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PROSPECTS

Regulation of the Osteoblast-Specific Transcription Factor, Runx2: Responsiveness to Multiple Signal Transduction Pathways

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Abstract The Cbfa1/Runx2 is an important transcription factor necessary for osteoblast differentiation and bone formation. However, the signaling pathways regulating Runx2 activity are just beginning to be understood. Inconsistencies between Runx2 mRNA or protein levels and its transcriptional activity suggests that posttranslational modification and/or protein-protein interactions may regulate this factor. Runx2 can be phosphorylated and activated by the mitogen-activated protein kinase (MAPK) pathway. This pathway can be stimulated by a variety of signals including those initiated by extracellular matrix (ECM), osteogenic growth factors like bone morphogenic proteins (BMPs) and fibroblast growth factor-2 (FGF-2), mechanical loading and hormones such as parathyroid hormone (PTH). Protein kinase A (PKA) may also phosphorylate/activate Runx2 under certain conditions. In addition, Runx2 activity is enhanced by protein-protein interactions as are seen with PTH-induced Runx2/AP-1 and BMP-mediated Runx2/Smads interactions. Mechanisms for interaction with Runx2 are complex including binding of distinct components such as AP-1 factors and Smads proteins to separate DNA regions in target gene promoters and direct physical interactions between Runx2 and AP-1/Smad factors. Post-translational modifications such as phosphorylation may influence interactions between Runx2 and other nuclear factors. These findings suggest that Runx2 plays a central role in coordinating multiple signals involved in osteoblast differentiation. J. Cell. Biochem. 88: 446–454, 2003.

Key words: Runx2; MAPK; PKA; signaling pathways; osteoblast

Cbfa1/Runx2 is a bone-related transcription factor homologous to the Drosophila protein, *Runt* [Ducy et al., 2000]. This protein is essential for the differentiation of osteoblasts from mesenchymal precursors and bone formation as homozygous *Cbfa1*$^{-/-}$ mice show a complete lack of functional osteoblasts and are devoid of mineralized bone or hypertrophic cartilage [Otto et al., 1997]. Mutations in the Cbfa1 locus in humans cause cleidocranial dysplasia, an autosomal dominant disease characterized by the absence of clavicles, open fontanelles, supernumerary teeth and short stature [Mundlos et al., 1997]. Runx2 can directly stimulate transcription of osteoblast-related genes such as those encoding osteocalcin (OCN), type I collagen, osteopontin (OPN) and collagenase 3 by binding to specific enhancer regions containing the core sequence, PuCCPuCA [Ducy et al., 1997; Selvamurugan et al., 1998; Kern et al., 2000]. Beyond this, the molecular mechanism of Runx2 action is unknown.

Although Runx2 is expressed exclusively in mineralized tissues and their precursors, in many cases there is a poor correlation between actual Runx2 mRNA or protein levels and the expression of osteoblast-related genes. Thus, during development Runx2 expression precedes osteoblast differentiation and OCN expression by several days [Ducy et al., 1997]. Also, in several osteoblast cell culture systems,

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Runx2 protein levels are not well correlated with expression of its target genes. For example, cultures of primary osteoblasts or of the MC3T3-E1 preosteoblast cell line do not exhibit major changes in Runx2 protein during in vitro differentiation even though expression of osteoblast marker genes like OCN, bone sialoprotein and alkaline phosphatase is dramatically increased [Xiao et al., 1998]. Likewise, although both TGF-β and BMP2 dramatically increase Runx2 expression in C2C12 myoblasts, only BMP treatment can induce osteoblast-specific gene expression [Lee et al., 2000]. Taken together, these studies indicate that Runx2-dependent transcription is not simply regulated by levels of the Runx2 protein. Rather, they imply that this transcription factor is regulated either by post-translational modification or by protein-protein interactions.

This article will present five representative examples of Runx2 regulation using studies from the authors' laboratory and the current literature. Specifically, we will discuss Runx2 activation by: (i) binding of the extracellular matrix (ECM) to cell surface integrins; (ii) FGF2; (iii) mechanical loading; (iv) PTH; and (v) BMPs. These examples, while not covering all potential types of Runx2 regulation, will give the reader an appreciation of the breadth of signaling pathways that can control this important transcription factor.

ECM-DEPENDENT ACTIVATION OF RUNX2-DEPENDENT TRANSCRIPTION

Osteoblasts must establish a type I collagen-containing ECM before they can differentiate and express osteoblast-related genes such as those encoding OCN, bone sialoprotein, alkaline phosphatase and the parathyroid hormone/parathyroid hormone-related protein receptor and, ultimately, mineralize [for review, see Franceschi, 1999]. The ECM signals to the differentiating preosteoblast by binding to β1 subunit-containing integrins (α2β1 and, possibly, α1β1) [Takeuchi et al., 1997; Xiao et al., 1998; Zimmerman et al., 2000]. Disruption of integrin signaling using either blocking antibodies or peptides that mimic the cell-binding domain of collagen completely blocks ECM-dependent differentiation. This observation is highly significant for understanding osteoblast metabolism since it is through integrins that cells sense their ECM environment and respond to changes in mechanical loading [Danen et al., 1998].

We showed that Runx2 at least in part mediates the response of preosteoblasts to matrix signals. Specifically, ECM production by murine MC3T3-E1 cells dramatically increases transcription of the OCN gene. This matrix response requires Runx2 and its cognate DNA binding site in the OCN promoter, osteoblast-specific element 2 (OSE2) [Xiao et al., 1997]. Interestingly, this Runx2-dependent increase in transcriptional activity is not accompanied by a significant change in Runx2 mRNA or protein although large increases are seen in the in vitro binding of Runx2 to OSE2 DNA as measured by gel retardation assays [Xiao et al., 1998].

As one of the primary transducers of integrin signals to the cell nucleus, the MEK/ERK branch of the mitogen-activated protein kinase (MAPK) pathway provides a plausible link between cell surface integrin activation and subsequent stimulation of Runx2-dependent transcription. We recently showed that U0126, a specific inhibitor of ERK1/2 phosphorylation by MEK, rapidly and specifically inhibits both ERK phosphorylation and ECM-dependent induction of the OCN gene. Similarly, BMP actions in osteoblasts also require matrix signals and can be blocked by U0126 [Xiao et al., 2002a].

In separate studies, we showed that over expression of a constitutively active MEK1 mutant induces both endogenous OCN mRNA and promoter activity and, further, that this response requires Runx2 and an intact OSE2 sequence. Finally, Runx2 phosphorylation increased after transfection of cells with constitutively active MEK1, the kinase immediately before ERK1/2 in the MAPK cascade [Xiao et al., 2000], thus providing evidence that a MAPK-dependent phosphorylation cascade regulates Runx2 activity. Although the specific amino acid residues in Runx2 necessary for MAPK responsiveness and phosphorylation have not been identified, we know that they reside in a 270 amino acid proline/serine/threonine-rich region (P/S/T domain) in the C-terminal portion of the molecule [Xiao et al., 2000; Franceschi et al., 2002].

In summary, Osteoblasts must be in contact with a collagen-containing ECM and bind to this matrix via interactions between type I collagen and specific β_1 integrins. Integrin liganding then activates MAPK and related pathways, which transduce signals to the nucleus. Finally, Runx2 is phosphorylated and activated by

MAPK, thereby allowing it to stimulate osteoblast differentiation by increasing transcription of osteoblast marker genes such as OCN.

FGF2 ACTIVATES RUNX2 VIA THE MAPK PATHWAY

FGF2 is an important in vivo regulator of skeletal development and growth. Intermittent FGF2 administration can restore bone mass in the overiectomized rat, a well-established model for postmenopausal bone loss [Liang et al., 1999]. Overexpression of FGF-2 in transgenic mice causes premature mineralization, achondroplasia and shortening of long bones while disruption of the *FGF-2* gene leads to decreased bone mass and bone formation [Coffin et al., 1995; Montero et al., 2000]. Furthermore, activating mutations in FGF receptors are associated with a series of craniosynostosis syndromes characterized by accelerated intramembranous bone formation in calvarial sutures. Of particular interest, activating mutations in FGFR1 up-regulate Runx2 and enhance differentiation of calvarial osteoblasts [Zhou et al., 2000]. FGF2 can also stimulate osteocalcin gene expression in MC3T3-E1 preosteoblast cells [Boudreaux and Towler, 1996].

Since a major route for FGF receptor signaling involves activation of the MEK/ERK branch of the MAP kinase pathway [Nugent and Iozzo, 2000], we initiated a series of studies to examine whether FGF2 induction of the osteocalcin gene required MAPK activity and Runx2 phosphorylation [Xiao et al., 2002b]. Initial studies demonstrated that FGF2 could rapidly induce ERK phosphorylation and stimulate OCN mRNA in MC3T3-E1 cells and in bone marrow stromal cells. FGF2 also stimulated activity of a 1.3 kb OCN promoter-luciferase reporter gene and this stimulation could be blocked by the MEK/ERK inhibitor, U0126. This stimulation was only seen in cells containing wild type Runx2 (a P/S/T domain mutant was unresponsive) and also required an intact Runx2 binding site in the OCN promoter. Metabolic labeling with [^{32}P]-orthophosphate showed that the level of Runx2 phosphorylation is increased by FGF2 treatment and that this response is also prevented by U0126.

Others have shown that FGF2 can also activate OCN transcription through an AP-1-like site that is immediately 5′ to the Runx2 binding site described above [Boudreaux and Towler, 1996]. The nuclear factor/s binding this site are currently unknown although their DNA binding activity increases with FGF2 treatment. Of further interest, the FGF2 response is synergistically stimulated by the PKA pathway activator, forskolin, which is known to increase the activity of AP-1-related nuclear factors such as c-Fos and c-Jun. Taken together, these results raise the intriguing possibility that cooperative interactions take place between Runx2 and AP-1 like factors (see below).

FGF2 has also been reported to stimulate the transcriptional activity of other osteoblast-related genes such as those encoding bone sialoprotein and interstitial collagenase (matrix metalloproteinase 1) although it is not known whether Runx2 is involved in either of these responses [Newberry et al., 1997; Shimizu-Sasaki et al., 2001]. Of interest, AP-1-like sites as well as MAP kinase activities were implicated in the regulation of both genes.

RUNX2 IS A TARGET OF MECHANOTRANSDUCTION

It is well established that mechanical loading plays an important role in the regulation of bone homeostasis and skeletal morphology during development and in postnatal life where it increases bone density and strength. In contrast, skeletal unloading in humans and rats, as seen during space flight, is associated with bone loss and compromised bone mechanical properties. Mechanical stimulation has also been examined in a variety of cells in vitro including epithelial cells, fibroblast, chondrocytes, and osteoblasts [for review, see Banes et al., 1995]. Mechanically strained osteoblasts express increased levels of osteopontin (OPN), OCN, and collagen I/ III mRNA [Harter et al., 1995; Carvalho et al., 1998].

Although the importance of mechanical loading in the development and maintenance of bone integrity is undisputed, the mechanisms underlying mechanotransduction through which osteoblasts sense and convert mechanical stimuli into cellular responses are largely unknown. Of particular interest, the MAP kinase pathway is one of the principle signal transduction cascades to be associated with mechanotransduction. Integrins, which connect the cytoskeleton to the extracellular matrix and mediate a variety of signaling cascades, may transduce mechanical stimuli into biochemical signals.

For example, MacKenna et al. showed that mechanical stretch activated ERK2 and JNK1, but not p38, in rat cardiac fibroblasts and that this activation required the presence of two integrins, $\alpha4\beta1$ and a non-$\alpha5\beta1$ integrin, as well as extracellular matrix (fibronectin) [MacKenna, 1998]. Similarly, application of mechanical force to osteoblasts specifically through $\alpha2$ and $\beta1$-containing integrins induces MAPK activation [Schmidt et al., 1998].

Two recent studies established an important link between Runx2 and mechanotransduction. Ziros and coworkers showed that Runx2 may act as a target for mechanical signals in human periodontal ligament (hPDL) cells (i.e., osteoblast-like cells which can differentiate toward osteoblasts in response to a variety of extracellular stimuli.) [Ziros et al., 2002]. Specifically, low level continuous mechanical stretching of hPDL cells dramatically increased binding of Runx2 to OSE2 DNA in gel retardation mobility shift assays, although a slight increase in Runx2 mRNA or protein was also observed. This stimulation was detected after as little as 30 min of stretching, peaked after 6 h, and lasted for at least 12 h. ERK1/2 phosphorylation was activated in a time-dependent manner in mechanically stretched hPDL cells and was well-correlated with the increase in Runx2 binding activity. Furthermore, the stretch-induced increase in Runx2 DNA binding activity was completely abolished by U0126, a specific inhibitor of ERK1/2 activation. Of particular interest, stretch-activated ERK physically interacted with Runx2 and could phosphorylate this transcription factor in vitro. In separate studies, Wang et al. provided evidence that both ERK activation and Runx2 phosphorylation are required for mechanical signaling in human and rat bone marrow stromal cells [Wang et al., 2002]. They showed that extracorporeal shock wave (ESW), an alternative non-invasive method for the promotion of bone growth and tendon repair, promoted stromal cell proliferation and differentiation to osteoblasts. Specifically, optimal ESW treatment of bone marrow stromal cells at 0.16 mJ/nm^2 for 500 impulses increased [^{3}H]-thymidine incoporation into DNA, alkaline phosphatase activity, OCN gene expression, and bone nodule formation. Of particular interest, ESW dramatically stimulated ERK-dependent Runx2 phosphorylation although it did not change the Runx2 protein levels. Thus, mechanical force may regulate osteoblast proliferation and differentiation as well as bone formation through MAPK-dependent Runx2 phosphorylation.

INVOLVEMENT OF RUNX2 IN PTH SIGNALING

Parathyroid hormone (PTH) is an important regulator of calcium homeostasis and has both anabolic and catabolic effects on osteoblasts and bone in vitro and in vivo. PTH functions by binding to the G-protein-coupled PTH-1 receptor (PTH1R). Binding of PTH to PTH1R activates two well-defined signal transduction pathways: (1) the protein kinase A (PKA) pathway in which stimulatory G-alpha proteins ($G\alpha_s$) activate adenylate cyclase with subsequent production of cAMP and activation of PKA; (2) the protein kinase C (PKC) pathway where $G\alpha_q$ activates phopholipase Cβ with subsequent formation of diacylglycerol, PKC activation and formation of 1,4,5-inositol trisphosphate. This later compound stimulates a rise in intracellular free Ca^{2+} and related signaling events. PKA and PKC pathways can regulate transcription factors such as cAMP response element binding proteins (CREBs), AP-1 family members [for review, see Karaplis and Goltzman, 2000] as well as Runx2 (below).

Studies on the collagenase 3 gene (*MMP13*) have been particularly informative for understanding how PTH regulates gene expression in bone. Systematic analysis of the MMP13 promoter showed that two conserved enhancer sequences are necessary for PTH responsiveness; a Runx2 binding site and an AP-1 site [Selvamurugan et al., 1998; Hess et al., 2001; D'Alonzo et al., 2002]. Mutations in either site that abrogated the binding of Runx2 or c-Fos/c-Jun, respectively, abolished PTH stimulation of promoter activity. Furthermore, the helical phasing between these two sites appears to be critical for the PTH response since insertion of additional bases interfered with promoter activation. Overexpression of both Runx2 and AP-1 (c-Fos and c-Jun) proteins increased PTH responsiveness suggesting that these factors cooperatively interact. Indeed, immunoprecipitation experiments provided direct evidence for a physical interaction between Runx2 and c-Fos/c-Jun in intact cells. Furthermore this interaction requires the runt domain of Runx2 and the leucine zipper domain of the two AP-1 factors [Hess et al., 2001; D'Alonzo et al., 2002].

Since PTH does not change Runx2 protein levels, posttranslational modification of this factor in the signaling pathway for PTH-mediated collagenase gene expression was suggested [Selvamurugan et al., 2000]. Analysis of mutations in the P/S/T domain of Runx2 fused to the DNA binding domain of the Gal4 yeast transcription factor localized a PTH responsive region to a PKA consensus phosphorylation site in the activation domain 3 (AD3) region of Runx2. This same site could be phosphorylated by purified PKA in vitro, although it is not yet known if it is also phosphorylated in intact cells. These results suggest that PTH stimulates the collagenase 3 promoter by a PKA-dependent pathway that phosphorylates Runx2 and up-regulates c-Fos and c-Jun via phosphorylation of CREB. It is not currently known whether phosphorylation affects interactions between Runx2 and c-Fos/c-Jun. This pathway is to be distinguished from the regulation of Runx2 by ECM or FGF2 which is mediated by the MEK/ERK MAP kinase pathway.

RUNX2 IS A TARGET OF BMP/SMADs SIGNALING

The bone morphogenetic proteins (BMPs) are the best-described inducers of osteoblast and chondrocyte differentiation, as well as bone and cartilage formation in vivo. All BMPs function by interacting with unique combinations of type I and type II BMP receptors. Signals initiated by binding of BMPs to their receptors are transduced using specific Smad proteins (i.e. the receptor-regulated Smads 1, 5, and 8 (R-Smads) and the common partner protein, Smad4 [Baker and Harland, 1997]). BMP regulation of osteoblast gene expression is complex, involving direct interactions of R-Smad-Smad4 complexes with enhancer sequences on target genes (Smad binding elements or SBEs), binding of Smads to other nuclear factors as well as up-regulation of separate transcription factors including Runx2 and Osterix (Osx) [Ducy et al., 1997; Watanabe and Whitman, 1999; Nakashima et al., 2002].

BMP treatment or overexpression of Runx2 have both been reported to induce osteoblast-specific gene expression in mesenchymal cells [Ducy et al., 1997; Xiao et al., 1999]. Since BMPs can increase Runx2 expression, one possible explanation for how BMPs act in bone would be to assume that Runx2 mediates their effects. Alternatively, BMPs may also activate other signaling pathways that, together with Runx2, stimulate gene expression. For example, consensus SREs in the promoter regions of target genes could bind the R-Smad–Smad4 complex and this complex could cooperatively interact with Runx2 bound to another region of the promoter. In fact, there is some precedent for this concept; TGF-β stimulation of the IgCα promoter is known to require interactions between Cbfa3, another *Runt*-related protein homologous to Runx2, and a protein complex containing Smad3/Smad4 bound to a separate SBE in this promoter [Hanai et al., 1999].

To specifically test for cooperativity between Runx2 and BMP signaling in osteoblast gene expression, we examined effects of BMP overexpression in the presence or absence of Runx2. For these studies, recombinant adenoviruses engineered to overexpress BMPs 2, 4, or 7 or Runx2 under the control of a CMV promoter were used to transduce the pluripotent C3H10T1/2 mesenchymal cell line [Franceschi et al., 2002]. Cells transduced with each virus were assayed for induction of osteoblast markers (alkaline phosphatase activity-ALP and OCN mRNA). Our data showed individual BMPs or combinations of BMPs only modestly stimulated ALP activity. A slightly higher level of induction was observed in cells transduced with the Runx2 virus alone. In contrast, co-transduction of cells with BMP and Runx2 viruses dramatically increased ALP activity relative to activities observed with either virus alone. Similarly, cells transduced only with BMP2 virus showed a small stimulation in Runx2 and OCN mRNA at d 3 and 6 while transduction with the Runx2 virus induced a somewhat higher level of OCN mRNA at all times examined. However, combination of both viruses led to an approximately 10-fold increase in OCN mRNA levels. In separate experiments, we saw a similar degree of synergy when control or Runx2 transduced cells were treated with saturating levels of recombinant BMP2 protein.

These studies strongly support the view that Runx2 and BMP signals cooperatively interact to stimulate osteoblast gene expression although they do not explore the basis for this cooperation. Possible explanations for the observed cooperativity include direct interactions between Runx2 and R-Smads, modulation of either R-Smad or Runx2 transcriptional activity or regulation of BMP receptor activity.

Nevertheless, the observation that BMPs dramatically stimulate osteoblast gene expression even in cells already overexpressing Runx2 suggests that some aspects of BMP action cannot be explained simply by upregulation of Runx2.

Recently, the laboratory of Dr. Benoit de Crombrugghe's identified another osteoblast-specific transcription factor, Osterix (Osx), in BMP2-treated C2C12 myoblast [Nakashima et al., 2002]. Gene deletion studies showed that Osx, like Runx2, is essential for osteoblast differentiation and bone formation. However, Osx is clearly downstream from Runx2 since it is not detected in $Runx2^{-/-}$ mice. Current evidence supports the view that Runx2 is required for early mesenchymal commitment to chondro/osteoblastic lineages as well as expression of phenotypic markers in differentiated cells while Osx functions to further define the differentiation potential of chondro/osteogenic precursors toward the osteoblast phenotype [Inada et al., 1999; Nakashima et al., 2002]. Consistent with this concept, Runx2 is expressed in condensed chondrogenic mesenchyme as early as embryonic day 12.5, well before osteoblasts or hypertrophic chondrocytes appear [Ducy et al., 1997]. Osx, in contrast, is absent from early chondrogenic cells, first appearing in differentiating chondrocytes and the perichondrium of E 13.5 embyros [Nakashima et al., 2002]. At later times, it is exclusively expressed in osteogenic cells. $Osx^{-/-}$ mice exhibit normal chondrocyte differentiation (i.e., undergo normal hypertrophy) while in $Runx2^{-/-}$ mice, neither osteoblasts nor hypertrophic chondrocytes are observed. Although we do not yet know whether Runx2 and Osx physically or functionally interact, Osx is clearly necessary for Runx2 transcriptional activity in vivo since the putative Runx2 target genes, osteocalcin, bone sialoprotein, osteopontin and Col1a1 are silent in $Osx^{-/-}$ animals, yet Runx2 is still expressed at normal levels [Nakashima et al., 2002]. Clearly, an important area for future studies will be to understand the relationship between these two factors in the control of gene expression in bone.

CONCLUSIONS

The five regulatory signals discussed in this article are shown in Figure 1 (i.e., those initiated by BMPs, ECM, FGF2, mechanical loading, or PTH/PTHrP). We believe that activation of Runx2 via phosphorylation is crucial for this factor to be transcriptionally active. Phosphorylation can be stimulated in several ways: (i) ECM binding to integrins on the cell surface activates focal adhesion kinase (FAK) and the MEK/ERK branch of the MAPK pathway; (ii) Activation of receptor tyrosine kinase (RTK) activity in the FGF2 receptor also activates the MEK/ERK pathway; (iii) Mechanical loading activates the MAPK pathway; (iv) The classic protein kinase A (PKA) pathway activated by the parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) receptor may also stimulate phosphorylation of Runx2 on sites distinct form those utilized by the MEK/ERK pathway. Alternatively, stimulation of the MAPK pathway via protein kinase C (PKC) is a potential route for cross-signaling from the PTH/PTHrP receptor via activation of Gq. The PKA pathway also up-regulates AP-1 related factors like c-Fos and c-Jun by phosphorylation of cAMP response element binding protein (CREBP). AP-1 factors regulate gene expression by binding to AP-1 sites in osteoblast-related genes as well as by interacting with Runx2. Finally, the BMP/Smad pathway controls Runx2 gene expression possibly by directly up-regulating the Runx2 gene. In addition, R-Smad-Smad4 heterodimers can directly interact with SBEs in regulatory regions of osteoblast-related genes as well as form complexes with Runx2. The function of Osx in this scheme remains to be determined, although it may play an important role in BMP action.

In our view, Runx2 can be considered a focal point for integration of a variety of signals affecting osteoblast activity. These signals include information about the extracellular matrix environment as detected through integrin-ECM interactions (i.e., Has an appropriate ECM been synthesized? Is the cell in contact with this ECM? Is the ECM experiencing mechanical loads?) and hormone/growth/differentiation factor levels in the extracellular milieu. (i.e., Are the combined signals from endocrine/juxtacrine/autocrine factors telling the osteoblast or preosteoblast to grow or differentiate? Lay down new bone matrix or resorb existing matrix?) These disparate signals can affect Runx2 activity by altering transcription factor levels as is the case for BMP induction of Runx2, phosphorylation state (mediated by MAPK as well as

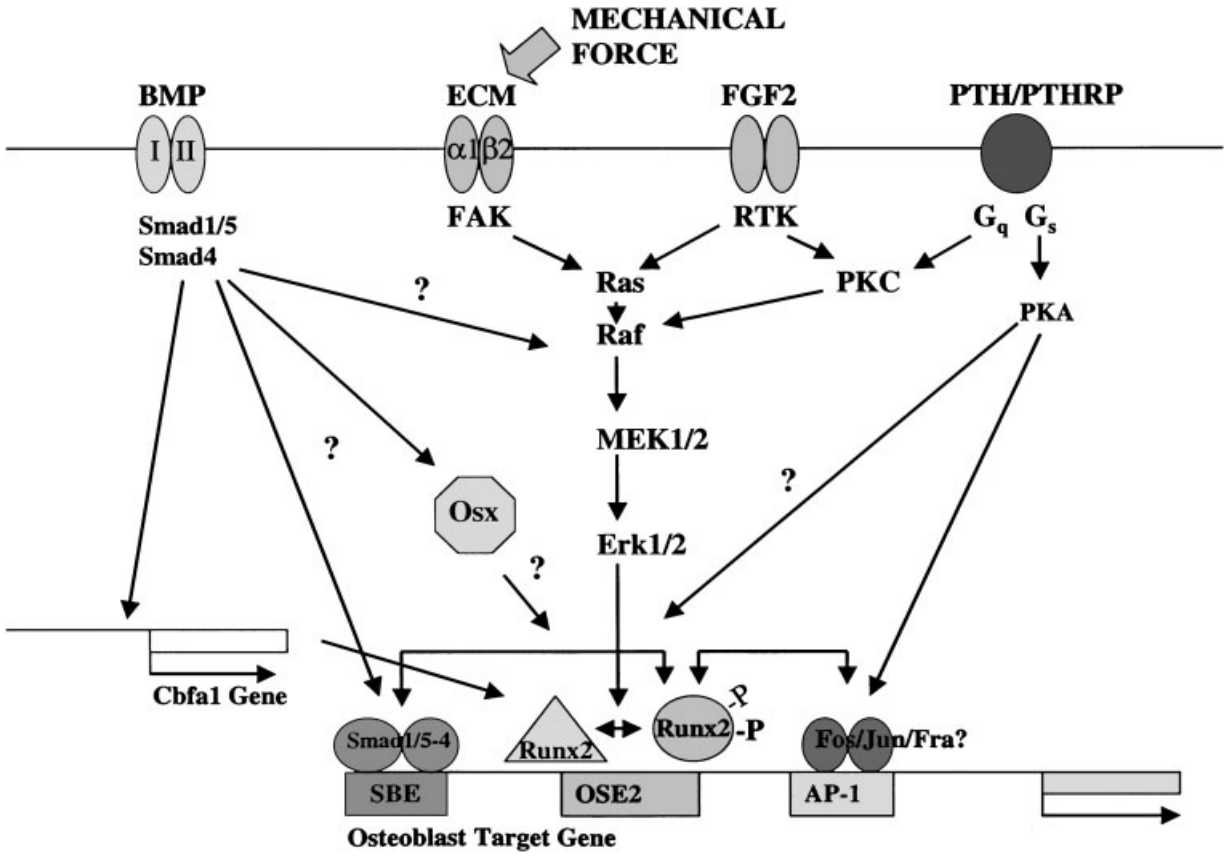

Fig. 1. Overview of signal transduction pathways affecting Runx2 activity. Refer to text for explanation.

possibly PKA-dependent pathways) or interactions between Runx2 and other transcription factors such as Smads and AP1-related factors and Osx.

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