The Protein Kinase C Pathway Plays a Central Role in the Fibroblast Growth Factor-stimulated Expression and Transactivation Activity of Runx2*

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Fibroblast growth factor (FGF)/FGF receptor (FGFR) signaling induces the expression of Runx2, a key transcription factor in osteoblast differentiation, but little is known about the molecular signaling mechanisms that mediate this. Here we examined the role of the protein kinase C (PKC) pathway in regulating Runx2 gene expression and its transactivation function. Treatment with FGF2 or FGF4, or transfection with a vector expressing a mutant FGFR2 that is constitutively activated in the absence of ligand, strongly stimulates Runx2 expression. Electrophoretic mobility shift assays also showed that FGF2 treatment increases the specific binding of Runx2 to the cognate response element in the osteocalcin gene promoter. Blocking PKC completely inhibited FGF2-induced Runx2 expression, whereas mitogen-activated protein kinase inhibitor had no effect. The FGF/FGFR-stimulated 6xOSE2 promoter activity was also blocked by inhibiting PKC, as was the FGF2 stimulation of the DNA-binding activity of Runx2. Experiments with PKC isoform-specific inhibitors and dominant negative isoforms of PKC indicate that PKCδ is one of key isoforms involved in the FGF2-stimulated Runx2 expression. In addition, experiments with Runx2-knockout cells showed that, although the PKC pathway largely regulates FGF2-stimulated Runx2 activity by up-regulating Runx2 expression, it also modifies Runx2 protein post-translationally and thereby increases its transcriptional activity. Thus, we show for the first time that FGF/FGFR signaling stimulates the DNA-binding and transcriptional activities of Runx2 as well as its expression, and these are largely regulated by the PKC pathway.

Fibroblast growth factors (FGFs)† are important autocrine/paracrine signaling molecules involved in intramembranous

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‡ The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; MEK1, MAPK/Erk kinase; PKC, protein kinase C; Runx2, Runt-related transcription factor 2; BSA, bovine serum albumin; α-MEM, α-minimal essential medium; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MOPS, 3-[morpholin]propanesulfonic acid; OSE2, osteoblast-specific core binding sequences; DAG, diacylglycerol.
Protein Kinase C Mediates the Activation of Runx2 by FGF2

EXPERIMENTAL PROCEDURES

Materials—Recombinant human FGF2 and the luciferase assay system were purchased from Promega (Madison, WI). Recombinant human FGFR2 was obtained from R&D Systems (Minneapolis, MN). FGF4 and FGF2 were purchased from Calbiochem (San Diego, CA). The p38 MAPK inhibitor SB203580 and the MEK1/2 inhibitor PD98059 were purchased from Tocris (Ballwin, MO) and were prepared as 25 and 50 mM stock solutions in Me2SO, respectively. The p38 MAPK inhibitor SB203580 and the MEK1/2 inhibitor PD98059 were purchased from Tocris (Ballwin, MO) and were prepared as 25 and 50 mM stock solutions in Me2SO, respectively.

Cell Culture—Osteoblast-like MC3T3-E1 cells (22) were maintained in α-MEM supplemented with 15% FBS, 100 μg/ml streptomycin, and 100 μg/ml penicillin, and 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO2. Murine premyoblast C2C12 cells (23) were maintained in DMEM containing 15% FBS, whereas rat osteosarcoma ROS17/2.8 cells (22) were cultured in DMEM with 10% FBS. Runx2+/- calvaria cell lines were maintained in α-MEM supplemented with 10% FBS (24). Cells were washed twice with phosphate-buffered saline (PBS) and then treated for 3 h with 10 ng/ml FGF2 or FGF4 in serum-free medium supplemented with 0.2% BSA. In some experiments, the cells were pretreated for 1 h with calphostin C (0.5 μM), GF109203X (1 μM), Go6976 (200 nM) or rotterlin (1 μM), inhibitors of protein kinase C, or 50 μM PD98059, a MEK1/2 specific blocker, or 25 μM SB203580, a p38 specific inhibitor, before they were treated with 10 ng/ml FGF2.

RNA Preparation and Northern Blot Analysis—Total RNA was prepared by single-step isolation as previously described (22), and the concentration was determined by spectrophotometry. Total RNA (10 μg) was then heated to 65 °C for 15 min in 50 μl of formamide, 0.02% formaldehyde, 40 mM MOPS, and 0.1 mg/ml ethidium bromide prior to gel electrophoresis on a 1% agarose gel containing 5.5% formaldehyde and 40 mM MOPS. The RNA was then blotted onto a Zeta-probe membrane in 20× SSC. The blot was air-dried and cross-linked by exposure to ultraviolet light. Runx2 cDNA was labeled with [α-32P]dCTP by using the Megaprim DNA labeling system kit (Amer sham Biosciences, UK). Prehybridization and hybridization were performed by using the ExpressHyb solution according to the manufacturer's protocol. After hybridization, the membrane was washed in 2× SSC/0.1% SDS at room temperature and then in 0.1× SSC/0.1% SDS at 50 °C and exposed to x-ray film at −70 °C with intensifying screens.

Transient Transfection and Luciferase Assay—Cells were transfected by following the protocol recommended by the manufacturers of the LipofectAMINE Plus reagent. Cells were cultured to a density of 1× 106 cells/well for 16–24 h and then transfected with the reporter vector (1 μg/well) and/or the expression vector (1 μg/well) in serum-free medium. Three hours later, the medium was replaced by medium containing 10% FBS and cultured overnight. The medium was then replaced with serum-free medium with or without FGF2, and the incubation was continued for 16–24 h. Cell lysates were prepared and luciferase activity was determined with the Luciferase Assay System kit (Promega). The luciferase activity was normalized with respect to the protein content as determined by the Bradford protein assay kit (Bio-Rad). Luminescence was measured by an AutoLumat LB953 instrument (EG&G Berthold, Walloc, Finland). For the forced expression of Runx2, we used the Runx2-type II expression vector, which transduces a 5′-7-kDa protein (25). Expression vector for constitutive active FGFFR2 (FR2Y340H) causing human Crouzon syndrome and dominant negative PKCδ was provided by Dr. Donoghue (26) and Dr. Soh (27), respectively. The promoter region of rat osteocalcin (−208/+23) was subcloned into pGL2-basic vector from −208 OC-CAT and −208 Runx mut vector (25).

Preparation of Nuclear Extract and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as previously described (28). For electrophoretic mobility shift assays, previously characterized wild type and mutant OSE2 oligonucleotides corresponding to the −156/−112 segment of the mouse osteocalcin promoter (29) were used. Probes were labeled with [α-32P]dCTP. Approximately 1 ng of labeled oligonucleotide probe was incubated with 10 μg of nuclear extract for 30 min at room temperature in the presence of 1× gel shift binding buffer containing 15 mM Tris, pH 7.5, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10% glycerol, and 1 μg of poly(dI-dC). Competition was performed with a 10- to 50-fold molar excess of unlabelled OSE2. Antibodies against Runx2 were preincubated with nuclear extracts and binding buffer for 30 min at 4 °C prior to the addition of the probe. Reactions were separated on 5% non-denaturating polyacrylamide gel.

Construction of the p6xOSE2-Luc Reporter Plasmid and Establishment of a Stable p6xOSE2-Luc-transfected Cell Line—To construct the p6xOSE2-Luc reporter plasmid containing six tandem repeats of the osteoblast-specific core binding sequences (OSE2), oligonucleotides including Runx2ACCPuCA were synthesized for sense and antisense in such a way as to create XmaI cohesive overhangs at each 5′-end as shown as follows: sense, 5′-CCGGCTGCAATCACCACACAGCAT- C-3′; antisense, 3′-CGAGTTAATGGTTGCTCAGAAGGC-5′. Annealed fragments containing OSE2 were multimerized by T4 DNA ligase. 6xOSE2 multimers were separated and purified by agarose gel electrophoresis and then cloned into the XmaI site of the pG-U2-promoter reporter vector (Promega). To confirm that the 6xOSE2 multimers were randomly repeated, the cloned fragment was sequenced. To establish a stable p6xOSE-Luc-transfected cell line, C2C12 myogenic stem cells were co-transfected with the p6xOSE2-Luc reporter vector and pcDNA3 in a 5.1 ratio with the LipofectAMINE reagent according to the manufacturer's instructions. Because FGF2-mediated stimulation of Runx2 expression, DNA binding, and transactivation function of Runx2 protein in C2C12 cells was not different from that in MC3T3-E1 cells (Fig. 1A), and these cells can be differentiated into osteoblasts in response to osteogenic signals (23, 24), C2C12 cells were selected as the host of this stable transfection. Stably transfected cells were selected in media containing 2.5 μg/ml G418 (Promega). The 6xOSE2 promoter activity of each clone was assayed by using the luciferase assay system. Once established, the clones were maintained in media containing 100 μg/ml G418.

Western Blot Analysis—Nuclear extracts were resolved by SDS-PAGE and electrotransferred to a polyvinyldene difluoride membrane. After transfer, the blot was washed with PBS for 5 min at room temperature and then incubated in blocking buffer (1× PBS containing 0.1% Tween 20 with 5% nonfat dry milk) for 1 h at room temperature. The blot was washed three times for 5 min each with 1× PBS. Primary antibody (Runx2) (24) was added to the blocking buffer at 1:2000 dilution. The blot was washed three times with PBS and incubated with the horseradish peroxidase-conjugated rabbit anti-mouse antibody (Advanced Biochemicals, Inc., Seoul, Korea) for 1 h at room temperature. After three washes, the signal was detected by ECL plus (Amer sham Biosciences, UK).

RESULTS

FGF/FGFR Signaling Increases Runx2 mRNA Levels—Murine osteoblast-like MC3T3-E1 cells, rat osteosarcoma ROS17/2.8 cells, and murine premyoblastic C2C12 cells were treated with recombinant human FGF2. Although the basal expression levels of Runx2 were different in each cell type, FGF2 treatment stimulated Runx2 expression in all three cell lines (Fig. 1A). It is noteworthy that each cell line expressed different Runx2 isoforms, because MC3T3-E1 cells mainly expressed the Runx2-type II isoform, ROS 17/2.8 cells mainly expressed the Runx2-type I isoform, and C2C12 cells expressed both isoforms equally. This isoform composition was not altered by FGF2 treatment. FGF4, another FGF family member, also increased Runx2 mRNA expression to a similar extent as FGF2 in C2C12 cells (Fig. 1B) and MC3T3-E1 cells (data not shown). MC3T3-E1 cells transfected with an expression vector expressing the constitutively active mutant of FGFR2 (FR2Y340H) also stimulated Runx2 mRNA expression, even in the absence of FGF treatment (Fig. 1C). Thus, Runx2 expression is up-regulated by FGF/FGFR signaling.

FGF2 Enhances the DNA-binding Activity and Transactivation Function of Runx2—To investigate whether FGF2-stimulated Runx2 expression increases the binding of Runx2 to its DNA binding sites, electrophoretic mobility shift assays were performed using a radiolabeled oligonucleotide Runx2 probe that hybridizes to the Runx2 binding site (OSE2) in the promoter of mouse osteocalcin. Thus, C2C12 cells were cultured in the presence or absence of FGF2, and their nuclear extracts were incubated with the probe. In untreated cells, considerable Runx2 DNA-binding activity was observed but FGF2 treatment strongly enhanced this (Fig. 2A, lanes 2 and 3). The
binding complex is Runx2-specific, because it could be competed out by adding a molar excess of unlabelled wild type OSE2 oligonucleotides as competitors (Fig. 2A, lanes 4 and 5) but was not affected by mutant competitors (Fig. 2A, lanes 6 and 7). Moreover, the binding complex was supershifted by a monoclonal antibody specific for the C-terminal portion of Runx2 (24) (Fig. 2A, lane 10). Runx2 bound specifically to the wild type probe but not to the mutant OSE2 sequence (Fig. 2A, lanes 8 and 9).

We next asked whether FGF/FGFR signaling might also enhance Runx2-mediated transactivation of downstream genes. A Runx2 binding site within the osteocalcin promoter is needed for the transactivation of the gene (30). Osteocalcin gene expression is induced by FGF2 (31), and it is likely that the induction is mediated by Runx2 binding to the osteocalcin gene promoter. We confirmed this by transfecting MC3T3-E1 cells with osteocalcin promoter constructs consisting of ~208 to +23 bp of rat osteocalcin proximal promoter that contains a Runx2 binding site, then incubating the cells with and without FGF2, after which reporter activities were determined. FGF2 treatment increased the reporter activity. When the cells were transfected with an osteocalcin promoter in which the Runx2 site was mutagenized, the FGF-mediated reporter activation was abolished (Fig. 2B).

To more accurately quantify the transcriptional activity of Runx2 protein that is induced by FGF/FGFR signaling, we used a more sensitive reporter system, namely, the p6xOSE2-luciferase reporter vector (p6xOSE2-Luc). When the p6xOSE2-Luc reporter plasmid was transiently transfected into osteoblastic cells (MC3T3-E1 cells) or non-osteoblastic cells (C2C12), the reporter activity was enhanced ~4-fold by FGF2 treatment (Fig. 2C). When the cells were co-transfected with p6xOSE2-Luc and the vector expressing the constitutively active mutant of FGFR2, FR2Y340H, both cell lines had 3- to 4-fold increases in luciferase activity even in the absence of FGF2 (Fig. 2D). In the Runx2+/− calvaria cell line (24) (hereafter referred to as Runx2+/− cells), which cannot generate endogenous Runx2, neither FGF2 treatment nor overexpression of FR2Y340H increased the reporter activity, as expected. MC3T3-E1 cells were co-transfected with the Runx2 expression vector, which expressed the p57 Runx2 product, together with the p6xOSE2-Luc reporter vector. The forced expression of Runx2 enhanced the basal activity of p6xOSE2-Luc promoter and the activity was further stimulated by FGF2 treatment, however, the extent of the increase by FGF2 treatment was not quite different from exogenous Runx2 overexpression (Fig. 2B).

The Protein Kinase C Pathway Plays a Crucial Role in FGF/FGFR-stimulated Runx2 Expression—We next asked which signaling pathway is responsible for the FGF/FGFR-mediated up-regulation of Runx2 expression. We first examined the involvement of the PKC pathway, because PKC is known to be activated by the FGF/FGFR signal (32, 33). Pharmacological down-modulation of PKC activity with calphostin C, a general PKC inhibitor, dramatically reversed the FGF2-induced increase in Runx2 mRNA levels (Fig. 3A). Another specific inhibitor of PKC α, δ, ε, and η, GF109203X, had a similar effect (Fig. 3B). In contrast, PD98059, an MEK1/2-specific blocker, or SB203580, a p38-specific inhibitor, did not alter FGF2-stimulated Runx2 mRNA expression (Fig. 3, C and D). Thus, FGF-mediated up-regulation of Runx2 mRNA expression occurs mainly through the activation of the PKC pathway.

FGF-mediated Increases in Runx2 Transcriptional Activity Correspond with PKC-mediated Increases in Runx2 Protein Levels—We examined the effects of inhibiting the PKC pathway on the Runx2 DNA binding activity in C2C12 cells. The inhibition of the PKC pathway by calphostin C completely blocked the FGF2-induced DNA binding of Runx2 (Fig. 4A). The inhibition of PKC activity with calphostin C almost completely abolished FGF2-mediated stimulation of p6xOSE2-Luc activity (Fig. 4B). Western blot analysis showed that both the increase in the Runx2 binding to OSE2 and the increase in the transcriptional activity of Runx2 correlate quite well with Runx2 protein levels, which are clearly regulated by the PKC pathway (Fig. 4C). Thus, the PKC pathway mediates the FGF/FGFR signal that up-regulates the expression and DNA binding and transactivation properties of Runx2.

PKCδ Mediates FGF2-stimulated Runx2 Expression—Until recently, at least 11 PKC isoforms have been identified (33), and previous work has shown that FGF2 differentially activates different set of isoforms in different cells (34, 35). PKC isoforms express in osteoblastic cells are α, β, δ, ε, and η isoforms (36), and those are also expressed in MC3T3-E1 cells that are used in this experiment. To narrow down the range of PKC isoforms that may be involved in Runx2 regulation by FGF2, we could rule out several PKC isoforms by using isoform-selective PKC inhibitors. Because FGF2-stimulated Runx2 expression was blocked by GF109203X (Fig. 3B), a specific inhibitor of PKC isoforms α, δ, and ε, we can rule out the involvement of the other isoforms in the regulation. The increase in Runx2 expression by FGF2 was significantly blocked by rottlerin (a PKCδ-specific inhibitor) but was not affected by Go6976, a specific inhibitor of PKCα and β1 (Fig. 5A). Consequently, PKCδ would be a candidate for mediating the effect of FGF2. Furthermore, rottlerin inhibited the activation of 6xOSE2-Luc promoter by FGF2 in a dose-dependent manner up to 12 μM (Fig. 5B). To confirm further the involvement of PKCδ isoform in the Runx2 expression by FGF2, C2C12 cells were transiently transfected with a dominant negative PKCδ (PKCδ-KR). The forced expression of PKCδ−KR abolished FGF2-stimulated Runx2 expression (Fig. 5C). Consistently, transient cotransfection of PKCδ−KR and p6xOSE2-Luc vectors into MC3T3-E1 cells also strongly down-regulated FGF2-stimulated 6xOSE2-Luc promoter activity (Fig. 5D).

Post-translational Modification of Runx2 Protein by PKC Contributes to FGF2-stimulated Runx2 Transcriptional Activity—The observations made so far strongly support the notion.
that the increase in the transcriptional activity of Runx2 depends on increases in Runx2 protein levels resulting from FGF-induced PKC activation that up-regulates Runx2 expression.

We noted, however, that the Western blot analysis revealed that protein levels of Runx2 were not completely abolished by calphostin C treatment. In contrast, the binding of Runx2 to OSE2 was completely inhibited by the blocker. In other words, Runx2 protein level was still maintained comparable to basal level in Western blot analysis but the Runx2 binding activity was completely disappeared by the PKC blocker. This discrepancy suggests that the PKC pathway may also be involved in a post-translational modification of Runx2 protein that is needed for it to conduct its DNA binding and/or transactivation activities. To investigate this, we used Runx2−/− cells and introduced a Runx2-expression vector together with the p6xOSE2-Luc reporter vector. The presence of the Runx2-expressing vector increased the basal reporter activity by about 5-fold. FGF treatment of the doubly transfected cells stimulated the reporter activity by 3-fold (i.e. 15-fold higher than the basal reporter activity in cells transfected with the p6xOSE2-Luc reporter vector only). Runx2−/− cells cannot express endogenous Runx2 in response to FGF stimulation, and thus the increase of Runx2-mediated reporter activity caused by adding FGF2 must be due to the FGF2-mediated post-translational modification of the exogenously introduced Runx2. That the PKC pathway mediates this post-translational modification is shown by pretreating the cells with calphostin C. Calphostin C treatment. In contrast, the binding of Runx2 to OSE2 was completely inhibited by the blocker. In other words, Runx2 protein level was still maintained comparable to basal level in Western blot analysis but the Runx2 binding activity was completely disappeared by the PKC blocker. This discrepancy suggests that the PKC pathway may also be involved in a post-translational modification of Runx2 protein that is needed for it to conduct its DNA binding and/or transactivation activities. To investigate this, we used Runx2−/− cells and introduced a Runx2-expression vector together with the p6xOSE2-Luc reporter vector. The presence of the Runx2-expressing vector increased the basal reporter activity by about 5-fold. FGF treatment of the doubly transfected cells stimulated the reporter activity by 3-fold (i.e. 15-fold higher than the basal reporter activity in cells transfected with the p6xOSE2-Luc reporter vector only). Runx2−/− cells cannot express endogenous Runx2 in response to FGF stimulation, and thus the increase of Runx2-mediated reporter activity caused by adding FGF2 must be due to the FGF2-mediated post-translational modification of the exogenously introduced Runx2. That the PKC pathway mediates this post-translational modification is shown by pretreating the cells with calphostin C. Calphostin C treatment had a little effect on the transactivating activity of exogenously introduced Runx2 in the doubly transfected cells, but it completely blocked the further increase in the reporter activity induced by FGF2 treatment (Fig. 6).

![Figure 2](image-url)  
**Fig. 2.** FGF/FGFR signaling enhances Runx2 DNA binding activity as well as transactivation function. A, sequence-specific Runx2 protein-DNA interactions at the mouse osteocalcin (OC) OSE2 element. C2C12 cells were treated with or without FGF2 for 12–24 h. Cells were harvested, and nuclear extracts were prepared and incubated with oligonucleotides corresponding to wild type (wt) or mutated OSE2 sites (mut) from the OC gene. The specificity and the identity of the complexes were shown by competition assays and supershift assays, respectively. B, MC3T3-E1 cells transiently transfected with wild type (wild) or mutated OC promoter constructs (mut; carrying a mutation in the Runx2 binding sites) were treated with or without FGF2. Reporter activities were determined 16–24 h after transfection. C, Runx2-deficient cells (Runx2−/−), non-osteoblastic C2C12 cells, and osteoblastic MC3T3-E1 (MC) cells were transfected with the p6xOSE2-Luc reporter vector. After overnight culture in medium containing FBS at normal concentrations, the medium was replaced with BSA-supplemented serum-free media with or without FGF2, and the cells were cultured an additional 16–24 h. The cells were then lysed, and the luciferase activities were determined. D, Runx2−/− cells, MC3T3-E1 cells, and C2C12 cells were transfected with the p6xOSE2-Luc reporter vector together with vectors expressing the constitutively active FGFR2 mutant (FR2Y340H) or empty vector as a control. Cells were cultured and luciferase activities were determined. E, MC3T3-E1 cells were transiently co-transfected with the Runx2 expression vector, which expresses the p57 Runx2 product, or the empty expression vector (mock), together with the p6xOSE2-Luc reporter vector. After overnight culture, the cells were treated with or without FGF2. The cells were harvested after 16–24 h, and luciferase activities were determined. The luciferase activity was normalized by the protein concentrations in the respective lysates.

![Figure 3](image-url)  
**Fig. 3.** FGF2-induced Runx2 expression is regulated by the protein kinase C (PKC) pathway but not by the MAPK pathway. A and B, C2C12 cells were treated with FGF2 with or without calphostin C (Cal C) (A) or GF109203X (GF) pretreatment (B). The cells were harvested 3 h later, and Runx2 mRNA expression was determined by Northern blot analysis. C and D, C2C12 cells were treated with FGF2 with or without PD98059 (PD) (C) or SB203580 (SB) (D) pretreatment. The cells were harvested 3 h later, and Runx2 mRNA expression was determined by Northern blot analysis. MeSO was used as a vehicle. Ribosomal RNA (rRNA) is shown as control for equivalent loading of RNA.
**Discussion**

**Runx2 Is a Downstream Target of FGF/FGFR Signaling**—During the development of the vertebrate skeleton, FGF/FGFR signaling appears to be closely involved in the determination of the shape and size of the skeleton (37). The importance of these molecules in skeletal development is supported by the association of several FGFR mutations with dominantly inherited human skeletal disorders (9, 37, 38), as mutations in FGFR1, FGFR2, and FGFR3 are linked to several craniosynostosis related syndromes characterized by premature fusion of the cranial sutures. These observations indicate that FGF/FGFR signaling accelerates bone growth by inducing the rapid differentiation of osteoprogenitor cells to bone cells. However, little is known about the underlying molecular mechanisms and the downstream target genes of FGF/FGFR signaling. In this matter, Zhou et al. (21) recently provided an important clue when they showed that Runx2, an essential transcription factor involved in osteoblast differentiation, could be a downstream target of FGF/FGFR signaling. They revealed that the introduction of the Pro-250 → Arg mutant of FGFR1, which is a constitutively active form of FGFR1, resulted in craniosynos-
Luciferase activities were determined. Me2SO was used as vehicle.

The cells were harvested after 16–24 h, and luciferase activities were determined. Me2SO was used as vehicle.

Although the different FGF ligands are specific for particular receptors (40) and FGFR1 and FGFR2 play distinct differentiation- and proliferation-related roles in the developing skull (41), it appears that FGF2, FGF4, and FGF8 and FGFR1 and FGFR2 may play equivalent roles in stimulating Runx2 expression.

More recently, Tsuji et al. (42) published the opposite observation that FGF2 treatment transiently suppresses Runx2 expression in ROS17/2.8 cells. We show here, however, that FGF2 treatment up-regulates Runx2 mRNA levels in these cells. This discrepancy between their study and ours may be explained by differences in the experimental conditions, particularly the length of FGF2 treatment and the presence of FBS in the medium. We have already shown earlier that Runx2 expression is maintained by BMP2 treatment but that the expression levels fluctuate after the immediate increase (24). This may be due to the fact that there are multiple Runx2 binding sites in the Runx2 promoter and Runx2 expression is stringently autoregulated by its own protein (43). FGF2-stimulated Runx2 expression appears to be an early event, because it peaks within 3 h of the initiation of treatment, after which expression in the presence of continuous FGF2 treatment fluctuates (data not shown). Tsuji et al. (42) treated their cells with FGF2 for 6–24 h and thus may not be documenting the initial response, rather, they may be measuring the fluctuations that arise from the auto-feedback regulation of the gene product. Moreover, they did not exclude FBS from the medium when they were treating the cells with FGF2. FBS contains many growth factors and thus should be eliminated to clearly demonstrate the effect of adding growth factors.

FGF Signaling Activates the PKC Pathway, Which Elevates Runx2 Activity by Increasing Its Transcription—Runx2 controls the expression of osteoblast differentiation-related genes by binding to OSE2 sites in the promoters of target genes (14). Although it has been reported that transforming growth factor β1 and bone morphogenetic protein 2 both induce Runx2 gene expression and stimulate the binding of Runx2 to its recognition site (24, 23), it was unclear whether FGF2-stimulated Runx2 expression is associated with an increase in the DNA binding activity of Runx2. Our experiments reported here show that FGF/FGFR signaling does increase Runx2 binding to the Runx2-binding consensus sequence in the osteocalcin promoter and that this activates the osteocalcin promoter. That mutation of the Runx2 binding site eliminated the FGF2-mediated increase in Runx2 DNA binding and activation of the osteocalcin promoter strongly suggest that FGF/FGFR-stimulated Runx2 activity results mainly from increases in Runx2 transcription.

We next determined which signaling pathway is responsible for the FGF2-mediated increase in Runx2 expression. There is a wealth of information regarding the signals that stimulate Runx2 activity in osteoblast differentiation, especially those transmitted by the transforming growth factorβ superfamily (24, 44, 45), but as yet, nothing is known about the signal transduction mechanisms through which FGF/FGFR controls the expression of Runx2. FGF binding to FGFR is known to stimulate receptor dimerization, tyrosine phosphorylation, and the activation of multiple signal transduction pathways, including those involving Ras, mitogen-activated protein kinases (MAPks), extracellular signal-regulated kinases (Erks), src, and p38 MAPks, phospholipase C, and protein kinase C (PKC) (46–48). We used specific inhibitors to investigate the role of some of these pathways in the FGF2-mediated increase of Runx2 expression. We found that, although FGF/FGFR signaling activates Erk1/2 and p38 MAPk in osteoblasts (10–12), inhibition of MEK1/2 or p38 MAPk did not block FGF2 stimulation of Runx2 expression in osteoblast-like MC3T3-E1 or non-osteoblast C2C12 cells (Fig. 3, C and D). However, the inhibition of the PKC pathway by selective and potent PKC inhibitors (calphostin C and GF109203X) and upstream phospholipase C-γ inhibitor (U73122, data not shown) dramatically decreased FGF2-stimulated Runx2 mRNA levels in MC3T3-E1 and C2C12 cells (Fig. 3, A and B). Because the Runx2 protein levels in FGF2-stimulated calphostin C-treated cells correlated fairly well with the mRNA levels (Fig. 4C), it appears that the PKC pathway activated by the FGF/FGFR signal acts to modulate Runx2 activity by up-regulating its transcription.

The PKCs have been classified into three groups based upon their ability to be activated by Ca2+ and diacylglycerol (DAG). The classic PKCs are activated by both Ca2+ and DAG and include the α, βI, βII, and γ isoforms. The Ca2+-independent but DAG-dependent isoforms (θ, δ, ε, and ρ) comprise the novel PKCs. Finally, the atypical PKCs, ζ, η, θ, and μ, are both Ca2+- and DAG-independent. Thus, it is difficult to reveal the role of each PKC isoform in this regulation. However, two lines of evidence point to the involvement of PKCδ in FGF2-stimulated Runx2 regulation. One is that rottlerin, a specific inhibitor of the δ subtype, suppressed the FGF2-stimulated Runx2 expression and its transcriptional activity (Fig. 5, A and B). The other is that the overexpression of the inactive PKCδ in C2C12 or MC3T3-E1 cells, which express PKCδ subtype (49), abolished the increase in Runx2 expression and 6xOSE2-Luc promoter activity by FGF2 (Fig. 5, C and D). Recent studies showed that FGF2 stimulated PKCδ activation in neuronal cells and pituitary tumor cells (50–52). Collectively, these results strongly indicate that PKCδ is a key mediator of FGF2-stimulated Runx2 regulation. Although we’ve shown the role of PKCδ in FGF2-stimulated Runx2 expression, we could not completely rule out the involvement of other PKC isoforms, because the treatment of rottlerin or the transfection of PKCδ-KR could not completely reversed the FGF2-stimulated Runx2 transcriptional activity (Fig. 5, B and D).

PKC Activation by FGF/FGFR Signaling Also Stimulates Runx2 Activity by a Post-translational Mechanism—Several lines of evidence indicate that the stimulation of the Runx2 expression would be the main component of the FGF2-stimu-
lated Runx2 transcriptional activity; first, the activation of FGF/FGFR signaling could not stimulated Runx2 transcriptional activity in Runx2–/– cells (Fig. 2, C and D). Second, forced expression of Runx2 is sufficient to increase Runx2 transcriptional activity even in the absence of FGF2 treatment (Fig. 2E). Third, the mRNA or protein induction level by FGF2 was quite well correlated with Runx2 transcriptional activity (Fig. 4). Moreover, there was a minor discrepancy between the protein level of Runx2 and its binding activity, because, although the PKC inhibitor reversed the FGF2-stimulated Runx2 protein level only to its basal level, it completely abolished the DNA binding activity and transactivating function of the Runx2 protein (Fig. 4, A and C). Despite this, there was a good correlation between Runx2 mRNA and protein levels. Thus, there must be an additional regulatory mechanism through which the PKC pathway controls the activity of Runx2 protein. To confirm this notion, we used Runx2–/– cells, because these cells cannot express endogenous Runx2 protein in response to FGF2 signaling. FGF2 treatment up-regulated the transactivating activity of the exogenously expressed Runx2 and the PKC inhibitor reversed this (Fig. 6). Thus, although the PKC pathway up-regulates Runx2 activity largely by elevating Runx2 expression, it also increases the transactivating function of Runx2 protein by an as yet undetermined post-translational modification.

Thus, our data show that the PKC pathway is involved in regulating both FGF/FGFR-stimulated Runx2 expression and the transcriptional activity of the Runx2 protein. Furthermore, MAPK pathways are not involved in Runx2 expression. However, we cannot completely rule out the possibility that MAPKs participate in the post-translational regulation of Runx2. Two reports indicate that the MAPK pathway regulates Runx2 activity selectively and rapidly blocks the ERK pathway by an as yet undetermined post-translational modification.

References—The FGF mutant found in patients with Crouzon syndrome was generously provided by Dr. Daniel Donoghue, Department of Chemistry and Biochemistry, University of California, San Diego, CA. OC208-CAT and OC208-CAT Runx mutant reporter constructs and Runx2-type II expression vector were provided by Drs. Gary S. Stein and Jane B. Lian, Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA. Dominant negative PKCδ expression vector (PRCO-KR) was generously provided by Dr. Jae-Won Soh, Department of Biochemistry & Molecular Biophysics and Herbert Irving Comprehensive Cancer Center, Columbia University, NY.
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