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Gene expression in normotopic and heterotopic human bone: increased level of *SP7* mRNA in pathological tissue

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Abstract Head injury-induced heterotopic ossification (HO) develops at vicinity of joints and in severe cases requires surgical intervention. Our previous study demonstrated high mRNA levels of osteocalcin (OC), type 1 collagen (COL1), osteonectin and RUNX2/CBFA1 in osteocytes and lining osteoblasts from non-evolutive HO compared to equivalent healthy cells from the proximal femoral shaft of patients receiving prosthesis. This allowed a first molecular characterisation of this pathological bone. The aims of this study is to extend the analysis to 10 more genes and determine those involved in the high OC mRNA level observed in pathological bone samples. RNAs were prepared from normotopic and heterotopic human bone samples digested by collagenase. After cDNA synthesis, mRNA levels were determined by real-time PCR and normalised using β actin and glyceraldehyde-3-phosphate dehydrogenase. OSTERIX/SP7 expression was observed for the first time in human adult bone biopsies. In HO samples higher levels of SP7 (four- to sevenfold increase) and 1α,25-dihydroxy vitamin D_3 receptor (VDR) (two- to threefold increase) were observed compared to control samples. Moreover, SP7 level was correlated to OC and RUNX2 levels. In control samples, OC and SP7 levels were correlated. Our study further confirms that the involvement of SP7 in bone physiology is not only limited to the developmental step. Moreover, our results support the hypothesis that in HO the high level of OC expression could be due not only to an increase in RUNX2, but also in SP7 or VDR or to an imbalance in their respective activities.

Keywords Osterix/SP7 · Osteocalcin · Heterotopic ossification · Real-time PCR

Abbreviations

($1,25(OH)_2D_3$)	1α,25-Dihydroxyvitamin D_3
AP-1	Activator protein-1
COL1	Type 1 collagen
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HO	Heterotopic ossification
OC	Osteocalcin
RT-PCR	Reverse transcriptase-polymerase chain reaction
RXR	Retinoid X receptor
SP7	Specificity protein 7
VDR	1α,25-Dihydroxyvitamin D3 receptor

Introduction

Heterotopic ossification (HO) is characterised by the formation of bone in sites where it is not normally present. In acquired forms, HO frequently occurs after neurological injury such as head injury. Commonly located near a joint,

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it may require surgical resection [1, 2]. Despite numerous studies based on the research of a humoral factor or the measurement of osteoblastic activity on cells isolated from HO and cultured, the biological mechanisms responsible for acquired HO formation remain to be determined [3, 4].

In a previous study, we showed the possibility to measure differences in gene expression between resected tissues without culturing step, using quantitative RT-PCR, while quantitative measurement of protein expression was not feasible due to the low amount of available human biopsies. We focused the study on osteocytes that, through its mechanosensor function, takes part in the modulation of the activity associated to bone remodelling and bony matrix maintaining [5]. On this cell population and compared to control samples, an over-expression in HO of *OC*, *RUNX2*, *COL1* and *Osteonectin* mRNAs was demonstrated [6]. The results raised several questions about the physiological as well as molecular origin of the observed over-expressions in osteocytes from HO. Because increasing observations suggest that osteocalcin, one of the very few osteoblast-specific proteins, has several features of a bone-derived hormone involved in regulation of energy metabolism [7], we focused on the transcription regulators of this gene.

Several transcription factors are known to be involved in osteoblastic differentiation and *OC* expression. *c-FOS*, *c-JUN*, *FRA-2* and *JUN D* are gene coding for activator protein-1 (AP-1) complexes that may have opposite effects on *OC* transcription [8]. The homeodomain containing transcription factors MSX2 and DLX5 is involved in skeletal development and regulation of *OC* transcription [9, 10]. Human glucocorticoid receptors (hGRs) mediate, in part, the negative actions of glucocorticoids on bone metabolism and *OC* gene expression [11, 12]. Retinoid X receptors/1α,25-dihydroxyvitamin D_3 receptor (RXR/VDR) heterodimers and VDR homodimers enhance *OC* promoter activity [13, 14]. Moreover, in osteoblast, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) regulates the expression of *RUNX2* [15].

The osterix/specificity protein7 (SP7) transcription factor is necessary for bone formation and for the differentiation of preosteoblasts into fully functioning osteoblasts in a step subsequent to RUNX2. In transfected cells, it activates *OC* and *COL1* expression [16]. However, the pattern of SP7 activation during preosteoblast differentiation and maturation has not been clearly defined. Some studies failed to detect *SP7* expression in mature bone and in primary osteoblasts from adults [17, 18], whereas others indicated that *SP7* may play a role in adults' bones [19, 20].

Using quantitative RT-PCR to compare mRNA levels, we searched among these 10 factors for the causes of the previously observed up-regulation of *OC* on osteocytes from HO versus control samples.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma Chemical (L'Isle d'Abeau, France) unless otherwise stated.

Specimens

All bone specimens are surgical waste according to the French law of March 4th 2002, and their use was approved by the "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lille" (October 2000). Samples of heterotopic bone were removed per-operatively from seven head-injured patients (three male and four female, 19–60 years, average age 37) who developed HO around the hip. Normal bone pieces used as control were obtained per-operatively from patients (five male and two female, 32–56 years, average age 48) receiving prosthesis of the hip joint, without fracture or osteoporosis but with femoral head osteonecrosis or hip osteoarthritis. Both were cases of local pathology, and biopsies were performed far from the diseased tissue.

Bone fragments were prepared as previously described [6]. A 15-min collagenase digestion allowed the elimination of almost all non-osteoblastic cells, preserving mainly osteocytes in the mineralised matrix with some remaining lining osteoblasts (Fig. 1).

RNA extraction, reverse transcription and real-time PCR experiments

Of treated bone fragments, 0.5–1 g were crushed in 5–10 ml of extraction reagent (Eurobio, Les Ulis, France). RNA extraction was then performed following recommendations of the manufacturer and gave 30–100 μg total RNA/g bone. After DNase I treatment (Roche Diagnostics, Meylan, France), 1 μg of each RNA sample was used for reverse transcription performed under standard conditions with Superscript II reverse transcriptase (Life technologies, Cergy Pontoise, France) and random hexamer primers (Amersham Pharmacia Biotech, Saclay, France) in a 20-μl final volume. Real-time PCR was performed using a LightCycler system (Roche Diagnostics) and Master SYBR Green I mix, according to the manufacturer's instructions. For each gene presented, preliminary experiments were performed in order to normalise the study. Efficiencies of PCR were optimised according to Roche Diagnostic's recommendations on a standard sample expressing all studied gene. Efficiencies of PCR ranged from 1.85 to 2.00. To confirm amplification specificity, PCR products were subjected to a melting curve analysis and subsequent gel

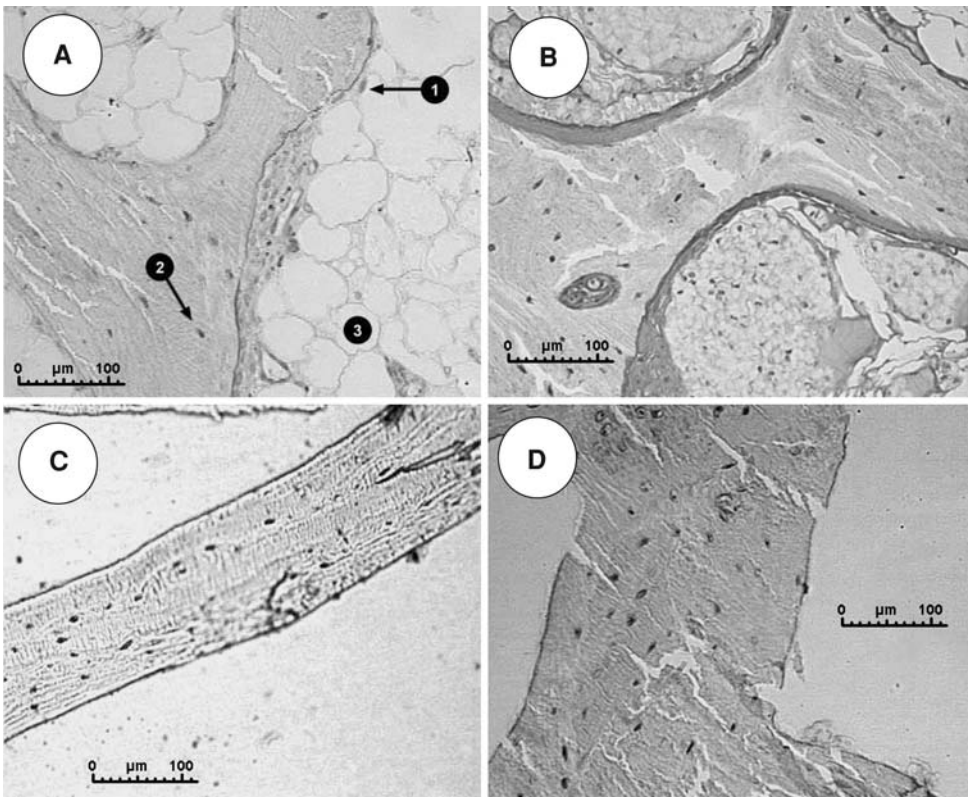

Fig. 1 Histological analysis. Bone sections of 8 µm stained by May Grünwald-Giemsa observed under light microscopy. 1: osteoblast; 2: osteocytes; 3: adipocytes. (**a**, **c**) Normal bone pieces obtained from the proximal femoral shaft. (**b**, **d**) Heterotopic ossification surrounding the hip. Observation of osteocytes, osteoblasts and bone marrow cells in bone before collagenase digestion (**a**, **b**). After 15 min of collagenase digestion, osteocytes represent 90–95% of cellular population (**c**, **d**)

electrophoresis; Table 1 lists all the primers used and the melting temperature of the corresponding PCR product. Quantification data are representative of two experiments realised in triplicate. The standard deviation of crossing point replicates was less than 0.12.

Statistical analysis

Statistical significance of mRNA level differences between the two groups was determined for each gene by a univariate analysis (Mann–Whitney *U*-test). Between genes showing a statistical significance ($P < 0.05$), correlation and significance of correlation within the same group were determined by the Pearson product moment correlation test.

Results

Quantitative RT-PCR was used to compare mRNA levels in osteocytes from HO versus osteocytes from normal bone. Crossing point values obtained with the two

Table 1 Primers data for the genes investigated

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Melting temperature (°C)
β actin	ATCTGGCACCACACCTTCTA	AGCTCGTAGCTCTTCTCCAG	92
GAPDH	GTTCCAATATGATTCCACCC	AGGGATGATGTTCTGGAGAG	89
C-FOS	CCAACTTCATTCCCACGGTC	CTCCCTCCTCCGGTTGC	92.5
C-JUN	GACTGCAAAGATGGAAACGA	GTTGCTGGACTGGATTATCA	92
FRA-2	GAACCTCGTCTTCACCTATC	AAGGAGTCTGATGATTGGTC	88
JUN D	GCCTCATCATCCAGTCCAACG	CTGGTTCTGCTTGTGTAAATCCTCC	90
MSX2	CAGAAGATGGAGCGGCGT	GCTCTGCAATGGAGAGGTA	90
DLX5	CCAGAGAAAGAAGTGACCGA	CCTGTGTTTGTGTCAATCCC	87
GRα	GTATGTTTCCTCTGAGTTACAC	GGGAATCTCCTTCTTGACCAG	83.5
RXRβ	CCATCCGCAAAGACCTTACA	CCCACTCAACAAGCGTGAAT	91
VDR	ACGCCCACCATAAGACCTA	ATTGGAGAAGCTGGACGAGT	88
SP7	ACAAAGAAGCCGTACTCTG	GGGTCATTAGCATAGCC	87

housekeeping genes used (*GAPDH* and *β actin*) were homogeneous within all the samples (data not shown), indicating they were expressed at constant levels and samples were comparable. No correlation was found between any patient factors and variations of mRNA levels in the two groups (e.g. age, gender, heterotopic bone dimensions or time from injury to biopsy). Radionuclide bone scans were performed on each HO patient before surgical resection and showed that HO were in the later stage of their development and shared bone remodelling activity similar to normotopic bone (data not shown).

SP7 mRNA levels

SP7 expression was detected in all samples. Moreover, it exhibited substantial and significant higher levels within the HO group versus the control one (7× and 4× standardising with *β actin* and *GAPDH*, respectively) (Fig. 2).

Nuclear receptor mRNA levels

Among the studied nuclear receptors, only *VDR* showed a slight increase of transcription level for HO patients (but only significant when normalised to *β actin*) but not *GRα* nor *RXRβ* (Fig. 2).

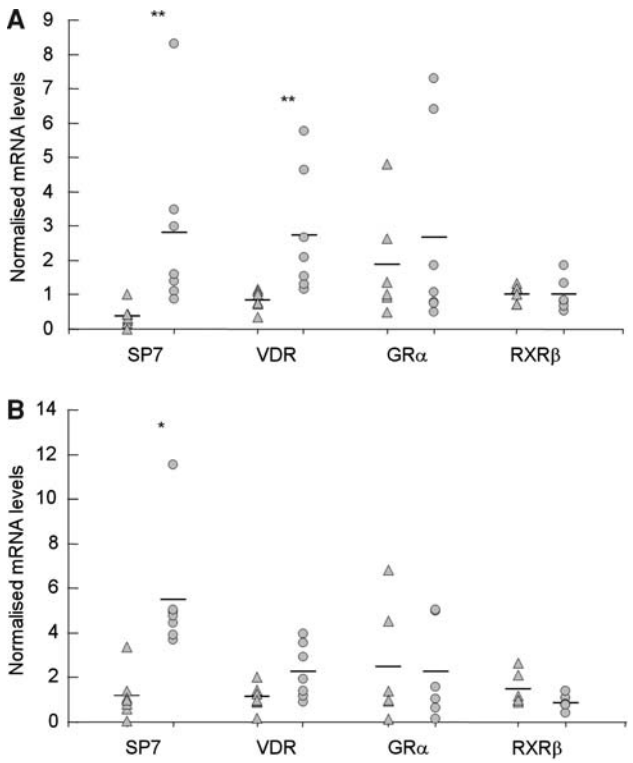

Fig. 2 mRNA levels of SP7 and nuclear receptors. Quantitative RT-PCR was performed on total RNA extracted from normotopic bone samples ($n = 7$) (triangles) and heterotopic bone samples ($n = 7$) (circles). Results were normalised to *β* actin (**a**) or GAPDH (**b**). Bars indicate mean values of each group. Significant differences between HO and control groups were noted. $^{*}P \leq 0.05$, $^{**}P \leq 0.001$

AP-1 family gene mRNA levels

No significant difference was found for the AP-1 family members analysed (*c-FOS*, *FRA-2*, *c-JUN* and *JUN D*) between the two groups (Fig. 3).

Homeodomain containing gene mRNA levels

No significant difference was observed for the two homeodomain containing transcription factors analysed (*MSX2* and *DLX5*) (Fig. 3).

mRNA level correlations between the differentially expressed genes

OC and *SP7* were both correlated in control and HO samples, while *OC*, *RUNX2* and *SP7* appeared highly correlated all together in HO samples only (Table 2).

Discussion

In the present study, we searched for molecular events linked to the specific genetic expression observed on pathological bone cells. For this purpose, we compared mRNA levels of some of the major factors controlling osteoblastic cells activity and OC expression, the osteoblast specific protein which has been recently described to be involved in energy metabolism regulation [7].

DLX5 and *MSX2* were assayed as they regulate directly *OC* transcription, with opposite effects [10]. A decrease in *OC* gene expression by GR binding to a negative GRE existing on the promoter has been described [12]. Interestingly, these transcription factors were not differentially expressed between the two groups.

Among the AP-1 members tested, c-FOS and c-JUN are known to be highly expressed in proliferating osteoblasts, where they contribute to decrease the *OC* promoter activity. On the contrary, co-transfection of *FRA-2* and *JUN D*, preferentially expressed in differentiating osteoblast, enhances *OC* expression [21].

The close expression levels obtained in the two groups for these factors seem to indicate the non-involvement of these genes in this study. Moreover, this homogeneity in transcript levels confirms that there were few differences in the bone development stage and in the remodelling activity between all the samples, as assessed by radionuclide bone scans, allowing associating more directly the observed differences to the pathology.

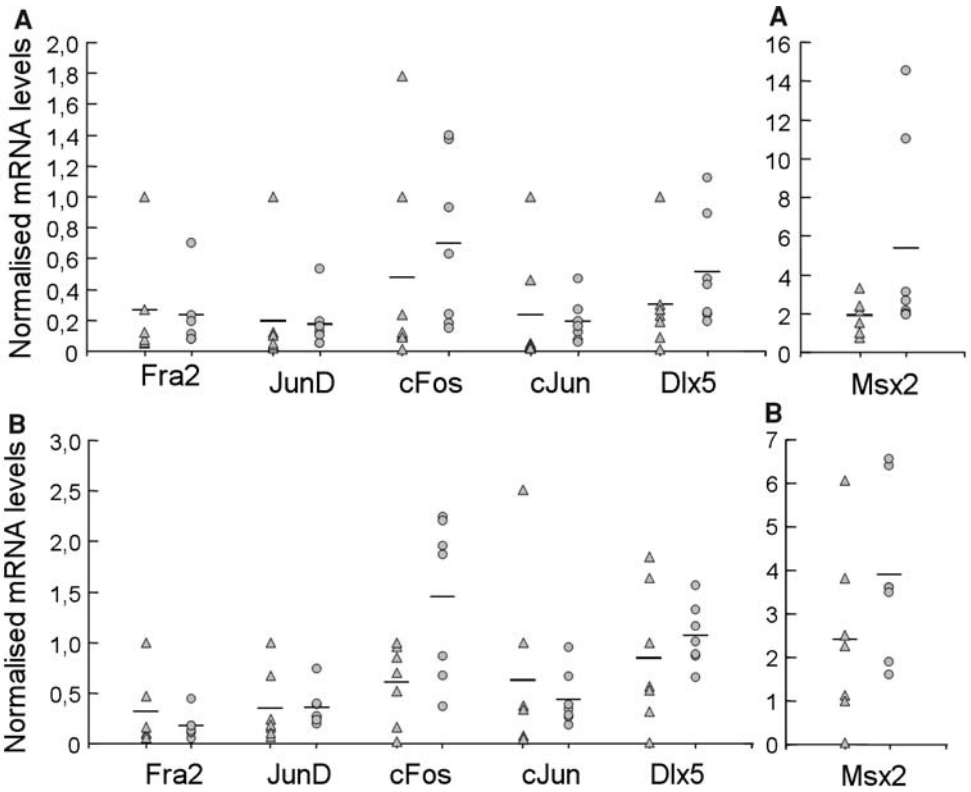

Fig. 3 mRNA levels of AP-1 and homeodomain containing genes. Quantitative RT-PCR was performed on total RNA extracted from normotopic bone samples ($n = 7$) (triangles) and heterotopic bone samples ($n = 7$) (circles). Results were normalised to β actin (**a**) or GAPDH (**b**). Bars indicate mean values of each group

Table 2 Correlation test of gene expression levels

(A) Correlation analysis of gene expression levels in control samples			
Control samples	*VDR*	*SP7*	*RUNX2*
OC	0.530	0.967**	0.675
RUNX2	0.573	0.545	
SP7	0.422		
(B) Correlation analysis of gene expression levels in HO samples			
HO samples	*VDR*	*SP7*	*RUNX2*
OC	0.036	0.951*	0.925*
RUNX2	0.287	0.969**	
SP7	0.123		

Pearson product moment correlation tests were done on differentially expressed genes. Correlation coefficients are shown, with P value for significant correlations. * $P < 0.005$, ** $P < 0.0005$

In contrast, we measured an increase in the level of *VDR* mRNA in HO patients. VDR is known as a strong and direct activator of *OC* transcription [22]. Paredes et al. published in 2004 that in osteoblastic cells VDR interacts directly with RUNX2 [23]. This interaction seems necessary for the activation of *OC* transcription. Since we previously observed an over-expression of *RUNX2* in HO samples, this new result led us to suppose that the combination of VDR and RUNX2 may be of singular importance for the regulation of *OC* over-expression in this pathological tissue.

Among RXR isoforms, RXRβ appears to be a major heterodimeric partner of VDR in differentiated human osteoblast-like cells [24, 25]. FRA2 and JUN D facilitate VDR/RXR binding to their regulatory elements on the *OC* promoter [26]. The lack of difference in mRNA level of *FRA2*, *JUN D* and *RXRβ* did not allow us to suppose the involvement of these factors in the *OC* up-regulation observed in HO samples.

For the first time, we showed that SP7 gene is expressed by osteoblastic cells in trabecular bone from human adults. Due to our sample preparation procedure, this suggests that SP7 is expressed in well-differentiated osteoblasts (bone lining cells and osteocytes), which is consistent with its described functions. But if it is well established that Sp7 is a key regulator of osteoblast differentiation, Sp7 function in bone metabolism is still controversial. Some recent studies conclude that Sp7 may play only a role in

embryonic but not in adult stem cells [27]. Previous studies also failed to detect *SP7* expression. Milona et al. did not observe *SP7* expression in primary cultures of osteoblasts from adults. However, these cultured osteoblasts expressed *RUNX2* and, as it is supposed by the authors themselves, loss of *SP7* expression may result from partial de-differentiation in the in vitro culture conditions [18]. In the other study, probably because of low sensitivity, Gao et al. could not detect *SP7* in adult tissue by Northern blot hybridisation with 1 μg of mRNA isolated from total bone [17]. But some recent results indicate that *SP7* may play a role in mediating endochondral ossification during bone repair [19] and even that ex vivo gene therapy with *SP7* is an useful approach in regenerating adult bone tissue [20]. Our results confirms the involvement of SP7 in bone physiology all lifelong. Moreover, we found that *SP7* gene was also significantly over-expressed on osteoblastic cells from head injury-induced HO, compared to cells from control samples. These high levels of *SP7, OC* and *COL1* mRNA are consistent with results obtained by Nakashima et al. [16].

Results obtained for each patient indicated considerable differences of mRNA level for the differentially expressed genes *SP7* and *VDR* analysed in this study, but also *RUNX2* and *OC* selected from the previous one [6]. This leads to the question of a possible coordinated action of these three transcription factors leading to very high levels of *OC* mRNA. The four genes' mRNA levels were submitted to the Pearson product moment correlation test, for the two kinds of samples (Table 2). The only correlation found within the control samples was between *OC* and *SP7*. In HO samples, *OC*, *RUNX2* and *SP7* genes' expressions appeared highly correlated all together. But we did not find any correlation for the *VDR* mRNA level. This could be explained in part by a preponderant role of RUNX2 and SP7 compared to VDR. The lack of obvious correlation in some cases is to be interpreted cautiously, but these results could indicate a major involvement of the SP7 transcription factor in *OC* expression. Interestingly, in an animal model of unloading, decreases of *OC* and *SP7* levels have been reported and were also found correlated [28].

In conclusion, quantitative RT-PCR analysis allowed us to measure mRNA level for several genes, the expression of which would be very difficult to analyse otherwise in limited amount of human biopsies. Specific over-expression of *RUNX2*, *SP7* and *VDR* and positive correlations determined between some of these genes suggest that in HO, the high level of *OC* mRNA could be preferentially due to at least one of these three transcription regulators or to an imbalance in their respective activities.

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