transduced colonies obtained, and three of them were randomly selected for further polymerase chain reaction confirmation and assays. Immunoblotting analysis revealed that lenti-hBMP-2/neo-infected ADSCs, in which induction was induced by the addition of doxycycline, produced noticeable levels of BMP-2, whereas BMP-2 was not produced in non-infected ADSCs or lenti-hBMP-2/neo-infected ADSCs without doxycycline induction (Figure 2A). Furthermore, the level of BMP-2 by ADSCs, as measured by ELISA in the supernatant and lysates of lenti-hBMP-2/neo-infected ADSCs, was 0.41 ng/mL in the absence of doxycycline; with doxycycline induction, the BMP-2 content increased by 234.15% to 1.37 ng/mL, suggesting a potent induction of BMP-2 production by doxycycline (Figure 2B). In order to determine if lentiv infection would interfere with ADSC growth, we further studied ADSCs infected with lenti-hBMP-2/neo. The results showed a similar growth pattern as the control ADSCs with viral vector infection only or with no infection. However, MTT assays indicated that the infected ADSCs in vitro exhibited similar, but relatively slow, rates of cellular proliferation (Figure 2C).

Lentiviral-mediated expression of BMP-2 potentiated the osteogenic differentiation of ADSCs

We further examined alkaline phosphatase activity, an early marker of osteogenic differentiation of ADSCs. We found that alkaline phosphatase levels in ADSCs were 7.31, 8.53, and 6.46 U/L at weeks 1, 2, and 3 of osteogenic induction, respectively (Figure 3A). Furthermore, we found that alkaline phosphatase levels in lenti-hBMP-2/neo-infected ADSCs were
BMP-2 promotes chondrogenesis of rat adipose tissue-derived stem cells (ADSCs). There were no significant differences in alkaline phosphatase levels between lentivirus-infected ADSCs and the non-infected ADSCs (P > 0.05). On the other hand, induction of BMP-2 by doxycycline in lentivirus-infected ADSCs greatly increased alkaline phosphatase levels at weeks 1, 2, and 3 of osteogenic induction, which were 5.68, 7.23, and 16.02 U/L, respectively. This clearly demonstrated that osteogenic induction of ADSCs was associated with increased alkaline phosphatase activity.

**Figure 3.** Lentiviral expression of bone morphogenetic protein 2 (BMP-2) potentiated the osteogenic differentiation of adipose-derived stem cells (ADSCs). **A.** Alkaline phosphatase levels of ADSCs. *Indicates a statistically significant difference between the indicated group and the SD ADSC/BMP-2+Dox group at each time point. †Indicates a statistically significant difference between the indicated group and the SD ADSC/BMP-2-Dox group. *Indicates a statistically significant within group difference between the given time point and the week 1. †Indicates a statistically significant within group difference between weeks 2 and 3. Pairwise multiple comparisons between groups were determined using the Student-Newman-Keuls method. Experiments were performed at least three times independently. **B.** Alizarin red S assay for detecting the degree of the mineralization (calcium content) of lentiviral infected ADSCs after BMP-2 stimulation. Representative photos of lentivirus-infected ADSCs with doxycycline and osteogenic induction (a), osteogenic induction only (b), and no doxycycline or osteogenic induction (c). **C.** Measurement of calcium content. Mineralization of likely biological significance began approximately 16 days after osteogenic induction. SD = Sprague-Dawley rats.

Using the Alizarin red S assay, we further studied whether BMP-2 could stimulate the mineralization of ADSCs. Representative images are shown in Figure 3B including lentivirus-infected ADSCs with doxycycline and osteogenic induction (a), osteogenic induction only (b), and no doxycycline or osteogenic induction (c). The calcium content in lentivirus-infected ADSCs was 0.78 ng/mL in the absence of doxycycline, which increased by 20.25% to 1.18 ng/mL with BMP-2 induction by doxycycline (Figure 3C). Thus, mineralization began approximately 16 days after osteogenic induction.
DISCUSSION

In this study, we demonstrated that osteogenesis of primary rat ADSCs could be effectively induced ex vivo, demonstrating that these cells could be expanded to provide a ready source of early-passage primary ADSCs. We further showed that the ADSCs could be induced to differentiate into adipocytes and chondrocytes, demonstrating their multipotent differentiation potential.

ADSCs are regarded as putative osteoblast progenitors and can be induced to differentiate into osteoblasts in vitro (Yang et al., 2004). Bone remodeling underlies the process of bone repair or osteogenesis in many bone disorders. Defective osteogenesis is characterized by reduced bone mass and deteriorated bone microstructures, which is associated with a noticeably increased risk of bone fractures, and may contribute to osteoporosis. Therefore, inducing differentiation of ADSCs may provide the basis for an alternative therapy for bone disorders that involve ongoing bone remodeling.

Under both normal and pathological conditions, multiple local and systemic signals derived from hormones, growth factors, and other agents control different aspects of bone remodeling. BMP-2 and insulin growth factor-1 have been shown to modulate the proliferation and differentiation of mesenchymal stem cells (Rumalla and Borah, 2001; Fukumoto et al., 2003; Presta et al., 2005; Toh et al., 2007). BMPs belong to the transforming growth factor-beta (TGF-β) superfamily of polypeptides, including TGF-β, activins/inhibins, and BMPs. Specifically, BMP-2 has been demonstrated to induce the differentiation of ADSCs into osteocytes, promote osteogenic differentiation, upregulate bone gene expression after mechanical injury, and improve bone repair after injury (Seong et al., 2010). BMPs bind to specific transmembrane type I and type II receptors and stimulate the intracellular mediators Smad-1, -5, and -8 (Herpin and Cunningham, 2007), which transmit the BMP signal into the nucleus to regulate target gene transcription (Lian et al., 2006). BMP-2 and BMP-7 (OP1) have been shown to promote bone healing in long bone defects in human studies (Yasko et al., 1992; Cook et al., 1994a,b, 1995; Zabka et al., 2001) and in animal models of spinal arthrodesis (Sandhu et al., 1997; Boden et al., 1998; Wang et al., 2003). Currently, BMP-2 is approved by the Food and Drug Administration in the United States for spinal fusion in degenerative disc disease and tibial fractures, and OP1 is approved as an alternative to autograft in recalcitrant long bone unions and in lumbar spinal fusions. In this study, we showed that lentiviral expression of BMP-2 could promote the proliferation and potentiate the osteogenesis of ADSCs. Furthermore, alkaline phosphatase levels increased slowly. Although our results indicated a statistical difference in alkaline phosphatase levels at 1 and 2 weeks, it was only at 3 weeks that an obvious difference of likely biological significance was noted. Thus, ADSCs initiated mineralization about 16 days after osteogenic induction.

Viruses derived from adeno-associated virus, such as adenovirus and lentivirus, are among the most successful viral vectors, and show great promise as viral vectors for gene transfer. Lentiviruses are also able to transduce a wide variety of cells, including non-dividing cells, and can integrate into the genome to provide sustained gene expression. With respect to bone growth, Miyazaki et al. (2008) showed that BMP-2-producing rat bone marrow cells created through lentiviral gene transfer induced sufficient spinal fusion in rats. Zhang et al. (2002) reported that lentiviral vectors were able to transduce human bone marrow-derived stromal cells through many cell divisions and during differentiation into adipocytes. Importantly, Hsu et al. (2007) found that lentiviral-mediated BMP-2 gene transfer enhanced the healing of segmental femoral defects in a rat model.
In the present study, we showed that lenti-hBMP-2/neo-infected ADSCs under doxycycline induction produced noticeable levels of BMP-2, whereas BMP-2 was not produced in non-infected ADSCs or lenti-hBMP-2/neo-infected ADSCs without doxycycline induction. Doxycycline has been successfully used in tetracycline-controlled transcriptional activation to regulate transgene expression in vivo and in cell cultures (Samtani et al., 2009). A prior study showed that a doxycycline-regulated lentiviral vector system with a novel tetracycline reverse transactivator rtTA2 (S)-M2 exhibited a tight control of gene expression in vitro and in vivo (Koponen et al., 2003). Tang et al. (2009) used a doxycycline-inducible gene expression system to establish a cell line that regulated the expression of the hepatitis B virus X protein.

There are some limitations of this study that should be considered. We did not examine if the expression of BMP-2 potentiated the adipogenic differentiation of ADSCs because the main purpose of this study was to explore the effect of BMP-2 on the chondrogenesis of ADSCs using an inducible lentiviral system. Secondly, we did not confirm the involvement of BMP-2 expression in the observed potentiation of osteogenic differentiation by the use of direct BMP-2 inhibitors (such as antibodies against the growth factor) or by inhibiting the BMP-2-mediated signal transduction pathways.

In conclusion, we have here demonstrated that ADSCs maintain vigorous growth ex vivo and possess stem cell-like properties. When infected with lenti-hBMP-2/neo, ADSCs can be induced to promote BMP-2 that increased the osteogenic differentiation of ADSCs. Notably, BMP-2 promotes chondrogenesis of adipose-derived stem cells with a lentiviral system in rats. This finding suggests that lentiviral-based gene therapy systems offer a clinical alternative to treat osteoporosis and resulting bone defects.

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Conflicts of interest

The authors declare no conflict of interest.

REFERENCES


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