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Research Article

HOX gene analysis in the osteogenic differentiation of human mesenchymal stem cells

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Abstract

Human bone marrow-derived mesenchymal stem cells (hMSCs) have the capacity to differentiate into osteoblasts during osteogenesis. Several studies attempted to identify osteogenesis-related genes in hMSCs. Although *HOX* genes are known to play a pivotal role in skeletogenesis, their function in the osteogenesis of hMSCs has not yet been investigated in detail. Our aim was to characterize the expression of 37 *HOX* genes by multiplex RT-PCR to identify the ones most probably involved in osteogenic differentiation. The results showed that the expression patterns of four *HOX* genes were altered during this process. In particular, the expression levels of *HOXC13* and *HOXD13* were dramatically changed. Real-time PCR and Western blot analysis were performed in order to further analyze the expression of *HOXC13* and *HOXD13*. The qRT-PCR results showed that transcription of *HOXC13* was up-regulated by up to forty times, whereas that of *HOXD13* was down-regulated by approximately five times after osteogenic differentiation. The Western blot results for the HOXC13 and HOXD13 proteins also corresponded well with the real-time PCR result. These findings suggest that HOXC13 and HOXD13 might be involved in the osteogenic differentiation of hMSCs.

Key words: human mesenchymal stem cells, *HOX* genes, osteogenic differentiation, stem cell differentiation, gene profiling.

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Introduction

Bone marrow-derived stem cells can be divided into two major types: hematopoietic stem cells and nonhematopoietic, or mesenchymal, stem cells. Human bone marrow-derived mesenchymal stem cells (hMSCs) have the capacity for self-renewal and multilineage differentiation. Under the appropriate conditions, they can also give rise to mesenchymal tissues such as muscle, bone, fat, and cartilage (Pittenger *et al.*, 1999). Due to their ability to differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes and myoblasts, hMSCs hold promise for clinical applications in regenerative medicine (Song *et al.*, 2006).

Because osteoblastic cells play a major role in the processes of normal bone growth, remodeling and fracture repair, many researchers have used the process of osteogenesis to study the differentiation and characteristics of stem cells (Kraus and Kirker-Head, 2006). To obtain osteoblastic cells, MSCs are incubated with a mixture medium containing dexamethasone, β-glycerophosphate and ascorbic acid for a period of 2~3 weeks (Bobis *et al.*, 2006).

HOX genes were initially identified by their homology with the Drosophila HOM genes (Levine *et al.*, 1984; Acampora *et al.*, 1989; Duboule and Dolle, 1989). These genes encode homeodomain transcription factors related to anterior-posterior axis patterning that takes place during embryonic development (van den Akker *et al.*, 2001). The

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homeodomain contains a 180-base-pair homeobox sequence that encodes a conserved 60 amino acid region and acts as a DNA-binding domain via a helix-turn-helix motif (Gehring *et al.*, 1994). In vertebrates, 39 *HOX* genes have been identified. These are distributed over four homologous HOX clusters termed HOXA, B, C, and D. These loci are located on four different chromosomal locations and are comprised of nine to eleven genes (Akin and Nazarali, 2005). It is well known that HOX proteins participate in many common developmental processes during normal embryogenesis. Several reports have indicated that *HOX* genes play a regulatory role in skeletogenesis (Goff and Tabin, 1997; Kanzler *et al.*, 1998; van den Akker *et al.*, 2001; Remacle *et al.*, 2004).

Although *HOX* genes are known to play an essential role in skeletal development and bone formation, there is no report regarding the screening of HOX groups that are involved in the osteogenesis of hMSCs. Thus, in the present study, the expression profile of *HOX* genes during osteogenic differentiation of hMSCs was investigated by multiplex PCR and the results showed significant changes in the expression of four of them during this process. Of these four genes, the expression of *HOXC13* and *HOXD13* showed the most dramatic changes. Therefore, the expression levels of HOXC13 and HOXD13 were evaluated by qRT-PCR and Western blot analysis, and the results were similar to the multiplex PCR result. This suggests that the other two genes (*HOXA1* and *HOXC11*) are also involved in osteogenesis.

Materials and Methods

Research protocol

The research protocol was reviewed and approved by the human ethical care committee at St. Mary's Hospital, Catholic University in Daejeon, Republic of Korea. The hMSCs were isolated from the bone marrow of six individuals, as described below (Choi *et al.*, 2006). All experiments were performed with hMSCs obtained after the third cell passage.

Flow cytometric analysis (FACS) of hMSCs

hMSCs were analyzed by FACS-Calibur (Becton Dickinson, San Jose, CA) as previously described (Choi *et al.*, 2006). FACS analysis was performed using fluorescein isothiocyanate (FITC)-conjugated anti-CD11b, CD29, CD34, CD45, CD73 and CD105 antibodies (BD Bioscience, San Diego, CA) to confirm that the phenotype of the hMSCs was maintained after expansion in the culture. The samples were incubated with antibodies against each surface marker for 30 min, and this treatment was followed by FACS.

Osteogenic differentiation

To induce osteogenic differentiation, hMSCs at the third passage were plated with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a 250-ml tissue culture flask (Nunc, Roskilde, Denmark). The cells were then incubated at 37 °C in 5% CO_2 for 24 h. The medium was replaced with high-glucose DMEM containing 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 0.3 mM ascorbic acid (Sigma, St. Louis, MO) for osteogenic differentiation. This osteogenic medium was replaced every 2 days for 21 days.

Alkaline phosphatase (ALP) staining

About 3 x 10^5 cells were seeded onto each well of a 6-well plate. After incubation for 12 h at 37 °C in 5% CO_2, the medium was replaced with osteogenic differentiation medium, replaced again every 2 days for periods of 10 and 21 days. The 10-day and 21-day differentiated and undifferentiated hMSCs were washed twice with ice-cold PBS (phosphate buffered saline), fixed with 2% paraformaldehyde/ 0.1 M sodium cacodylate for 10 min, and washed with 0.1 M cacodylic acid. The cells were incubated with ALP substrate solution (5 mg naphthol AS-TR phosphate in 25 mL water plus 10 mg Fast red TR in 24 mL of 0.1 M Tris buffer, pH 9.5) for 1 h at room temperature. Cells were photographed using a Nikon TE-300 (Tokyo, Japan) inverted light microscope.

von Kossa staining

Approximately 3 x 10^5 cells were seeded onto each well of a 6-well plate. After incubation for 12 h at 37 °C in 5% CO_2, the medium was replaced with osteogenic differentiation medium and thereafter replaced every 2 days for periods of 10 and 21 days. Day-10 and day-21 differentiated and undifferentiated hMSCs were washed with distilled water, fixed with 4% formalin, and then treated with 5% silver nitrate. Then the cells were exposed to UV light for 1 h, 5% thiosulfate was added, and the cells were placed at room temperature after a washing step with distilled water. The samples were photographed with a Nikon TE-300 (Tokyo, Japan) inverted light microscope.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed as described by Jee *et al.* (2006). After the induction of osteogenic differentiation for 21 days, total RNA was isolated from the cells using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Two micrograms of total RNA were reverse-transcribed in order to synthesize cDNA, using an AccuPower RTPReMix kit (Bioneer, Inc., Rockville, MD). The subsequent PCR amplification was performed with 1 μL of RT reaction mixture, using the following thermocycling profile: 1 cycle at 94 °C for 5 min, followed by 30 cycles of 92 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, and a final cycle at 72 °C for 10 min. The primer sequences used are listed in Table 1. The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an internal control. The PCR

Table 1 - Primer sequences.

Symbol	Name	RefSeq	Primer
ALP	Alkaline phosphatase	NM_000478	5'-ATCTCGTTGTCTGAGTACCAGTCC 5'-TGGAGCTTCAGAAGCTCAACACCA
BSP	Bone sialoprotein	NM_004967	5'-ATCATAGCCATGGTAGCCTTGT 5'-AATGAAAACGAAGAAAGCGAAG
OCN	Osteocalcin	NM_199173	5'-GCCGTAGAAGCGCCGATA GGC 5'-ATGAGAGCCCTCACACTCCTC
HOXC13	Homeobox C13	NM_017410	5'-CTGTCCTCTAGGGCCAAGGAGTTCGCC TTCTACC 5'-GTAGCCTTCGACGGGGATGAGGGCGTC GTGAC
HOXD13	Homeobox D13	NM_000523	5'-TTCTGCTGCCCAACCTGACTTTGTAGT TCTG 5'-GAGCACTGCCTGCCTTTCCACTTGCCT CAGGGCAA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	5'-CGAGATCCCTCCAAAATCAA 5'-TGCTGTAGCCAAATTCGTTG

products were run on a 1% agarose gel and then analyzed under UV light after staining with ethidium bromide. The gel was photographed and then quantitatively measured by scanning densitometry. The experiments were performed with three different RNA samples.

Immunoblotting analysis

Immunoblotting analysis was performed as previously described (Jee *et al.*, 2007). After incubation with osteogenic differentiation medium for 21 days, the cells were lysed on ice for 30 min in RIPA buffer (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1% NP-40, 0.1% SDS, 1 mM DTT and 1 mM PMSF) containing a mixture of protease inhibitors (Roche, Mannheim, Germany). The insoluble materials were separated using 10% polyacrylamide gels containing 10% sodium dodecyl sulfate (SDS), 1.5 M Tris-HCl, 0.035% N, N, N', N'-tetra-methylenediamine and 7 mg ammonium persulfate. The separated proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) at 36 mA in a transfer buffer that contained 39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol. The membranes were sequentially incubated with anti-OPN (osteopontin), OCN (osteocalcin), HOXC13 monoclonal antibodies (mAb; Abnova, Taipei, Taiwan), and HOXD13 mAb (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilutions. A horseradish peroxidase-conjugated anti-rabbit IgG was used as a secondary antibody at a dilution of 1:1500 (Pierce Biotechnology, Rockford, IL). Detection was performed with an electrochemiluminescence detection reagent (Amersham Biosciences, Uppsala, Sweden). In some cases, the Western blots were stripped and re-blotted with antibody, according to the manufacturer's instructions.

Multiplex PCR of *HOX* genes

Multiplex PCR was performed using the GeneXP Human HOX Assay Kit (Seegene, Seoul, Republic of Korea). After the induction of osteogenic differentiation for 21 days, total RNA was isolated from the cells using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Two micrograms of total RNA were reverse-transcribed in order to synthesize cDNA using an AccuPower RTPReMix kit (Bioneer, Inc., Rockville, MD). The synthesized cDNAs were used as templates for multiplex PCR,according to the manufacturer's instructions (http://www.seegene.com). PCR was carried out under the following conditions: 1 cycle at 94 °C for 15 min, followed by 40 cycles of 94 °C for 0.5 min, 63 °C for 1.5 min, and 72 °C for 1.5 min, and a final cycle at 72 °C for 10 min. *GAPDH* was used as an internal control. Electrophoresis was carried out on a 2% agarose gel. The multiplex PCR products were analyzed with the Alpha EaseFC software (Alpha Innotech, San Leandro, CA). The experiment was performed six times on each individual (Table 2).

Real-time quantitative PCR analysis (qPCR)

After the induction osteogenic differentiation for 21 days, total RNA was isolated using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Two micrograms of total RNA were reverse-transcribed in order to synthesize cDNA, using an AccuPower RTPReMix kit (Bioneer, Inc., Rockville, MD). For relative quantification, the reactions were performed in a total volume of 20 µL, containing 15 µL of LightCycler® FastStart DNA Master SYBR Green 1 (Roche Diagnostics, Mannheim, Germany), 10 ng of cDNA, and 10 pmol of each primer. Real-time quantitative PCR was carried out with specific primers, in a LightCycler Instrument (Roche Diagnostics, Mannheim, Germany). The samples were analyzed in triplicate. The primer sequences used are listed in Table 1. *GAPDH* was used as an internal control. For quantification, the data were analyzed using the LightCycler analysis software (Roche Diagnostics, Mannheim, Germany). Relative quantification of target gene expression was evaluated using the comparative C_T method (Wang *et al.*, 2004). The ΔC_T value was determined by subtracting the target C_T of each sample from its respective *GAPDH* C_T value. Calculation of ΔC_T involves using the mean ΔC_T value of the control gene as an arbitrary con-

Table 2 - *HOX* multiplex PCR. A total of thirty-seven *HOX* genes were examined using multiplex RT-PCR. The relative expression values are expressed as mean ± SEM. *P* values were calculated using ANOVA.

Gene	Relative expresion value			p	Gene	Relative expresion value			p
	Day 0	Day 10	Day 21			Day 0	Day 10	Day 21	
HOXA1	100	110.8 ± 4.0	166.6 ± 9.7	0	*HOXC4*	100	112.5 ± 2.9	112.7 ± 8.1	0.262
HOXA2	100	62.8 ± 5.6	95.8 ± 7.7	0.004	*HOXC5*	100	109.6 ± 9.5	138.9 ± 15.1	0.066
HOXA3	100	93.8 ± 6.0	112.6 ± 12.5	0.389	*HOXC6*	100	111.2 ± 11.2	162.4 ± 24.2	0.76
HOXA4	100	143.8 ± 14.7	146.4 ± 11.4	0.054	*HOXC8*	100	100.0 ± 2.9	97.7 ± 7.2	0.939
HOXA5	100	74.4 ± 5.1	92.7 ± 12.7	0.226	*HOXC9*	100	101.6 ± 3.8	100.3 ± 7.1	0.982
HOXA6	100	105.7 ± 8.7	130.3 ± 12.5	0.119	*HOXC10*	100	93.8 ± 4.8	90.1 ± 3.3	0.644
HOXA7	100	83.7 ± 3.0	82.8 ± 6.1	0.217	*HOXC11*	100	136.6 ± 9.6	100.6 ± 9.0	0.01
HOXA9	100	100.2 ± 4.6	121.3 ± 10.8	0.804	*HOXC12*	100	118.1 ± 5.3	130.8 ± 10.5	0.095
HOXA10	100	95.5 ± 2.0	98.5 ± 5.3	0.804	*HOXC13*	100	91.3 ± 5.3	191.0 ± 10.5	0
HOXA11	100	96.0 ± 3.2	123.5 ± 11.2	0.104	*HOXD1*	100	106.7 ± 6.2	94.0 ± 7.4	0.452
HOXA13	100	85.3 ± 6.5	93.2 ± 3.1	0.197	*HOXD3*	100	112.2 ± 6.8	110.1 ± 13.1	0.648
HOXB1	100	90.4 ± 7.7	85.9 ± 7.7	0.348	*HOXD4*	100	148.1 ± 10.5	131.1 ± 16.4	0.067
HOXB3	100	105.4 ± 3.3	93.8 ± 6.8	0.395	*HOXD8*	100	104.4 ± 2.8	113.7 ± 3.5	0.05
HOXB4	100	77.3 ± 9.4	76.5 ± 5.0	0.164	*HOXD9*	100	78.3 ± 6.8	90.3 ± 6.8	0.068
HOXB5	100	94.7 ± 4.8	97.5 ± 9.6	0.834	*HOXD10*	100	106.5 ± 11.7	122.7 ± 15.4	0.508
HOXB6	100	98.2 ± 5.0	115.5 ± 3.0	0.057	*HOXD11*	100	101.6 ± 3.8	100.3 ± 7.1	0.982
HOXB7	100	96.9 ± 5.7	109.6 ± 8.4	0.118	*HOXD12*	100	104.5 ± 8.1	118.6 ± 10.2	0.35
HOXB9	100	87.5 ± 5.5	124.3 ± 11.0	0.046	*HOXD13*	100	69.4 ± 6.3	49.6 ± 7.4	0
HOXB13	100	77.9 ± 3.8	86.0 ± 7.5	0.1					

stant to subtract from all other ΔC_T mean values. Fold-changes in gene expression of the target gene were equivalent to $2^{-}\Delta\Delta CT$. The values obtained were then entered into a Student's *t* test. *P* values less than 0.05 were considered significant.

Statistical analysis

To investigate differentially expressed *HOX* genes during osteogenic differentiation from hMSCs, the data obtained from multiplex PCR were examined by variance analysis (ANOVA) with SPSS 12.0 software for Windows (SPSS, Chicago, IL). Tukey's HSD test was used for post hoc comparisons. For all statistical tests, an error probability of $p < 0.05$ was regarded as significant.

Results

Characterization of hMSCs

In an effort to explore the characterization of hMSCs, flow cytometry was used to examine the expression of the surface antigens CD11b, CD29, CD34, CD45, CD73, and CD105 in the isolated hMSCs. The isolated hMSCs were submitted to FACS analysis and found to be positive for CD29 (68 ± 2.5%), CD73 (96.9 ± 2.7%) and CD105 (91.5 ± 2.5%), and negative for CD11b, CD34 and CD45. These results show that the hMSCs were successfully isolated and that the culture-expanded hMSCs maintained their phenotype (Figure 1).

Osteogenic differentiation

ALP and von Kossa staining were used to examine the differentiation of hMSCs into osteoblasts in the osteogenic medium. Although ALP staining at day 10 showed a weak color signal, the intensity of ALP activity increased remarkably by day 21. The intensity of von Kossa staining also peaked at day 21 (Figure 2A). RT-PCR was performed using osteogenic markers to confirm hMSC osteogenesis (Table 1). The mRNA expression levels of the osteogenic markers, which included bone sialoprotein (BSP), OCN and ALP, were significantly higher at day 21 than at day 0 (Figure 2B). Immunoblot analysis was performed using OCN and OPN in order to obtain further confirmation of osteogenesis. The results of von Kossa staining and RT-PCR were identical to the result observed with ALP and showed that the expression of the OCN and OPN proteins increased as differentiation progressed (Figure 2C). All of the corresponding results confirmed that the hMSCs were successfully differentiated into osteoblasts.

Analysis of *HOX* gene expression using multiplex PCR

Multiplex PCR was used to assess the expression levels of *HOX* genes during osteogenic differentiation. The expres-

sion patterns of the 37 *HOX* genes were screened at day 0, day 10, and day 21 in both undifferentiated and differentiated hMSCs. The expression of the 37 *HOX* genes at the level of transcription is listed in Table 2. The *HOXA1, HOXA4, HOXA6, HOXA9, HOXA11, HOXB9, HOXC5, HOXC6, HOXC12, HOXC13, HOXD4,* and *HOXD10* genes were up-regulated under the osteogenesis-induced condition. On the other hand, *HOXB4* and *HOXD13* were down-regulated during osteogenesis. The other *HOX* genes showed no significant changes in their mRNA expression levels.

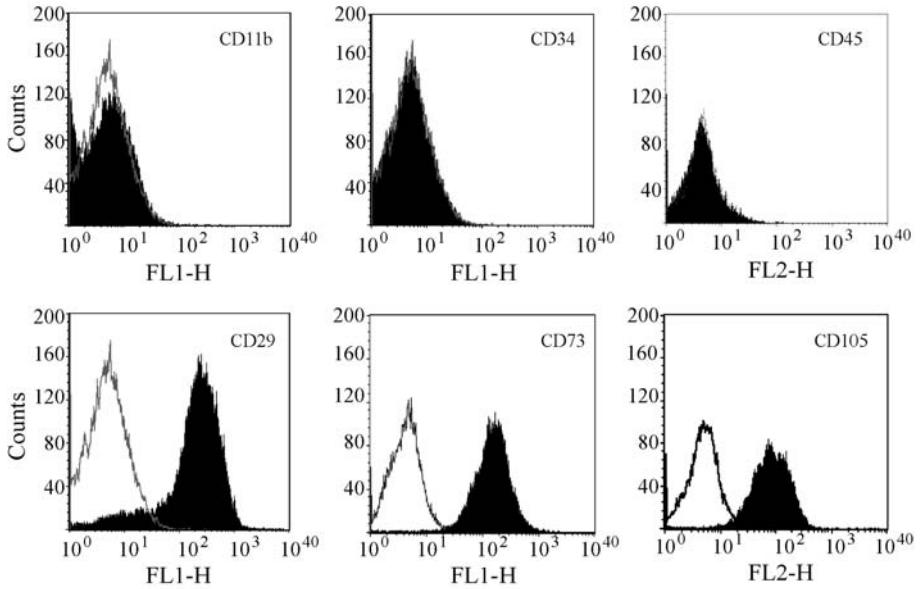

Figure 1 - Phenotypic characterization of hMSCs using flow cytometric analysis. FACS analysis showed that the cells were negative for CD11b, CD34 and CD45 expression and positive for CD29, CD73 and CD105, which are phenotypes currently known to be characteristic of hMSCs. The gray line indicates the control of the CD marker isotypes.

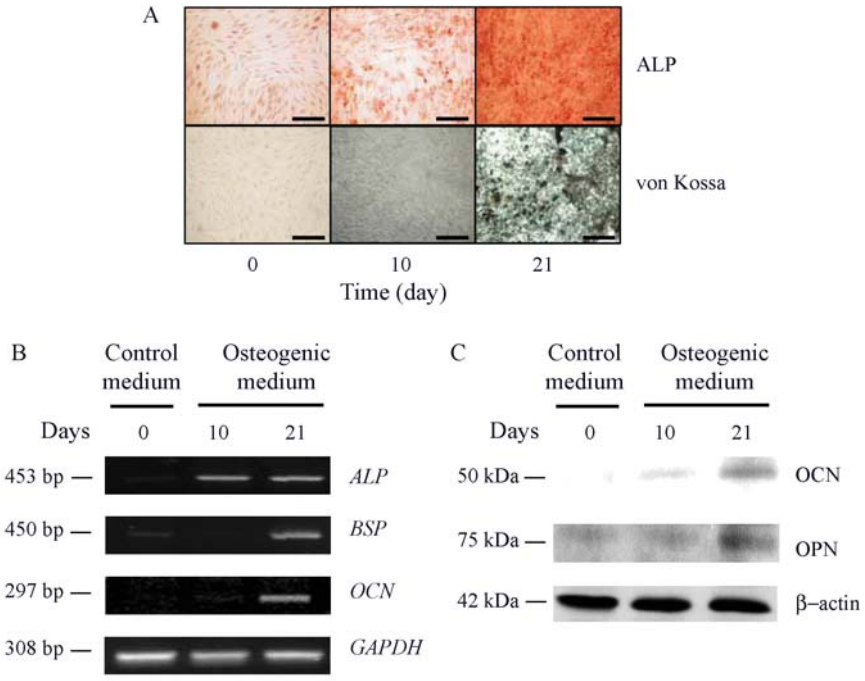

Figure 2 - Osteogenic differentiation of hMSCs. (A) ALP staining and von Kossa staining. Osteogenic differentiation was confirmed by ALP staining. The cells stained positively for endogenous ALP activity during 21 days of culture in osteogenic media. The von Kossa staining also showed an increase in calcium deposition during differentiation. Scale bar = 30 μm. (B) Expression of osteogenesis-specific genes (*ALP*, *BSP*, and *OCN*) was observed by RT-PCR at day 0, 10 and 21 of the culture period. *GAPDH* was used as control. The expression levels of the osteogenesis-specific genes increased during osteogenic differentiation. The early osteogenesis marker *ALP* showed increased expression at day 10, and the expression of late osteogenesis markers (*BSP*, *OCN*) was observed at day 21. (C) Immunoblotting analysis of OCN and OPN expression. Whole-cell proteins obtained on day 0, day 10 and day 21 were blotted onto a nitrocellulose membrane. The expression levels of the OCN and OPN proteins increased during osteogenic differentiation. β-actin was used as control.

Statistical analysis revealed that four *HOX* genes showed significant differences in expression at the transcription level. The *HOXA1*, *HOXC11* and *HOXC13* genes were found to be up-regulated. The expression of *HOXC13* was unaltered between day 0 and day 10 and only increased after day 10. The expression of *HOXA1* gradually increased for 21 days, but the increase in the expression of *HOXC13* was more dramatic. The mRNA level of *HOXC11* fluctuated during osteogenesis. The expression of *HOXC11* increased during the first 10 days of osteogenic differentiation, but then decreased over the next 11 days (Figure 3A). The expression of *HOXD13* was down-regulated during the osteogenesis of hMSCs. The mRNA level of *HOXD13* decreased gradually over the 21-day period (Figure 3B).

Expression of *HOXC13* and *HOXD13*

The expression of *HOXC13* and *HOXD13* showed the most dramatic change after 21 days of differentiation. The expression of *HOXC13* increased by approximately 91%, whereas that of *HOXD13* decreased by 50% after osteogenesis. Real-time quantitative PCR and immunoblotting analysis were carried out in order to further confirm the increased expression of *HOXC13* and *HOXD13*. The results of qPCR showed that the expression of *HOXC13* was five times higher at day 10 and forty-two times higher at day 21 than in the undifferentiated state, respectively, whereas the mRNA expression of *HOXD13* showed a five-fold decrease at day 10 (Figure 4). These qPCR results of the *HOX* genes were in agreement with those of multiplex PCR.

The expression levels of these two *HOX* genes were then submitted to immunoblot analysis to further evaluate their protein level in the osteogenic differentiation of hMSCs. The results showed increased expression of the HOXC13 protein and decreased expression of the HOXD13 protein after 21 days of differentiation (Figure 5). This result was in agreement with those of multiplex and real-time PCR.

Discussion

Many factors are known to regulate osteogenesis (Bobis *et al.*, 2006). The important factors involved in osteogenic regulation include bone morphogenetic protein (BMP),

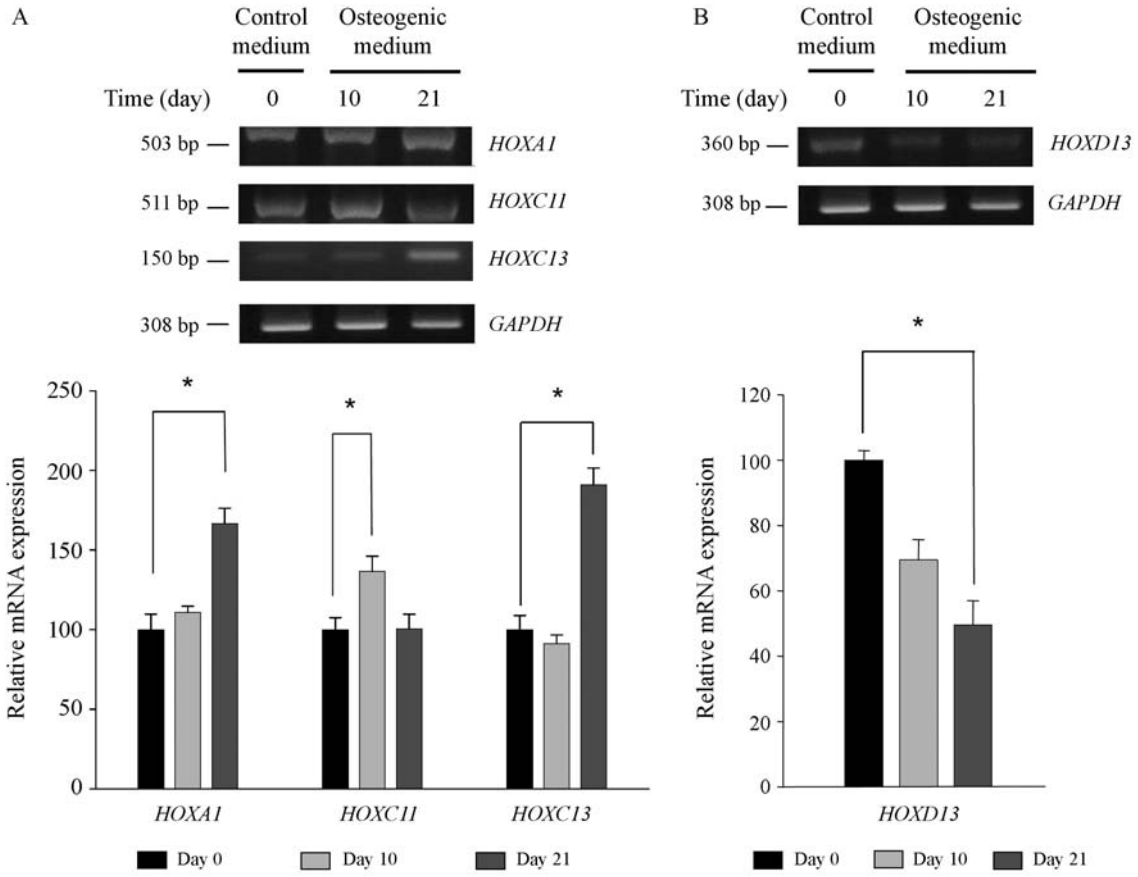

Figure 3 - *HOX* multiplex PCR. (A) The mRNA expression of *HOXA1*, *HOXC11* and *HOXC13* increased during osteogenesis. The *HOXC13* gene showed the most significant up-regulation. The expression of *HOXA1* gradually increased during osteogenic differentiation. The expression of *HOXC11* increased by approximately 30% and then returned to the expression level observed on day 0. (B) The mRNA expression of *HOXD13* clearly decreased during osteogenic differentiation. The expression of *HOXD13* decreased by approximately 70% on day 10; on day 21, the expression of *HOXD13* was half the value observed on day 10. *GAPDH* was used as control. The graphs represent the mean ± SEM of six separate experiments. Asterisk (*) indicates a significant increase between two samples ($p < 0.05$).

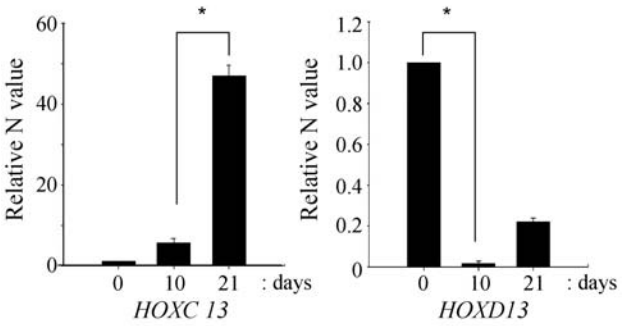

Figure 4 - Real-time PCR analysis of *HOXC13* and *HOXD1*. The data were presented as fold changes relative to day 0. The mRNA expression of *HOXC13* was five times higher on day 10 and 42 times higher on day 21 compared to the expression in a control. The expression of *HOXD13* decreased rapidly at day 10 and slowly increased at day 21. The real-time PCR data were normalized with *GAPDH* expression. Asterisk (*) indicates a significant increase between two samples ($p < 0.05$).

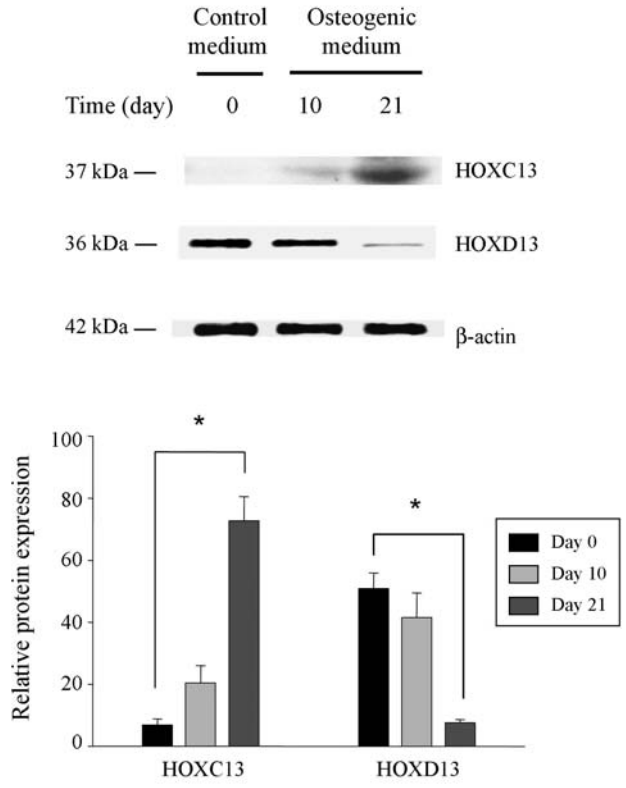

Figure 5 - Immunoblotting analysis of HOXC13 and HOXD13. Whole cell proteins obtained on day 0, day 10 and day 21 were blotted onto a nitrocellulose membrane. The protein level of HOXC13 showed a significant increase on day 21, whereas that of HOXD13 decreased gradually during osteogenic differentiation. β-actin was used as control. Asterisk (*) indicates a significant increase between two samples ($p < 0.05$).

transforming growth factor (TGF), insulin-like growth factor (IGF), brain-derived growth factor (BDGF), fibroblast growth factor (FGF), leptin and parathyroid hormone-related peptide (PTHrP). These proteins regulate the expression of signals needed for bone remodeling. In addition, many reports have suggested that various transcription factors participate in osteogenesis. Among them, Cbfa1/Runx2, Osterix, ΔFosB, Fra-1, Aj18, Osf1, Msx2, Dlx5 and TWIST have been shown to play pivotal roles.

Several studies have also reported that *HOX* genes are involved in osteogenesis. These reports showed that HOXA2 plays several important roles in the process of skeletogenesis (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993; Kanzler *et al.*, 1998). Another study found, using quantitative RT-PCR (Dobreva *et al.*, 2006), that the expression of HOXA2 was up-regulated during osteogenesis. HOXA10 has been shown to contribute to osteogenic lineage determination (Hassan *et al.*, 2007). HOXC8 was reported to be involved in the regulation of osteogenesis through bone morphogenic protein (BMP) pathways (Juan *et al.*, 2006). However, no significant changes in the expression of *HOXA2*, *HOXA10* and *HOXC8* were observed in the present study. The differences in these results may be due to the fact that HOXA2 may have been induced during mouse embryogenesis, and HOXA10 and HOXC8 expression were likely induced by BMP. However, in the present study, mesenchymal stem cells were used, and osteogenic differentiation was induced *in vitro* using dexamethasone, β-glycerophosphate and ascorbic acid.

Knowledge regarding the expression patterns of the *HOX* genes during osteogenic differentiation may reveal the signal pathway of osteogenesis and may also help in the potential therapeutic application of hMSCs. However, a report regarding the expression profile of *HOX* genes during osteogenesis has not yet been published. In the present report, 37 *HOX* genes were investigated in order to determine their expression patterns during the osteogenesis of hMSCs. For this purpose, we performed multiplex PCR, real-time PCR and Western blot analysis. Based on the results, we suggest that four *HOX* genes, *HOXA1*, *HOXC13*, *HOXC11* and *HOXD13*, might be involved in the osteogenic differentiation of hMSCs. *HOXA1* is a key gene in skull development, and it is a retinoic acid (RA) direct target gene (Ijichi and Ijichi, 2002). Mice with mutations in the HOXA1 hexapeptide motif show skeletal defects (Remacle *et al.*, 2004). Similar results were reported by Martinez-Ceballos *et al.* (2005), who showed that the disruption of the *HOXA1* gene results in abnormal ossification of the skull. Andrews *et al.* (1994) reported that osteogenic protein-1 (OP-1), a member of the TGF-β superfamily, induces HOXA1. In addition, recent microarray analyses revealed that BSP and Col1a1, both key markers of osteogenesis, are the target molecules of HOXA1 (Martinez-Ceballos *et al.*, 2005). The results of multiplex PCR showed that *HOXA1* was significantly increased during osteogenesis. The results of the present study and those of previous reports suggest that HOXA1 is an important factor involved in the osteogenesis of hMSCs.

In the present study, the expression of *HOXC13* showed the largest increase. However, there are no previous reports suggesting a relationship between *HOXC13* and osteogenesis. Kulessa *et al.* (2000) reported that the over-

expression of the BMP inhibitor resulted in the down-regulation of *HOXC13* expression in mutant mice. Based on the findings of the present study, it seems likely that *HOXC13* contributes to the osteogenesis of hMSCs via the BMP pathway.

The *HOXC11* gene encodes a transcription factor known to be involved in the definition of segment identities along the anterio-posterior axis. The expression of *HOXC11* is detected in the mesenchyme posterior to the region forming the femur and fibula (Hostikka and Capecchi, 1998). There is a report suggesting that *HOXC11* is involved in chondrogenesis, which is regulated by BMP2 and BMP7 (Papenbrock *et al.*, 2000). However, there is no clear evidence that *HOXC11* contributes to osteogenesis, thus *HOXC11* may be related to the osteogenesis of hMSCs. In particular, *HOXC11* may only be involved in the early stages of the osteogenic process from the hMSCs stage to the osteoblast progenitor cell stage, and not from the osteoblast progenitor cell stage to the osteoblast stage, once the expression level drops after day 10 (Figure 3A).

Williams *et al.* (2005) recently demonstrated that the interaction between the mouse HOXD13 protein and Smad1 might reciprocally antagonize the expression of Runx2, which is a key molecule in mammalian osteogenesis (Williams *et al.*, 2005). This implies that the expression of HOXD13 may decrease as osteogenesis progresses, which is in agreement with the results of the present study. In light of previous reports on *HOXD13* and of the present results, it is likely that the decrease in HOXD13 expression during osteogenesis is required for the promotion of osteogenic differentiation (Shi *et al.*, 1999; Yang *et al.*, 2000; Liu *et al.*, 2004; Williams *et al.*, 2005; Li *et al.*, 2006).

There are few studies regarding the *HOX* genes involved in the differentiation of hMSCs. The results of the present study show that the mRNA expression levels of four *HOX* genes noticeably changed during the osteogenic differentiation of hMSCs. Although the roles of the four genes in the osteogenic differentiation of hMSCs have yet to be clarified, the present study represents a first step elucidating the relationship between *HOX* gene expression and the differentiation of hMSCs, making part of the signalling pathway in osteogenic differentiation from hMSCs. Functional studies, such as a gene siRNA-mediatd gene silencing or gene transfection, are needed in order to further investigate the role of the *HOX* genes in osteogenic differentiation.

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