



Endogenous and exogenous bone morphogenetic protein-2 play an important role in chondrocytic differentiation and maturation in rat cord blood-derived mesenchymal progenitor cells

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ABSTRACT

Bone morphogenetic protein-2 (BMP-2) strongly induces chondrogenesis in the presence of transforming growth factor beta-1 (TGF- β 1) or –3 in bone marrow (BM), synovium, adipose, and muscle-derived mesenchymal stem cells (MSCs). However, the effect of BMP-2 on umbilical cord derived (UCB)-derived MSCs, which are potentially useful for cartilage repair, has not been elucidated. In this study, the effect of endogenous and exogenous BMP-2 on rat umbilical cord blood-derived mesenchymal progenitor cells (UCB-MPCs) was evaluated. When cells were cultured in medium supplemented with only 10% FBS, type II procollagen (COL2A1) transcripts and collagen II protein were detectable after 2 weeks in UCB-MPCs, but not in BM-MPCs. The BMP antagonist, noggin, suppressed gene expression of COL2A1 and SOX-9 in UCB-MPCs. Exogenous BMP-2 promoted chondrogenic differentiation of UCB-MPCs in a dose-dependent manner, even in the absence of exogenous TGF- β 1. UCB-MPCs expressed both type IIA and IIB procollagen in medium supplemented with only 10% FBS. Notably, exogenous BMP-2 was able to stimulate COL2A1 expression, particularly that of the type IIB transcripts, and also induce chondrogenic differentiation in rat UCB-MPCs. Interestingly, TGF- β 1 inhibited BMP-2-induced COL2A1 splicing. As our results suggest that exogenous BMP-2 plays an important role for the spontaneous differentiation of chondrogenic lineages in rat UCB-MPCs, exogenous BMP-2 may effectively promote cartilage repair when UCB-MPCs are used.

Key words: Bone morphogenetic protein-2, Chondrogenesis, Mesenchymal progenitor cells, Rat cord blood

Umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) have the potential to differentiate into multi-lineage cells, including mesenchymal lineage cells, but can also form neurons, skeletal muscle, and myocardium (Bicknese *et al.* 2002, Erices *et al.* 2000, Goodwin *et al.* 2001, Kakinuma *et al.* 2003, Rosada *et al.* 2003, Ma *et al.* 2005, Koblas *et al.* 2009, Liu *et al.* 2010). Berg *et al.* (2009) reported that equine UCB-derived MSCs appear to possess greater chondrogenic potential than bone marrow (BM)-derived MSCs, suggesting UCB-derived cells may be

effective for cartilage repair. Despite their potential, factors that accelerate chondrogenic differentiation in UCB-MSCs are not well characterized.

Endogenous and exogenous growth factors play important roles for chondrogenic differentiation of MSCs. In particular, bone morphogenetic protein-2 (BMP-2) strongly induce chondrogenesis in the presence of transforming growth factor beta-1 (TGF- β 1) or –3 in BM, synovium, and adipose-derived MSCs (Sekiya *et al.* 2005, Lee *et al.* 2009, Ronziere *et al.* 2010). Gaining a better understanding of BMP-2 regulation of chondrogenic differentiation in UCB-derived MSCs may provide critical information for the utilization of these cells in cartilage repair. However, the effect of BMP-2 on UCB-derived MSCs has not been elucidated.

We previously isolated mouse and rat UCB-derived mesenchymal progenitor cells (UCB-MPCs) (Naruse *et al.* 2004, Uchida *et al.* 2010) and demonstrated that rat UCB-MPCs have higher chondrogenic differentiation potential than BM-derived mesenchymal cells (Naruse *et al.* 2004). In the present study, we investigated the effects of

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endogenous and exogenous BMP-2 on chondrogenic differentiation and maturation of rat UCB-derived MPCs.

MATERIALS AND METHODS

Preparation of UCB-MPCs from circulating fetal rat blood: Rat UCB was collected from the end of umbilical cords of rat fetuses on gestation day 18 (Naruse *et al.* 2004). After total cell counts of the harvested nucleated cells were counted using the Turk staining method, the cells were suspended in α -MEM supplemented with 10% fetal bovine serum (FBS) and antibiotic mixture, and 2×10^6 cells were plated in each well of a primaria 6-well culture plate. UCB-MPCs were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C, and the culture medium was replaced after the first 24 h and then every 3 to 4 days.

Preparation of bone marrow-derived mesenchymal progenitor cells: BM-MPCs were isolated from femurs of 8-week-old Wistar rats (Uchida *et al.* 2007). Similar to the preparation of UCB-MPCs, 2×10^6 BM-MPCs in supplemented α -MEM were added to each well of a 6-well plate and incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cells were subcultured every week and harvested after 3 weeks for evaluation.

Preparation of chondrocytes from rat costal cartilage: For the preparation of chondrocytes, costal cartilage was aseptically excised from 8-week-old Wistar rats (Mukaida *et al.* 2005). After removing the surrounding soft tissue and growth plates, cartilaginous tissue was digested with trypsin (2.5 mg/ml in PBS) for 1 h at 37 °C to further remove superficial fibrous tissue, and the digested material was discarded. The remaining tissue was then incubated in DMEM containing 2 mg/ml type II collagenase in a 25-cm² tissue culture flask (at 37 °C overnight). After repeated pipetting to detach cells, separated cells were passed through 100 μ m sieves, pelleted by centrifugation, and dispersed in DMEM supplemented with 0.2 mg/ml ascorbate 2-phosphate, antibiotics-antimycotic, 10% FBS, and 25 mM HEPES, pH 7.4. When the cultures reached confluency, total RNA was extracted from the cells, as described below.

Growth factor: All recombinant human growth factors and recombinant mouse Noggin-Fc chimera had been used for previous rat studies (Simionescu *et al.* 2005, Kawabata *et al.* 2007). To examine the effects of exogenous BMP-2 and noggin on chondrogenic differentiation, and the effects of BMP-2 and TGF- β 1 on COL2A1 splicing, UCB-MPCs were cultured in supplemented α -MEM for 2 weeks and then cultured in α -MEM containing either 500 ng/ml BMP-2, 10

ng/ml TGF- β 1, 5 mg/ml noggin, or 500 ng/ml BMP-2 + 10 ng/ml TGF- β 1 for 7 days at 37 °C.

RT-PCR analysis: Five mg of total RNA was extracted from cells at several time points using ISOGEN and reverse transcribed using SuperScript II reverse transcriptase (Uchida *et al.* 2007). Polymerase chain reaction was carried out with primers specific for COL2A1, Sry-type high-mobility group box transcription factors (SOX)-5, -6, and -9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The self-designed primers against SOX-5 and -6 are listed in Table 1. All other primers were designed based on previously published cDNA sequences reported in the literature (Mukaida *et al.* 2005, Naruse *et al.* 2004, Uchida *et al.* 2007) and were purchased from a peptide synthesis company.

Immunostaining: After 3 weeks of culture, UCB-MPCs were fixed, washed, and treated with 250 U/ml hyaluronidase at 37 °C for 10 min. The cells were then incubated at 4 °C for 18 h with rabbit polyclonal antibody against rat type II COL2A at 1:200 dilutions. A biotin-labeled goat anti-rabbit IgG antibody was added as the secondary antibody, followed by either horseradish peroxidase-conjugated streptavidin or Texas red-conjugated streptavidin. Color development was performed for 5 min in the presence of diaminobenzidine and H₂O₂. Prior to immunofluorescence microscopy, cells were counterstained with DAPI.

RESULTS AND DISCUSSION

We first investigated the role of endogenous BMP-2 on spontaneous differentiation of MPCs. When cells were cultured in medium supplemented with only 10% FBS, COL2A1 gene expression and collagen II proteins were detectable after 2 weeks in UCB-MPCs, but not BM-MPCs (Figs 1, 2). Notably, the areas where cells condensed and formed layers were heavily stained by collagen II antibody (Fig. 1). As SOX-5, -6, and -9 play important roles for chondrogenesis and may be regulated by BMP-2 (Uusitalo *et al.* 2001, Fernandez-Lloris *et al.* 2003, Lee *et al.* 2003), we also examined the expression of these genes in MPCs. The BMP antagonist, noggin, suppressed gene expression of both SOX-9 and COL2A1 in UCB-MPCs (Fig. 2C). Taken together, these results suggest that BMP-2 is a key factor for the spontaneous differentiation of UCB-MPCs into chondrogenic lineages.

Previous studies have demonstrated that exogenous BMP-2 stimulates osteoblastic differentiation of BM-derived MSCs. However, MSCs from BM can only differentiate into chondrocytic cells in the presence of growth factors such as

Table 1. Primers designed for this study

Target Gene	Accession number	Primer pair sequence (sense/antisense)
SOX-5	NM_001014060	5'-GACCTCTGACGGCAAATCAC-3' 5'-CTGTTCACTACGGCCACCTT-3'
SOX-6	NM_001024751	5'-TCTCAACTCTCCTGCCCTGT-3' 5'-CCAGGTTCTCAAAGCGTGTT-3'

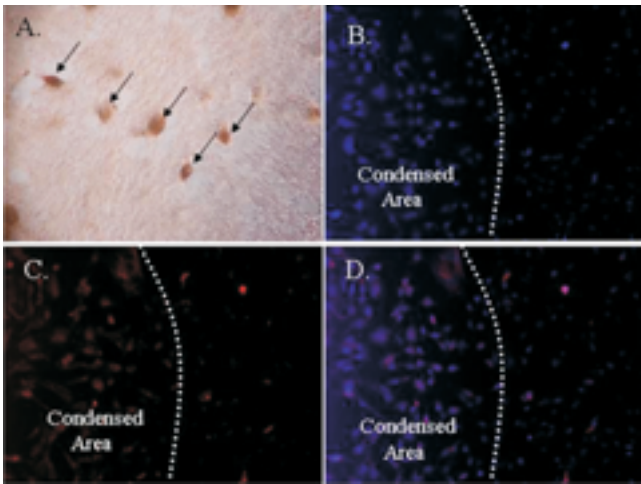


Fig. 1. Immunocytochemistry for collagen II in rat umbilical cord blood-derived mesenchymal progenitor cells. (A) collagen II (brown, arrows), (B) nuclei (blue), (C) collagen II (red), and (D) merged image (collagen II + nuclei). Dotted lines were drawn at the boundary position between condensed and non-condensed areas within cells.

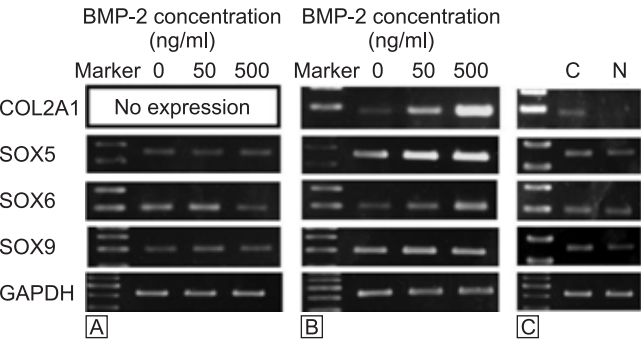


Fig. 2. Effect of exogenous BMP-2 and noggin on chondrogenic differentiation of mesenchymal progenitor cells (MPCs) on day seven. (A) Effect of exogenous BMP-2 (500 ng/ml) on bone marrow (BM)-derived BM-MPCs, (B) effect of exogenous BMP-2 (500 ng/ml) on umbilical blood cord (UCB)-derived MPCs, and (C) effect of exogenous noggin (5 ng/ml) on UCB-MPCs. C, control (α -MEM), N, noggin.

TGF- β 1 and - β 3 (Roark *et al.* 1994, Moses *et al.* 1996). We next investigated whether exogenous BMP-2 accelerates chondrocytic differentiation in UCB-MPCs. We found that exogenous BMP-2 promotes COL2A1 gene expressions in a dose-dependent manner in UCB-MPCs, even without treatment with exogenous TGF- β 1 (Fig. 2). Roark and Greer (1994) reported that TGF- β has the greatest effect on mesenchymal cells that have not yet undergone cell condensation, a critical event in early cartilage differentiation, whereas BMP-2 is the most effective after cells have condensed or differentiated. Therefore, the different effect of BMP-2 on UCB-MPCs and BM-MPCs may reflect the differentiation stage of these cells, because UCB-MPCs were able to condense and differentiate into chondrocytic cell even

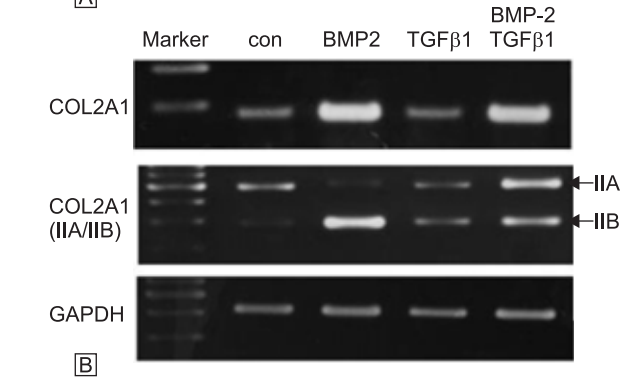
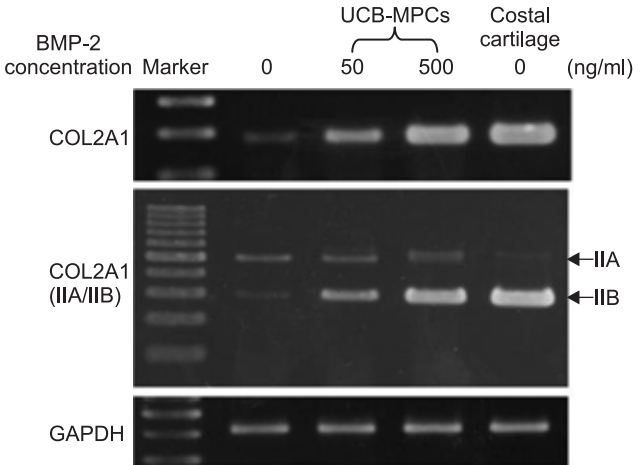


Fig. 3. Effects of BMP-2 and TGF- β 1 on COL2A1 splicing in rat umbilical cord blood-derived mesenchymal progenitor cells. (A) Effects of BMP-2 (500 ng/ml), and (B) effects of BMP-2 (500 ng/ml) and TGF- β 1 (10 ng/ml) after 7 days of culture.

without exogenous TGF- β 1. Type IIB procollagen is synthesized by mature chondrocytes and represents the major extracellular matrix component of cartilage. When pre- and immature chondrocytes differentiate into mature chondrocytes, the mRNA splice form of COL2A1 switches from type IIA to IIB procollagen (Nah *et al.* 1991, Ng *et al.* 1993, Sandell 1994, Sandell *et al.* 1994, Nalin *et al.* 1995). In the present study, we also observed that costal cartilage-derived chondrocytes mainly expressed the type IIB isoform (Fig. 3A). In contrast, UCB-MPCs expressed both type IIA and type IIB (Fig. 3A), indicating that UCB-MPCs differentiate into immature chondrocytes in medium supplemented only with 10% FBS. However, the presence of exogenous BMP-2 was able to stimulate COL2A1 expression in UCB-MPCs, particularly that of the type IIB transcript (Fig. 3A, B). These results suggested that BMP-2 stimulates not only chondrogenesis, but also chondrocytic maturation in UCB-MPCs. Sekiya *et al.* (2005) reported that BMP-2 was more effective for chondrogenic differentiation of BM-derived mesenchymal cells than BMP-4 and -6 in chondrogenic

medium containing TGF- β and dexametasone. In addition, Melhorn *et al.* (2007) suggested that BMP-2 and TGF- β 1 were beneficial for differentiation of adipose-derived stem cells into chondrocytes. Therefore, we investigated whether BMP-2 stimulated chondrogenesis in UCB-MPCs cultured in medium containing TGF- β . Interestingly, when the expression profiles of cell were examined after 7 days of culture, TGF- β 1 inhibited BMP-2-induced COL2A1 splicing (Fig. 3B). Our results suggested that TGF- β 1 inhibits UCB-MPCs differentiation into mature chondrocytes by influencing the alternative splicing of COL2A1 by BMP-2, which may explain the findings of recent studies that TGF- β 1 inhibits chondrocyte maturation in mesenchymal cells (Kawamura *et al.* 2005, Mello *et al.* 2006). The present observations suggested that BMP-2 and TGF- β 1 may act in a sequential manner to regulate UCB-MPCs through the various stages of cartilage differentiation.

In conclusion, we have demonstrated that exogenous BMP-2 is able to induce chondrogenic differentiation in rat UCB-MPCs and promote chondrocytic maturation due to the stimulation of COL2A1 expression, particularly that of type IIB transcripts. As our results suggested that endogenous BMP-2 plays an important role for the spontaneous differentiation of chondrogenic lineages in rat UCB-MPCs, exogenous BMP-2 may effectively promote cartilage repair when UCB-MPCs are used.

The BMP antagonist, noggin, suppressed gene expression of COL2A1 and SOX-9 in rat umbilical cord blood-derived mesenchymal progenitor cells (UCB-MPCs). Exogenous BMP-2 promoted chondrogenic differentiation of UCB-MPCs in a dose-dependent manner, even in the absence of exogenous TGF- β 1. Notably, exogenous BMP-2 was able to stimulate COL2A1 expression, particularly that of the type IIB transcripts, and also induce chondrogenic differentiation in rat UCB-MPCs. Interestingly, TGF- β 1 inhibited BMP-2-induced COL2A1 splicing. As our results suggested that exogenous BMP-2 plays an important role for the spontaneous differentiation of chondrogenic lineages in rat UCB-MPCs, exogenous BMP-2 may effectively promote cartilage repair when UCB-MPCs are used.

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