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Establishment and Characterization of a Stable Cell Line to Evaluate Cellular Runx2 Activity

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Abstract Runx2 is an essential transcription factor for osteoblast differentiation from early commitment step to final differentiation. Based on its crucial role in osteoblast differentiation, the transcriptional activity of Runx2 protein implies more valuable information for osteoblast differentiation than any other parameters, such as Runx2 mRNA or protein level. Thus, a sensitive, specific, and consistent method to determine the Runx2 transcriptional activity has long been expected. Here we suggest a stable cell line that carries 6xOSE2-Luciferase reporter vector would be a good evaluation system to determine biological Runx2 transcriptional activity. The proliferation rate, cell shape, and the myogenic differentiation potential of the cloned cell line were similar to those of parental premyoblastic C2C12 cells. The cells specifically responded to Runx2 modulating agent such as FGF2. The stable cell line responded 5–6 folds more sensitively than the transiently transfected cells with Runx2. Though overexpression of any Runx gene stimulated the luciferase activity, Runx2 enhanced the reporter activity the highest. Collectively, the 6xOSE2-luc stable cells would be a good biological evaluation system to assess the activity of extracellular Runx2 modulating stimulations as well as the signal transduction pathways involved in the stimulations. J. Cell. Biochem. 91: 1239–1247, 2004.

Key words: runt-related transcription factors; osteoblast-specific element 2; fibroblast growth factor 2; C2C12

Runt-related transcription factor 2 (Runx2), previously known as Cbfa1/Pebp2αA/AML3, plays an essential role in osteoblast differentiation [Ducy et al., 1997; Komori et al., 1997]. Runx2-knockout animals display complete absence of bone because of arrested osteoblast maturation [Komori et al., 1997] and heterozygotes develop cleidocranial dysplasia, a disorder characterized by delayed ossification [Mundlos et al., 1997; Otto et al., 1997]. Several reports show that Runx2 regulates the expression of bone marker genes during osteoblast differentiation [Banerjee et al., 1997; Ducy et al., 1997; Ji et al., 1998]. In our previous report, we showed the expression pattern of Runx2 during intramembranous and endochondral bone formation, which indicate that Runx2 is required for all steps of osteoblast differentiation, namely, from the initial commitment to the final differentiation events [Park et al., 2001; Choi et al., 2002]. These observations collectively indicate that Runx2 plays a key role in osteoblast differentiation.

Although the mechanism by which Runx2 controls osteoblast differentiation and skeletal bone formation is not fully revealed, this transcription factor regulates osteogenic marker genes such as osteocalcin (OC), osteopontin (OP), bone sialoprotein (BSP), collagenase 3, osteoprotegerin, and α1(I) and α2(I) collagen. Promoters of these genes contain common consensus sequences, 5′-(Pu/T)ACCPuCPu-3′ or 5′-PyGPyGGT(Py/A)-3′, referred as nuclear

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matrix protein 2 (NMP2)-binding site or osteoblast-specific *cis*-acting elements 2 (OSE2) [Ducy and Karsenty, 1995; Merriman et al., 1995], which is also recognized by highly conserved runt domain of other Runx proteins [Meyers et al., 1993; Geoffroy et al., 1995]. Runx2 binds to the conserved OSE2 element and stimulated transcriptional activity of reporter gene in the multimerized OSE2 promoter-driven reporter system. However, mutations of common consensus sequences in OSE2 elements abolished Runx2 binding as well as its transcriptional activity [Geoffroy et al., 1995; Ducy et al., 1997; Kim et al., 2003]. These results indicate that Runx2 transcriptional activity is basically dependent on the binding of runt domain and cognate *cis*-acting element. Moreover, post-translational modifications of the Runx2 protein also greatly influence on the binding or transcriptional activity [Xiao et al., 2002; Kim et al., 2003]. Thus, the Runx2 activity could not be simply determined by its mRNA or protein level.

In the present study, we have constructed multimerized OSE2-driven reporter vector, established a stable cell line expressing 6xOSE2-driven luciferase. We compared general cellular activity of the cell with that of parental cell and assessed usefulness of the cell line for the determination of Runx2 transcriptional activity.

MATERIALS AND METHODS

Materials

Recombinant human FGF2 and luciferase assay system were purchased from Promega (Madison, WI). G418, LipofectAMINE PLUS reagent, Dulbecco's modified eagle medium (DMEM), and alpha minimum essential medium (α-MEM) were from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was from HyClone (Logan, UT). Zeta-probe blotting membranes and ExpressHyb hybridization solution were from Bio-Rad (Hercules, CA) and Clontech (Palo Alto, CA), respectively. The p38 MAPK inhibitor SB203580 and the MEK1 inhibitor PD98059 were purchased from Tocris (Ballwin, MO) and calphostin C, PMA, U73122, and forskolin were from Sigma Chemical Co. (St. Louis, MO). Polyclonal Runx1 and Runx3, and monoclonal Runx2 antibodies were generously provided from Dr. Suk-Chul Bae in Chungbuk National University, Korea.

Vector Construction

To construct p6xOSE2-luc reporter vector, complimentary oligonucleotides containing sequences for osteoblast-specific element 2 (OSE2) of mouse OC promoter were synthesized (5′-CCGGGCTGCAATCACCAACCACAGCATC-3′ and 5′-CCGGGATGCTGTGGTTGGTGATTG-CAGGC-3′). Annealed oligonucleotides of OSE2 element were multimerized by unidirectional ligation. 6xOSE2 multimer was isolated and cloned into XmaI site of pGL3-promoter reporter vector (Promega). To confirm tandemly repeated 6xOSE2 multimer, cloned fragments were sequenced.

Cell Culture and the Construction of Stable Cell Lines

Premyoblastic C2C12 cells were maintained in DMEM supplemented with 15% FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. In order to determine the effect of each agent, cells were washed twice with phosphate-buffered saline (PBS) to exclude the serum effect, and pretreated for 1 h with each inhibitor in serum-free medium supplemented with 0.2% BSA and treated with 10 ng/ml FGF2 for 16–24 h. Runx2−/− calvarial cell lines were maintained in α-MEM supplemented with 10% FBS. To establish the p6xOSE2-luc-stably transfected cell line, C2C12 cells were cotransfected with p6xOSE2-luc reporter vector and pcDNA3.0 in a 5:1 ratio by using LipofectAMINE PLUS reagent as manufacturer's instructions. Stably transfected clones were selected in media containing 2.5 mg/ml of G418 (Gibco). Individual G418-resistant clones were isolated by using separation rings, transferred, and subcultured. Stable cell clones were screened by luciferase assay. Once established, clones were maintained in media containing 200 μg/ml of G418.

Transient Transfection and Luciferase Assays

For transient transfection assays, 1×10^5 cells were plated per well in 6 well plates and transfected 24 h after seeding with 0.5 μg of p6xOSE2-luc or 0.5 μg of other expression vectors using lipofectAMINE PLUS reagent. After 24–48 h of transfection, cells were harvested and assayed for luciferase activity using Luciferase Reporter Assay kit according to the manufacturer's instructions.

Northern Blot Analysis and Electrophoretic Mobility Shift Assays (EMSA)

Isolation of total RNA and Northern blot analysis were done as previously described [Kim et al., 2003]. Full-length coding region of Runx2-type I was used for the hybridization probe. Nuclear extracts were prepared and EMSA were performed as previously described [Kim et al., 2002]. Previously characterized wild type OSE2 oligonuleotides corresponding to the −156/−112 segment of the mouse OC promoter [Ducy and Karsenty, 1995] were used as EMSA probes. For supershift assay, a specific antibody against each Runx protein was incubated with nuclear extract in the binding buffer for 20 min at room temperature prior to the addition of the probes.

MTT Assay

MTT assay is based on the cleavage of the yellow tetrazolium salt, MTT, to purple formazan crystals by the mitochondrial metabolic activity of cells; the assay is used for the quantitative determination of cell proliferation and viability. C2C12 and 6xOSE2-C2C12 cells were cultured for 24 or 48 h after plating 4×10^4 cells/well in 24 well plates. We treated 50 μl of 5 mg/ml MTT (dimethyl thiazolyldiphenyltetrazolium bromide) in 1 ml culture medium and cells were returned to the incubator for additional 4 h. The reaction was stopped by the removal of the medium, and 200 μl of DMSO and 50 μl glycin buffer (0.1 M glycin, 0.1 M NaCl, pH 10.5) was added to the cells. The degree of MTT conversion was measured immediately with spectrophotometer at 570 nm.

RESULTS

Establishment of Stable Cell Lines With 6xOSE2-Luciferase Reporter

Parental C2C12 cells originated from embryonic skeletal muscle so that the cells were prone to differentiate myoblasts. The cells express relatively low level of Runx2 but have a potential to transdifferentiate into osteoblast when stimulated by osteogenic factors [Katagiri et al., 1994; Lee et al., 1999, 2000]. Cells with stable integration of the reporter genes were selected in media containing 2.5 mg/ml of G418 (see Materials and Methods). Nineteen clones resistant to G418 were selected and basal luciferase activity was measured (Fig. 1A). Among them, 18 clones had detectable reporter activity although the level of basal activity was variable. Stable cell lines expressing 6xOSE2-luc were referred to as 6xOSE2-C2C12 hereafter. The 18 clones with basal luciferase activity were plated and treated with FGF2 which is known to stimulate Runx2 expression as well as its transcriptional activity by posttranslational level [Kim et al., 2003]. Figure 1B showed that the FGF2 responsiveness was variable in each cell clone. Clones 3, 5, and 29 showed a 41.1, 15.5, and 7.8 fold increase by FGF2, respectively while clones 7, 20, and 28 showed relatively lower 2.1, 2.5, and 2.2 fold increase, respectively. There were little response in clones 1, 15, 19, and 22. To further characterize 6xOSE2-C2C12, we focused on clone 3 since this showed the highest response to FGF2.

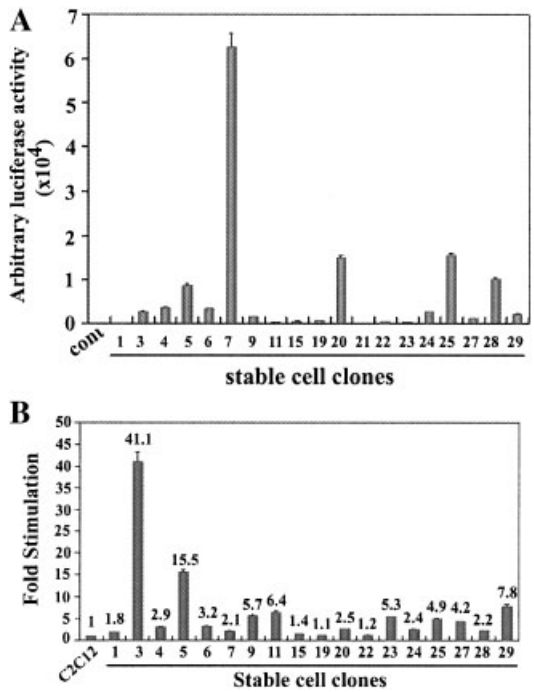

Fig. 1. The establishment of 6xOSE2-C2C12 stable cells. **A**: After 6xOSE2-luc reporter genes were transfected into C2C12 cells, 19 G418 resistant clones were selected, and basal luciferase activities were measured. Cont denotes the basal activity of C2C12 lysate without any reporter genes. **B**: 18 clones which have detectable luciferase activity were treated with 10 ng/ml FGF2. After 24 h, cells were lysed and assayed for luciferase activity. Fold stimulation of FGF2/Cont of each clone is indicated on top of each graph bar.

6xOSE2-Stable Cell Line Retains the Characters of C2C12 Cells

Since stable transfection results in a random incorporation of plasmid into chromosomal

DNA and this may change the characteristics of the cells, we thus compared 6xOSE2-stable cell lines with parental C2C12 cells. The proliferation of 6xOSE2-C2C12 cells was not affected by the chromosomal incorporation of reporter vector when compared to C2C12 cells (Fig. 2A). In addition, 6xOSE2-C2C12 cells still retain a potential of myotube formation (Fig. 2B) when cultured with 5% FBS, which is quite similar that of parental C2C12 cells [Lee et al., 1999]. Northern blot analysis indicated that the induction level of Runx2 by FGF2 in the stable cells was quite comparable that of parental C2C12 cells (Fig. 2C). Collectively, 6xOSE2-stable cell line still maintained some typical characteristics of parental C2C12 cells.

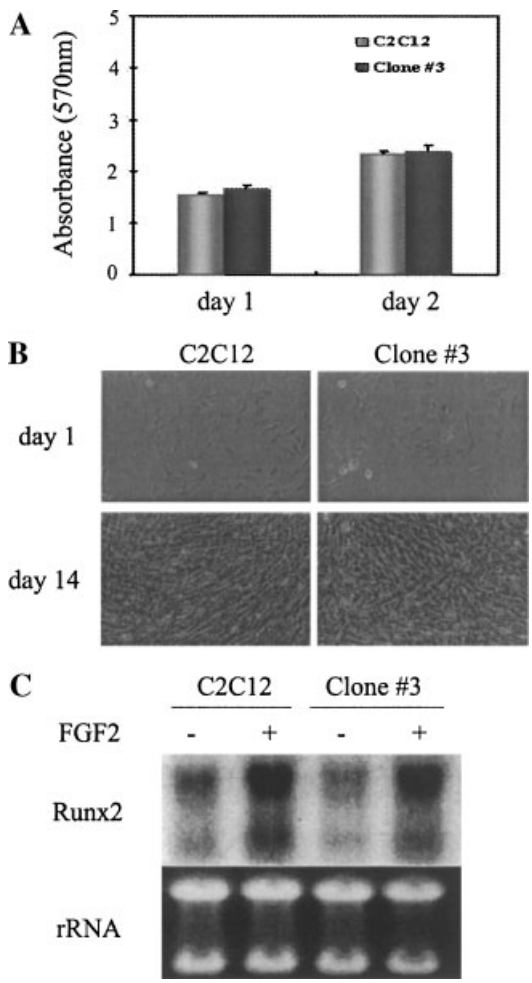

Fig. 2. 6xOSE2-C2C12 cells maintain pre-myoblastic features like C2C12 cells. **A**: C2C12 and 6xOSE2-C2C12 cells (clone #3) were cultured for 24 or 48 h after plating 4×10^4 cells/well in 24 well plates. At indicated time points, MTT assay was preformed and absorbance was measured at 570 nm. **B**: C2C12 and 6xOSE2-C2C12 cells (clone #3) were plated at 1×10^5 cells/well in 6 well plates. Morphology of the cells was photographed on day 1 and 14. **C**: C2C12 and clone #3 were treated with 10 ng/ml FGF2 in serum free medium upon confluency. Three hours post treatment, cells were harvested and total RNAs were prepared. Runx2 expression was determined by Northern blot analysis. Ethidium bromide stained ribosomal RNA (rRNA) is shown as control for equal loading.

The Stimulation of Runx2 Activity by FGF2 Is Prominent in Proliferating Cells

In order to understand the usefulness of the stable cell line for the determination of Runx2 activity in long term cultures, we tested their responses at various cell densities and time points after plating. Various numbers of 6xOSE2-C2C12 cells (0.2×10^5, 1×10^5, or 1×10^6 cells/well) were plated in 6 well plates. Enhancement of FGF2-mediated luciferase activity was decreased with the increase in the cell density (Fig. 3A). Similarly, we observed the higher level of FGF2-stimulated Runx2 activity during the active proliferation stage, and this response to FGF treatment was dramatically decreased after the cell reached to the confluence (Fig. 3B).

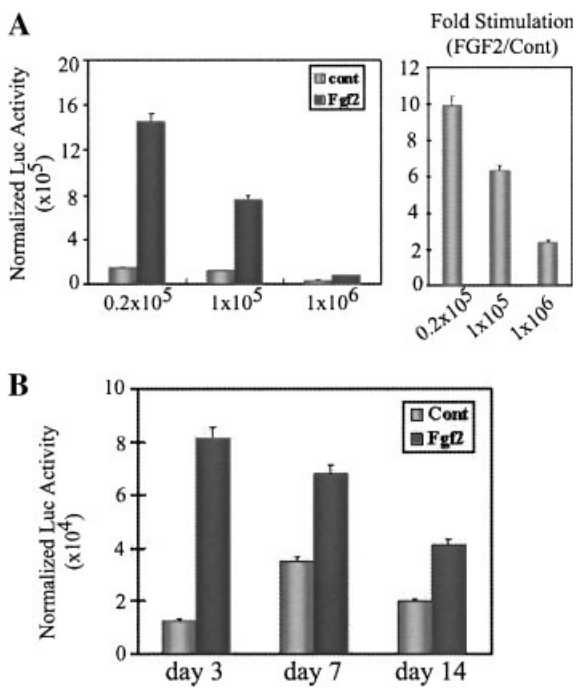

Fig. 3. The stimulation of Runx2 activity by FGF2 is prominent in proliferating cells. **A**: 6xOSE2-C2C12 cells were plated at 0.2×10^5, 1×10^5, or 1×10^6 cells in 6 well plates and were treated with 10 ng/ml FGF2 after 24 h. Cells were harvested 24 h later, and luciferase activities were determined. **B**: 6xOSE2-C2C12 cells were plated at 1×10^5 cells in 6 well plates and harvested at indicated time points (day 3, 7, and 14) after treatment with 10 ng/ml FGF2 final 24 h. The luciferase activity was normalized by the total protein in the respective lysates.

The Basal Promoter Activity of 6xOSE2-C2C12 Cells Decreased With Passages

Except for the homologous recombination, most of the stably transfected genes are prone to lose their specific character with passages. In the 6xOSE2-C2C12 cells, the basal luciferase activity was gradually decreased with the passages (Fig. 4A). Even if the basal luciferase activity was decreased with the cell passages, the cells still showed consistent and reproducible fold-induction of reporter activity by FGF2 stimulation (data not shown). When the higher passage cells with decreased basal activity (approximately over 10–15 passages) were reselected at a higher concentration (2.5 mg/ml) of G418, the basal activity of luciferase was recovered back to the original level (Fig. 4B). For this reason, we cultured the cell in a higher concentration of G418 until the experimental use.

All Runx Proteins Commonly Bind to OSE2 But 6xOSE2-luc More Specifically Reflects Runx2 Activity

The Runx family of transcription factor proteins consists of three known members (Runx1–3) of which the amino terminal part comprises the DNA-binding runt domain, a region of 128 amino acids with conserved sequence homology to the *Drosophila* transcription factor runt. Since all Runx proteins have the same recognition sequences, we investigated how the 6xOSE2-luc reporter responds to different Runx transcription factors. Gel mobility shift and supershift assay confirmed that all three Runx proteins commonly bind to OSE2 (Fig. 5A). When the expression vector of each Runx family member and reporter vector were transfected into C2C12 cells, the reporter activity was stimulated commonly by the forced expression of each Runx gene but was increased to the highest by Runx2 (about 10 folds) while Runx1 and Runx3 stimulated 5 and 3 folds, respectively (Fig. 5B). Runx2 specific stimulation was commonly determined in other cell types such as ROS 17/2.8 and MC3T3-E1 (data not shown). To determine the specificity of Runx-mediated activation of the 6xOSE2-luc reporter gene, *c-fos* and *c-jun*, components of activator protein 1 (AP1) which recognizes the consensus sequence (TGAG/CTCA), were transiently transfected into Runx2 knockout cells (Runx2−/− cells). Neither c-Fos nor c-Jun activated the promoter, indicating that 6xOSE2-luc specifically responds to Runx factors (Fig. 5C).

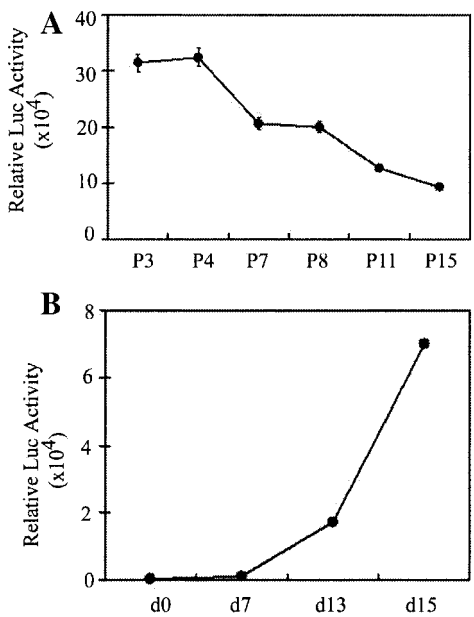

Fig. 4. The recovery in basal promoter activities in 6xOSE2-C2C12 cells after reselection. **A**: 6xOSE2-C2C12 cells were cultured and passed under the treatment of 200 μg/ml G418. Cells were harvested at passage number 3, 4, 7, 8, 11, and 15 and then luciferase activities were determined. **B**: Stable cells with decreased basal promoter activity were plated and treated for several days with 2.5 mg/ml of G418. Cells were harvested at indicated days to determine luciferase activity.

The 6xOSE2-C2C12 Cell Clone Is a Useful System for the Screening of Signaling Pathways Involved in Runx2-mediated Activation or Suppression

In our previous study, we demonstrated that protein kinase C (PKC) pathway plays a pivotal role in Runx2 protein activation by FGF2 stimulation [Kim et al., 2003]. To explore the other signaling pathways involved in the regulation of Runx2 activity, we used 6xOSE2-C2C12 cell system and several pathway specific inhibitors or dominant negative expression vectors. 6xOSE2-C2C12 cells were treated with each pathway inhibitor for 1 h prior to the stimulation by FGF2. After 24 h of FGF2 treatment, cells were harvested and luciferase activity was measured. FGF2-mediated induction of Runx2 activity was downregulated by calphostin C, U73122, PD98059, or SB203580 which are the specific inhibitors of PKC, phospholipase Cγ (PLCγ), Erk MAPK, and p38 MAPK, respectively (Fig. 6A). However, forced expression of dominant negative MEKK1 which is known to inhibit SPAK/JNK pathway did not

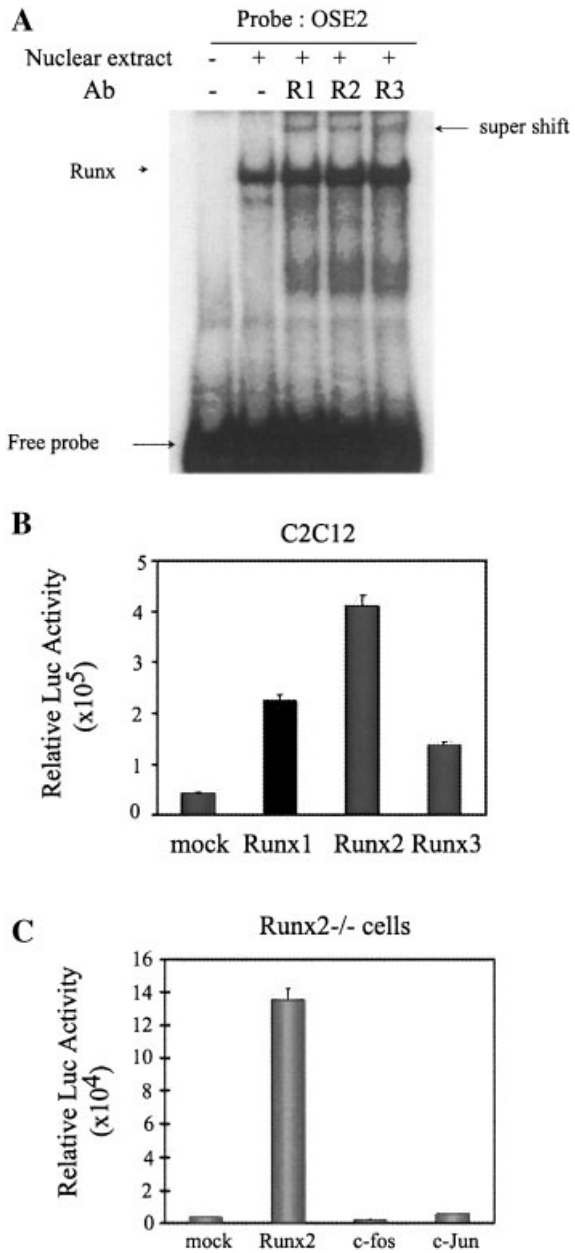

Fig. 5. 6xOSE2-luc reporter responds to all three Runx transcription factors. **A**: Nuclear extracts were prepared from C2C12 cells. Electrophoretic mobility shift assays were performed with 5 μg nuclear extracts and ^{32}P-labeled OSE2 oligonucleotides. The arrow head indicates the Runx complex. R1, R2, and R3 indicate specific antibodies for Runx1, Runx2, and Runx3, respectively. **B**: Expression vectors for each Runx were transfected into C2C12 cells. After 24 h, cells were harvested and luciferase activity was determined from the cell lysates. Mock indicated the empty vector transfected cells as a control. The luciferase activity was normalized by the protein concentrations in the respective cell lysates. **C**: Runx2−/− cells were transiently transfected with Runx2-type II isoform, c-fos, c-jun, or empty vector as a control. The luciferase activity was normalized by the protein concentrations in the respective cell lysates.

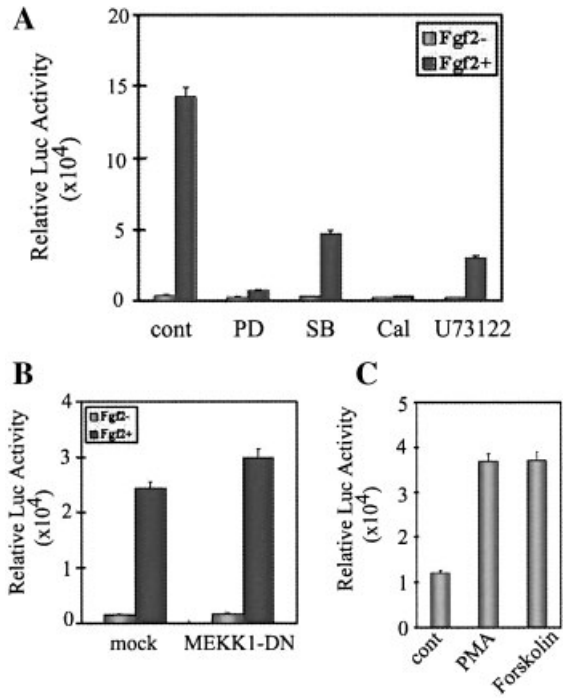

Fig. 6. The involvement of various signaling pathways in FGF2-mediated regulation of Runx2 activity. **A**: 6xOSE2-C2C12 cells were treated with FGF2 for 16–24 h with or without 50 μM PD98059 (PD), 25 μM SB203580 (SB), 0.5 μM calphostin C (Cal), and 3 μM U73122 pretreatment. DMSO was treated as vehicle. The luciferase activity was normalized by protein concentrations in the respective cell lysates. **B**: 6xOSE2-C2C12 cells were transiently transfected with vectors expressing the dominant negative MEKK1 (MEKK1-DN) or empty vector as a control. After overnight culture, the cells were treated with or without FGF2 and were harvested after 16–24 h to determine luciferase activities. The luciferase activity was normalized as described above. **C**: 6xOSE2-C2C12 cells were treated with 100 nM PMA or 2 μM forskolin. The cells were harvested after 16–24 h culture and luciferase activity was determined from the lysate.

change FGF2-stimulated Runx2 activity (Fig. 6B). We next examined the Runx2 activity after the treatment of PMA and forskolin, activator of PKC and protein kinase A (PKA), respectively. The activation of PKC or PKA pathway stimulated 6xOSE2-luc promoter activity (Fig. 6C).

DISCUSSION

Since Runx2 is an essential transcription factor for skeletal development and osteoblast differentiation [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997], the Runx2 activity is the utmost important parameter to be examined in monitoring osteoblast differentiation. Runx2

binds to the consensus sequences (PuACCACA) on the promoter of target genes such as OC, OP, BSP, and other bone marker genes, and stimulates the transcription of these genes [Ducy et al., 1997]. However, since the levels of Runx2 mRNA and protein are not always correlated with the transcriptional activity of Runx2 protein [Xiao et al., 1998; Sudhakar et al., 2001], and the Runx2 transcription itself is autoregulated by its own protein [Drissi et al., 2000], the direct measurement of Runx2 activity is more effective and critical parameter for monitoring osteoblast differentiation. In the present study, we established and characterized a cell line harboring 6xOSE2-luc reporter for this purpose.

The General Characteristics of the Stable Cell Clone Were Similar to Those of C2C12 Cells

As demonstrated in several gene targeting studies, the homologous recombination of heterologous genes is permanently maintained through innumerable cell division. However, stable transfection by non-homologous recombination method results in a nonspecific incorporation of foreign gene into host chromosomes, which in turn may result in changes of the original cell characters. In that case, the use of stable cell line causes a misinterpretation of the experimental results. Therefore, it is extremely important to compare characters of the stable cells with those of the parental cells. In this context, the 6xOSE2-C2C12 cell clone satisfies three criteria; it retains a comparable cell shape and proliferation rate, a predetermined differentiation phenotype (myotube formation), and the FGF2-responsiveness (Runx2 activation).

Runx2 Is Mainly Activated by FGF2 in Proliferation Stage of Osteoblast Differentiation

During the cranial suture development, FGF2 expression is mainly found in sutural mesenchyme in which Runx2-type I isoform (Pebp2aA/Cbfa1) expression is dominant [Park et al., 2001] and the active proliferation of osteoprogenitor cells occurs. Moreover, the application of FGF2 stimulated Runx2 expression as well as cell proliferation [Kim et al., 1998, 2003]. Similar to these biological situations, 6xOSE2-luc promoter responded to FGF2 in proliferating stage of the stable cells, suggesting that these stable cells can reflect Runx2 activity similar to that in the physiological and developmental conditions of osteoblasts.

OSE2 Commonly Responds to all Runx Proteins

Runx2 response element in OC promoter was first identified in rat as an osteogenic tissue specific nuclear matrix protein binding site [Merriman et al., 1995] and in mouse as a osteoblast specific factor 2 binding site [Geoffroy et al., 1995], respectively. The binding sites of Runx2 and Runx1 have an identical core binding sequence [Bae et al., 1993, 1994; Meyers et al., 1993, 1995; Ogawa et al., 1993a,b]. However, it has not been reported that Runx3 binds directly to OSE2. In this experiment, we assumed that the expression level of each Runx protein would be similar because all Runx genes were subcloned in the same expression vector, pcDNA3.1-His vector, so that their expressions were controlled under the same promoter. Our gel mobility shift and supershift assays showed that Runx1, 2, and 3 commonly bind with OSE2 of mouse OC promoter without significant differences in affinity. Even though the forced expression of each Runx commonly stimulated 6xOSE2-driven luciferase activity, the OSE2 responded more specifically to Runx2. Therefore, the result indicates that the common core binding sequence is good enough for the binding of all Runx proteins but adjacent sequences probably determine transcriptional specificity of each Runx protein.

The binding of these Runx proteins to OSE2 oligonucleotides is specific since the binding complex on OSE2 oligonucleotides was neither shifted by AP1 protein nor supershifted by AP1 antibodies (data not shown). Its Runx-specificity was also confirmed by cotransfection experiments of other transcription factors. Forced expression of Runx2 dramatically enhanced 6xOSE2-luc promoter activity but c-fos and c-jun did not affect the promoter activity. For this experiment, we used Runx2−/− cells because AP1 can interact physically with Runx2 and synergistically stimulates Runx2 function [Hess et al., 2001; D'Alonzo et al., 2002]. Actually, forced expression of AP1 components stimulated slightly 6xOSE2-luc promoter activity without cotransfection of Runx2 in C2C12 cells (data not shown). It can be explained by that Runx2 is endogenously expressed in C2C12 cells and can interact with exogenously introduced AP1 components [Kim et al., 2003]. Recent reports indicated that Cbfβ, a common partner of Runx1, 2, and 3 for their transactiva-

tion function [Ogawa et al., 1993a,b; Wang et al., 1996], is essential for skeletal development [Kundu et al., 2002; Yoshida et al., 2002]. However, consistent with the results of Harada et al. [1999], the cotransfection of Cbfβ with each Runx did not have additional effects on the stimulation of 6xOSE2-luc promoter activity over the expression of each Runx alone (data not shown). Even if this artificial promoter system has such a limitation that it could not reflect all the physiological events of Runx activation, the 6xOSE2-luc stable cell is a useful monitoring system for Runx2 activity.

Various Signaling Pathways Are Involved in the Regulation of Runx2 Activity

Extracellular signals are transmitted to the nucleus in a variety of ways by activating several kinases. FGFR induces receptor dimerization, intrinsic tyrosine phosphorylation, and activation of multiple signal transduction pathways, including those involving MAPKs, Src, PLCγ, and PKC [Kuo et al., 1997; Maher, 1999]. Our previous report showed that FGF/FGFR-stimulated Runx2 expression and activity are mediated by PKC pathway using these stable cell lines [Kim et al., 2003]. Although previous study focused on the PKC pathway in the regulation of Runx2 by FGF/FGFR signaling, the results presented here showed that several signaling pathways are involved in the regulation of FGF/FGFR-stimulated Runx2 activity. FGF2-stimulated 6xOSE2 promoter activity was downregulated by calphostin C, U73122, PD98059, or SB203580 which are the specific inhibitors of PKC, PLCγ, Erk MAPK, and p38 MAPK, respectively. 6xOSE2 promoter activity was also stimulated by PKA activator, forskolin. This result is correlated with recent report that PTH-stimulated collagenase 3 gene expression is regulated by Runx2 via PKA pathway [Selvamurugan et al., 2000; Hess et al., 2001; D'Alonzo et al., 2002]. These results suggest that the regulation of Runx2 activity is very sophisticated, thus the individual regulation by each signaling pathway, cross-talk between them, and an eventual comprehensive understanding of the regulation of Runx2 activity should be further investigated.

Collectively, 6xOSE2-C2C12 stable cell is a very efficient and useful system for screening of the transcriptional activity of Runx2 protein. Furthermore, given the importance of Runx2 in osteoblast differentiation and bone formation, it will be a useful system for the screening of new osteogenic agents for the treatment of osteoporosis and bone fracture. In addition, it would be also a good system to understand signaling pathways involved in the regulation of Runx2 activity.

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REFERENCES

Bae SC, Yamaguchi-Iwai Y, Ogawa E, Maruyama M, Inuzuka M, Kagoshima H, Shigesada K, Satake M, Ito Y. 1993. Isolation of PEBP2 alpha B cDNA representing the mouse homolog of human acute myeloid leukemia gene, AML1. Oncogene 8:809–814.

Bae SC, Ogawa E, Maruyama M, Oka H, Satake M, Shigesada K, Jenkins NA, Gilbert DJ, Copeland NG, Ito Y. 1994. PEBP2 alpha B/mouse AML1 consists of multiple isoforms that possess differential transactivation potentials. Mol Cell Biol 14:3242–3252.

Banerjee G, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, Lian JB. 1997. Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. J Cell Biochem 66:1–8.

Choi KY, Lee SW, Park MH, Bae YC, Shin HI, Nam SH, Kim YJ, Kim HJ, Ryoo HM. 2002. Spatio-temporal expression patterns of Runx2 isoforms in early skeletogenesis. Exp Mol Med 34:426–433.

D'Alonzo RC, Selvamurugan N, Karsenty G, Partridge NC. 2002. Physical interaction of the activator protein-1 factors c-Fos and c-Jun with Cbfa1 for collagenase-3 promoter activation. J Biol Chem 277:816–822.

Drissi H, Luc Q, Shakoori R, Chuva De Sousa Lopes S, Choi JY, Terry A, Hu M, Jones S, Neil JC, Lian JB, Stein JL, Van Wijnen AJ, Stein GS. 2000. Transcriptional autoregulation of the bone related CBFA1/RUNX2 gene. J Cell Physiol 184:341–350.

Ducy P, Karsenty G. 1995. Two distinct osteoblast-specific *cis*-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol 15:1858–1868.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Geoffroy V, Ducy P, Karsenty G. 1995. A PEBP2 alpha/AML-1-related factor increases osteocalcin promoter activity through its binding to an osteoblast-specific *cis*-acting element. J Biol Chem 270:30973–30979.

Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M. 1999. Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem 274:6972–6978.

Hess J, Porte D, Munz C, Angel P. 2001. AP-1 and Cbfa/runt physically interact and regulate parathyroid hormone-dependent MMP13 expression in osteoblasts through a new osteoblast-specific element 2/AP-1 composite element. J Biol Chem 276:20029–20038.

Ji C, Casinghino S, Chang DJ, Chen Y, Javed A, Ito Y, Hiebert SW, Lian JB, Stein GS, McCarthy TL, Centrella M. 1998. CBFa (AML/PEBP2)-related elements in the TGF-beta type I receptor promoter and expression with osteoblast differentiation. J Cell Biochem 69:353–363.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Kim HJ, Rice DP, Kettunen PJ, Thesleff I. 1998. FGF-, BMP-, and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. Development 125:1241–1251.

Kim HJ, Lee MH, Kim HJ, Shin HI, Choi JY, Ryoo HM. 2002. Okadaic acid stimulates osteopontin expression through de novo induction of AP-1. J Cell Biochem 87: 93–102.

Kim HJ, Kim JH, Bae SC, Choi JY, Kim HJ, Ryoo HM. 2003. The protein kinase C pathway plays a central role in the FGF-stimulated expression and transactivation activity of Runx2. J Biol Chem 278:319–326.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Kundu M, Javed A, Jeon JP, Horner A, Shum L, Eckhaus M, Muenke M, Lian JB, Yang Y, Nuckolls GH, Stein GS, Liu PP. 2002. Cbfbeta interacts with Runx2 and has a critical role in bone development. Nat Genet 32:639–644.

Kuo WL, Chung KC, Rosner MR. 1997. Differentiation of central nervous system neuronal cells by fibroblast-derived growth factor requires at least two signaling pathways: Roles for Ras and Src. Mol Cell Biol 17:4633–4643.

Lee MH, Javed A, Kim HJ, Shin HI, Gutierrez S, Choi JY, Rosen V, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Ryoo HM. 1999. Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor beta1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. J Cell Biochem 73:114–125.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC. 2000. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20:8783–8792.

Maher P. 1999. p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. J Biol Chem 274:17491–17498.

Merriman HL, van Wijnen AJ, Hiebert S, Bidwell JP, Fey E, Lian J, Stein J, Stein GS. 1995. The tissue-specific nuclear matrix protein, NMP-2, is a member of the AML/CBF/PEBP2/runt domain transcription factor family: Interactions with the osteocalcin gene promoter. Biochemistry 34:13125–13132.

Meyers S, Downing JR, Hiebert SW. 1993. Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: The runt homology domain is required for DNA binding and protein–protein interactions. Mol Cell Biol 13:6336–6345.

Meyers S, Lenny N, Hiebert SW. 1995. The t(8;21) fusion protein interferes with AML-1B-dependent transcriptional activation. Mol Cell Biol 15:1974–1982.

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Gole WG, Henn W, Knoll JHM, Owen MJ, Mertelsmann R, Zabel BU. 1997. Mutations involving the transcription factor cbfa1 cause cleidocranial dysplasia. Cell 89:773–779.

Ogawa E, Inuzuka M, Maruyama M, Satake M, Naito-Fujimoto M, Ito Y, Shigesada K. 1993a. Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel *Drosophila* runt-related DNA binding protein PEBP2 alpha. Virology 194:314–331.

Ogawa E, Maruyama M, Kagoshima H, Inuzuka M, Lu J, Satake M, Shigesada K, Ito Y. 1993b. PEBP2/PEA2 represents a family of transcription factors homologous to the products of the *Drosophila* runt gene and the human *AML1* gene. Proc Natl Acad Sci USA 90:6859–6863.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Park MH, Shin HI, Choi JY, Nam SH, Kim YJ, Kim HJ, Ryoo HM. 2001. Differential expression patterns of Runx2 isoforms in cranial suture morphogenesis. J Bone Miner Res 16:885–892.

Selvamurugan N, Pulumati MR, Tyson DR, Partridge NC. 2000. Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor alpha1. J Biol Chem 275:5037–5042.

Sudhakar S, Li Y, Katz MS, Elango N. 2001. Translational regulation is a control point in RUNX2/Cbfa1 gene expression. Biochem Biophys Res Commun 289:616–622.

Wang Q, Stacy T, Miller JD, Lewis AF, Gu TL, Huang X, Bushweller JH, Bories JC, Alt FW, Ryan G, Liu PP, Wynshaw-Boris A, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. 1996. The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. Cell 87:697–708.

Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. 1998. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem 273:32988–32994.

Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT. 2002. Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. J Biol Chem 277:36181–36187.

Yoshida CA, Furuichi T, Fujita T, Fukuyama R, Kanatani N, Kobayashi S, Satake M, Takada K, Komori T. 2002. Core-binding factor beta interacts with Runx2 and is required for skeletal development. Nat Genet 32:633–638.