Osteoarthritis and Cartilage 23 (2015) 1421–1431

Osteoarthritis and Cartilage

Ucma, a direct transcriptional target of Runx2 and Osterix, promotes osteoblast differentiation and nodule formation

Y.-J. Lee † §, S.-Y. Park ‖, S.-J. Lee † §, Y.C. Boo † §, J.-Y. Choi ‡ §, J.-E. Kim † § *

† *Cell and Matrix Research Institute, Department of Molecular Medicine, Kyungpook National University School of Medicine, Daegu, Republic of Korea*
‡ *Cell and Matrix Research Institute, Department of Biochemistry and Cell Biology, Kyungpook National University School of Medicine, Daegu, Republic of Korea*
§ *BK21 Plus KNU Biomedical Convergence Program, Department of Biomedical Science, Kyungpook National University, Daegu, Republic of Korea*
‖ *Department of Biochemistry, School of Medicine, Dongguk University, Gyeongju, Republic of Korea*

ARTICLE INFO

Article history:
Received 11 August 2014
Accepted 28 March 2015

Keywords:
DNA microarray
Ucma
Osteoblast differentiation
Nodule formation

SUMMARY

Objective: Runt-related transcription factor 2 (Runx2) and Osterix (Osx) are the master transcription factors in bone formation. Nonetheless, genes acting downstream of both Runx2 and Osx have yet to be fully characterized. Here, we investigate the downstream targets of both Runx2 and Osx in osteoblasts.
Materials and methods: DNA microarray analysis was conducted on calvarial RNA from wild-type, *Runx2* heterozygous, *Osx* heterozygous, and *Runx2*/*Osx* double heterozygous embryos. Expression and transcriptional responses of the selected target gene were analyzed in MC3T3-E1 osteoblastic cells.
Results: The expression of unique cartilage matrix-associated protein (*Ucma*) was decreased in *Runx2*/*Osx* double heterozygous embryos. In contrast, *Ucma* expression was increased in osteoblasts overexpressing both Runx2 and Osx. *Ucma* expression was initiated mid-way through osteoblast differentiation and continued throughout the differentiation process. Transcriptional activity of the *Ucma* promoter was increased upon transfection of the cells with both *Runx2* and *Osx*. Runx2-and Osx-mediated activation of the *Ucma* promoter was directly regulated by Runx2-and/or Sp1-binding sites within its promoter. During osteoblast differentiation, the formation of mineralized nodules in *Ucma*-overexpressing stable clones occurred earlier and was more enhanced than that in the mock-transfected control. Mineralized nodule formation was strongly augmented in the cells cultured in a medium containing secretory Ucma proteins.
Conclusion: Ucma is a novel downstream gene regulated by both Runx2 and Osx and it stimulates osteoblast differentiation and nodule formation.

Introduction

The bone morphogenetic protein (BMP) signaling pathway is essential for the regulation of osteogenesis. BMP subtypes bind to their receptors and activate Smads, which directly regulate target gene expression[1]. In osteoblasts, BMP target genes include a number of transcription factors, including distal-less homeobox 3 (*Dlx3*), *Dlx5*, activating transcription factor 4, Runt-related transcription factor 2 (*Runx2*), and Osterix (*Osx*)[2]. Runx2 and Osx are master transcriptional regulators of osteogenesis. Runx2 plays an essential role in the differentiation of mesenchymal progenitor cells into osteoblasts. A *Runx2* deficiency in mice leads to a complete lack of both endochondral and intramembranous bone formation due to the arrest of osteoblast differentiation[3,4]. Osx is also important for osteoblast differentiation and bone formation[5]. Despite normal cartilage development, *Osx*-null mutants do not undergo bone formation. Although *Runx2* is expressed in *Osx*-null mutants, *Osx* is not expressed in *Runx2*-null mice, indicating that *Osx* is downstream of Runx2 in osteoblast differentiation[5]. However, this is still under debate. While *Runx2* and *Osx* heterozygous mice display no bone phenotypes, *Runx2*/*Osx* double heterozygous embryos showed reduced bone length and absence of several bones[6]. These

* Address correspondence and reprint requests to: J.-E. Kim, Department of Molecular Medicine, Kyungpook National University School of Medicine, 101 Dongin-dong, Jung-gu, Daegu 700-422, Republic of Korea. Tel: 82-53-420-4949; Fax: 82-53-426-4944.
E-mail addresses: koreapuz@nate.com (Y.-J. Lee), psyoon@chol.com (S.-Y. Park), sjlee8807@nate.com (S.-J. Lee), ycboo@knu.ac.kr (Y.C. Boo), jechoi@knu.ac.kr (J.-Y. Choi), kjeun@knu.ac.kr (J.-E. Kim).

http://dx.doi.org/10.1016/j.joca.2015.03.035
1063-4584/

embryos also had expanded hypertrophic chondrocytes and reduced mineralization of bone. BMP target genes, *Runx2* and *Osx*, regulate various other genes. Osteocalcin and bone sialoprotein, which are markers of osteoblast differentiation, are directly controlled by a complex Runx2-mediated mechanism[7,8]. During bone formation, Osx regulates the transcription of genes encoding the special AT-rich sequence-binding protein (Satb2) and the vitamin D receptor through GC-rich-binding sites[9,10]. Osx also controls the interleukin 1-α and collagen genes through an Sp1-binding site in their promoters[5,11,12]. Underscoring the importance of Runx2 and Osx, these results indicate that downstream genes regulated by Runx2, Osx, or both may be required for osteoblast differentiation.

To search for such genes that are important during osteogenesis, DNA microarray analysis was performed on calvarial RNA isolated from wild-type, *Runx2* heterozygous, *Osx* heterozygous, and *Runx2*/*Osx* double heterozygous embryos. Among the 26 genes classified as regulators of osteogenesis and altered by *Runx2* and/or *Osx* deficiency, unique cartilage matrix-associated protein (*Ucma*) was markedly downregulated in double heterozygous embryos compared to the embryos of other genotypes. Ucma is a novel secreted factor present in fetal and juvenile growth plate cartilage as well as in trabecular bone[13–15]. In developing long bones, *Ucma* is expressed in resting chondrocytes[13,14] and in osteoblasts and osteocytes of trabecular bone[15]. On the other hand, its function and regulatory mechanisms remain unclear. In this study, we explored the role of *Ucma* in osteoblast differentiation and suggest that *Ucma* is a novel gene that is regulated by both Runx2 and Osx during osteoblast differentiation and bone formation.

Materials and methods

Animals and microarray materials

All animal experimental procedures were conducted with the approval from the animal ethics committee of Kyungpook National University. As described previously in Ref. 6, $Osx^{+/-}$ mice were crossed with $Runx2^{\Delta C/+}$ mice to generate four types of embryos: $Runx2^{+/+}$;$Osx^{+/+}$ (Wild-type, WT), $Runx2^{\Delta C/+}$;$Osx^{+/+}$ ($Runx2^{het}$), $Runx2^{+/+}$;$Osx^{+/-}$ (Osx^{het}), and $Runx2^{\Delta C/+}$;$Osx^{+/-}$ double heterozygous embryos ($Double^{het}$) (Fig. S1). To collect calvariae from embryos on embryonic day 18.5 (E18.5), we prepared three pregnant females and the numbers of embryos collected from each female are listed in Table I. Calvarial tissues of the same genotype were pooled for each pregnant female. Total RNA was isolated from the calvariae and cultured cells, using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For microarray analysis of calvarial RNA, whole pooled samples from the three sets were pooled again to attain sufficient concentrations of RNA. The cDNA synthesis was performed using the SuperScript® III First-Strand Synthesis System (Invitrogen). Gene expression profiles were analyzed using the GeneChip® Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA).

Table I
The samples and populations relevant to the study

	No. of embryos from each genotype				Analysis methods
	WT	Osx^{het}	$Runx2^{het}$	$Double^{het}$	
1st female	4	1	4	2	RT-PCR, qRT-PCR
2nd female	1	1	5	2	RT-PCR, qRT-PCR
3rd female	1	2	3	2	RT-PCR, qRT-PCR
Total	6	4	12	6	Microarray

RT-PCR and quantitative real-time PCR (qRT-PCR)

Total RNA extracted from the calvariae and cultured cells was used to measure mRNA expression levels of the genes under study. The sequences of the primers used in this study are listed in Table II. Information regarding methods is provided in the Supplementary data.

Cell culture and western blotting

The Supplementary data provide information regarding methods.

Construction of the mouse Ucma promoter and reporter plasmids

To construct the mouse *Ucma* promoter, the transcription initiation site of the mouse *Ucma* gene was determined using 5′ rapid amplification of cDNA ends (5′ RACE), in accordance with the manufacturer's instructions (Invitrogen). The 5′-flanking region (−980 to +51) of the mouse *Ucma* gene was amplified from the genomic DNA of ATDC5 cells by using PCR and was cloned into the NheI–XhoI sites of the pGL3/basic vector (Promega) to generate the *Ucma* reporter plasmid pUcma-980. Three 5′-deletion mutants of the mouse *Ucma* promoter (−459 to +51, −222 to +51, and −41 to +51) were amplified using PCR and cloned into the NheI–XhoI sites of the pGL3/basic vector. Mutations were introduced into the Osx- and/or Runx2-binding sites within the mouse *Ucma* promoter using the QuikChange site-directed mutagenesis kit (Stratagene). The primer sequences are listed in Table III.

The reporter assay

MC3T3-E1 cells were seeded in 12-well culture plates at a density of 8×10^4 cells/well. The next day, the cells were transfected with either the pGL3/basic or the pUcma-luc vector, together with either a Runx2 or an Osx expression vector by using Lipofectamine (Invitrogen). At 48 h after transfection, luciferase activity was measured using the Dual Luciferase assay system (Promega). The transfection efficiency was determined in each sample using co-transfection with a plasmid encoding Renilla

Table II
List of primers for RT-PCR and qRT-PCR

Name		Sequence
Osx	S	5′-CGTCCTCTCTGCTTGAGGAA-3′
	AS	5′-CTTGAGAAGGGAGCTGGGTA-3′
Runx2	S	5′-CCGCACGACAACCGCACCAT-3′
	AS	5′-CGCTCCGGCCCACAAATCTC-3′
Ucma	S	5′-GATGCCTCCAATTTCCTCAA-3′
	AS	5′-GGCATCAGAACAGAGGGTGT-3′
Osteocalcin	S	5′-CCTCAGTCCCCAGCCCAGATCC-3′
	AS	5′-CAGGGCAGAGAGAGAGGACAGG-3′
Osteopontin	S	5′-GGCATTGCCTCCTCCCTC-3′
	AS	5′-CGAGGCTGTAAAGCTTCTCC-3′
Col2a1	S	5′-CACACTGGTAAGTGGGGCAAGACCG-3′
	AS	5′-GGATTGTGTTGTTTCAGGGTTCGG G-3′
Sox9	S	5′-CTCTGTTTTCCCTCCCTCCT-3′
	AS	5′-TCCTCGCTCTCCTTCTTCAG-3′
Aggrecan	S	5′-GAAGACGACATCACCATCCAG-3′
	AS	5′-CTGTCTTTGTCACCCACACATG-3′
GAPDH	S	5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′
	AS	5′-CATGTAGGCCATGAGGTCCACCAC-3′

Table III
DNA sequences of oligonucleotides used in this study

Name	Sequence
5′ RACE–PCR	
Ucma (S)	5′-GACTTCCAAGATACCTCACC-3′
Ucma-1 (AS)	5′-GTCCTCTCTTCCTGCTCATC-3′
Ucma-2 (AS)	5′-ACTTCATCTCGGGACTTAGG-3′
Ucma-3 (AS)	5′-TGTTTCACACTACACAGGAG-3′
Cloning of Ucma promoter and its deletion constructs	
pUcma-980 (S)	5′-AAAAGCTAGCCTGGCTCCCTTTGAGGTG-3′
pUcma-459 (S)	5′-AAAAGCTAGCAGGAGATCCCATAGAAGTC-3′
pUcma-222 (S)	5′-AAAAGCTAGCACTCAGGCTGAGTATTTC-3′
pUcma-41 (S)	5′-AAAAAGCTAGCCTCTCCCCCAGTGGCATC-3′
pUcma+51 (AS)	5′-AAAACTCGAGCTGCCCAGTAAGTCCTCAC-3′
Primers for site-directed mutagenesis	
mGC	5′-GACAGTTTAAAACTGCAAATGAAAGTTAAAACAAGC TCCACTTC-3′
mSp1	5′-ACCACAGCCAGGACAAATAAAACTCTCCCCCAGTGG-3′
mRunx2	5′-AGCAGCCAATGGAAAGTACAGCCAGGACCCCTCC-3′
Primers for ChIP assay	
p1	5′-GGCCAAAAACATCTCCAGTG-3′ and 5′-CACAGAAGTGGAGCTTGTGG-3′
p2	5′-GCTTCTCCCACTTGGACTCA-3′ and 5′-CAGGCCAGAACACTGGAGAT-3′
p3	5′-TCCCTGGGAACAGTGTATCAG-3′ and 5′-CATATGGGGCCAGAGAAAGAA-3′

Cloning of Ucma promoter and its deletion constructs. (The underlined nucleotides indicate NheI or XhoI site.) Primers for site-directed mutagenesis. (The underlined nucleotides indicate mutated binding sites.)

luciferase, and all luciferase activity data were normalized to those of Renilla luciferase.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using the UPSTATE Kit (Millipore, Billerica, MA, USA). The Supplementary data provide information regarding the method. PCR was performed with the specific primers listed in Table III.

Transient transfection and establishment of stable cell clones

To construct the mouse Ucma expression vector, Ucma cDNA was amplified from ATDC5 cells by PCR using the following primers: 5′-TTTTTGAATTCGACTTCCAAGATACCTCACC-3′ and 5′-TTTTTCTCGAGGATGTTTTGGCGGTTGTAG-3′. The PCR product was digested with *EcoRI–XhoI* and cloned into the same sites of the pcDNA3.1 vector (Invitrogen) containing the FLAG epitope. A shRNA target sequence for mouse Ucma (accession number: NM_026754), shUcma: 5′-CGAGTTTGAGAACTTCGTG-3′, was selected using the OligoEngine siRNA design tool. Forward and reverse oligonucleotides (sense-loop-antisense) were synthesized, annealed, and cloned into the BglII–HindIII sites of the pSuper/puro vector (OligoEngine, Seattle, WA, USA). For transient transfection, 293FT cells were seeded in 6-well culture plates at a density of 3×10^5 cells/well. Five hours after transfection, the cells were serum-starved for 48 h, and the supernatants and cell lysates were collected for analysis by western blotting. To achieve stable transfection, MC3T3-E1 cells were transfected with either the Ucma expression vector or the knockdown vector, and the transfectant clones were selected in media containing 500 μg/mL G418 or 1 μg/mL puromycin. Individual antibiotic-resistant colonies were isolated after 3 weeks of culture, and eventually, two clones with strong Ucma overexpression or knockdown were selected. Negative control clones were randomly selected after transfection with an empty vector and were designated as the mock control.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical significance of the differences among the groups was determined using ANOVA or Student's *t* test. Cell culture experiments were performed independently at least three times and each experiment was performed in duplicate. A P value <0.05 was considered to denote statistical significance.

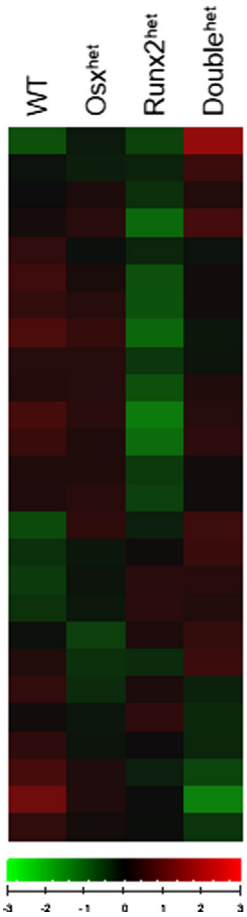
Results

Differential gene expression in Runx2-and/or Osx-deficient calvaria

To compare the differential expression of genes during osteogenesis under conditions of *Runx2* and/or *Osx* deficiency, DNA microarray analysis was performed using calvarial RNA isolated from WT, Runx2^{het}, Osx^{het}, and Double^{het} embryos at E18.5 (Fig. S2). Twenty-six genes that are classified as regulators of osteogenesis showed differences in expression, with a fold change of at least 1.5 under conditions of *Runx2* and/or *Osx* deficiency (Fig. 1). In comparison to the embryos of other genotypes, the expression of Indian hedgehog, Dentin sialophosphoprotein (*Dspp*), 3′-phosphoadenosine 5′-phosphosulfate synthase 2 (*Papss2*), and *Ucma* was downregulated in Double^{het} embryos. In particular, compared to the WT, *Ucma* was downregulated by 50% in Runx2^{het} and Osx^{het} embryos, and by 80% in Double^{het} embryos. The expression and function of *Dspp* and *Papss2* have been studied in bone, but few studies have explored the role of Ucma. Thus, we focused on Ucma in the present study.

Ucma expression in osteoblasts

To validate the disrupted *Ucma* expression detected by microarray analysis, RT-PCR and qRT-PCR were performed using calvarial mRNA. Compared to WT, *Ucma* expression decreased in the Runx2^{het} and Osx^{het} animals, and further decreased in the Double^{het} animals [Fig. 2(A)]. Moreover, *Ucma* expression was significantly reduced in the *Osx* knockout (KO) and *Runx2* KO animals [Fig. 2(B)]. To minimize the influence of differences in residual cartilage of the calvariae, we examined the expression of cartilage genes. The expression of *Col2a1*, *Sox9*, and *aggrecan* was identical in the calvarial tissue samples [Fig. S3(A) and (B)]. Furthermore, in rib cage cartilage, *Ucma* and *Col2a1* expressions was not significantly different [Fig. S3(C)]. Expression of Ucma and Col2a1 in rib cage cartilage was higher than that in the calvaria, but their expression was detected in calvarial tissue [Fig. S3(D) and (E)]. To analyze the regulation of *Ucma* expression



Probe ID	Gene symbol	WT	Osx^{het}	Runx2^{het}	Double^{het}	Full name
10434860	Ostn	1.000	1.483	1.105	4.448	Osteocrin
10427461	Ptger4	1.000	0.921	0.890	1.473	Prostaglandin E receptor 4 (subtype EP4)
10540248	Mitf	1.000	1.132	0.788	1.193	Microphthalmia-associated transcription factor (Alx)
10499358	Bglap2	1.000	1.130	0.466	1.380	Bone gamma carboxyglutamate protein 2
10421737	Tnfsf11	1.000	0.756	0.643	0.741	Tumor necrosis factor (ligand) superfamily, member 11
10542929	Calcr	1.000	0.772	0.422	0.723	Calcitonin receptor
10423363	Ank	1.000	0.909	0.453	0.787	Progressive ankylosis
10523693	Dmp1	1.000	0.840	0.321	0.574	Dentin matrix protein 1
10499363	Bglap1	1.000	0.991	0.604	0.776	Bone gamma carboxyglutamate protein 1
10368289	Enpp1	1.000	1.018	0.514	0.980	Ectonucleotide pyrophosphatase
10591739	Acp5	1.000	0.815	0.293	0.797	Acid phosphatase 5, tartrate resistant
10423894	Tm7sf4	1.000	0.840	0.358	0.914	Transmembrane 7 superfamily member 4
10607499	Phex	1.000	0.996	0.612	0.897	Phosphate regulating endopeptidase homolog, X-linked
10523717	Spp1	1.000	1.063	0.595	0.916	Secreted phosphoprotein 1
10540265	Mitf	1.000	2.009	1.388	2.261	Microphthalmia-associated transcription factor
10572456	Jund	1.000	1.197	1.345	1.808	Jun proto-oncogene related gene d
10459512	Mc4r	1.000	1.318	1.708	1.704	Melanocortin 4 receptor
10382228	Axin2	1.000	1.202	1.633	1.542	Axin2
10500011	Tuft1	1.000	0.708	1.175	1.393	Tuftelin 1
10499354	Bglap-rs1	1.000	0.655	0.672	1.188	Bone gamma carboxyglutamate protein, related seq 1
10491477	Sox2	1.000	0.620	0.873	0.661	SRY-box containing gene 2
10476874	Pax1	1.000	0.885	1.225	0.780	Paired box gene 1
10355706	Ihh	1.000	0.741	0.799	0.653	Indian hedgehog
10523683	Dspp	1.000	0.751	0.573	0.433	Dentin sialophosphoprotein
10469058	Ucma	1.000	0.556	0.474	0.204	Unique cartilage matrix-associated protein
10462507	Papss2	1.000	0.815	0.749	0.563	3'-phosphoadenosine 5'-phosphosulfate synthase 2

Fig. 1. Genes affected by Runx2 and Osx during osteogenesis. Microarray analysis was performed with pooled RNA isolated from embryos with the same genotype. Expression of 26 osteogenic genes was altered between wild-type (WT), and Runx2^{het}, Osx^{het}, and Double^{het} genotypes. Upregulation or downregulation of genes under conditions of *Runx2* and/or *Osx* deficiency is presented as fold change in gene expression relative to wild-type, which was set as 1.0. Expression of the *Ucma* gene was significantly decreased in Double^{het} embryos compared to Runx2^{het} and Osx^{het} embryos.

by Runx2 and Osx, MC3T3-E1 cells were transfected with vectors encoding *Runx2*, *Osx*, or both. *Ucma* expression increased in osteoblasts overexpressing either *Runx2* or *Osx*. In addition, *Ucma* expression further increased upon overexpression of both *Runx2* and *Osx* [Fig. 2(C)]. These results suggest that Runx2 and Osx function together to regulate *Ucma* expression in MC3T3-E1 osteoblasts. To examine the change in Ucma expression during osteoblast differentiation, we cultured MC3T3-E1 cells for 30 days in differentiation medium. Osteoblast differentiation was confirmed by measuring the expression of differentiation markers, the patterns of which were identical when measured by RT-PCR or qRT-PCR (Fig. 3). *Runx2* expression was consistently high at all stages of differentiation. *Osx* expression was observed at the beginning of differentiation; it gradually increased throughout the differentiation process. A late-stage marker of osteoblast differentiation, osteocalcin, was highly expressed at the end of the differentiation process. Weak *Ucma* expression was detected on day 5 of differentiation, and then was clearly detected from the mid-stage of differentiation until osteoblast differentiation was complete.

Transcriptional activity of the mouse Ucma promoter is responsive to Runx2 and/or Osx

To test whether Runx2 and/or Osx contribute to the transcriptional activity of *Ucma*, we cloned the mouse *Ucma* promoter. The transcription initiation site of the mouse *Ucma* gene was determined using 5′ RACE. The 5′ RACE product was purified and sequenced to identify the transcription initiation site (Fig. S4). MC3T3-E1 cells transfected with pUcma-980 and either the *Runx2* or the *Osx* expression vectors exhibited increased luciferase activity in a dose-dependent manner [Fig. 4(B)]. Transcriptional activity of the *Ucma* promoter increased further when both vectors were transfected into the cells. This result suggested that the effects of Runx2 and/or Osx on the activation of *Ucma* transcription were dose-dependent.

We performed TFSEARCH (Searching Transcription Factor Binding Sites, version 1.3) analysis of the 1-kb promoter sequence of Ucma to identify transcription factor binding sites. Osx, a member of the Sp family, contains a DNA-binding domain that binds GC-rich sequences and Sp1 elements to control target gene expression[5,11,12]. AML elements of target genes are putative Runx2-binding sites (PuACCPuCA or TGTGGT)[16,17]. Within the *Ucma* promoter, two Sp1-binding sites, two GC-rich regions and three Runx2-binding sites were identified [Fig. 4(A) and Fig. S4]. Therefore, the transcriptional activity of *Ucma* may be regulated by Runx2 and Osx through these binding sites. To determine which regions of the *Ucma* promoter are essential for Runx2-and Osx-mediated activation, 5′-truncated mutants of the *Ucma* promoter were generated and examined using a luciferase assay [Fig. 4(C)]. Deletion of the region between positions −222 and −41 significantly reduced Runx2-and Osx-induced *Ucma* promoter activity [Fig. 4(C)]. This result indicated that the −222/−41 region in the *Ucma* promoter was responsive to Runx2 and Osx.

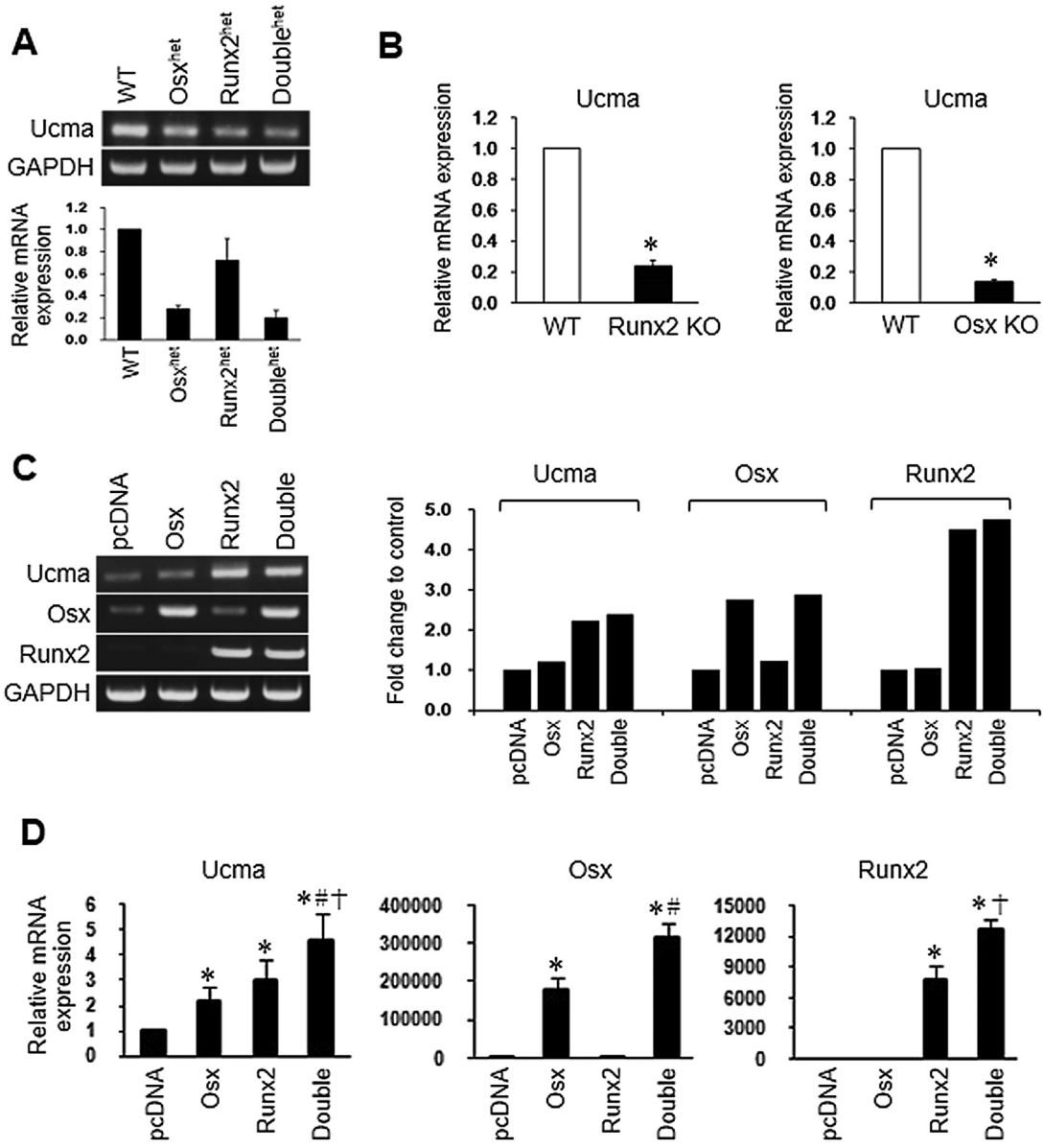


Fig. 2. Alteration of *Ucma* expression by *Runx2* and/or *Osx* gene deficiency or overexpression. (A) Reduced expression of *Ucma* mRNA in Double^{het} embryos. Calvarial tissues of the same genotype in each pregnant female were pooled and RNA was isolated. Three independent RT-PCR assays were performed using RNA collected from each pregnant female; a representative image of RT-PCR data is shown. Reduced expression of *Ucma* in Double^{het} embryos was confirmed by qRT-PCR. (B) Reduced expression of *Ucma* in *Runx2* KO and *Osx* KO embryos by qRT-PCR. The results are presented as mean ± SD. *Mean values were significantly different from the wild-type ($P < 0.05$). (C, D) Ucma induction upon Runx2 and/or Osx overexpression in MC3T3-E1 osteoblastic cells. MC3T3-E1 cells were transfected with either Runx2 or Osx expression vectors, or both vectors (designated "Double"). *Ucma* mRNA expression was strongly increased in the Double samples according to RT-PCR (C) and qRT-PCR (D). The intensity of individual bands after RT-PCR [shown in Fig. 2(C)] was determined using the Image J software. The data were normalized to *Gapdh* and calculated as a fold change relative to control pcDNA only, which was set to 1.0. Relative expression levels shown in Fig. 2(D) were plotted against those of respective genes in control pcDNA only data, which were set to 1.0. The data are presented as mean ± SD of three independent experiments. *Mean values were significantly different from pcDNA only ($P < 0.05$). #Mean values were significantly different from Osx only ($P < 0.05$). †Mean values were significantly different from Runx2 only ($P < 0.05$).

The −222/−41 region of the *Ucma* promoter contains one Runx2-binding site, one GC-rich region, and one Sp1-binding site. To determine whether these sites were response elements for Runx2-and/or Osx-mediated transcriptional activation of *Ucma*, we performed site-directed mutagenesis [Fig. 4(D)]. Runx2-and Osx-mediated activation of the *Ucma* promoter was strongly attenuated after mutation of the Runx2-binding site and/or the Sp1-binding site within the −222/−41 region [Fig. 4(D)]. However, mutation of the GC-rich region did not affect Runx2-or Osx-mediated transcription. These results indicated that the Runx2-and Sp1-binding sites within the −222/−41 region played a crucial role in Runx2-and Osx-responsive *Ucma* transcription, respectively. To determine whether Runx2 and Osx bind directly to their respective binding sites within the −222/−41 region, we performed a ChIP assay. After precipitation of chromosomal DNA by using an anti-Osx or anti-Runx2 antibody, the promoter region harboring the Runx2-and Sp1-binding sites was amplified successfully, indicating that Runx2 and Osx directly bound to their respective response elements within the promoter [Fig. 4(E) and Fig. S5]. We were not able to amplify the GC-rich region of the *Ucma* promoter from the precipitated DNA. The potential Runx2 and Osx binding sites within the Ucma promoter are conserved among different species including human, rabbit, mouse, rat, guinea pig, and dog (Fig. S5). In particular, they are completely conserved

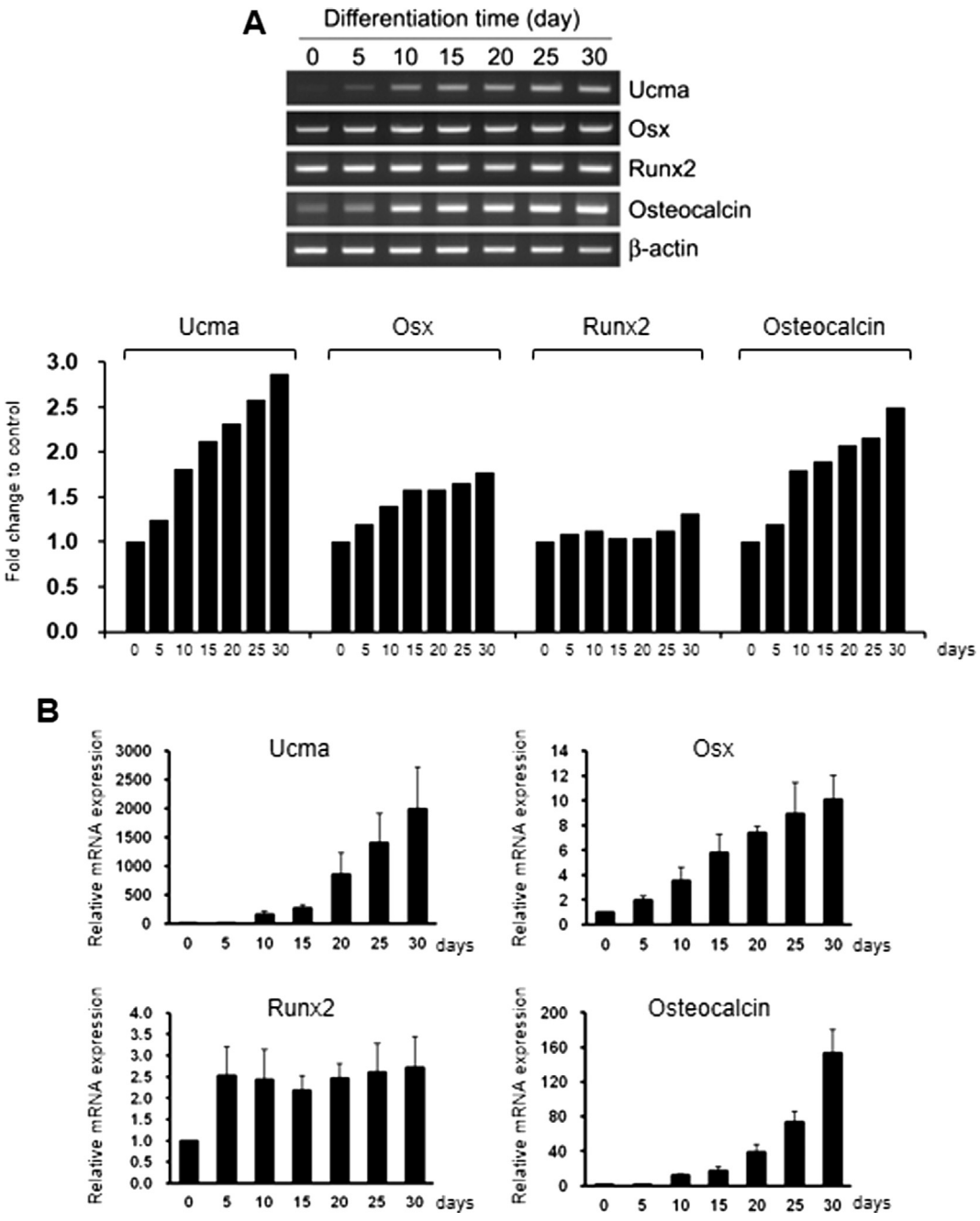


Fig. 3. Increased expression of *Ucma* during osteoblast differentiation. At the indicated time points, expression of *Ucma* and marker genes was analyzed using RT-PCR (A) and qRT-PCR (B). After induction of *Runx2* and *Osx* expression, *Ucma* was clearly induced mid-way through osteoblast differentiation and continued during the course of the differentiation process. *Osteocalcin* was expressed at the late stage of osteoblast differentiation. The intensity of the individual bands in the RT-PCR image shown in Fig. 3(A) was determined using the Image J software, and the data that were normalized to *Gapdh* were calculated as a fold change relative to control (differentiation day 0), which was set to 1.0. Relative expression levels shown in Fig. 3(B) were plotted against those of the respective genes on differentiation day 0, which were set to 1.0. The data are presented as mean ± SD of three independent experiments.

among rodents. Thus, we concluded that Runx2 and Osx bound directly to the Runx2-and Sp1-binding sites within the −222/−41 region of the mouse *Ucma* promoter, respectively, and activated *Ucma* transcription.

Ucma partially increases osteoblast differentiation

To determine whether Runx2-activated and Osx-activated *Ucma* expression are required for osteoblast differentiation, we generated Ucma-overexpressing stable clones. Compared to the mock control, in an Ucma-overexpressing stable clone, the mRNA expression of *Ucma* was strong throughout the entire period of cell differentiation. The major marker of late-stage differentiation in osteoblasts, osteocalcin expression, was elevated at the early stages of osteoblast differentiation [Fig. 5(A) and (B)]. An identical pattern was observed in another stable clone [Fig. S6(A)]. The expression level of the other osteoblast marker gene, *osteopontin*, also increased in the Ucma-overexpressing cells [Fig. S6(D) and (E)]. Alkaline phosphatase (ALP) staining also indicated that differentiation was enhanced in Ucma-overexpressing osteoblasts

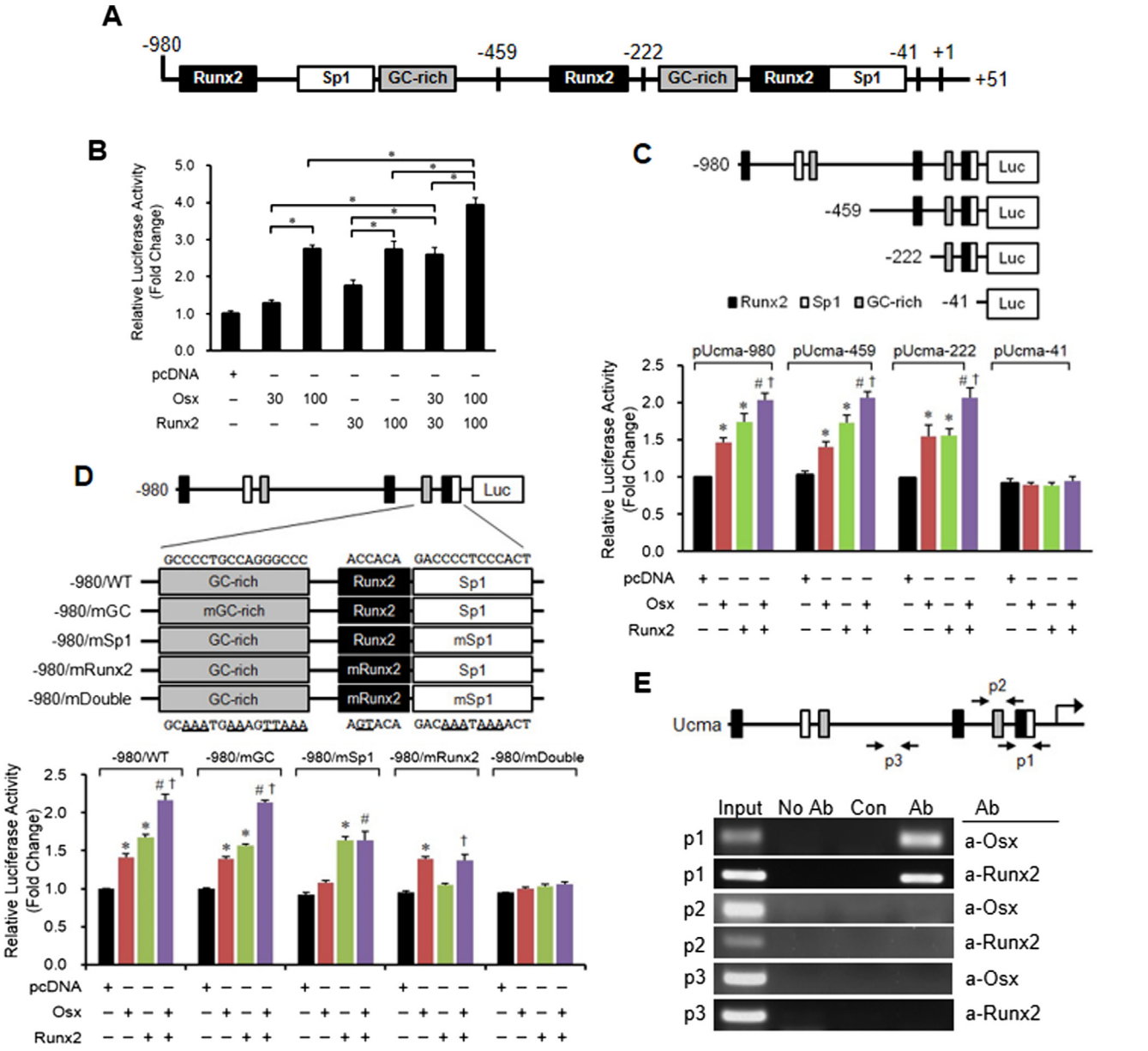


Fig. 4. Ucma transcriptional activity activated by Runx2 and/or Osx. (A) Putative binding sites of Runx2 and Osx were identified in the promoter region of Ucma by using the TFSEARCH software. Within the 1-kb *Ucma* promoter, two Sp1-binding sites, two GC-rich regions, and three Runx-binding sites were found. (B) MC3T3-E1 cells were co-transfected with pUcma-luc and vectors expressing the transcription factors, Runx2 and/or Osx, at the concentration of 30 and/or 100 ng, respectively. *Ucma* transcription was activated by either Osx or Runx2 in a dose-dependent manner. Transcriptional activity of *Ucma* was highly increased by the addition of both Runx2 and Osx. (C) Identification of the Runx2 and Osx-responsive regions in the mouse *Ucma* promoter. The full-length mouse *Ucma* promoter (pUcma-980) or one of three 5′ deletion mutants (pUcma-459, -222, and -41) was transiently cotransfected with either Runx2 and/or Osx expression vectors or the pcDNA empty vector into MC3T3-E1 cells. Luciferase activity was normalized to that of Renilla luciferase in each sample. Relative luciferase activity was expressed as fold induction over the *Ucma*/luciferase reporter activity in the presence of the pcDNA control vector. A significant decrease in Runx2-and Osx-induced *Ucma* transcriptional activity was observed after deletion of the regions between positions −222 and −41 of the *Ucma* promoter. The results are presented as mean ± SD of three independent experiments. *Mean values were significantly different from pcDNA only data ($P < 0.05$). #Mean values were significantly different from Osx only data ($P < 0.05$). †Mean values were significantly different from Runx2 only data ($P < 0.05$). (D) Identification of the Runx2-and Osx-responsive regions in the mouse *Ucma* promoter using site-directed mutagenesis. Four mutations (-980/mGC, -980/mSp1, -980/mRunx2, and -980/mDouble) within the −222/−41 region of the mouse *Ucma* promoter were generated as shown in the diagram. *Ucma* transcriptional activity was significantly reduced after mutation of the Runx2-and/or Sp1-binding sites. The results are presented as mean ± SD of three independent experiments. *Mean values were significantly different from pcDNA only data ($P < 0.05$). #Mean values were significantly different from Osx only data ($P < 0.05$). †Mean values were significantly different from Runx2 only data ($P < 0.05$). (E) Schematic representation of the Runx2-and Sp1-binding sites in the *Ucma* promoter indicated by the primer sets used in the ChIP assay. Chromatin from MC3T3-E1 cells that were transfected with Runx2 or Osx expression vector was subjected to immunoprecipitation with an anti-Osx antibody (a-Osx), anti-Runx2 antibody (a-Runx2), or isotype-matched control antibody (Con). The direct binding of Runx2 and Osx to the *Ucma* promoter was determined by PCR.

[Fig. S6(B)]. In addition, the formation of mineralized nodules started earlier and was more frequent in the Ucma-overexpressing stable clone than in the mock control [Fig. 5(C) and Fig. S6(C)]. These results demonstrated that Ucma-overexpressing osteoblasts differentiated faster than did the mock control cells, meaning that osteoblasts were more mature as a result of increased *Ucma* expression.

Ucma is secreted after removal of its signal peptide[13,14]. After transient transfection of 293FT cells with the Ucma expression vector, Ucma was detected in the medium as well as in cell lysates

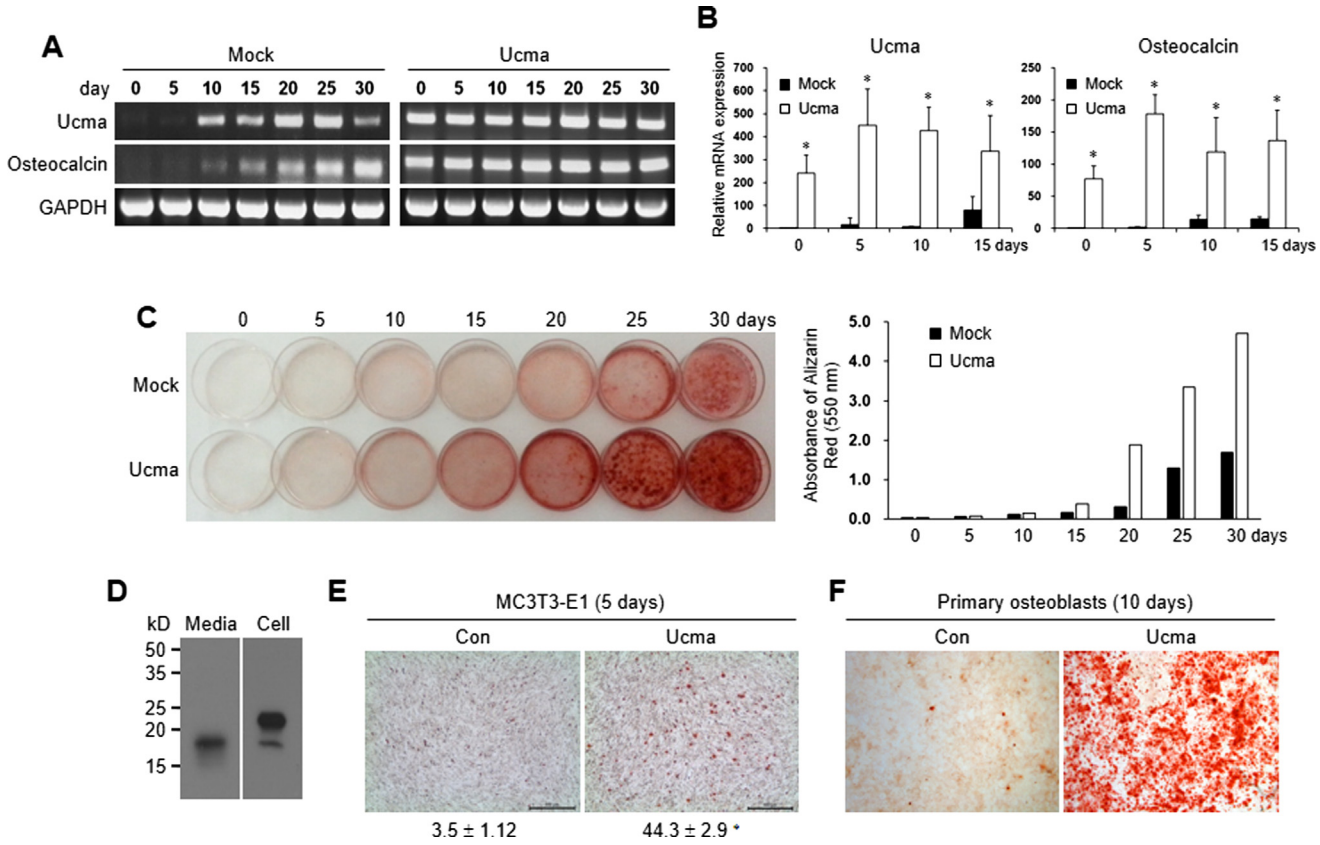


Fig. 5. Ucma-induced osteoblast differentiation. (A) In osteoblasts overexpressing Ucma, *Ucma* and *osteocalcin* expression levels were elevated during the entire period of osteoblast differentiation by RT-PCR. (B) Compared to the mock control, the expression of *Ucma* and *osteocalcin* was elevated at the early stages of differentiation as confirmed by qRT-PCR in *Ucma* overexpressing osteoblasts. Relative expression levels were plotted against those of respective genes on differentiation day 0 of the mock control, which were set to 1.0. *Mean values were significantly different from the mock control at indicated time points ($P < 0.05$). (C) Alizarin red-S staining and OD value analysis were used to evaluate the level of mineralized nodule formation. Mineralized nodule formation started earlier and was more frequent in Ucma-overexpressing osteoblasts compared to the mock control. (D) Ucma protein was detected in the culture medium and cell lysates of 293FT cells transiently transfected with *Ucma*. (E) Increased formation of mineralized nodules was observed in MC3T3-E1 osteoblastic cells grown for 5 days in a medium containing the secreted Ucma protein. The large and thick mineralized nodules stained by alizarin red S were counted in several visual fields (more than five fields per well) of culture plates. Relative mean ± SD for the number of mineralized nodules are shown underneath the images. Scale bar = 500 μm *$P < 0.05$. (F) Increased formation of mineralized nodules was observed in primary osteoblast cells cultured in a medium containing the secreted Ucma protein for 10 days. All cell culture experiments were performed independently in triplicate. A representative result is shown.

[Fig. 5(D)], indicating that Ucma was produced in the cells and subsequently secreted into the culture supernatant. To determine whether the secretion of Ucma is important for osteoblast differentiation or nodule formation, we added the cell culture medium containing Ucma to MC3T3-E1 osteoblasts for 5 days of differentiation. The mineralized nodule formation and *osteocalcin* expression were significantly increased in cells cultured in medium containing secreted Ucma, compared to that observed in osteoblasts cultured in control medium from the pcDNA-transfected cells [Fig. 5(E) and Fig. S7(A)]. Enhanced mineralization was also evaluated by ALP staining [Fig. S7(B)]. The ability of secreted Ucma to enhance differentiation was also confirmed in primary osteoblast cells [Fig. 5(F) and Fig. S7(C)–(E)]. This result suggested that the Ucma stimulated osteoblast differentiation and nodule formation.

Ucma is necessary for Runx2-or Osx-dependent osteoblast differentiation

To determine whether the Ucma knockdown has any effect on Runx2-or Osx-dependent osteoblast differentiation, Ucma shRNA was generated and Ucma knockdown was tested in 293FT cells co-transfected with Ucma expression vector by western blotting [Fig. 6(A)]. Immunoblotting showed that Ucma protein levels were remarkably decreased in shUcma cells. Then, we silenced Ucma with the verified Ucma shRNA in MC3T3-E1 osteoblasts. Osteoblast differentiation was significantly reduced in MC3T3/shUcma cells, as observed by *osteocalcin* expression [Fig. 6(B)]. Nonetheless, nodule formation and osteocalcin gene expression were partially rescued in MC3T3/shUcma cells cultured for 10 days in a medium containing secreted Ucma protein [Fig. 6(C) and (D)]. Even though Runx2 or Osx was overexpressed, the expression of *Ucma* and *osteocalcin* were not increased in MC3T3/shUcma cells compared to MC3T3/Mock cells [Fig. 6(E)]. These results indicate that Ucma is required for Runx2-and Osx-dependent osteoblast differentiation.

Discussion

Runx2 and Osx, master transcription factors of osteogenesis, are upregulated by BMP signaling during osteogenesis. *Runx2* expression and function are controlled through BMP-activated Smad signaling, and BMP2 up-regulates *Osx* expression in osteoblasts. According to the results from KO mouse models[5], *Osx* is a downstream target of Runx2 during osteogenesis. *Osx* expression, however, is not upregulated in Runx2-overexpressing osteoblasts, as shown in Fig. 2(C). Several reports have demonstrated that BMP2-induced *Osx* expression is mediated by both Runx2-dependent and independent pathways *in vitro*. BMP2-induced *Runx2* or *Osx* expression is mediated by Dlx5[18,19]. In addition, *Osx* expression is

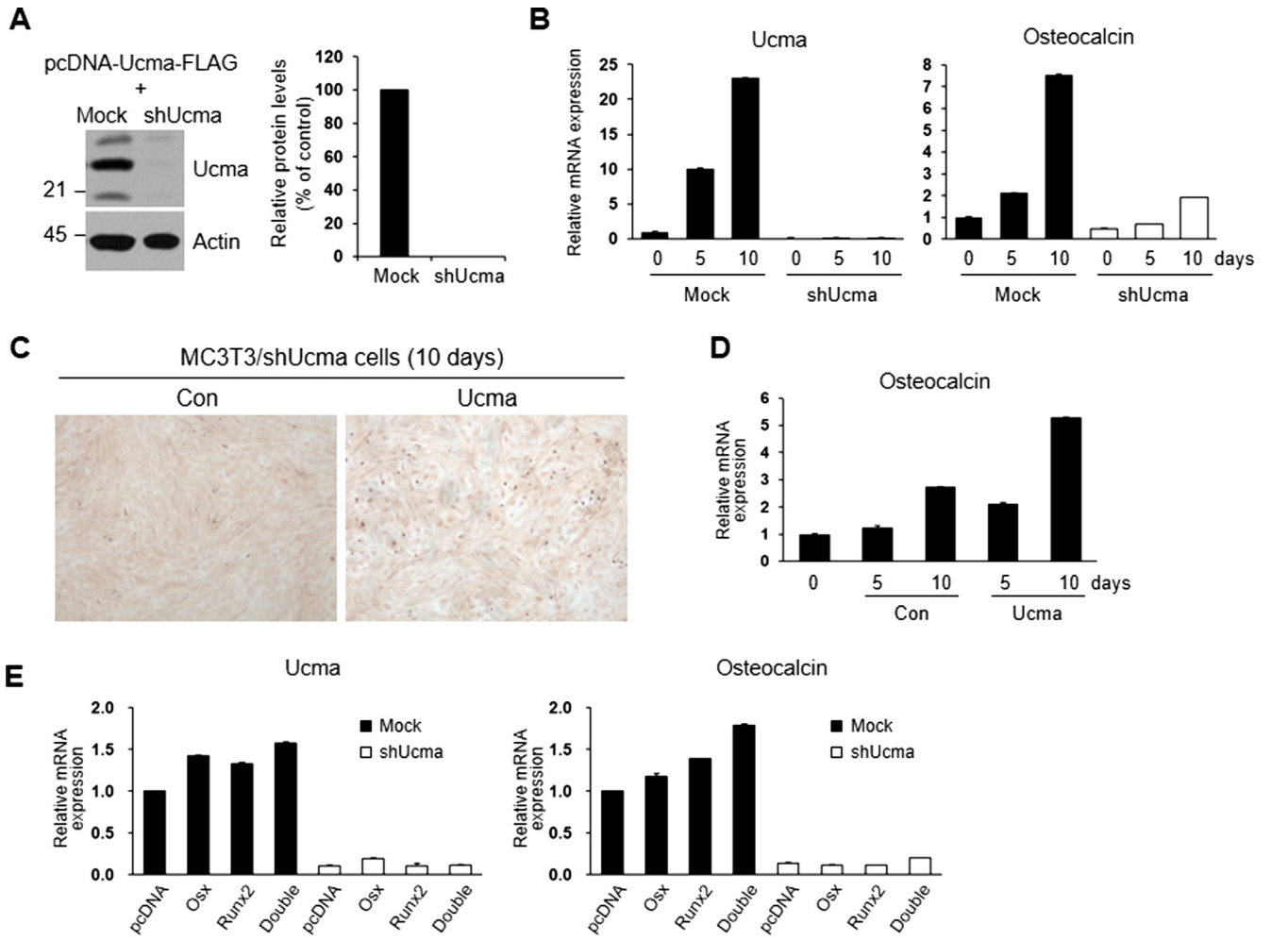


Fig. 6. The effect of Ucma knockdown on osteoblast differentiation. (A) Ucma protein levels in mock and shUcma cells were analyzed by western blotting. Immunoblot intensities for Ucma/β-actin were quantitated by densitometry. Intensities of mock cells were set at 100%. A representative result is shown. (B) Ucma knockdown by shRNA resulted in the reduction of osteocalcin in MC3T3/shUcma cells. The mRNA expression of *Ucma* and *osteocalcin* in MC3T3/mock and MC3T3/shUcma cells were evaluated by qRT-PCR. Relative expression levels were plotted against those of respective genes on differentiation day 0 of the mock control, which were set to 1.0. (C) Nodule formation increased in MC3T3/shUcma cells cultured in a medium containing the secreted Ucma protein for 10 days. (D) The expression of *osteocalcin* was increased in MC3T3/shUcma cells grown in a medium containing the Ucma protein during differentiation as confirmed by qRT-PCR. Relative expression levels were plotted against those of *osteocalcin* on differentiation day 0 of MC3T3/shUcma cells, which were set to 1.0. (E) The mRNA expression of *Ucma* and *osteocalcin* did not change in MC3T3/shUcma cells overexpressing Runx2 or Osx compared to MC3T3/mock cells. Relative expression levels were plotted against those of respective genes in control pcDNA only data of the mock control, which were set to 1.0.

still induced by BMP-2, but not by blocking Dlx5 in Runx2-deficient cells, and is not induced in Runx2-overexpressing C2C12 cells[19]. Together, these results suggest that Dlx5 independently regulates the expression of *Runx2* and *Osx*. Another study also confirmed that *Osx* expression is induced by BMP-2 in Runx2-deficient cells, and this induction is inhibited by an *Msx2* knockdown or by the expression of an inhibitory Smad, suggesting that *Osx* is regulated *via* both Runx2-dependent and Runx2-independent pathways[20]. These observations indicate that *Osx* expressed in bone may not be regulated by Runx2 exclusively. This may be the reason that Osx expression is not upregulated by Runx2 overexpression. Runx2 and Osx, however, are still responsible for the regulation of additional genes downstream of BMP signaling[7–12]. Recent studies have shown that NEL-like 1 (*Nell-1*) is downstream of both Runx2 and Osx during osteoblast differentiation[21,22]. In *Runx2* heterozygotes, aberrant osteoblastic gene expression and calvarial defects are reversed by *Nell-1* overexpression[21]. *Nell-1* transcription is negatively regulated by Osx, indicating that *Nell-1*, controlled by the crosstalk between Runx2 and Osx, may perform an important function in osteoblast differentiation[22]. Nevertheless, genes that are targeted by both Runx2 and Osx during the regulation of osteoblast differentiation and function are still unknown. A better understanding of the regulation of osteogenesis by Runx2-and Osx-controlled genes may facilitate the development of new bone anabolics.

To identify the genes downstream to both Runx2 and Osx that act as transcriptional regulators during osteogenesis, we conducted DNA microarray analysis. Among the 26 genes that were differentially expressed during osteogenesis as a result of a *Runx2* and/or *Osx* deficiency, expression of *osteocrin* and jun proto-oncogene related gene d (*JunD*) was upregulated, while expression of *Dspp*, *Papss2*, and *Ucma* was downregulated in Double^{het} embryos. Osteocrin is a signaling molecule that modulates the osteoblast phenotype and bone growth[23], and Papss2 promotes ALP activity and mineralization in osteoblasts[24]. Even though Dspp in bone is not as highly expressed as that in tooth, regulatory mechanisms governing DSPP expression are involved in both tissues[25]. JunD regulates bone sialoprotein expression, which is detected in human osteotropic cancers[26]. The few studies mentioned above showed that *osteocrin*, *JunD*, *Dspp*, and *Papss2* might be closely linked to

osteogenesis. Compared to these genes, the expression of *Ucma* was observed in osteoblasts and osteocytes, but its function remains unclear in osteogenesis. Nonetheless, there have been few studies on whether Runx2 and/or Osx regulate the expression of *Ucma* during osteoblast differentiation and bone formation.

Surmann-Schmitt and colleagues[14] reported that *Ucma* is expressed at the early stages of osteoblast differentiation and that the recombinant protein interferes with osteoblast differentiation, suggesting that Ucma inhibits osteogenic differentiation. However, Viegas and colleagues[15] observed the expression of Ucma in osteoblasts and osteocytes, as well as in chondrocytes, and provided information based on tissue-specific EST data, arguing for the cartilage-specific expression of Ucma. In the present study, Ucma expression and regulation were observed in osteoblasts. *Ucma* expression was monitored during 30 days of differentiation. *Ucma* expression was negligible in MC3T3-E1 cells before differentiation, but began on day 5 and increased continuously throughout the differentiation period. At the early stage of osteoblast differentiation, *Ucma* expression is quite low in spite of the upregulation of Runx2 and Osx. This finding indicates that neither Runx2 nor Osx alone is sufficient, and that either higher levels of Runx2 and Osx expression, or other factors, are needed to increase Ucma expression. Double^{het} embryos have a reduced area of mineralized bones and delayed bone formation compared to Runx2^{het} and Osx^{het} embryos[6]. This result also supports the findings that low levels of Runx2 and Osx expression reduced the transcriptional activity of Ucma, finally resulting in the reduction of osteoblast differentiation and mineralization. Nevertheless, Ucma expression in osteoblasts can be a controversial issue. Col2 is believed to be a chondrocyte-specific marker. Even though quite low when compared to expression in rib cartilage, Col2a1 expression was still observed in calvaria, as shown in Fig. S3. This result suggests either that calvariae are contaminated with cartilage with chondrocytes or that some osteoblastic cell populations in calvariae are derived from chondrocytes.

Ucma has a conserved cleavage site for subtilisin-like proprotein convertases (SPCs). Among SPC family members, SPC4 and PC6A are attached to heparin sulfate proteoglycans, which are abundant in cartilage matrix, indicating that they are responsible for the cleavage of Ucma in cartilage[27]. Several SPCs also are expressed in cartilage including furin, SPC6, and SPC7[28,29]. Surmann-Schmitt and colleagues[14] generated and used Ucma-C protein (C-terminal fragment of Ucma) that is proteolytically processed by a furin-like protease and found in the entire cartilage matrix. However, it was not studied well for the SPCs abundant in osteoblasts or bone. In the present study, full-length Ucma (approximately 20 kD) was detected in cell lysates and the cleaved form of the signal peptide of Ucma (approximately 16 kD) was detected in cell medium. However, the smallest cleavage form of Ucma (approximately 12 kD), resulting from processing by convertases, was not observed during or after secretion. This suggests that SPCs for Ucma processing are cell type dependent or that uncleaved Ucma plays a different role from Ucma-C, which is processed in chondrocytes. An interesting study showed that Ucma is required for skeletal development in zebrafish; specifically, an *Ucma* knockdown results in severe growth retardation and impaired skeletal development[30]. Secreted Ucma proteins accumulate in soft tissue, including the skin and vascular system, to affect calcifications, indicating that Ucma plays a role in mineral formation[31]. A recent report showed that the sequence variants found in the Ucma basal promoter are associated with Paget's disease of the bone[32]. Together, these findings suggest that Ucma regulates osteoblast differentiation and mineralization. In the present study, formation of mineralized nodules during differentiation is markedly enhanced in *Ucma*-overexpressing osteoblasts and in cells cultured in a medium containing the secreted Ucma protein. Moreover, in *Ucma*-overexpressing osteoblasts, the expression of osteocalcin seems to be upregulated. Even though it is not clear whether Ucma directly increases osteocalcin expression, it is obvious that Ucma-overexpressing osteoblasts are more mature than mock control cells. As a result of increased Ucma expression, osteoblasts differentiate faster than do mock control cells; this finding was confirmed by the increased expression of osteocalcin, which is a major marker of late-stage differentiation in osteoblasts. Increased expression of osteopontin also proved that Ucma-overexpressing osteoblasts are more mature than mock control cells. On comparing Ucma and osteocalcin expression during osteoblast differentiation, the expression patterns of Ucma and osteocalcin were found to be similar; however, additional experimental evidence must be gathered to conclude that Ucma is a late stage marker for osteoblast differentiation.

Eitzinger and colleagues[33] reported that skeletal development is normal in *Ucma*-deficient mice and that Ucma is not necessary for endochondral ossification during development. Although Ucma does not have a decisive function in skeletal development, our results demonstrate that increased *Ucma* expression promote osteoblast differentiation, thereby resulting in enhanced bone formation. Further refinement of mouse models of osteoblast-specific Ucma-overexpression should help researchers understand the *in vivo* function of Ucma in the promotion of bone formation. Consequently, our results suggest that Ucma may be involved in homeostasis and enhancement of the skeleton under certain pathological conditions, such as aging, disease, or fractures.

Author contributions

YJ Lee participated in study design, data collection, and in the drafting and approval of the manuscript.

SY Park, SJ Lee, YC Boo, and JY Choi participated in the study design, data collection, data analysis, and approval of the manuscript.

JE Kim participated in the study design, data analysis, and in the drafting and approval of the manuscript.

Conflict of interest

None.

Acknowledgments

This study was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (HI13C1874).

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2015.03.035.

References

1. Massagué J, Seoane J, Wotton D. Smad transcription factors. Genes Dev 2005;19:2783–810.
2. Lian JB, Stein GS, Javed A, van Wijnen AJ, Stein JL, Montecino M, *et al*. Networks and hubs for the transcriptional control of osteoblastogenesis. Rev Endocr Metab Disord 2006;7:1–16.
3. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, *et al*. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755–64.

4. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Roseweel IR, *et al.* Cbfa, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 1997;89:765–71.
5. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, *et al.* The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002;108:17–29.
6. Baek JE, Choi JY, Kim JE. Skeletal analysis and differential gene expression in Runx2/Osterix double heterozygous embryos. Biochem Biophys Res Commun 2014;451:442–8.
7. Frendo JL, Xiao G, Fuchs S, Franceschi RT, Karsenty G, Ducy P. Functional hierarchy between two OSE2 elements in the control of osteocalcin gene expression in vivo. J Biol Chem 1997;273:30509–16.
8. Franceschi RT, Ge C, Xiao G, Roca H, Jiang D. Transcriptional regulation of osteoblasts. Cells Tissues Organs 2009;189:144–52.
9. Tang W, Li Y, Osimiri L, Zhang C. Osteoblast-specific transcription factor osterix (Osx) is an upstream regulator of satb2 during bone formation. J Biol Chem 2011;286:32995–3002.
10. Zhang C, Tang W, Li Y, Yang F, Dowd D, MacDonald P. Osteoblast-specific transcription factor osterix increases vitamin D receptor gene expression in osteoblasts. PLoS One 2011;6:e26504.
11. Cao Y, Jia SF, Chakravarty G, de Crombrugghe B, Kleinerman ES. The osterix transcription factor down-regulates interleukin-1 alpha expression in mouse osteosarcoma cells. Mol Cancer Res 2008;6:119–26.
12. Wu YF, Matsuo N, Sumiyoshi H, Yoshioka H. Sp7/Osterix is involved in the up-regulation of the mouse pro-α1(V) collagen gene (Col5a1) in osteoblastic cells. Matrix Biol 2010;29:701–6.
13. Tagariello A, Luther J, Streiter M, Didt-Koziel L, Wuelling M, Surmann-Schmitt C, *et al.* Ucma-A novel secreted factor represents a highly specific marker for distal chondrocytes. Matrix Biol 2008;27:3–11.
14. Surmann-Schmitt C, Dietz U, Kireva T, Adam N, Park J, Tagariello A, *et al.* Ucma, a novel secreted cartilage-specific protein with implications in osteogenesis. J Biol Chem 2008;283:7082–93.
15. Viegas CS, Simes DC, Laizé V, Williamson MK, Price PA, Cancela ML. Gla-rich protein (GRP), a new vitamin K-dependent protein identified from sturgeon cartilage and highly conserved in vertebrates. J Biol Chem 2008;283:36655–64.
16. Jiménez MJ, Balbín M, López JM, Alvarez J, Komori T, López-Otín C. Collagenase 3 is a target of Cbfa1, a transcription factor of the runt gene family involved in bone formation. Mol Cell Biol 1999;19:4431–42.
17. Zheng Q, Zhou G, Morello R, Chen Y, Garcia-Rojas X, Lee B. Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. J Cell Biol 2003;162:833–42.
18. Lee MH, Kim YJ, Kim HJ, Park HD, Kang AR, Kyung HM, *et al.* BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. J Biol Chem 2003;278:34387–94.
19. Lee MH, Kwon TG, Park HS, Wozney JM, Ryoo HM. BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. Biochem Biophys Res Commun 2003;309:689–94.
20. Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, *et al.* BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. J Biol Chem 2008;283:29119–25.
21. Truong T, Zhang X, Pathmanathan D, Soo C, Ting K. Craniosynostosis-associated gene nell-1 is regulated by runx2. J Bone Min Res 2007;22:7–18.
22. Chen F, Zhang X, Sun S, Zara JN, Zou X, Chiu R, *et al.* NELL-1, an osteoinductive factor, is a direct transcriptional target of Osterix. PLoS One 2011;6:e24638.
23. Thomas G, Moffatt P, Salois P, Gaumond MH, Gingras R, Godin E, *et al.* Osteocrin, a novel bone-specific secreted protein that modulates the osteoblast phenotype. J Biol Chem 2003;278:50563–71.
24. Wang W, Li F, Wang K, Cheng B, Guo X. PAPSS2 promotes alkaline phosphates activity and mineralization of osteoblastic MC3T3-E1 cells by crosstalk and Smads signal pathways. PLoS One 2012;7:e43475.
25. Qin C, Brunn JC, Cadena E, Ridall A, Tsujigiwa H, Nagatsuka H, *et al.* The expression of dentin sialophosphoprotein gene in bone. J Dent Res 2002;81:392–4.
26. Detry C, Lamour V, Castronovo V, Bellahcène A. CREB-1 and AP-1 transcription factors JunD and Fra-2 regulate bone sialoprotein gene expression in human breast cancer cells. Bone 2008;42:422–31.
27. Farach-Carson MC, Hecht JT, Carson DD. Heparan sulfate proteoglycans: key players in cartilage biology. Crit Rev Eukaryot Gene Expr 2005;15:29–48.
28. Zheng M, Streck RD, Scott RE, Seidah NG, Pintar JE. The developmental expression in rat of proteases furin, PC1, PC2, and carboxypeptidase E: implications for early maturation of proteolytic processing capacity. J Neurosci 1994;14:4656–73.
29. Constam DB, Calfon M, Robertson EJ. SPC4, SPC6, and the novel protease SPC7 are coexpressed with bone morphogenetic proteins at distinct sites during embryogenesis. J Cell Biol 1996;134:181–91.
30. Neacsu CD, Grosch M, Tejada M, Winterpacht A, Paulsson M, Wagener R, *et al.* Ucmaa (Grp-2) is required for zebrafish skeletal development. Evidence for a functional role of its glutamate γ-carboxylation. Matrix Biol 2011;30:369–78.
31. Viegas CS, Cavaco S, Neves PL, Ferreira A, Joao A, Williamson MK, *et al.* Gla-rich protein is a novel vitamin K-dependent protein present in serum that accumulates at sites of pathological calcifications. Am J Pathol 2009;175:2288–98.
32. Michou L, Conceição N, Morissette J, Gagnon E, Miltenberger-Miltenyi G, Siris ES, *et al.* Genetic association study of UCMA/GRP and OPTN genes (PDB6 locus) with Paget's disease of bone. Bone 2012;51:720–8.
33. Eitzinger N, Surmann-Schmitt C, Bösl M, Schett G, Engelke K, Hess A, *et al.* Ucma is not necessary for normal development of the mouse skeleton. Bone 2012;50:670–80.