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Regulation of Osteoblast Differentiation by Nurr1 in MC3T3-E1 Cell Line and Mouse Calvarial Osteoblasts

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Abstract The orphan nuclear receptor Nurr1 is primarily expressed in the central nervous system. It has been shown that Nurr1 is necessary for terminal differentiation of dopaminergic (DA) neurons in ventral midbrain. The receptor, however, is also expressed in other organs including bone, even though the role of Nurr1 is not yet understood. Therefore, we investigated the role of Nurr1 in osteoblast differentiation in MC3T3-E1 cells and calvarial osteoblasts derived from Nurr1 null newborn pups. Our results revealed that reduced Nurr1 expression, using Nurr1 siRNA in MC3T3-E1 cells, affected the expression of osteoblast differentiation marker genes, osteocalcin (OCN) and collagen type I alpha 1 (COL1A1), as measured by quantitative real-time PCR. The activity of alkaline phosphatase (ALP), another osteoblast differentiation marker gene, was also decreased in Nurr1 siRNA-treated MC3T3-E1 cells. In addition, Nurr1 overexpression increased OCN and COL1A1 expression. Furthermore, consistent with these results, during osteoblast differentiation, the expression of osteoblast marker genes was decreased in primary cultured mouse calvarial osteoblasts derived from Nurr1 null mice. Collectively, our results suggest that Nurr1 is important for osteoblast differentiation. J. Cell. Biochem. 99: 986–994, 2006.

Key words: Nurr1; osteoblast differentiation; osteocalcin; collagen type I alpha 1; ALP

Bone formation is essential for skeletal growth, remodeling, and repair. These processes involve the synthesis and deposition of mineralizing extracellular matrix by osteoblasts. The differentiation and proliferation of osteoblasts can be modulated by numerous transcriptional factors, cytokines, growth factors, and hormones [Ducy and Karsenty, 1998; Marie, 2003; Nakashima et al., 2003]. The coordinate action of these factors results in the expression of several genes that are characteristics of osteoblast differentiation and function, such as alkaline phosphatase (ALP), osteocalcin (OCN), COL1A1, osteopontin (OPN), and bone sialoprotein that are expressed sequentially during the process of osteogenesis [Stein et al., 1996; Marie, 2001].

The nuclear receptor, Nurr1, is an orphan member of the steroid-thyroid hormone receptor superfamily [Maruyama et al., 1998; Eells et al., 2000]. During development, Nurr1 is expressed at high levels in the ventral mesencephalon, where dopaminergic (DA) neurons are being generated [Xiao et al., 1996; Zetterstrom et al., 1996]. Targeted disruption of the Nurr1 gene in Nurr1 null mice arrests the DA neuron precursors in development and prevents expression of DA neuron specific proteins [Zetterstrom et al., 1997; Castillo et al., 1998; Castro et al., 2001]. These findings indicate that Nurr1 is involved in neuronal differentiation in developing nervous system. Most studies have focused on Nurr1 function in nervous system; however, Nurr1 is also, for example, expressed in hepatocytes, synoviocytes, and endothelial cells [Bandoh et al., 1997; Murphy et al., 2001; Ohkubo et al., 2002; Liu et al., 2003]. In addition, Nurr1 expression is induced by various stimuli such as inflammatory stimuli [Pei et al., 2005], corticotropin-releasing hormone [Kovalovsky et al., 2002], and reperfusion injury [Ohkubo et al., 2002] in various tissue. In bones,

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Nurr1 is expressed in osteoblasts and osteoblastic cell lines [Tetradis et al., 2001; Lammi et al., 2004], and is known to be induced by parathyroid hormone, both in in vitro and in vivo system [Tetradis et al., 2001; Pirih et al., 2005].

Nurr1 can bind as a monomer to a cis acting sequence AAAGGTCA, NBRE responsive element [Murphy et al., 1996]. The crystallographic study by Wang et al. [2003] showed that the ligand-binding domain of Nurr1 could not accommodate any ligand, in contrast to the other members of the superfamily, and, thus, Nurr1 is assumed to regulate gene expression in a ligand independent manner. The binding of Nurr1 is specific since certain mutations of this element affect promoter activity induced by Nurr1 [Murphy and Conneely, 1998]. In vitro, Nurr1 can also form a transcriptionally active heterodimer with the 9-cis retinoic acid receptor (RXR) [Wallen et al., 2003].

Recently, Nurr1 has been reported in bone remodeling when regulating OPN [Lammi et al., 2004]. However, little is known about the role of Nurr1 in osteoblasts. Moreover, sequence analysis of some genes important for osteoblastic differentiation shows potential NBRE in their 5′ distal promoter regions. Consistent with our analysis, Nurr1 has been reported to regulate *OPN* and *OCN* genes in osteoblasts, through its binding to NBRE in their promoters [Lammi et al., 2004; Pirih et al., 2004]. Therefore, we investigated the role of Nurr1 in osteoblast differentiation. First, we have shown that the expression of Nurr1 in MC3T3-E1 cells was reduced using Nurr1 siRNA, and then we measured the effect on the expression of osteoblast differentiation genes, such as OCN, COL1A1, and ALP. In addition, the effect of Nurr1 overexpression, using Nurr1 expression plasmid, on osteoblast differentiation was evaluated. Moreover, the expression of osteoblast differentiation marker genes in primary cultured mouse calvarial osteoblasts derived from Nurr1 null mice was compared with those from WT mice and has shown for the first time that Nurr1 plays an important role in osteoblast differentiation in vivo.

MATERIALS AND METHODS

MC3T3-E1 Cell Cultures

MC3T3-E1, a mouse osteoblastic cell line, was obtained from American Type Culture Collection (ATCC). Stock cultures were grown in α-MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Biosource) but in the absence of ascorbic acid to maintain the cells in an undifferentiated state. Differentiation was initiated by adding 50 μg/ml of ascorbic acid to the medium at desired time.

Primary Mouse Calvarial Cultures

Nurr1 KO mice were generated as previously described [Castillo et al., 1998]. The genotype of each pup was determined by PCR of tail DNA using specific probes for the Nurr1 gene and neomycin resistance gene [Castillo et al., 1998]. Since the Nurr1 KO mice die shortly after birth (P0), calvaria were isolated from each WT and KO P0 mice. Calvaria-derived osteoblasts were isolated by the sequential collagenase digestion method [Garcia et al., 2002]. In brief, calvaria were dissected away from loosely adherent connective tissues and minced under aseptic conditions. Sequential digestions were conducted in Dulbecco's phosphate buffered saline (DPBS), containing 1 mg/ml collagenase (Invitrogen) solution for 10 min at a time. Cells released at between 2 and 5 digestions were pooled and collected by centrifugation and cultured in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 1 week, the cells were used for ALP activity, and total RNA was isolated for quantitative real-time PCR.

Transfection of siRNA

Nurr1 siRNA was designed by a computer program provided by the Oligoengine company and a randomly scrambled sequence (scrambled siRNA) was used as a negative control. MC3T3-E1 cells were seeded the day before transfection at a concentration of 1×10^4 cells/cm^2 in an undifferentiated state. The next day, cells were transfected with Nurr1 siRNA or scrambled siRNA at a concentration of 100 nM using Oligofectamine (Invitrogen) according to manufacturer's protocol (day 0). As shown in Figure 1, differentiation was induced 1 day after transfection (day 1) by changing medium containing 50 mg/ml of ascorbic acid. On day 3, total RNA was isolated for quantitative real-time PCR analysis. Some of cultures were transfected again with 100 nM Nurr1 siRNA or scrambled siRNA, and maintained until day 8 in differentiation medium. On day 8, total RNA

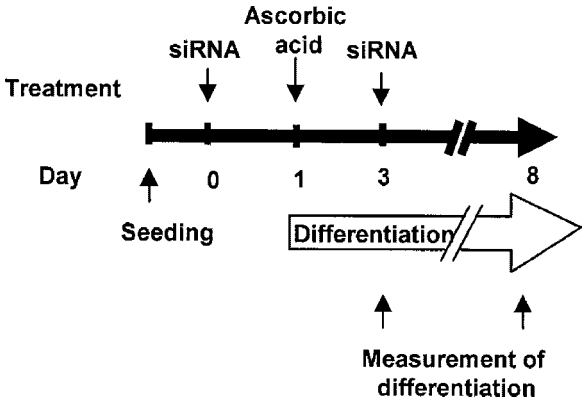

Fig. 1. Outline of treatment protocol: Nurr1 siRNA was transfected at days 0 and 3. Ascorbic acid was added at day 1 and present for the duration of the experiment. Analysis of osteoblast differentiation is depicted.

was isolated for quantitative real-time PCR analysis. ALP activity and ALP staining were also assayed on day 8.

Transfection of Nurr1

Nurr1 expression plasmid was prepared by cloning the coding region of Nurr1 into pcDNA3.1 expression vector (Invitrogen). MC3T3-E1 cells were seeded the day before transfection at a concentration of 1×10^4 cells/cm^2 in an undifferentiated state. Cells were transfected with Nurr1 or empty vector using FuGene transfection reagent (Roche) according to manufacturer's protocol. Differentiation was induced 1 day after transfection by changing medium containing 50 μg/ml of ascorbic acid and maintained in differentiation medium for further 7 days. After 7 days, total RNA was isolated for quantitative real-time PCR.

TaqMan Quantitative Real-Time PCR Analysis

Total RNA was extracted from MC3T3-E1 and primary calvaria cells using Trizol reagent (Invitrogen), and reverse transcribed to cDNA using oligo-dT and Superscript reverse transcriptase (Invitrogen) according to manufacturer's instructions.

TaqMan quantitative real-time PCR analysis was performed as we reported previously [Lee and Nikodem, 2004]. Optimal primers and the TaqMan probes for Nurr1, S16, OCN, and COL1A1 were designed using Primer Express v1.0 (Perkin-Elmer Applied Biosystems, Inc.). Primers and probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems. Forward and reverse primer sequences for Nurr1 (GeneBank NM013613) are 5′-AAC ATC GAC ATT TCT GCC TTC TC-3′ and 5′-TCT TGG GTT CCT TGA GCC C-3′, respectively. The TaqMan probe for Nurr1 was labeled with FAM fluorochrome with the following sequence: 5′-TGC CCT GGC TAT GGT CAC AGA GAG ACA-3′. S16 was used as an internal standard. Forward and reverse primer sequences for S16 (GeneBank NM013647) are 5′-GAT CGA GCC GCG CG-3′ and 5′-CAA ATC GCT CCT TGC CCA-3′, respectively. The TaqMan probe for S16 was labeled with VIC fluorochrome with the following sequence; 5′-CTG CAG TAC AAG TTA CTG GAG CCT GTT TTG CT-3′. The probe and primer sets Mm00443057_g1 (Nurr1) and Mm00835090_g1 (S16) (TaqMan® Gene Expression Assays; Applied Biosystems, Inc.) was used for the TaqMan quantitative real-time PCR analysis of the RNA from primary cultured calvarial cells.

For the Taqman analysis of OCN and COL1A1, a universal probe (UniPrimer™) was used. This probe recognized a specific sequence (Z-sequence), which had been incorporated into the primers. Forward and reverse primer sequences for OCN (GeneBank NM007541) are 5′-GTG AGC TTA ACC CTG CTT GTG A-3′ and 5′-ACT GAA CTT GAC CGT ACA TGC GTT TGT AGG CGG TCT TC-3′, respectively. Forward and reverse primer sequences for COL1A1 (GeneBank NM007742) are 5′-CCT GAG TCA GCA GAT TGA GAA CA-3′ and 5′-ACT GAA CTT GAC CGT ACA CCA GTA CTC TCC GCT CTT CCA-3′, respectively.

TaqMan PCR reactions were performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in a reaction volume of 30 μl containing 1× universal master mix, 100 nM of each of the forward and reverse primers, and fluorescent probes for Nurr1 and S16. The temperature cycling program was set at 2 min initial incubation at 50°C followed by 10 min at 95°C, and 40 cycles with 15 s at 95°C and 1 min at 60°C. Plasmids containing the cDNA were used as a template to generate a standard curve. The expression of each gene was normalized with S16 expression.

Assay of ALP Activity and ALP Staining

To assess ALP activities, cells were rinsed with phosphate-buffered saline (PBS) and lysed

in 0.01% sodium dodesyl sulfate (SDS). Following clarification by centrifugation, cell lysates were assayed for ALP activity using the Alkaline Phosphate Assay Kit (Sigma Diagnostics) according to manufacturer's instructions. A standard curve was prepared with *p*-nitrophenol (Sigma). Each value was normalized with the protein content of cell lysate, measured by BCA protein assay protocol (Pierce).

For ALP staining, cells were rinsed with PBS and fixed in 100% methanol for 1 h at room temperature. Cells were rinsed with PBS and stained with 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphoate (Sigma) plus 0.3 mg/ml nitroblue tetrazolium chloride (Sigma) in 0.1 M Tris-HCl, 0.01 N NaOH, 0.05 M $MgCl_2$ for 2 h in the dark [Sowa et al., 2002].

Animal Guidelines

All procedures were performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals in research, and approved by the Animal Care Committee of the National Institute for Diabetes and Digestive and Kidney Diseases.

Statistics

All data are presented as the mean ± SEM. Data were evaluated for statistical significance using analysis of variance (ANOVA) and a $P < 0.05$ was considered significant. Each experiment was repeated three times with similar results.

RESULTS

To investigate the role of Nurr1 in osteoblast differentiation, we employed the MC3T3-E1 cell line that is derived from mouse calvaria and primary cultures that we generated from calvaria of Nurr1 null newborn mice and WT. The MC3T3-E1 osteoblast cell line is undifferentiated in the absence of ascorbic acid, but differentiation can be easily induced by adding the ascorbic acid into the medium [Sudo et al., 1983]. These cells express sequentially osteoblast marker proteins when cultured with ascorbic acid and, constitutively, Nurr1 [Franceschi and Lyer, 1992; Wang et al., 1999; Mizutani et al., 2001; Lammi et al., 2004]. Thus, this cell line is appropriate for investigating the role of Nurr1 in osteoblast differentiation.

Figure 1 outlines the treatment of MC3T3-E1 cells, using ascorbic acid and Nurr1 siRNA to induce cell differentiation and inhibition of Nurr1 expression, respectively. First, we evaluated the efficiency of Nurr1 siRNA in the culture. Since the stability of siRNA is 4–5 days, we transfected the cells at days 0 and 3 for long-term culture (Fig. 1). Figure 2 showed that the addition of Nurr1 siRNA decreased Nurr1 expression by 60%. The scrambled siRNA, a negative control, however, had no effect. This demonstrated the specificity of the Nurr1 siRNA sequence. Moreover, the level of expression of GAPDH, the housekeeping gene, was not changed in either Nurr1 siRNA or the scrambled siRNA transfected cells, indicating the specificity and the lack of a toxic effect.

Next, we assessed the role of Nurr1 in osteoblast differentiation after Nurr1 siRNA treatment (Fig. 3). The MC3T3-E1 cells were maintained in an undifferentiated condition and differentiation was induced by adding ascorbic acid to the medium at day 1 after the first siRNA transfection (day 0) with the second addition of siRNA Nurr1 at day 3 (Fig. 1). The culture was maintained for an additional 7 days to induce differentiation. Osteoblast differentiation was assessed by measuring the mRNA levels of OCN and COL1A1 since these genes are expressed in high levels in differentiated osteoblasts [Wang et al., 1999]. The levels of OCN and COL1A1 expression were measured at days 3 and 8 after siRNA transfection, corresponding to days 2 and 7 after differentiation was induced. The expression of these genes was

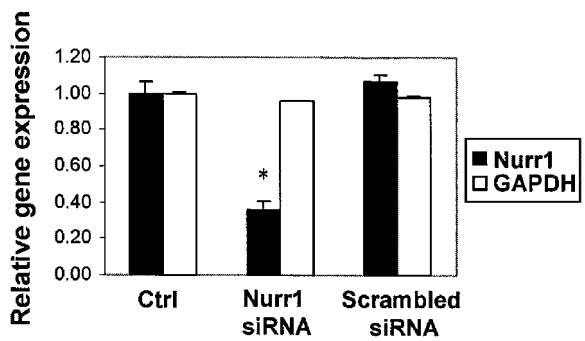

Fig. 2. Effect of Nurr1 siRNA on Nurr1 and GAPDH expression in MC3T3-E1 osteoblast cells. MC3T3-E1 osteoblast cells were transfected with 100 nM Nurr1 siRNA or scrambled siRNA as shown in Figure 1. Nurr1 and GAPDH expression was measured by quantitative real-time PCR analysis at day 8. Data obtained for Nurr1, GAPDH expression, were normalized to S16 expression and are presented relative to untreated controls (Ctrl). * denotes significant difference ($P < 0.05$) from the untreated control.

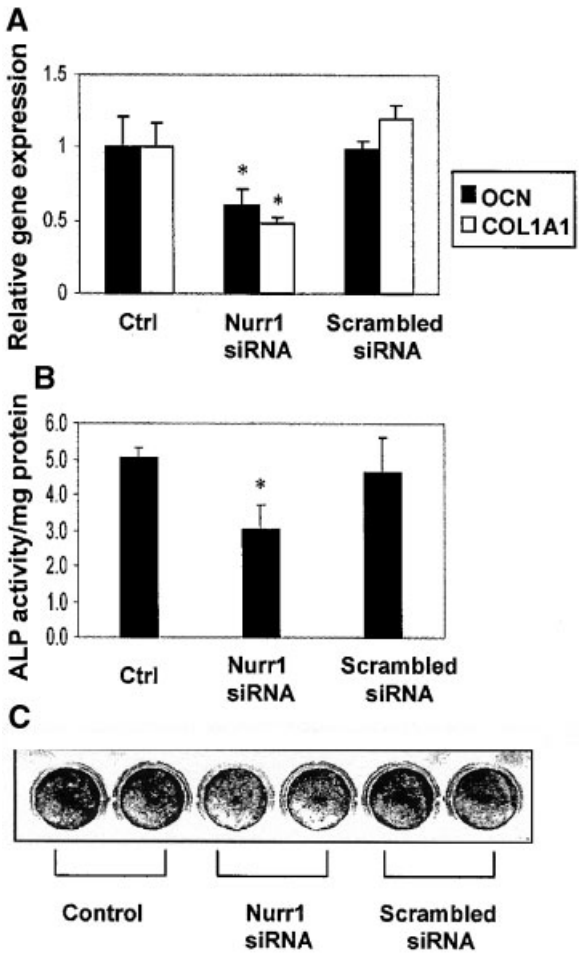

Fig. 3. Effect of reduced Nurr1 mRNA level on osteoblast differentiation marker genes expression, OCN, COL1A1, and ALP in MC3T3-E1 osteoblast cells. MC3T3-E1 osteoblast cells were transfected with 100 nM Nurr1 siRNA or scrambled siRNA, and differentiation was induced as described in Materials and Methods and shown in Figure 1. At day 8, total RNA was isolated and mRNA expression of OCN, COL1A1, and S16 was measured by quantitative real-time PCR analysis (**A**). Data obtained for OCN and COL1A1 expression were normalized to S16 expression and are shown relative to untreated controls. * denotes significant difference ($P < 0.05$) from the untreated control. ALP activity (**B**) and ALP staining (**C**) were analyzed as described in Materials and Methods.

very low at day 3 due to the low degree of differentiation (data not shown). At day 8, however, the expression in the control cultures was greatly increased. In the control culture, the expression levels of OCN and COL1A1 were 30- and ninefold higher, respectively, when compared to levels at day 3 (data not shown), indicating that differentiation was well induced at this time. The transfection of the cells with Nurr1 siRNA significantly decreased the level of Nurr1 mRNA (Fig. 2). Consequently, the expression of OCN and COL1A1 at day 8 was decreased 60% and 50%, respectively, compared to control (Fig. 3A). To confirm further that decreased Nurr1 expression inhibits osteoblast differentiation, ALP, another osteoblast differentiation marker enzyme, was examined. ALP activity and ALP staining were measured at day 8 after differentiation was induced. The ALP activity was decreased in Nurr1 siRNA-transfected cells, whereas it was not affected in scrambled siRNA-transfected cells (Fig. 3B). ALP staining also confirmed that transfection of Nurr1 siRNA, but not scrambled siRNA, decreased the number of ALP-positive nodules formed (Fig. 3C). This data further suggests that osteoblast differentiation is affected by decreased Nurr1 in MC3T3-E1 cells.

Next, we examined whether overexpression of Nurr1 affects osteoblast differentiation. MC3T3-E1 cells were transiently transfected with Nurr1 expression plasmid, and subsequently analyzed its effect on OCN and COL1A1 mRNA expression by real-time TaqMan analysis. As shown in Figure 4, Nurr1 overexpression increased OCN and COL1A1 expression about twofold, compared to empty-vector treated control.

Taken together, these results suggest that Nurr1 is involved in osteoblast differentiation in MC3T3-E1 cells.

We have previously shown that Nurr1 null newborns die shortly after birth, and DA

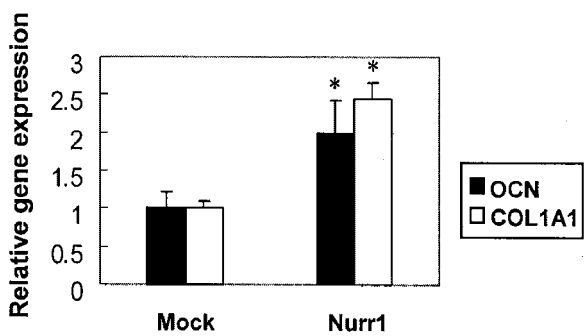

Fig. 4. Effect of overexpression of Nurr1 on osteoblast differentiation marker genes expression, OCN and COL1A1 in MC3T3-E1 osteoblast cells. MC3T3-E1 osteoblast cells were transfected with Nurr1-expression (Nurr1) or empty vector (Mock), and differentiation was induced as described in Materials and Methods. At day 8, total RNA was isolated and mRNA expression of OCN, COL1A1, and S16 was measured by quantitative real-time PCR analysis. Data obtained for OCN and COL1A1 expression were normalized to S16 expression and are shown relative to untreated controls. * denotes significant difference ($P < 0.05$) from the untreated control.

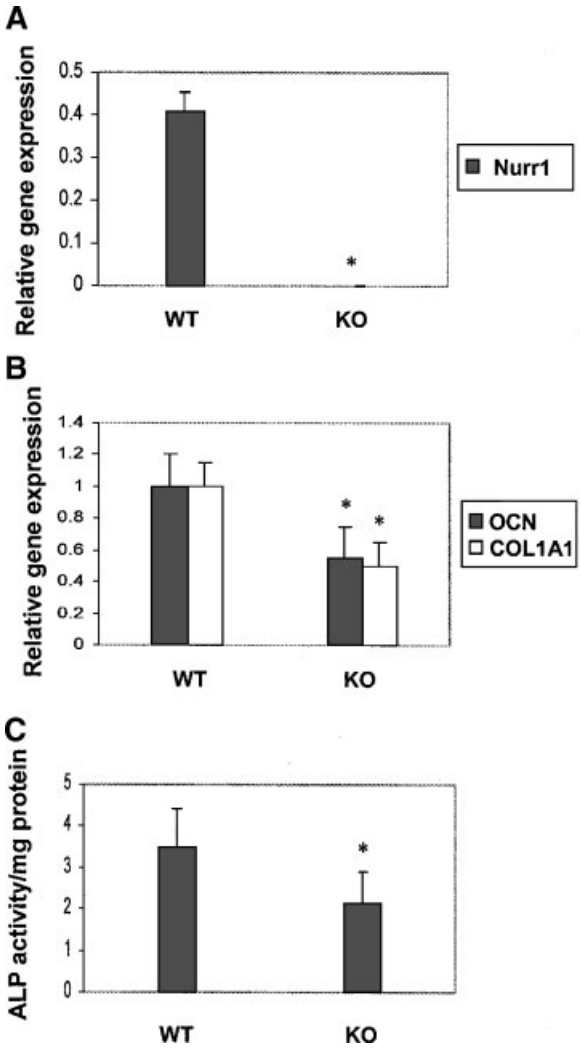

Fig. 5. Expression of osteoblast differentiation marker genes in primary mouse calvarial osteoblasts derived from WT and Nurr1 null newborns. Osteoblasts were isolated from calvaria of WT and Nurr1 null newborn pups, and cultured in differentiation medium. After 8 days, total RNA was isolated and the expression of nurr1 (**A**), OCN and COL1A1 (**B**) was measured by quantitative real-time PCR analysis. Data obtained for nurr1, OCN, and COL1A1 expression were normalized to S16 expression and are presented relative to WT controls. ALP activity (**C**) was measured in cell lysate as described in Materials and Methods. * denotes significant difference ($P < 0.05$).

neurons precursors did not terminally differentiate [Castillo et al., 1998]. To investigate the role of Nurr1 in osteoblast differentiation in vivo, we prepared primary cultures from calvaria of WT and Nurr1 null newborn pups. The cultures were maintained in differentiation media and grown for 8 days to induce differentiation. The expression of Nurr1, OCN, and COL1A1 in both WT and Nurr1 null mouse-derived primary cultured calvarial cells was measured by real-time TaqMan analysis. As expected, primary cultured calvarial cells derived from WT mouse express Nurr1, whereas Nurr1 was undetectable in primary cultured calvarial cells derived from Nurr1 null mouse (Fig. 5A). In addition, consistent with the Nurr1 siRNA experiment, the expression of marker genes, OCN and COL1A1 was significantly reduced in primary cultures from Nurr1 null mice compared to cultures from WT mice, 45% and 50% respectively (OCN and COL1A1 expression levels normalized to S16 expression were 0.26 ± 0.05 and 1.5 ± 0.22 for WT and 0.14 ± 0.04, and 0.74 ± 0.22 for KO, respectively) (Fig. 5B). The ALP activity was also decreased in primary cultured mouse calvarial cells derived from Nurr1 null mice compared to those from WT mice (Fig. 5C). These results suggested the role of Nurr1 in osteoblast differentiation.

Taken together, our present study, as assessed by the treatment of the MC3T3-E1 osteoblast cell line with Nurr1 siRNA, Nurr1 overexpression, and primary calvaria cultures from Nurr1 null mice, showed that Nurr1 transcription factor plays a role in osteoblast differentiation.

DISCUSSION

Osteoblasts differentiation is controlled by multiple transcription factors at various stages [Yang and Karsenty, 2002]. Cbfa1 and Osx have been recently identified as zinc-finger containing proteins, the osteoblast-specific transcription factors acting as developmental regulators of cell differentiation [Ducy et al., 1997; Nakashima et al., 2002]. Loss of either one of them leads to severe impairment of bone formation. Other transcriptional factors such as Dlx5, Msx2, Fos, and Twist are not bone-cell specific but are predominantly expressed in the skeleton during development. Even though these transcriptional factors have a function to control skeletal cell proliferation and differentiation, and regulate osteoblast gene expression [Karenty and Wagner, 2002], however, a single loss one of these factors does not always impair bone formation in vivo. Several other genes such as caspases, stanniocalcin 1, and β-catenin are also reported to be involved in osteoblast differentiation [Bain et al., 2003; Mogi and Togari, 2003; Yoshiko et al., 2003].

Herein, we suggest the role of Nurr1, another zinc-finger transcription factor in osteoblast

differentiation, using the MC3T3-E1 osteoblast cell line and primary cultured mouse calvarial osteoblasts derived from WT and Nurr1 null mice. Our data shows that in the MC3T3-E1 cell line, decreased expression of Nurr1 by the treatment with Nurr1 siRNA leads to reduced expression of osteoblast differentiation marker genes such as OCN, COL1A1, and ALP. Similarly, primary cultured mouse calvarial osteoblasts derived from Nurr1 null mice revealed lower levels of the expression of these differentiation marker genes compared to those derived from WT. COL1A1 and ALP are molecular markers of early-stage differentiation, whereas, late-stage differentiation is marked by expression of OCN. Therefore, our results indicate that reduced expression of Nurr1 might affect both early- and late-stage differentiation.

Nurr1 can directly regulate gene expression by binding to NBRE in a promoter of target genes and can also form a transcriptionally active heterodimer with RXR [Murphy et al., 1996]. Several genes including tyrosine hydroxylase and dopamine transporter have been reported to be regulated by Nurr1 [Murphy et al., 1996; Murphy and Conneely, 1998; Kim et al., 2003]. Recently, Lammi et al. [2004] showed that Nurr1 regulates the OPN gene in osteoblasts, by direct binding of Nurr1 to the promoter.

We performed sequence analysis of employing Genomatrix Suite (Mat Inspector, Quandt 1995) and identified potential Nurr1 binding sites within 5 kb of the 5′ regions of *OCN*, *COL1A1*, and *ALP* genes. *OCN* and *ALP* genes at positions 4246/4228 and 4241/4223, respectively, contain consensus NBRE sequences shown to bind Nurr1 and mediate transactivation [Murphy et al., 1996; Murphy and Conneely, 1998]. In addition, we have also identified several putative Nurr1 binding sites in these genes. The COL1A1 gene also harbors several potential Nurr1 binding sites within 5 kb of the promoter. Thus, it is conceivable that in vivo Nurr1 might regulate expression of these genes at the level of transcription. However, considering distal locations of NBRE in osteoblast genes whose expression is affected by Nurr1, the possibility exists that during osteoblast differentiation, the effect of Nurr1 might be secondary. In this case the Nurr1 would regulate transcription, of a not yet identified master gene of osteoblast differentiation that, in turn, would regulate expression of osteoblast genes. Nevertheless, Nurr1 plays a role in osteoblast differentiation as we have shown using siNurr1 RNA in MC3T3-E1 cells and primary cultures of calvarial cells from Nurr1 null mice.

Interestingly, Sato et al. [2001] reported high incidence of hip fractures in Parkinson's disease patients. Nurr1 is essential for differentiation of DA neurons and mutations in Nurr1 can cause DA dysfunction, associated with Parkinson's disease [Le et al., 2003]. Our present results support the notion that Nurr1 might be involved in bone fracture in Parkinson's patients. Therefore, we hypothesize that Nurr1 might affect the osteoblast differentiation in the adult system.

Collectively, our present study, as assessed by the experiment using Nurr1 siRNA, overexpression of Nurr1, and primary calvarial cultures from Nurr1 null mice, demonstrated that in vitro Nurr1 is involved in osteoblast differentiation. Further studies are required to address the regulation mechanism(s) of Nurr1 in osteoblast differentiation in vivo. Nurr1 conditional knockout should help to clarify this question.

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