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Osteogenic differentiation of C2C12 myogenic progenitor cells requires the Fos-related antigen *Fra-1* – A novel target of Runx2

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ABSTRACT

Bone formation is a developmental process requiring the differentiation of mesenchymal stem cells into osteoblasts. It is established that Runx2 tightly regulates osteoblast differentiation and bone formation. Fos-related antigen *Fra-1* is an essential factor for bone formation. Current evidence does not support a relationship between *Fra-1* and Runx2 in osteogenesis. Here, we explored the possibility that Runx2 regulates *Fra-1* expression during osteogenic differentiation of C2C12 myogenic progenitor cells. Expression of *Fra-1* was induced rapidly after activation of Runx2 in a Tet-on stable C2C12 cell-line (C2C12/Runx2^{Dox} sub-line). Transient transfection assay showed that Runx2 activates *Fra-1* promoter-reporter activity, suggesting that *Fra-1* may be a direct target of Runx2. To determine the minimal region of the *Fra-1* promoter that was activated by Runx2, a series of *Fra-1* promoter deletion constructs were made. By transient transfection assay, we defined the minimal region to the proximal 342 bp (−84 to +258). Two potential Runx2-binding sites (at positions +139 and +208) were predicted within this region. Mutation of the Runx2 motif at position +208 significantly decreased *Fra-1* promoter activity compared to wild type, whereas mutation of Runx2 at position +139 had no effect. Electrophoretic mobility shift assay (EMSA) demonstrated the existence of one atypical Runx2-binding element at position +208, and chromatin immunoprecipitation (ChIP) assay revealed that Runx2 bound to the native *Fra-1* promoter *in vivo* via this site. Finally, forced expression of *Fra-1* resulted in upregulation of alkaline phosphatase (ALP), a marker of early osteoblast differentiation. Together, these results indicate that *Fra-1* is a direct target of Runx2 during osteogenic differentiation of C2C12 myogenic progenitor cells.

1. Introduction

Runt-related transcription factor 2 (Runx2/Cbfa1/AML-3/PEBP2aA), a member of the runt-domain gene family [1], is expressed in mesenchymal condensations during early embryonic development in mouse [2]. Runx2 is also expressed in prehypertrophic chondrocytes and terminally differentiated osteoblasts [3,4]. Extensive analyses have revealed that during both embryogenesis and postnatal life, Runx2 is essential for osteoblast and hypertrophic chondrocyte differentiation, and bone formation [3,5,6]. The identification of Runx2 target genes can provide insights into the mechanisms by which Runx2 controls osteogenesis. However, the Runx2-targeted genes that have been identified thus far are limited.

The dimeric transcription factor activator protein-1 (AP-1) is typically composed of members of the Fos (c-Fos, FosB, *Fra-1* and Fra-2) and Jun family of proteins (c-Jun, JunB and JunD) [7]. All four members of the Fos family, and in particular *Fra-1*, may play important roles in bone development. Mice lacking c-Fos exhibit osteoporosis [8], and this osteoporosis can be rescued when overexpression of *Fra-1*[9], which is a downstream target of c-Fos present in osteoclast progenitors [10]. *Fra-1* transgenic mice develop progressive osteosclerosis [11], and also exhibit severe lipodystrophy [12], whereas embryo-specific *Fra-1* knockout mice show an osteopenic phenotype [13]. Importantly, transgenic mice overexpressing ΔFosB (a splice variant of FosB) or Fra-2, display a severe osteosclerotic phenotype that is observed in *Fra-1* transgenic mice [14,15]. These observations thus suggest that *Fra-1* plays an essential role in osteogenesis. However, detailed mechanisms responsible for regulating *Fra-1* expression during osteogenesis have not been completely delineated.

Abbreviations: Runx2, runt-related transcription factor 2; *Fra-1*, Fos-related antigen 1; AP-1, activator protein-1; ALP, alkaline phosphatase; OC, osteocalcin; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; Dox, doxycycline.

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In this study, we have investigated the relationship between *Fra-1* and Runx2 during osteogenic differentiation of C2C12 myogenic progenitor cells. Our results revealed that *Fra-1* is a Runx2-responsive gene during this process of differentiation, and that is also essential for Runx2-induced early osteoblast differentiation of C2C12 cells.

2. Materials and methods

2.1. Primers and probes

All primers and probes are available in Supplementary materials.

2.2. Construction of C2C12-Flag-Runx2 Tet-on cell line

The cDNA encoding mouse Runx2 (MASNSL isoform, type 2) was obtained by RT-PCR, and the Flag epitope was inserted during the PCR amplification. The Flag-Runx2 cDNA was then cloned into the EcoR V/Xba I-digested pTRE-Tight Vector (Clontech, Mountain View, CA, USA), and the resulting plasmid (pTRE-Tight–Flag-Runx2) was used as the response plasmid for construction of C2C12-Flag-Runx2 Tet-on cell line (C2C12/Runx2^{Dox} sub-line). The detailed experimental procedures can be obtained from the Tet-On Advanced Inducible Gene Expression System User Manual (Clontech; PR973293).

2.3. Cell culture

C2C12/Runx2^{Dox} cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Tet System Approved FBS (Clontech). Hygromycin B and G418 were purchased from Invitrogen (Carlsbad, CA, USA) and added into the medium at the concentration of 500 μg/ml and 800 μg/ml, respectively. Doxycycline (Dox) (Clontech) was used at 10 μg/ml and an equal volume of distilled water was used as the negative control (−Dox). To examine the effect of *Fra-1* on Runx2-induced osteogenic differentiation, C2C12/Runx2^{Dox} cells were transfected with *Fra-1* expression vector (pcDNA3.1-*Fra-1*) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. Reverse transcription PCR (RT-PCR)

Total RNA was extracted by Trizol reagent (Invitrogen). Reaction mixture (20 μl) containing 1 μg of total RNA which was reverse transcribed to cDNA in the usage of RT Ace reverse transcriptase (Toyobo, Osaka, Japan). Real-time quantitative PCR (Real-time qPCR) was performed on the cDNA using primers specific for *Runx2*, *Fra-1*, alkaline phosphatase (*Alp*), osteocalcin (*OC*), and 18S rRNA. PCR was carried out using the Real Time PCR Detection System Rotor-Gene 6000 (Corbett Research, Mortlake, NSW, Australia). Relative expression levels were calculated as ratios normalized against those of 18S rRNA, which was described in details previously [16].

2.5. In vitro osteoblastogenesis and cell staining

C2C12/Runx2^{Dox} cells were cultured in DMEM containing 10 μg/ml Dox, 10 mM disodium β-glycerophosphate and 50 μg/ml ascorbic acid. The medium was changed every 3 days. For ALP and Alizarin red staining, the detailed procedures were described previously [17].

2.6. Construction of reporter plasmids and luciferase assay

C2C12 genomic DNA was used as the template for PCR to generate the pGL3/01 (−942/+258) construct, which was used as the template for the other constructs. These PCR products were cloned into the Kpn I/Hind III sites of the pGL3-Basic vector (Promega, Madison, WI, USA), yielding the promoter reporter plasmids. The *Fra-1* promoter mutation constructs (pGL3/08M1, and pGL3/08M2) were generated by the PCR-based site-directed mutagenesis kit (Takara) using pGL3/08 plasmid as template. *Osteocalcin* (*OC*) promoter fragment (−392/+47) containing a bonafide Runx2-binding site was also inserted into the Kpn I/Hind III sites of the pGL3-Basic vector, yielding the positive reporter pGL3-OC. Promoter reporter plasmids DNA were transfected into cells using the Lipofectamine 2000 (Invitrogen). pTRL-TK (Promega) was co-transfected as an internal control to evaluate transfection efficiency. After 48 h of transfection, cells were harvested and analyzed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from C2C12/Runx2^{Dox} cells treated with Dox (10 μg/ml) according the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). EMSA was performed using the EMSA kit (Promega). For supershift assays, an antibody against Flag-Runx2 (anti-Flag antibody, Sigma) or normal mouse IgG (Sigma) was added to the reaction mixture and incubated 25 min before the addition of the labeled probe. All DNA–protein complexes were resolved by electrophoresis on 5% native polyacrylamide gels and transmembrane to Immobilon-Ny+ (Millipore, Billerica, MA, USA).

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed according to the manufacturer's instructions (Active Motif). The protein–DNA complexes were incubated with 5 μg of the anti-Flag antibody (Sigma) or normal mouse IgG (Sigma) and rotated at 4 °C for 12 h, and then were incubated with protein G beads. The complexes were eluted with buffer containing 1% SDS and 0.1 M $NaHCO_3$, and crosslinks were reversed at 65 °C. DNA was recovered by phenol–chloroform extraction and ethanol precipitation and then subjected to PCR analysis. Amplified products were electrophoresed through 2% agarose gel and visualized by Goldview staining.

2.9. Statistical analysis

Statistical significance was evaluated by Student's *t* test. A p value of less than 0.05 was considered statistically significant. All the data were expressed as mean ± SD ($n = 3$).

3. Results

3.1. Fra-1 expression was up-regulated during osteogenic differentiation of C2C12 cells

Identification of Runx2 target genes can provide novel insights into the mechanisms whereby Runx2 functions in osteogenesis. C2C12 cells are capable of transdifferentiating into osteoblasts [18]. Thus, we engineered a C2C12/Runx2^{Dox} sub-line, which conditionally expresses Runx2 in response to Dox. This sub-line was then used to identify Runx2-regulated genes during osteogenic differentiation of C2C12 cells. We found that Runx2 overexpression enhances ALP activity and matrix mineralization in C2C12 cells

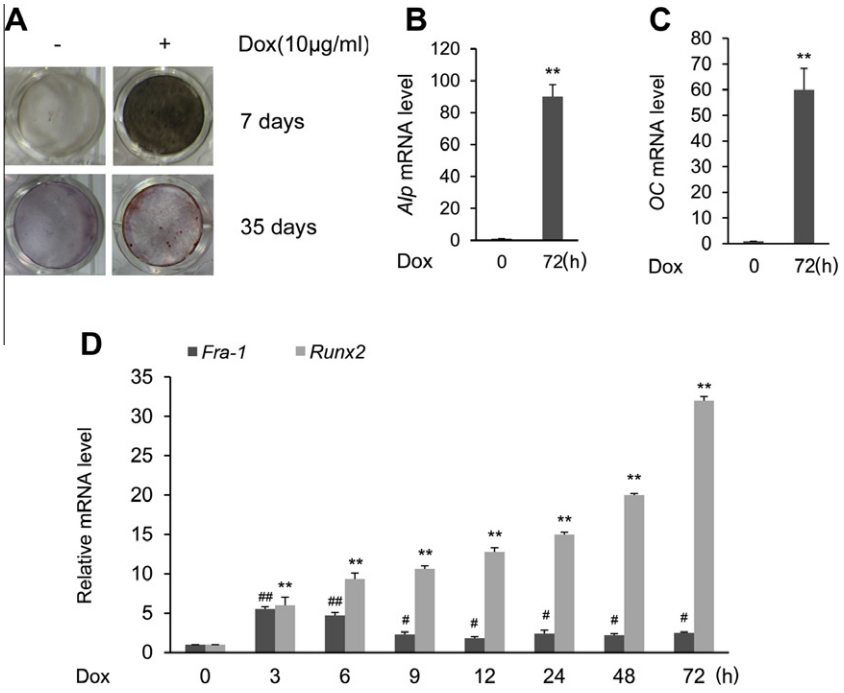


Fig. 1. Effect of Runx2 on *Fra-1* expression. (A) Induction of osteoblast differentiation by Runx2 in C2C12 cells. C2C12/Runx2^{Dox} cells were treated with Dox (10 μg/ml) for the times indicated. ALP activity and matrix mineralizing activity were measured by ALP (upper panel) and Alizarin red staining (lower panel), respectively. (B and C) C2C12/Runx2^{Dox} cells were treated with Dox (10 μg/ml) for 72 h. The mRNA level of *Alp* (B) and OC (C) was determined by real-time qPCR and normalized to 18S rRNA. (D) C2C12/Runx2^{Dox} cells were treated with Dox (10 μg/ml) for the times indicated (0–72 h). The mRNA level of *Fra-1* was examined by real-time qPCR and normalized to 18S rRNA. All data are presented as the mean ± S.D. from three independent experiments. $^{**}p < 0.01$ compared with untreated cells (0 h); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with untreated cells (0 h).

(Fig. 1A), which indicated the induction of osteoblast differentiation by Runx2 in C2C12 cells. Additionally, upregulation of osteoblast marker genes by Runx2, such as *Alp* and *OC*, was confirmed by real-time qPCR analysis (Fig. 1B and C).

Fra-1 is an essential factor for osteoblastdifferentiation and bone formation [11–13]. However, to date, evidence does not support a relationship between *Fra-1* and Runx2 in osteogenesis. Thus, we examined the expression of *Fra-1* by real-time qPCR in Dox-treated C2C12/Runx2^{Dox} cells at different times (0–72 h). Our results revealed that *Fra-1* expression was rapidly induced following activation of Runx2 in the early induction phase (3 h and 6 h, Fig. 1D), and declined to moderate levels in the late induction phase (9–72 h, Fig. 1D). Additionally, Runx2 mRNA expression increased gradually during treatment with Dox (Fig. 1D). Collectively, these observations indicate that the *Fra-1* gene was activated rapidly in response to Runx2 expression during this process of differentiation.

3.2. The Fra-1 promoter was responsive to Runx2

The observation that the *Fra-1* gene could be activated rapidly in response to Runx2 expression led us to conclude that *Fra-1* may be a direct target of Runx2. To confirm this conclusion, we generated a luciferase reporter construct driven by 1.2 kb *Fra-1* promoter (pGL3/01, −942/+258). As a positive control, an *osteocalcin* (*OC*) promoter fragment (−392/+47) containing a bona fide Runx2-binding site was cloned into the pGL3-Basic vector (pGL3-OC). After transient transfection of the reporter constructs into C2C12/Runx2^{Dox} cells in the presence of Dox (10 μg/ml), luciferase activities were determined. An approximate 65- to 11-fold increase in luciferase activity was observed in pGL3-OC- and pGL3/01-transfected cells, respectively, as compared with pGL3-Basic-transfected sample (Fig. 2A). These data revealed that the increased luciferase activity was dependent on the induction of Runx2 expression after Dox treatment, suggesting that Runx2 transcriptionally activated *Fra-1*.

3.3. Identification the Runx2-binding site in the promoter of the Fra-1 gene

We showed that Runx2 activated *Fra-1* promoter activity. However, it was unclear which region within the *Fra-1* promoter was responsible for Runx2 activation. To explore this question, deletion analysis and transient transfection assays were carried out to determine the responsible region within the 1.2 kb *Fra-1* promoter required for Runx2 activation. A series of *Fra-1* 5′ promoter deletion constructs with a fixed 3′ end at the +258 position (relative to the transcription start site) were generated. Although the repressive activity was found between nucleotides −83 and −189, all of the deletion constructs were significantly activated by Runx2 (Fig. 2B).The Runx2 activation in the pGL3/08 reporter construct was dramatically increased by 60-fold as compared to control (Fig. 2B). This observation suggested that the most proximal 342 nucleotide of the *Fra-1* gene was required for Runx2 activation. Thus, we examined the −84 to +258 region of the *Fra-1* promoter to search for the putative Runx2-binding sites using the TFSEARCH database [19] and the MatInspector program [20]. We identified two potential Runx2-binding sites at +139 to +147 (Runx2-I) and +208 to +216 (Runx2-II), respectively (Fig. 2C). Additionally, two partially overlapping binding sites for the TATA-box binding protein (TBP) were identified (Fig. 2C). To test which binding site was responsible for activation of the *Fra-1* promoter by Runx2, we generated two mutants of pGL3/08 promoter reporter named pGL3/08M1(Mut-I) and pGL3/08M2(Mut-I/II), in which the core sequence required for binding by Runx2 was replaced with a nonsense sequence. Transfection of the pGL3/08M2 mutant

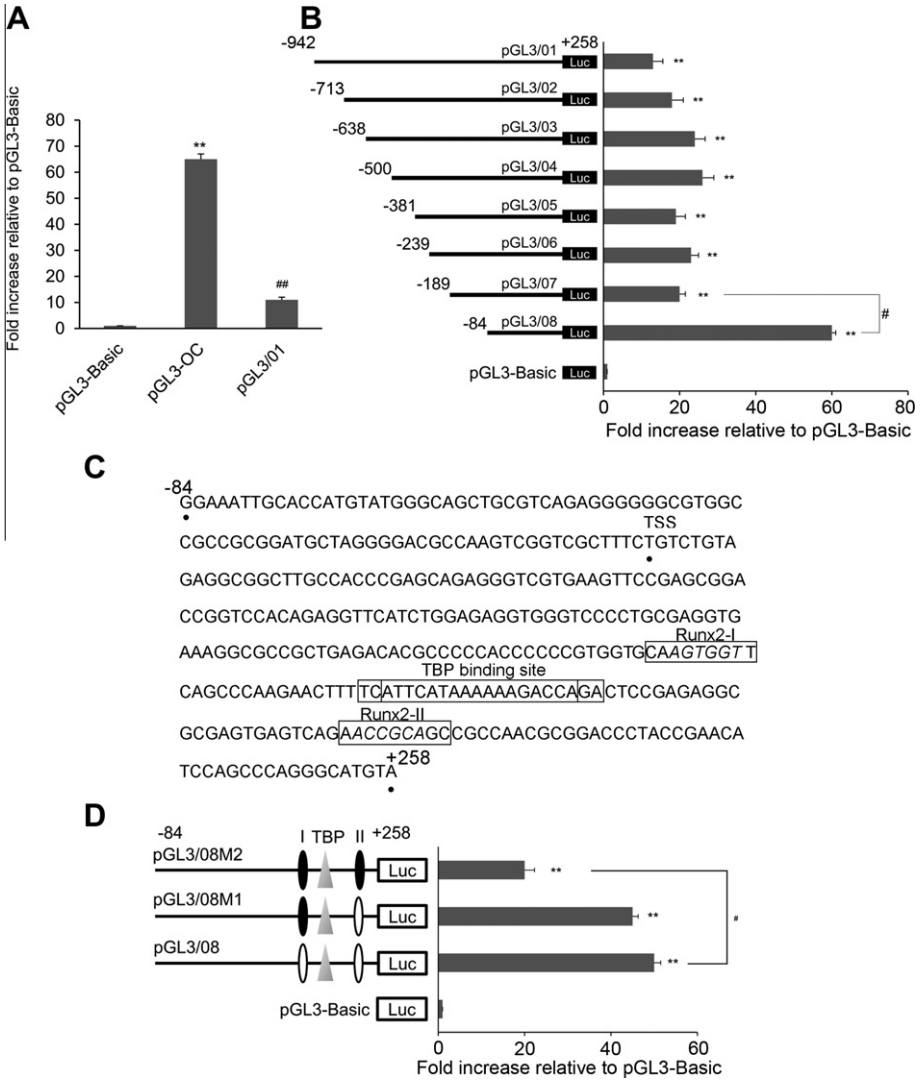


Fig. 2. Runx2-binding site is located within 342 bp of the *Fra-1* promoter. (A) Runx2 activated the *Fra-1* promoter. A 1.2 kb *Fra-1* promoter luciferase reporter (pGL3/01), and a 439 bp OC promoter luciferase reporter (pGL3-OC), were co-transfected with the Renilla luciferase expression vector into C2C12/Runx2^{Dox} cells. $^{**}p < 0.01$, $^{\#\#}p < 0.01$ compared with pGL3-Basic-transfected cells. (B) Deletion analysis of *Fra-1* promoter reporter activated by Runx2. Various constructs of the 5′-flanking region fused to the firefly luciferase reporter vector were co-transfected with the Renilla luciferase expression vector into C2C12/Runx2^{Dox} cells. $^{**}p < 0.01$ compared with pGL3-Basic-transfected cells, $^{\#}p < 0.05$ compared with pGL3/08-transfected cells. (C) Location of the putative Runx2- and TBP-binding sites within the 342 bp region of the *Fra-1* promoter. (D) The Runx2-binding site located at +208 (II) was responsible for activation of the *Fra-1* promoter reporter by Runx2. Both the pGL3/08M1 and pGL3/08M2 mutants were co-transfected with the Renilla luciferase expression vector into C2C12/Runx2^{Dox} cells. $^{**}p < 0.01$ compared with pGL3-Basic-transfected cells,$^{\#}p < 0.05$ compared with pGL3/08-transfected cells. The two putative Runx2-binding sites are indicated by open ovals; the mutated Runx2 sites are shown as black ovals. The filled triangle indicates the putative TBP-binding site. The transfected cells were treated with Dox (10 μg/ml) for 48 h, and luciferase activities were measured. Firefly luciferase activity was normalized to Renilla luciferase activity, and relative luciferase activities were presented as-fold increase over the pGL3-Basic vector. All data are the product of the mean ± S.D. from three independent experiments. Key: I, Runx2-I; II, Runx2-II.

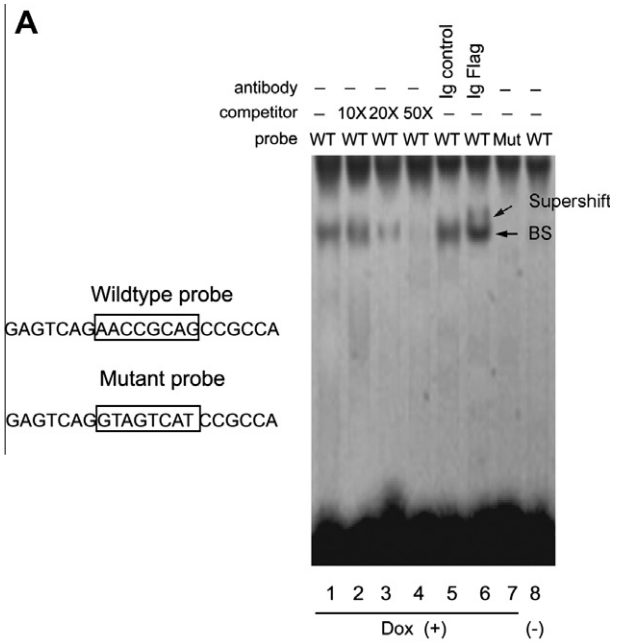
resulted in significantly reduced reporter activation by Runx2 (Fig. 2D). By contrast, the pGL3/08M1 mutant reporter was activated by Runx2 at a similar level than was seen in the wild-type pGL3/08 reporter. Together, these observations suggested that the predicted Runx2-II site within the *Fra-1* promoter region, from nucleotides −84 to +258, was responsible for activation of the *Fra-1* promoter by Runx2.

3.4. Runx2 directly binds to Runx element within Fra-1 promoter

Having demonstrated that the predicted Runx2-II site between the −84 and +258 regions of the *Fra-1* promoter was critical for *Fra-1* promoter activation by Runx2, we next determined whether Runx2 could physically bind to this site directly. With an EMSA, a labeled probe containing Runx2-II produced a significant shift (DNA–protein complex) in the nuclear extract of Dox-treated C2C12/Runx2^{Dox} cells (Fig. 3A, BS in lane 1), which was not seen in the nuclear extract of C2C12/Runx2^{Dox} cells (Fig. 3A, lane 8). This shift was abolished by 10- to 50-fold molar excess of unlabeled probe (competitor) (Fig. 3A, lanes 2–4). The labeled probe containing a 8 bp mutation also abolished this shift (Fig. 3A, lane 7). These results suggested that the specific binding by a nuclear factor to Runx2-II. Furthermore, the anti-Flag antibody but not the mouse IgG antibody supershifted the complex (BS), which indicated that the bound protein in the complex was Runx2 (Fig. 3A, lanes 5 and 6). Thus, these data strongly suggest that Runx2 specifically bound to the Runx2 motif within the *Fra-1* promoter.

3.5. Runx2 associates with the native Fra-1 promoter in vivo

We have demonstrated that Runx2 specifically binds to the *Fra-1* promoter *in vitro*. However, it remains unknown whether Runx2



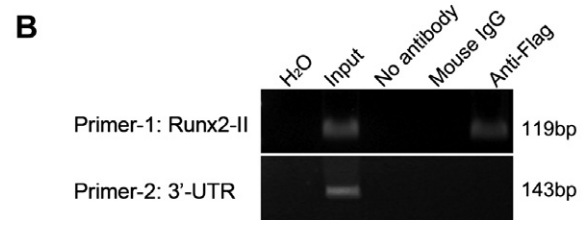


Fig. 3. Runx2 associates with the *Fra-1* promoter. (A) EMSA shows the interaction between the *Fra-1* site (Runx2-II) and Runx2 in Dox-treated C2C12/Runx2^{Dox} cells. The bottom arrow indicates the DNA–protein complex (BS), and the top arrow indicates the Runx2-supershifted complex. Key: WT, wild-type; Mut, mutant. (B) ChIP analysis was performed to confirm the interaction of Runx2 with the *Fra-1* promoter in Dox-treated C2C12/Runx2^{Dox} cells. PCR was performed with primer-1 specific for the mouse *Fra-1* site (Runx2-II). The primer-2 for a non-specific region locating the *Fra-1* 3′UTR was utilized for the control reaction in this ChIP analysis.

binds to the native *Fra-1* promoter *in vivo*. Therefore, ChIP assay was performed to determine whether Runx2 could associate with the *Fra-1* promoter during Runx2-induced osteogenic differentiation of C2C12 cells. In this ChIP assay, two pairs of primers were designed. Primer-1 was designed to amplify a 119 bp fragment of the *Fra-1* promoter flanking the Runx2-II site, and primer-2 was designed to amplify a 143 bp fragment of the *Fra-1* 3′ untranslated region (3′UTR) in the absence of any potential Runx2-binding site. The anti-Flag antibody specifically enriched the regions containing Runx2-II (Primer-1, Fig. 3B). By contrast, no positive signals were detected in any of the negative controls, including the PCR control (H_2O) or the immunoprecipitation control. Additionally, Runx2 did not associate with the *Fra-1* 3′UTR (Primer-2, Fig. 3B). The data suggested that Runx2 associates with the *Fra-1* promoter *in vivo*.

3.6. Fra-1 positively regulates Runx2-induced osteogenic differentiation of C2C12 cells

Since Runx2 upregulates *Fra-1*, it was necessary to establish the functional activity of *Fra-1* on Runx2-induced osteogenesis. *Fra-1* expression vector was used for transfection of C2C12/Runx2^{Dox} cells, followed by Dox treatment for 3 days. Real-time qPCR data revealed that *Fra-1* overexpression augments the expression of *Alp*, a defined marker of early osteoblast differentiation. However,

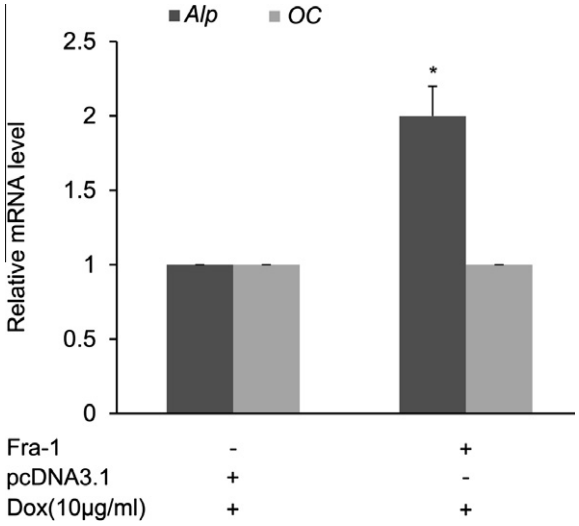


Fig. 4. Upregulation of *Fra-1* promotes Runx2-induced early osteoblast differentiation. The *Fra-1* expression vector (pcDNA3.1-*Fra-1*) was transfected into C2C12/Runx2^{Dox} cells. The cells were harvested after treatment with Dox (10 μg/ml) for 72 h. The mRNA level of *Alp* and *OC* was then determined by real-time qPCR and normalized to 18S rRNA. All data are described as the mean ± S.D. from three independent experiments. $^{*}p < 0.05$ compared with pcDNA3.1-transfected cells.

expression of OC, a marker of late osteoblast differentiation, was not affected (Fig. 4). This finding suggested that Runx2-mediated transcriptional activation of *Fra-1* was essential for Runx2-induced early osteoblast differentiation of C2C12 cells.

4. Discussion

To date, Runx2 remains the earliest of the knowntranscriptional regulators that serve critical roles in osteogenesis. The identification of Runx2 target genes provides greater insight into the mechanisms by which Runx2 regulates osteogenesis. The major finding of our study was that *Fra-1* transcription was directly transactivated by Runx2 during osteogenic differentiation of C2C12 cells. Moreover, upregulation of *Fra-1* contributed to Runx2-induced early osteoblast differentiation. To our knowledge, this is the first direct demonstration of the crucial role of Runx2 in the activitation of the *Fra-1* promoter.

In this report, we have identified *Fra-1* as a gene targeted by Runx2 during osteogenic differentiation of C2C12 cells. This is supported by two lines of evidence. First, the expression of *Fra-1* was rapidly induced after expression of Runx2 in the early induction phase (Fig. 1D), suggesting that the transcriptional activation of *Fra-1* is dependent upon Runx2 expression. It further suggested that *Fra-1* may be a direct target of Runx2. Second, a direct regulation of *Fra-1* transcription by Runx2 was evident in the ability of Runx2 to activate the *Fra-1* promoter-reporter constructs (Fig. 2A). This observation indicated that the data obtained from the RNA expression studies are likely due to the effects of Runx2 expression on *Fra-1* gene transcription.

At the molecular level, Runx2 activates or represses gene expression following specific interactions with the Runx-binding motif present in the promoter regions of Runx2-regulated genes [2,21]. To define the mechanism by which Runx2 directly regulates *Fra-1* transcription, we cloned and identified the mouse *Fra-1* promoter region spanning from position −942 to +258. Deletion analyses revealed that the most proximal 342 nucleotide of the *Fra-1* gene (−84/+258) was required for Runx2 activation (Fig. 2B). Indeed, two Runx2-binding motifs were identified within this region (Fig. 2C). Moreover, deletion analysis (Fig. 2B) also showed that the

activity of the longer construct (pGL3/07) was lower than that of pGL3/08. This observation implied the presence of at least one transcriptional repressor in the −83 to −189 region. Two partially overlapping binding sites for nuclear transcription factor kappa B (NF-kappa B) were predicted within this region by computer analysis. Lee et al. [22] have previously demonstrated that activation of NF-kappa B is essential for TNF-alpha-mediated inhibition of osteogenic differentiation, indicating the negative effect of NF-kappa B on osteogenesis. Chang et al. [23] reported that inactivation of NF-kappa B activity in osteoblasts augments expression of *Fra-1*, which can contribute to bone matrix mineralization, and this finding suggests a role for NF-kappa B in blocking *Fra-1* expression. Based on these findings, we speculated that the transcriptional repressor in the −83 to −189 region could be NF-kappa B. The detailed mechanism whereby NF-kappa B appears to directly regulate *Fra-1* transcription requires further experimentation and verification.

Comprehensive studies described herein demonstrate that the Runx2 motif located at position +208 is essential for the transcriptional activation of the *Fra-1* promoter by Runx2. Mutation of the Runx2 motif at position +208 (pGL3/08M2) served to dampen Runx2-directed activation of the *Fra-1* promoter by approximately 50%. However, mutation of the Runx2 motif at position +139 (pGL3/08M1) had no effect on *Fra-1* promoter activity. Moreover, EMSA analysis using nuclear extract from Dox-treated C2C12/Runx2^{Dox} cells demonstrated specific binding of Runx2 for the Runx2 motif at position +208 (Fig. 3A). However, the data obtained from these artificial *in vitro* systems may not necessarily reflect the regulation and function of the endogenous gene. To address this concern, we used ChIP analysis to corroborate the EMSAs and transient transfection data. This study clearly demonstrated that Runx2 is specifically associated with the *Fra-1* promoter during osteogenic differentiation of C2C12 cells (Fig. 3B). Together, our findings reveal that *Fra-1* could be a direct target of Runx2 during osteogeneis. This observation supports the notion that *Fra-1* is essential for Runx2-induced early osteoblast differentiation (Fig. 4).

An interesting finding from this work was the trend in expression of *Fra-1* during Runx2-induced osteogenic differentiation. *Fra-1* expression was rapidly induced by Runx2 within 6 h after Dox treatment (Fig. 1D), and thereafter declined to moderate levels within 72 h (Fig. 1D). The mechanisms by which Runx2 caused this expression trend remain to be determined. Here, we found that *Fra-1* overexpression promoted Runx2-induced early osteoblast differentiation (Fig. 4), supporting the observation that the expression of *Fra-1* was rapidly induced after expression of Runx2 in the early induction phase (Fig. 1D). The expression of *Fra-1* remained at a moderate level within 72 h, and this may be necessary for the maintenance of osteoblast differentiation. Moreover, *Fra-1* is also involved in bone matrix mineralization of osteoblasts *in vitro* [23], and loss- and gain-of-function studies in mice have demonstrated that *Fra-1* is essential for bone formation [11,13]. Collectively, these findings suggest that *Fra-1* plays an important role in the regulation of the functional activity of osteoblasts. Based on these observations, we conclude that the expression of the *Fra-1* gene may be either induced at high levels or retained at moderate levels in the late stage of osteoblast differentiation. Further studies are warranted to firmly confirm this conclusion.

In summary, we performed *in vitro* and *in vivo* experiments and demonstrated that the AP-1 family member *Fra-1* is a direct target of the transcription factor Runx2 during osteogenic differentiation of C2C12 myogenic progenitor cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.033.

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