Role of Retinoic Acid-Related Orphan Receptor- α in Differentiation of Human Mesenchymal Stem Cells along with Osteoblastic Lineage

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Key Words

Retinoic acid-related orphan receptor- α · Bone sialoprotein · Dentin matrix protein 1 · Human mesenchymal stem cells · Osteoblast differentiation · Mineralization

Abstract

Objective: In a human mesenchymal stem cell (hMSC) model of osteoblast differentiation, we identified expression of the retinoic acid-related orphan receptor- α (ROR α) gene as a candidate osteogenesis-related gene using gene expression profiling. ROR α is a member of the nuclear receptor family of intracellular transcription factors and plays multifunctional roles in tissue development and differentiation, inflammation metabolism and circadian rhythms. However, the important role of ROR α in the differentiation of hMSC along the osteoblastic lineage is not yet fully understood. **Methods:** To investigate the role of ROR α during osteoblast differentiation, we eliminated ROR α from hMSCs with a small interfering RNA molecule (siRNA), and investigated the effects of suppressing ROR α by RT-PCR analysis. **Results:** We showed that suppressing the expression of ROR α by the

siRNA inhibited the expression of both bone sialoprotein and dentin matrix protein 1. It was discovered coincidentally that the inhibitory effect of ROR α resulted in failure of mineralization and bone nodule formation during the course of osteogenesis. **Conclusion:** The results of this study indicate that ROR α must be an imperative modulator of bone sialoprotein and dentin matrix protein 1 involved in osteoblast differentiation and bone formation downstream of Wnt/ β -catenin signaling and just prior to the start of mineralization.

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Introduction

Differentiation of mesenchymal stem cells (MSCs) into the osteoblastic lineage is believed to be a stage-specific process involving many signaling pathways, hormones, growth factors, cytokines, and osteoblast-specific transcription factors. Recently, the Wnt/ β -catenin, and Indian hedgehog (IHH) signaling pathways in particular, have been shown to be essential for osteoblastic differentiation [1–7] along with the transcription factors runt-re-

lated transcription factor 2 (RUNX2) [8, 9] and osterix (OSX) [10]. It is noteworthy that β -catenin is a central player in the canonical Wnt pathway, and is indispensable for osteoblast differentiation. In the evaluation of bone development in β -catenin-conditional knockout mice and in mice with β -catenin inactivation, it has been demonstrated that β -catenin activity is essential in an early step of osteoblast differentiation and for inhibition of chondrocyte differentiation [1–7]. Several groups have indicated that Wnt signaling functions downstream of IHH signaling during differentiation into the osteoblast lineage [5, 6]. These data demonstrate that differentiation into the osteoblast lineage requires IHH and Wnt/ β -catenin signaling to promote osteogenic activity and block chondrogenic activity [3, 5, 6].

Osteoblastic differentiation *in vitro* is characterized by three distinct stages of cellular activity: proliferation, extracellular matrix (ECM) maturation and mineralization [11]. Following the stages of proliferation and matrix maturation, nodule cells need ECM proteins belonging to the SIBLING (small integrin-binding ligand N-linked glycoprotein) family [11, 12] to initiate matrix mineralization. Of these noncollagenous ECM proteins, bone sialoprotein (BSP) is one of the most abundantly expressed mineralized tissue-specific protein that appears to function in the initial stage of mineralization of bone. BSP is a critical factor for expression of the osteoblastic phenotypes of bone marrow cells [12], and some studies have suggested that BSP is a key regulator of osteoblast differentiation and mineralization [12–17]. Recently, an *in vitro* study demonstrated that expression of the gene encoding BSP is controlled by the retinoic acid-related orphan receptor- α (ROR α) during osteoblast differentiation [18]. The critical role of ROR α in bone metabolism was highlighted by an *in vivo* study which showed that ROR α -deficient mice were osteopenic. In that study, they showed that total mineral content of bone and the bone density are significantly reduced in ROR α -deficient mice as compared with the observations in wild-type mice [18, 19]. On the other hand, several studies have demonstrated that ROR α recruits β -catenin as a co-activator to bind to ROR response elements in a targeted promoter region [20, 21]. Following binding of β -catenin with the ROR α /co-activator complexes [21], the activation of β -catenin by Wnt signaling might influence the transcriptional activity of ROR α . It is considered that ROR α may act downstream of Wnt [19] in brain development [21, 22].

ROR α is a nuclear receptor-type transcription factor and plays a key role in the regulation of cellular differen-

tiation and the development of several tissues [19]. It is widely expressed throughout the body, and each isoform shows a tissue-specific distribution [23–25]. ROR α mRNA has been detected in the liver, brain, skeletal muscle, kidney tubules, lung, gut, skin, heart, vessels, whisker follicles and pancreas [24, 26–30]. While mainly ROR α 1 and ROR α 4 are expressed in numerous tissues, ROR α 2 and ROR α 3 are expressed abundantly in the testes. These observations suggest that each ROR α isoform may play a different role in different tissues or cell types [29]. In addition, ROR α has been shown to regulate the expression of genes involved in both myogenesis and adipogenesis. ROR α is multifunctional in that it has been shown, as described above, to be involved in brain and lymphocyte development, maintenance of circadian rhythms, mesenchymal differentiation, and bone formation [19, 30]. Although a previous study has suggested that ROR α may regulate osteoblast function and activity [18], further studies are needed to determine the functional role of ROR α in osteoblast differentiation and bone development [19, 30]. Thus, we focused on the critical role of ROR α and investigated its effect on bone formation in a human MSC (hMSC) differentiation model.

In this study, we initially examined the gene expression profiles during differentiation of hMSCs into the osteoblastic lineage and selected ROR α as a candidate human osteogenesis-related gene. We further investigated the effects of ROR α knockdown during osteogenesis. We hypothesized that ROR α is indispensable for the differentiation of hMSCs into the osteoblastic lineage, and ROR α functions as a modulator of bone development downstream of Wnt signaling through BSP-dependent pathways.

Materials and Methods

hMSC and Osteogenic Culture

hMSCs prepared from fresh bone marrow obtained by routine iliac crest aspiration from human donors were obtained from Osiris Therapeutics (Baltimore, Md., USA), and prepared according to Jaiswal et al. [31]. hMSCs, derived from three different healthy donors, were cultivated according to standard protocol using low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. hMSCs for osteogenic culture were obtained from cryopreserved samples and grown to confluence in 185 cm² flasks, passaged once, and plated at 1.0×10^6 cells/100-mm dish. Cells were cultured for 14 days according to previously published *in vitro* differentiation protocol [32]. Osteogenic differentiation of hMSCs was performed using osteogenic induction (OS) medium containing 0.1 μ M dexamethasone, 0.05 mM ascorbic acid and 10 mM β -glycerophosphate, and the OS medium was changed every 2 days.

Table 1. Human-specific primer pairs used for RT-PCR analysis

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
IGF2	TGCATTGCTGCTTACC	GTCCCTCTCTCGGACTTG
LEPR	TGTTGTGAATGTCTTGTGCC	TACTCCAGTCACTCCAGATTCC
ROR α 1–4	GTGCGACTTCATTTTCCTCCAT	GCTTAGGTGATAACATTTACCCATCA
ROR α 1	ACCCCGCTGAACCAGGAA	CTGCATACAAGCTGTCTCTC
ROR α 4	GATGTATTTTGTGATCGCAG	CTGCATACAAGCTGTCTCTC
COL1	CTGACCTTCCTGCGCCTGATGTTT	GTCTGGGGCACCAACGTCCAAG
OPN	TTGCTTTTGCCTCCTAGGCA	GTGAAAACCTTCGGTTGCTGG
OCN	ATGAGAGCCCTCACACTCCTC	GGGCCGTAGAAGCGCCGATAG
ALP	CCACGTCTTCACATTTGGTG	AGACTGCGCCTGGTAGTTGT
BMP-2	CCCACCTGGAGGAGAAACAA	GCTGTTTGTGTTTGGCTTGA
RUNX2	GCCTTCAAGGTGGTAGCCC	CGTTACCCGCCATGACAGC
OSX	CCTCAGGCTATGCTAATGATTA	GCAGAAGAGCCAGTTGCAGA
IHH	TCAGCGATGTGCTCATTTTC	ACACTGGTGGTGGAGGATGT
BSP	GCTCAGCATTTTGGGAATGGC	CTGCATTGGCTCCAGTGACAC
DMP-1	CCCTTGGAGACAGTGAGTC	AGTGCCCAAGATAACCACCAG
DSPP	TGTCGCTGTTGTCCAAGAAG	GACAATGGCAGCCAAAGAAT
GAPDH	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTTCAGC

Gene Expression Profiling during *in vitro* Osteogenesis of hMSCs

At days 0, 3, 7 and 14, total RNA was isolated from *in vitro* hMSCs along with osteogenic differentiation, and ^{32}P -labelled probes were synthesized. Atlas human 1.2 gene arrays (Clontech) were hybridized with donor-specific cDNA probes and were analyzed using GeneSpring Software (SiliconGenetics) enabling normalization at each time point. The expression level for each probe set was plotted to determine the reproducibility of the array-based hybridization signals and to compare with gene expression levels of day 0 used as controls.

Implant Preparation

We used implants of hydroxyapatite/tricalcium phosphate (HA/TCP; 60/40) matrix that has been previously described [33]. The cylindrical implants (3.2 mm diameter, 5 mm long) were cut from HA/TCP blocks. Central to the cylinder, a 1-mm in diameter, 3-mm-long channel was cut. The cylinders were ultrasonically cleaned and sterilized by dry heat at 250°C for 4 h. hMSCs cultured in cell culture flask were trypsinized and loaded onto implants. Subsequently, HA/TCP implants were incubated with 1.5×10^5 hMSCs/implant to allow attachment to occur. This matrix is well defined in its ability to support hMSC osteogenesis [33–35]. Then cylinders were implanted into SCID mice. At days 3, 7, 14 after implantation, RNAs were extracted. mRNA from ectopically formed bone for up to 14 days following hMSCs implantation in SCID mice was isolated and used to program RT-PCR assays to confirm *in vitro* gene expression events.

SCID Mouse Model

All animal experimentation was performed by means of protocols and facilities approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. SCID mice, 7 weeks of age, were obtained from Harlan Laboratories. General anesthesia was induced with Domitor (Orion-Farmos), Ketaset (Fort Dodge Animal Health). One prepared implant was placed in each subcutaneous pouch in the four dorsal quadrants. All grafts were harvested and randomly assigned to RT-PCR analysis. Harvested grafts were placed in RNA extraction buffer and homogenized. Similarly, cells adherent to culture dishes underwent lysis by means of RNA extraction buffer. RNA was isolated from cultured cells and tissues adherent to retrieved implants by an acid-phenol method.

RNA Isolation and RT-PCR Analysis

The various extracted RNA was quantified by UV spectrophotometry. For RT-PCR reactions, 1 μg total RNA was used to program reactions by means of primers for insulin-like growth factor 2 (IGF2), human leptin receptor (LEPR), ROR α , collagen type 1 (COL1), osteopontin (OPN), osteocalcin (OCN), alkaline phosphatase (ALP), bone morphogenic protein 2 (BMP-2), RUNX2, OSX, IHH, BSP, dentin matrix protein (DMP-1), dentin sialophosphoprotein (DSPP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Reactions with Taq DNA Polymerase (Invitrogen) were performed according to suppliers' instructions, with annealing performed at 55–60°C for various primer pairs. All amplifications were performed for 23–40 cycles. All human-specific primer sequences are listed in table 1.

ROR α Suppression by Small Interfering RNA Molecule

hMSCs were cultured in a 6-well plate at density of 4.0×10^3 cells/well. After reaching $\sim 80\%$ of confluence, small interfering RNA molecule (siRNA) transfections were started with DMEM of serum free medium, using siRNA transfection kit (siPORT Amine transfection agents; Ambion, Applied Biosystems). After 24 h of siRNA transfections, the serum free medium was replaced to OS medium, and that point was designated day 0. hMSC differentiation into osteoblast lineage was observed for up to 14 days. Prior to siRNA transfections, we designed two different target-specific sequences to knockdown ROR α gene and synthesized the sense and antisense RNA strands of siRNA, using siRNA construction kit (Ambion, Applied Biosystems). These different siRNA target sequences and design tools are as follows: target siRNA-1 sequence, 5'-AAGCCAUCAGGAUGACCACACG-3'; sense, 5'-AACGTGTGTCATCCTGATGGCCCTGTCTC-3'; antisense, 5'-AAGCCATCAGGATGACACACGCCTGTCTC-3'; target siRNA-2 sequence, 5'-AAUAUGUGACUACACACCAGC-3'; sense, 5'-AAGCTGGTGTGTAG TCACATACCTGTCTC-3'; antisense, 5'-AAGCCATCAGGATGACACACGCCTGTCTC-3'. For hMSCs, the detailed protocols for siRNA synthesis and transfections were followed as instructions of Ambion Silencer siRNA system.

ALP Activity and Mineralization Assays

ALP staining and alizarin red staining were performed to assess osteoblast differentiation and bone nodule formation, respectively. hMSCs were placed on 6-well plates, and the cultures were fed with OS medium (day 7 and 14) after confluence with DMEM (day 0). To assess ALP activity in osteoblast differentiation, MSCs were washed with PBS, fixed with 70% ethanol for 1 min and consequently stained with alkaline phosphatase detection kit (Sigma Diagnostics). To detect mineralized nodule formation, hMSCs were also fixed in methanol for 15 min, and stained with 2% Alizarin Red solution for 5 min at 20°C. After washing three times with distilled water, these staining images were obtained by scanning plates on a scanner.

Results

Identification of ROR α as an Osteogenesis-Related Gene Expression

We examined the gene expression profile during in vitro differentiation of hMSCs along the osteoblastic lineage by cDNA array analysis of 1,176 genes (fig. 1), and selected three genes, namely IGF2 (5.3-fold change at day 3), LEPR (5.2-fold change at day 7) and ROR α (5.6-fold change at day 14) as candidate genes showing upregulation during osteogenesis (fig. 2a, b). For validation of the in vitro and in vivo gene expression patterns, undifferentiated hMSCs adherent to culture dishes and adherent to HA/TCP implantation blocks were examined. Differentiation of both implant-adherent and culture-dish-adherent hMSCs was evaluated by RT-PCR for up to 14 days. As ROR α was found to be consistently expressed during the course of differentiation of hMSCs into the osteoblast

lineage (fig. 2a), we focused on investigation of the functions of ROR α as an osteogenesis-related gene in hMSCs during osteoblast differentiation. Gene expression on common regions of ROR α isoforms 1–4 as well as BSP and OPN was upregulated upon induced differentiation into the osteoblastic lineage (fig. 2c). Expression of ROR α isoforms 1 and 4, but not ROR α isoforms 2 and 3 (data not shown), was confirmed in hMSCs differentiating into the osteoblastic lineage.

ROR α Knockdown by siRNA Inhibits BSP Expression

To identify the optimal dose of siRNA to inhibit the expression of ROR α , we tested various doses (1–20 nM) of ROR α -siRNA (siRNA-1 and siRNA-2 cocktail) transfection. The results showed that siRNA at a concentration of 20 nM for 24 h could produce ROR α knockdown (fig. 3a). Whereas almost complete knockdown of ROR α 1 was obtained at day 14 after OS with both siRNA-1 and siRNA-2 treatments, only slight inhibition of ROR α 4 was observed at day 14 after OS with siRNA-2 treatment (fig. 3b). We also found significant downregulation of BSP gene expression in response to knockdown of ROR α 1. On the other hand, no consistent inhibitory effect of ROR α knockdown was found on the OPN gene (fig. 3b).

ROR α Knockdown Inhibits Osteoblast Differentiation and Bone Nodule Formation

To investigate the inhibitory effect of ROR α knockdown on osteoblast differentiation and bone nodule formation, hMSCs were treated with siRNA-1 and siRNA-2, and with cells treated with only siRNA transfection reagents used as controls, during differentiation of hMSCs into the osteoblastic lineage. ALP activity and alizarin red staining were performed on days 0, 7 and 14. These assays were evaluated after 24 h of siRNA transfections, and that point was designated day 0. After the serum-free medium was replaced with OS medium, hMSCs differentiation into osteoblast lineage was observed for up to 14 days.

In the 6 wells examined as controls without ROR α -siRNA treatment, significant increase in ALP (an early-stage osteoblast differentiation marker enzyme) activity was observed at days 7 and 14, and mineralized nodule formation detectable by alizarin red staining was observed at day 14 (fig. 4a). The results confirmed that the hMSC differentiation model into the osteoblastic lineage functioned normally in the model employed, and osteoblast differentiation and formation of bone nodules were clearly observed during the course of osteogenesis. In both of the 6-well plates treated with siRNA-1 and siRNA-2 for up to 14 days (fig. 4b, c) ROR α knockdown

Fig. 1. Scatter plot analysis of gene expression levels during the course of osteogenesis. All 1,176 genes are represented in this plot. The results of scatter plot analysis and the number of up- and

downregulated genes of days 3, 7 and 14 are shown. The parallel lines flanking the diagonal indicate 3.0-fold upregulated, 1.0-fold equality and 0.3-fold downregulated changes in gene expression.

significantly reduced the ALP activity and mineralized nodule formation.

Targeted Knockdown of ROR α Inhibits Induced Osteoblast Differentiation

To detect the expression pattern of osteoblast-specific markers under the condition of ROR α knockdown, we performed RT-PCR assays using ROR α -siRNA tools, as described above. We observed that while ROR α knockdown was associated with severe impairment of bone nodule formation and mineralization (fig. 4b, c), there was no downregulation of most osteoblast-specific markers, such as COL1, OPN, OCN, ALP, BMP-2, RUNX2, OSX or IHH (fig. 5a, b). In contrast, loss of ROR α sup-

pressed the expression of BSP, which is a major osteoblast-specific marker (fig. 5b). Simultaneously, loss of ROR α also suppressed the expression of DMP-1 and DSPP (fig. 5b). Only the expression patterns of BSP, DMP-1 and DSPP that belong to the SIBLING family were completely opposite to those of most osteoblast markers.

Discussion

Using gene expression profile during osteogenesis of hMSCs in vitro, we have identified ROR α as a candidate of osteogenesis-related gene. In this study, we used two siRNAs to knockdown the ROR α genes during differen-

Fig. 2. Expression analysis of targeted mRNA during the course of osteogenesis. Total RNA was extracted at the indicated days (0, 3, 7 and 14) and assessed in RT-PCR assays. **a** Results of RT-PCR assays showing the expression of selected candidate genes IGF2, LEPR and ROR α in vitro and in vivo. In vitro results are shown of experiments in which hMSCs were induced to differentiate into osteoblasts on dishes without (day 0 used as controls) or with OS media for up to 14 days. In vivo results are shown of experiments in which hMSCs with HA/TCP implantation blocks ectopically formed bone at 3–14 days after implantation in SCID mice model. **b** In vitro-differentiated hMSCs were induced with OS media for up to 14 days. Scale bar, 100 μ m. **c** Results of RT-PCR assays showing the expression of osteoblast marker genes BSP, OPN and ROR α , and control gene GAPDH under osteogenic induction for up to 14 days.

Fig. 3. ROR α knockdown by siRNA. **a** ROR α -siRNA was transfected at 1–20 nM siRNA oligo concentration. Results of RT-PCR assays showing the expression level of ROR α mRNA and GAPDH with different concentration of ROR α -siRNA oligo compared with control. **b** Results of RT-PCR assays showing the expression of BSP, OPN, ROR α 1 and ROR α 4 genes treated with two different siRNA target sequences under OS for up to 14 days.

tiation of hMSCs into osteoblasts (fig. 3b). These siRNAs were employed to gene-specific knockdown for ROR α 1 and ROR α 4. In fact, siRNA-2 inhibited both ROR α 1 and ROR α 4 expression; however, siRNA-1 inhibited only ROR α 1 expression. Moreover, siRNA-1 and siRNA-2 interfered with ALP activity and mineralization of hMSCs (fig. 4b, c). These results indicated that ROR α 1,

but not ROR α 4 was associated with ALP activity and mineralization in hMSCs differentiation.

Immunocytochemical study indicated that ALP proteins were localized in both the calcified nodules and matrix vesicles of bones [36]. In our study, ALP showed the highest activity at day 14 accompanied with nodule formation (fig. 4a) under the control condition without

Fig. 4. ROR α knockdown inhibits bone nodule formation. Representative images of qualitative ALP and alizarin red staining in hMSCs under OS for up to 14 days. **a** Osteoblast-mediated mineralization was formed in control without ROR α -siRNA treatment. **b** Osteoblast-mediated mineralization was inhibited by siRNA-1 treatment. **c** Osteoblast-mediated mineralization was inhibited by siRNA-2 treatment.

ROR α -siRNA; therefore, there is strong evidence that ALP was closely related with bone nodule formation. The inhibition of ALP activity and mineralization by knock-down of ROR α was remarkably observed (fig. 4), whereas the ALP mRNA expression did not change after knock-down of ROR α (fig. 5). We suspect that this disparity is due to the influence of ALP mRNA translation by ROR α suppression. This issue of a disparity between ALP activity and mRNA expression needs further investigation to clarify the inhibitory mechanism induced by ROR α -siRNA.

Although the expression of OPN was not affected by ROR α knockdown at all, loss of ROR α completely blocked the expression of BSP (fig. 3b). Previous functional study on ROR α provided experimental evidence that ROR α regulates the mouse BSP promoter in bone-derived cells

[18]. Furthermore, it is believed that ROR α binds selectively to the consensus response element A/GGGTCA found in several genes, including 5-lipoxygenase, cellular retinoic acid-binding protein, the inhibitor of cyclin-dependent kinases, and the rat BSP gene [37–39]. Thus, it has been established beyond doubt that transcription of the BSP gene is controlled by the actions of ROR α during osteoblast differentiation of hMSCs. In a recent study, BSP-deficient mice were found to have impaired bone growth and mineralization, and also dramatically reduced bone formation [16]. Moreover, it has been demonstrated that reduction in BSP expression in osteoblasts producing BSP-targeting shRNA significantly reduces matrix mineralization as a product of slowed or altered osteoblast differentiation [15]. Our results are in agreement with most previously published data [12–17, 34] and

Fig. 5. Targeted knockdown of ROR α inhibits induced osteoblast differentiation. Expression analysis of targeted gene mRNA level under OS for up to 14 days. **a** Results of RT-PCR assays showing expression of osteoblast marker gene mRNA (COL1, OPN, OCN, ALP, BMP-2, RUNX2, OSX) without (control) or with ROR α -

siRNA1 treatment under OS for up to 14 days. **b** Results of RT-PCR assays showing the expression of IHH, DMP-1 and DSPP mRNA without (control) or with ROR α -siRNA1 treatment under OS for up to 14 days.

Fig. 6. Model for ROR α 's role in osteogenesis through multiple signaling cascades. ROR α modulates BSP and DMP-1 to initiate ECM mineralization and to activate osteoblast differentiation downstream of Wnt/ β -catenin signaling. Scale bar, 100 μ m.

also lend support to the notion that BSP plays a fundamental role in the initiation of mineralization and bone nodule formation during osteoblast differentiation.

More interestingly, our data show that osteoblast differentiation and mineralization did not occur consistently following blockade of ROR α expression (fig. 3b, 4, 5a), whereas most osteoblast-specific markers (OPN, OCN, ALP, BMP-2, RUNX2, OSX and IHH) were upregulated at day 14 of osteogenesis induction (fig. 5a, b). In comparison with the profile of these markers, only the gene expression of BSP, DMP-1 and DSPP of the SIBLING family were inhibited by ROR α knockdown (fig. 5b). Therefore, our results suggest that in addition to its effect on BSP expression, ROR α can also directly or indirectly modulate DMP-1 and DSPP expressions to induce mineralization of the bone matrix components (fig. 6). In the early stage of osteoblast differentiation, β -catenin is required downstream of IHH signaling and OSX expression [5] and Wnt signaling functions downstream of IHH [3]. Furthermore, β -catenin is required either downstream of IHH and BMP signaling, or provides an essential independent signal for osteoblastogenesis [7]. Our findings that suppression of ROR α did not affect the expression of various osteoblast markers indicate that ROR α functions as a modulator of the expression of some members of the SIBLING family downstream of Wnt/ β -catenin and IHH signaling, along with RUNX2 and OSX (fig. 6). On the other hand, the role of DMP-1, which is a member of the SIBLING family, is to initiate hydroxyapatite nucleation [40] and function as an important reg-

ulatory molecule in the biomineralization process [41–45], just like BSP [12–17, 34]. In addition, a recent study provided evidence to suggest the function of DMP-1 as a transcriptional regulator of the DSPP gene [46]. The results of studies on DMP-1 null mice [47, 48] suggest that other compensatory mechanisms might be responsible for bone and dentin formation during early development [46]. More specifically, it has been established beyond doubt that not only DMP-1, but also BSP-mediated regulatory mechanisms are required downstream of ROR α to promote mineralization during osteoblastogenesis.

Of tissue regeneration strategies, cell-based therapies for bone tissue engineering are imperative to clearly elucidate the molecular mechanisms of bone development and formation. Therefore, it is important to identify the functional roles of target molecules along various signaling pathways during differentiation of mesenchymal stem cells into the osteoblastic lineage. There is strong evidence to suggest that osteoblast differentiation of mesenchymal stem cells needs Wnt/ β -catenin and IHH signaling with both the osteoblast-specific transcription factors RUNX2 and OSX, as previously reported [1–10]. From other aspects of bone formation, BSP is a major noncollagenous protein of the bone matrix components and is a crucial factor for osteoblast differentiation. However, whatever the signaling pathways and noncollagenous matrix proteins involved, ROR α appears to play the role of a key modulator of BSP and DMP-1 during bone formation and mineralization.

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