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Proteomic analysis on effectors involved in BMP-2-induced osteogenic differentiation of beagle bone marrow mesenchymal stem cells

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Abstract

Objective: To identify the protein regulation profile of recombinant human bone morphogenetic protein-2 (rhBMP-2)-induced osteogenic differentiation in beagle bone marrow stem cells (BMSCs).

Methods: Beagle BMSCs were isolated and cultured with or without rhBMP-2. Two-dimensional gel electrophoresis was used to determine the differences in protein expression in rhBMP-2-induced and non-induced BMSCs. Real-time PCR and western blotting analyses were used to verify the expression patterns of selected proteins.

Results: After the induction, the osteogenic differentiation of beagle BMSCs was activated successfully. Nine and 11 proteins were found to be down- and up-regulated by rhBMP-2, respectively. The increase in Lim and SH3 domain protein 1(LASP1) and the decrease in ferritin were verified by real-time PCR and western blotting analyses.

Conclusions: Among the 20 rhBMP-2-regulated factors, there is empirical evidence supporting the involvement of LASP1 and ferritin in osteogenic differentiation. LASP1 plays an important role in the regulation of the activity of the cytoskeleton, and ferritin is an important molecule in cellular iron homeostasis. Further studies focused on these 20 proteins will help elucidate the molecular mechanism(s) through which rhBMP-2 induces osteogenic differentiation of BMSCs.

Keywords: BMSCs, Osteogenic differentiation, rhBMP-2, Proteomics

Introduction

The repair of large segmental bone defects caused by trauma or disease is a very difficult process in orthopedic clinics. Sources of autologous bone grafts are limited and allogeneic grafts may be rejected and might induce infection. The effect of artificial bone products for this purpose is still uncertain. Thus, the use of autologous bone marrow stem cells (BMSCs) to build/repair bone tissue and to treat bone defects has been proposed as a new treatment option in recent years. BMSCs are a subset of non-hematopoietic bone marrow stromal cells. They are easy to obtain and culture, and can be amplified *in vitro* and transformed to multi-line ages without loss of genetic stability [1]. BMSCs have a multi-potent differentiation potential *in vitro* under certain conditions.

BMSCs can differentiate into adipocytes, osteoblasts, chondrocytes, and vascular smooth muscle, skeletal muscle, heart, endothelial, nerve, and liver cells, among others [2]. Thus, BMSCs have become the most promising seed cells for bone tissue engineering [3].

Bone morphogenetic proteins (BMPs) are bone growth factors that are known to induce osteogenesis [3]. These growth factors themselves are sufficient to induce bone formation [3]. Under certain conditions, they can induce undifferentiated mesenchymal cells to transform to bone cells and promote the proliferation of bone cells [3]. BMPs are currently considered the most important factor in osteogenesis [4,5]. The effect of recombinant human BMP-2 (rhBMP-2) on osteogenic differentiation of BMSCs is well-studied, and rhBMP-2 has already been applied widely in animal experiments and clinical applications [2]. In previous studies, we have also tested the utility of tissue-engineered bone containing rhBMP-2 and BMSCs in repairing bone defects in rabbits [6,7].

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However, the molecular mechanisms of BMP-2-induced osteogenic differentiation are currently not known.

The source of BMSC specimens are mainly mice, rats, and humans. Human BMSCs are in limited supply and it is difficult to carry out studies with human BMSCs. BMSCs from mice or rats are quite different from human BMSCs in physiology. Beagle is considered an ideal large animal model widely used in pre-clinical toxicological experiments, basic medical research, radiation sickness treatment, and experimental surgery. Therefore, in this study, we established a model for BMSC primary cell culture and rhBMP-2-induced osteogenic differentiation using Beagle dogs. Proteomic analysis was used to identify proteins regulated by rhBMP-2. The results provide an improved understanding of the rhBMP-2-induced osteogenic differentiation of BMSCs.

Material and methods

Culture and induction of differentiation of BMSCs

According to standard bone marrow puncture procedures, 5 to 10 mL of bone marrow was aspirated from the posterior superior iliac spine of beagles. Heparin was added to the bone marrows to block coagulation and then the marrows were diluted with an equal volume of phosphate-buffered saline (PBS). The diluted bone marrows were layered onto lymphocyte separation medium (Sigma-Aldrich) at a ratio of 2:1, followed by centrifugation at $400 \times g$ for 30 min. After centrifugation, the white layer between the two layers generated after centrifugation was collected and washed twice with PBS. Cells were seeded in culture plates at a density of 1×10^6 cells/well and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in an atmosphere of 5% CO₂ and 95% humidity. After 72 h of incubation, the culture medium was changed once every 3 days. At the third generation, cells were trypsinized, and 3×10^6 cells were plated in 100 mm culture dishes. The cells were stimulated with 20 mg/L rhBMP-2 (Hangzhou Jiuyuan Gene Engineering Co., Ltd., China) for one week, and the medium was changed once every three days. The morphology of the cells was observed everyday using an inverted phase-contrast microscope (IX81; Olympus, Japan). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Southern Medical University (Approval No. 120333, 114522).

Gomori alkaline phosphatase staining

Gomori alkaline phosphatase staining was performed on cultured BMSCs as per the instructions of BCIP/NBT chromogenic alkaline phosphatase staining kit (Beyotime, China). After fixation, cells were washed with PBS three times, the BCIP/NBT staining working solution was added, and the cells were incubated at room temperature

for 30 min. The BCIP/NBT staining working solution were then removed, and the cells were washed twice with distilled water to terminate the color reaction. The cells were observed and photographed using an inverted phase-contrast microscope (IX81; Olympus, Japan).

Two-dimensional (2D) gel electrophoresis

Dry cells (100 mg) were lysed by adding 400 μ L of lysis buffer (8 M urea, 4% CHAPS, 0.48% Tris base, 40 mM DTT, and 10 mM phenylmethylsulfonyl fluoride). The lysis mixture was incubated at room temperature for 60 min and centrifuged at 15,000 rpm for 60 min at 4°C. After centrifugation, the supernatant was collected and quantified with a 2D QUANT protein Assay Kit (GE, USA). The supernatant containing 300 μ g of total cellular protein (about 20 μ L) was mixed with the sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% ampholytes, and a small amount of bromophenol blue) to a final volume of 450 μ L. Two-dimensional gel electrophoresis (2-DE) was performed mainly according to the method described in a previous report [8] using a 24-cm pH4-7 linear DryStrip (GE, USA). After rehydration, isoelectric focusing (highest current set at 50 μ A/gel, 20°C) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. After electrophoresis, mass spectrometry-compatible silver staining was applied [9]. The gel was scanned using a UMAX Power Look 1100 transmission scanner to obtain images, and the images were analyzed with PDQuest 7.1.0 software. The analysis included the detection of protein spots, quantification, background removal, and cross-gel analysis. After comparison of the two gels, protein spots corresponding to a difference in expression greater than 2.5 times were selected for matrix-assisted laser desorption/ionization-time-of flight (MALDI-TOF) mass spectrometry.

In-gel digestion and MALDI-TOF mass spectrometry

The protein spots were excised from gels into 1–2 mm² blocks. After in-gel digestion, MALDI-TOF-TOF mass spectrometry was carried out using an ABI4700 (Applied Biosystems, USA) system. The obtained data were analyzed with the PMF Mascot database (<http://www.matrixscience.com/>). The parameters used were: species, mammal; trypsin digestion (the number of maximum missed cleavages was set to 1); carbamidomethylation of cysteine residues was set as a fixed modification; and mass error, 100 ppm.

Real-time quantitative PCR

The RNA extracted from BMSCs (1 μ g) was first reverse-transcribed into cDNA (PrimeScript; Takara, China). The cDNA was then used as the template for real-time quantitative PCR analysis (Maxima SYBR Green/ROX qPCR Master Mix; Thermo Scientific, Pittsburgh, PA). The primers for quantifying the mRNA

Table 1 Sequences of primers used in real-time quantitative PCR

Gene	Gen bank accession no.	Primers
GAPDH	NM_001003142.1	F: 5'-ATCATCAGCAATGCCTCCTG-3' R: 5'-ATGGACTGTGGTCATGAGTC-3'
LASP1	XM_859075.2	F: 5'- GTGCGCTACAAGGAGGAGTT-3' R: 5'- CTCTGGAGTTCGGGTGTGTC-3'
Ferritin light chain	NM_001024636.1	F: 5'-AGGCCCTTTTGGATCTTCAT-3' R: 5'-CAGGTGGTCACCCATCTTCT-3'
Ferritin heavy chain	NM_001003080.1	F: 5'-CTGGAGCTCTACGCCTCTA-3' R: 5'-TGGTTCGAGTTCATCAG-3'

expression of Lim and SH3 domain protein 1 (LASP1), ferritin light and heavy chains, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as the internal control, are listed below (Table 1). The average $\Delta\Delta CT$ of LASP1, ferritin light and heavy chains, and GAPDH from samples analyzed in triplicate was calculated [10,11].

Western blotting

BMSCs in the exponential growth phase were lysed with radio-immunoprecipitation assay (RIPA) buffer. The expression level of LASP1 and ferritin light and heavy chains in BMSC lysates were analyzed by western blotting. The protein concentration of cell lysates was examined

before resolving the total cellular protein (50 μ g/lane) using SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and the membranes were then blocked using 5% skim non-fat milk in Tris-buffered saline (TBS). The membranes were then incubated with primary antibodies specific for LASP1 (ab117806; Abcam, USA), ferritin light chain (ab69090; Abcam, USA), and ferritin heavy chain (ab65080; Abcam, USA). The membranes were washed three times in TBS containing 0.1% Tween 20 and then incubated with the appropriate secondary antibodies. The protein-antibody complexes were detected using a chemiluminescence reagent kit (ECL; Pierce Bio, USA). The protein-specific bands were analyzed by semi-quantitative densitometry.

Statistical analysis

The data obtained from real-time quantitative PCR and the density of protein bands from western blotting analysis were analyzed with SPSS12.0 software (SPSS Inc., Chicago, IL). The data shown are the mean \pm SD. The comparison of quantitative data was carried out using independent sample *t*-test and a value of $p < 0.05$ was considered to be statistically significant.

Results

rhBMP-2-induced osteogenesis in BMSCs

The BMSCs were cultured and treated with rhBMP-2 as described in materials and methods. The cell morphology was analyzed by observation under a microscope. The

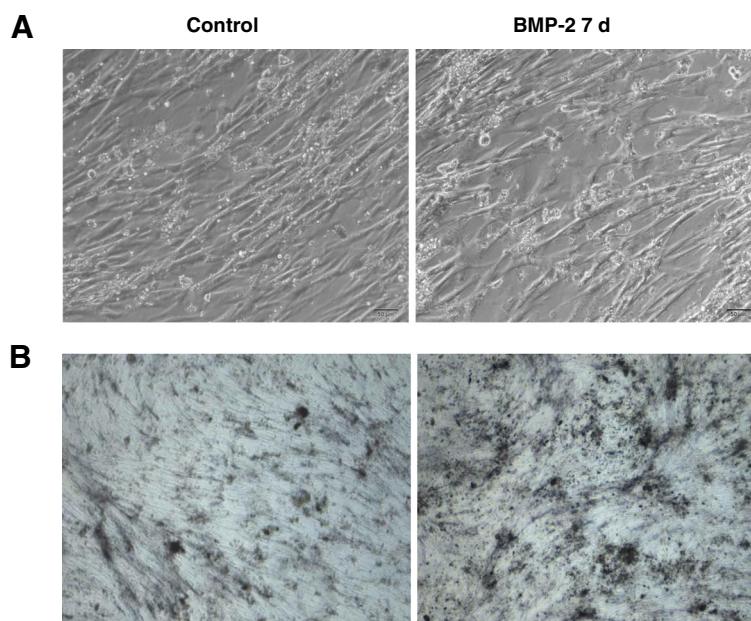


Figure 1 rhBMP-2-induced BMSC osteogenic differentiation. (A) Cell morphology of BMSCs without or with rhBMP-2 induction (labeled as control and BMP-2, respectively). The magnification is 100X. (B) Alkaline phosphatase staining was used to determine the status of osteogenic differentiation of BMSCs, without or with treatment with rhBMP-2. The image magnification is 100X.

non-induced cells were short, with spindle-like fusiform shapes. At day 7 of culture, the number of cells increased, the size of the cells enlarged, and the cells became elongated (Figure 1A, control). During continued culture, the cells remained long and spindle-shaped. After induction with rhBMP-2, the shapes of the BMSCs began changing gradually. Seven days after induction, the cell morphology drastically changed from long spindle-like cells to cubic cells (Figure 1A, BMP-2). The osteogenesis potential of rhBMP-2-induced BMSCs was evaluated by staining for alkaline phosphatase activity, a marker for osteoblastic differentiation. When BMSCs were treated with rhBMP-2 for 7 days, a lot of dark blue dots appeared, indicating the presence of alkaline phosphatase activity (Figure 1B, BMP-2). In BMSCs that had not been treated with rhBMP-2, only a few alkaline phosphatase spots were detected (Figure 1B, control). These results show that BMSCs from beagles can be successfully cultured and that osteogenes can be induced by treatment with rhBMP-2.

Two-dimensional gel electrophoresis

The protein extracts from non-induced and rhBMP-2 induced BMSCs were examined by two-dimensional gel electrophoresis followed by silver staining. The images of the silver-stained gels were clear and without background interference (Figure 2A, control and BMP-2). The protein spots were completely separated and similar results were obtained in all three independent trials of the experiment. An average of 1125 protein spots could be identified in the gel images from the three independent trials. The matching protein spots in the gels corresponding to the control (non-induced) and rhBMP-2-treated samples were considered to originate from the same proteins. The results showed that the percentage of matched proteins from the two groups of cells was approximately 90.84%.

After manual comparison and PDQuest 7.1.0 software analysis of the protein spots, 20 proteins with significant differences of expression levels between the control and induced cells were selected for further analysis. The

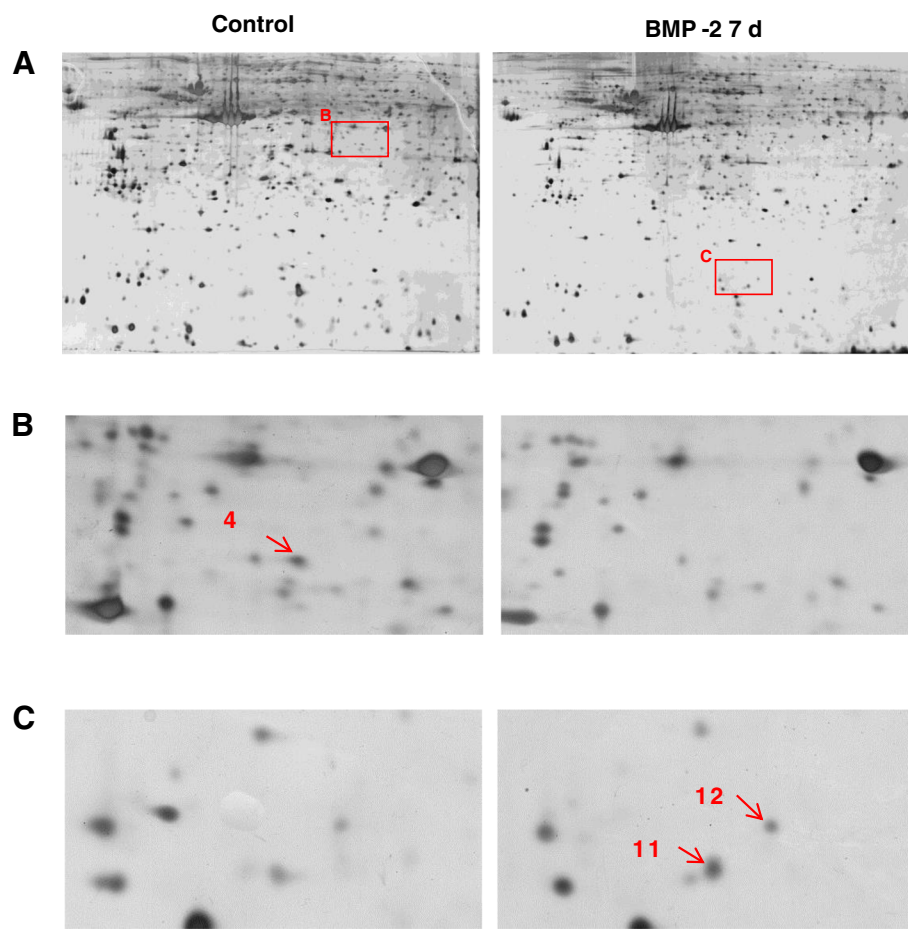


Figure 2 Proteomics analysis of protein expression profiles in rhBMP-2-induced BMSCs. (A) 2D gel electrophoresis maps of BMSCs without or with rhBMP-2 induction (labeled as control and BMP-2, respectively). (B and C) Close-up images of cropped sections of 2-DE maps shown in (A). Protein number 4, LASP1; Number 11 and 12, ferritin light and heavy chains.

Table 2 The proteins identified by proteomics analysis

Name	Accession number	Protein score	Mass	pI	Peptides identified	% Peptide coverage	rhBMP-2 induction/Control
1 Eukaryotic translation initiation factor 3 subunit K isoform 1	gij57036583	95	25342.6	4.81	YNPENLATLER ALTNLPHTDFTLCK GIDRYNPENLATLER CMIDQAHQEERPIR	25.7	0.3
2 Heat shock protein beta-6	gij57038366	100	17382.1	5.95	HFSPEEIAVK VGDHVEVHAR HEERPDEHGVIAR	21	<0.1
3 Annexin A8 isoform 1	gij73953207	102	36827.5	5.53	VFEERYER FITILCTR NLHSYFAER ILVCLLQGSR EGVIIELASR GIGTNEQAIIIDLTR DLTETLQSELSGKFER NLHSYFAERLYYALK GSPHFNPEPDAAELYTAMK AQFGKDLTETLQSELSGK	39	<0.1
4 LIM and SH3 domain protein 1 isoform 2	gij73966152	89	30162.4	6.33	EPAAPVSIPR QQSELQSQVR IPYCNAHYPK GFSVADTPELQR LKQSELQSQVR GKGFSWADTPELQR QSFTMVADTPENLR TQDQISNIKYHEEFEK	38	<0.1
5 Glucose-6-phosphate isomerase	gij296080693	94	63147	8.42	VDHQTGPIVWG KTFTTQETIT RSGDWKGYTGCETHAMLP YDQ	7.5	<0.1
6 RAD23 homolog B	gij57094213	99	43171	4.77	A PTPVPALAPT QAYFACEKNEN AAKQEKPAEK VMVTKPKAVS	10.6	<0.1
7 Fibronectin 1	gij345797318	99	262625	5.46	LEPGTEYTI RARIAGYRLT CSTTSNYEQDQ STSQTAPDAP YWGGETWEKPYQG LSISPSDNWVLTNLL	3	<0.1
8 Poly(rC) binding protein 1	gij222352151	95	37498	6.66	SSSPEVKGYWA NSSMTNSTAASRPP	7	<0.1
9 Signal transducer and activator of transcription 1	gij74005006	68	87335	5.74	FHAVEPYTK LWYNMLVTE RQQSACIGGPPN VIDLETSLPWV	6	<0.1

Table 2 The proteins identified by proteomics analysis (Continued)

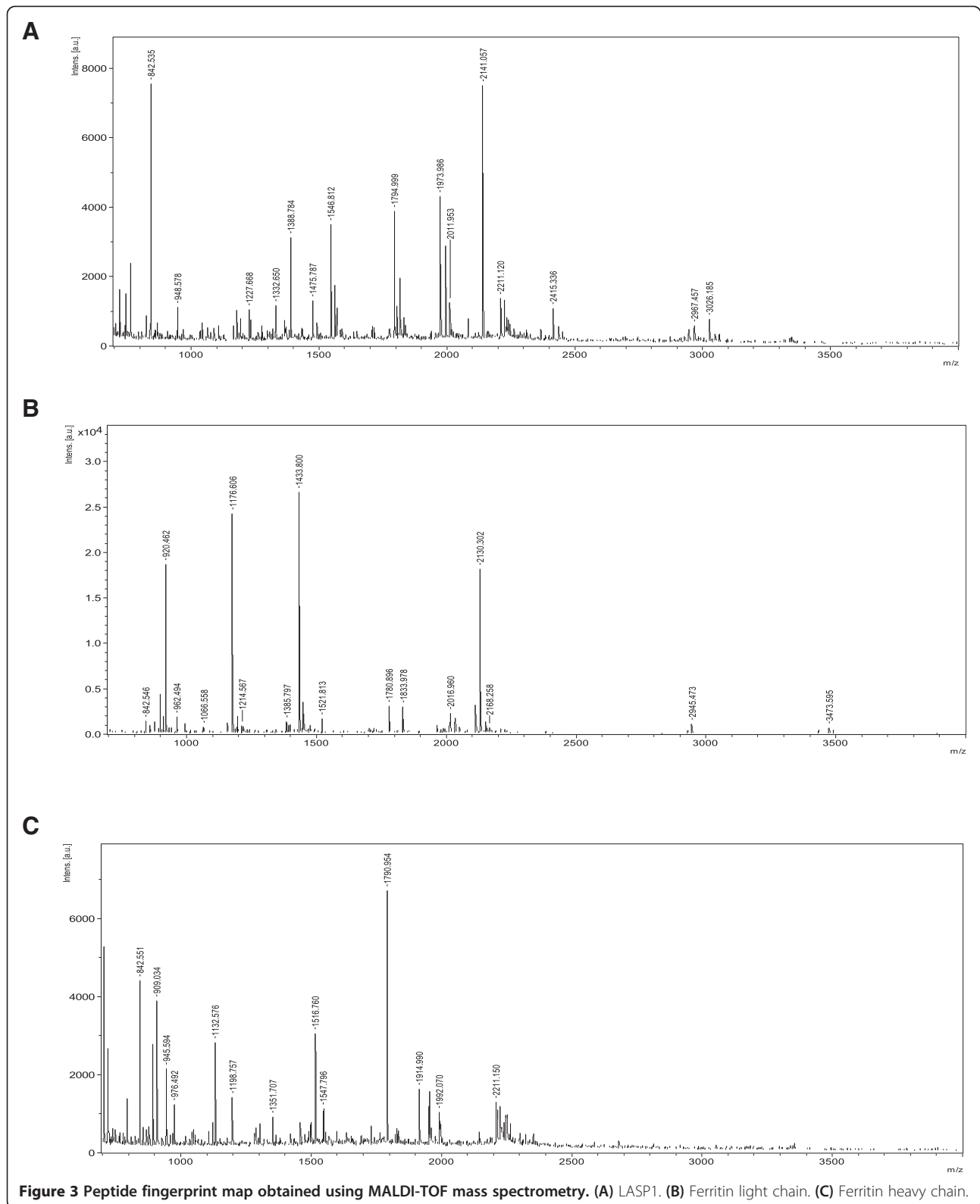
10	Retinol-binding protein 4	gij73998292	100	30177	8.48	QEELCLAR RLPSV FHPGR DPNGLPLEAQK QRQEELCLAR LIVHNGYCDGR LIVHNGYCDGRSEPNTL LLNLDGTCADSYSFVFSR KDPEGLFLQDNIVA EFSVDENGR GNDDHWIIDTDYDTYAVQYSCR	49	>10
11	Ferritin light chain	gij66864897	100	20139.2	5.66	MGDHLTNLRR ELAEKREGAER DDVALEGVGHFFR QNYSTEVEAAVNR LATPQAGLGEYLFER SLNQALLDLHALGSAR ASYTYLSLGFYFDR RLATPQAGLGEYLFER ADPHLCDFLENHFLDEEVK	73	4
12	Ferritin heavy chain	gij302393573	100	21408.4	5.53	ELGDHVTNLR SVNQSLLELHK YFLHQSHEER IFLQDIKKPDR SIKELGDHVTNLR QNYHQDSEAAINR SIKELGDHVTNLRK YFLHQSHEEREHAEK LATDKNDPHLCDFIETHYLNEQVK	66	3
13	Annexin A4	gij55742853	96	36075.2	5.72	VLVLSAGGR FLTVLCSR NKSAYFAER SDTSFMFQR DEGNFLDDALMR NHLLHVFDEYKR NRNHLLHVFDEYK SLYSFIKGDTSGDYR AEIDMMDIRESFKR SLYSFIKGDTSGDYRK NRNHLLHVFDEYKR DLMDDLKSELSGNFER GGTVKPASGFSA TEDAQTLR VLVLSAGGRDEGNFLDDALMR	60	>10
14	Proteasome activator complex subunit 3, isoform 6	gij73965646	84	29601.6	5.69	YPHVEDYR LKVDSFRER YPHVEDYRR IAKYPHVEDYRR	45	3

Table 2 The proteins identified by proteomics analysis (Continued)

						RLDECEEFQGTK		
						TVSEEAASYLDQISR		
						ITSEAEDLVANFFPK		
						TVTEIDEKEYISLR		
						IEDGNNFGVSIQEETVAELR		
15	Adenosylhomocysteinase isoform 1	gij73991635	100	48260.4	5.98	RIILAEGR	14.8	3
						FDNLYGCR		
						YPQLLSGIR		
						VNIKPVDR		
						SKFDNLYGCR		
						VAWAGYGDVKGKCAQALR		
16	Histidyl-tRNA synthetase, cytoplasmic, isoform 1	gij73949318	73	57880.3	5.44	IFSIVEQR	8	>10
						YDLTVPFAR		
						DQGGELLSLR		
						KVPCVGLSIGVER		
17	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	gij119618005	87	40,095	9.45	SWSVLNKE	10	4
						DAAPKMYKEEGL		
						IFNGFSVTLKEDGVRG		
18	ATP-binding cassette, sub-family B (MDR/TAP), member 1	gij 51094928	101	141479	9.06	EGLMPNTL	6	3
						LSLEVKKG		
						AVKEANAYDFI		
						GLQPAFAIIFSKIIG		
						LTLVILAISPVLGLS		
						AGNLEDLMSNITNRSDIND		
19	DEAD (Asp-Glu-Ala-Asp) box helicase 1	gij 148666036	66	82432	6.80	ERLGKNHI	7	>4
						CLHGDRKPHERKQN		
						GCYLDIDKGHVKFSK		
						AIGSDGLCCQSREVKE		
20	Isocitrate dehydrogenase 1 (NADP+), soluble	gij119590847	99	46659	6.53	EVSITIEA	11	3
						SPNGTIRNI		
						GTQKVTVLVHNF		
						DDMVAQAMKSEGGFI		
21	Filamin B, beta	gij 410336835	73	278164	5.47	LEFLDRES	2.5	3
						EAGEGDVSV		
						PHVWKVFFAG		
						ECSDNGDGTCS		
						ILVKYNDKHIPGS		
						KQPAKFTVDTISAG		

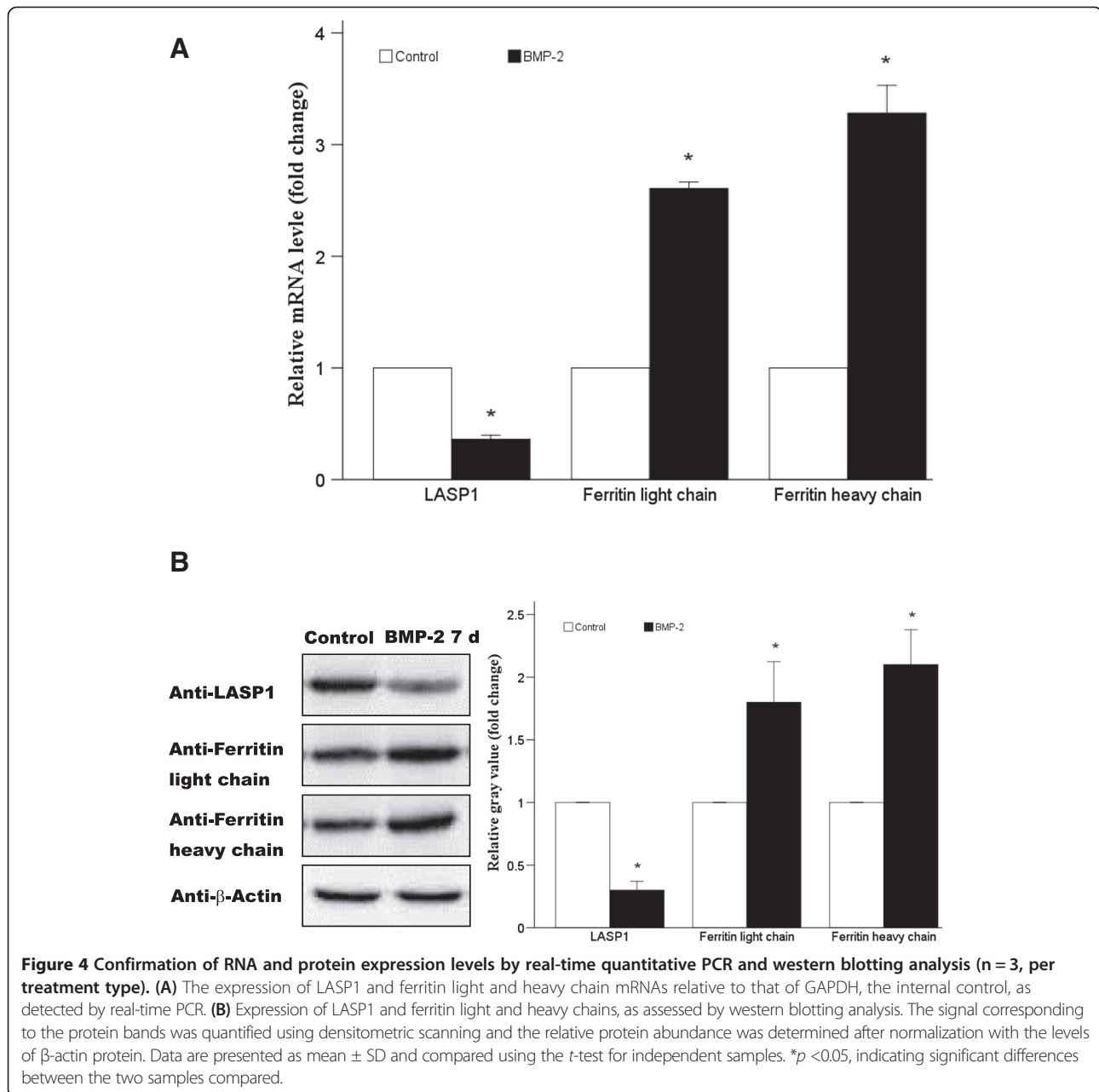
differences were categorized as proteins that were up-or down-regulated by 2 times or more, and proteins that were either down-regulated (9 proteins) or up-regulated (11 proteins) in the BMP-2-induced cells were analyzed further by MALDI-TOF mass spectrometry for protein identification (Table 2). The proteins numbered as 4, 11,

and 12 in Table 2 were identified as LASP1 isoform 2, ferritin light chain, and ferritin heavy chain (Figure 2B and C, marked by red arrows). Mass spectrometry peptide fingerprint peaks of these three proteins are shown in Figure 3A-C, respectively. The expression of the mRNAs corresponding to these three proteins in



control and BMP-2-treated cells was examined by real-time quantitative PCR. Compared with the expression levels in the control cells, the expression of LASP1 decreased, while

that of ferritin light and heavy chains increased, in the rhBMP-2-induced cells ($p < 0.05$) (Figure 4A). The expression levels of these three proteins in the non-induced and



induced cells were also examined by western blotting. Consistent with the expression data for the mRNA, LASP1 protein levels were higher in control cells, while the levels of ferritin light and heavy chains was higher in the BMP-2-treated cells (Figure 4B). Densitometric analysis of protein bands from the western blots also indicated the same pattern of expression as that from the real-time quantitative PCR analysis of mRNA expression ($p < 0.05$) (Figure 4B).

Discussion

In this study, beagle BMSCs were successfully isolated and cultured. The BMSCs were stimulated with rhBMP-2 to

induce osteogenic differentiation. After 7 days of stimulation, osteogenic differentiation of BMSCs was seen, confirmed by the morphological changes in BMSCs and the appearance of alkaline phosphatase activity, a marker for osteoblastic differentiation. The protein expression profiles of untreated control and rhBMP-2-treated BMSCs were characterized using 2D gel electrophoresis. Comparison of the two protein expression profiles indicated that 9 proteins were up-regulated and 11 proteins were down-regulated after stimulation of BMSCs with rhBMP-2. Based on previously published reports and bioinformatics analysis, two proteins, LASP1 and ferritin

(light and heavy chains), were selected for further analysis as candidate proteins involved in osteogenic differentiation induced by BMP-2. The results from real-time quantitative PCR and western blotting analyses confirmed the down-regulation of LASP1 and the up-regulation of ferritin in rhBMP-2-induced BMSCs.

LASP1 protein contains one LIM, one SH3, and two actin domains, and was first discovered in 1996 as a protein that is over-expressed in breast cancer [12]. Studies about LASP1 have almost all concentrated on LASP1 expression in tumors. LASP1 is involved in regulating cellular functions associated with actin-cytoskeleton-associated membrane rearrangements [13-15]. One study showed that aberrant expression of LASP1 can affect the formation of cartilage tissue [16]. To our knowledge, ours is the first study demonstrating a decrease in LASP1 expression during the process of rhBMP-2-induced osteogenic differentiation.

Ferritin is an important molecule in iron homeostasis in cells [17]. Iron overload can inhibit osteoblast differentiation and induce ferritin expression. Iron-provoked inhibition of osteoblast activity is mediated by ferritin and its ferroxidase activity [18]. We found that the expression of ferritin light and heavy chains was increased in BMSCs after induction of differentiation by rhBMP-2. The possible role for the up-regulation of ferritin might be that when stem cells in bone marrow are stimulated by rhBMP-2, over-expression of ferritin causes the enrichment of iron, thereby inhibiting osteogenic differentiation. However, the concentration of iron ions in the culture medium is maintained at steady levels, hence osteogenic differentiation is not inhibited under *in vitro* culture conditions, even though ferritin expressed is increased upon stimulation with rhBMP-2. The extent and exact mechanism(s) through which the expression of LASP1 and ferritin are altered upon stimulation with BMP-2 need to be addressed in further mechanistic studies of osteogenic differentiation of BMSCs.

In summary, rhBMP-2 induces osteogenic differentiation of BMSCs, decreases LASP1 expression, and increases ferritin expression. LASP1 is a key factor involved in the regulation of the cytoskeleton and ferritin controls cellular iron homeostasis, and the expression changes in these proteins likely play an important role in the process of rhBMP-2-induced osteogenic differentiation. The results of this study provide additional information regarding the role of changes in expression of specific proteins in driving BMP-2-induced osteogenic differentiation and indicate the need for further studies to elucidate the specific mechanism(s) through which BMP-2 promotes differentiation of BMSCs.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JH carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. MH carried out the immunoassays. DJ participated in the sequence alignment. HZ participated in the design of the study and performed the statistical analysis. YL conceived of the study, and participated in its design and coordination and helped to draft the manuscript. BY participated in the molecular genetic studies and the sequence alignment. All authors read and approved the final manuscript.

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