BMP-2 and Insulin-like Growth Factor-I Mediate Osterix (Osx) Expression in Human Mesenchymal Stem Cells via the MAPK and Protein Kinase D Signaling Pathways*

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Genetic studies place the transcription factor Osterix (Osx) downstream of Runx2, but limited information is available about Osx regulation during osteoblastic differentiation. An important role for bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor-1 (IGF-I) on Osx expression and the requirement for p38 for the BMP-2-mediated effect was reported previously by our group. In this study, we continued to investigate the molecular mechanisms by which BMP-2 and IGF-1 regulate Osx expression during osteoblast lineage progression. BMP-2-induced Osx expression required all three MAPK components (Erk, p38, and JNK), whereas BMP-2 required p38 and JNK signaling. As a common mediator of growth factor signaling, we also investigated the involvement of protein kinase C/D (PKC/D) signaling. BMP-2- and IGF-I-mediated Osx expression was blocked in response to a PKD inhibitor. A selective inhibitor of conventional PKCs had no effect on the BMP-2-mediated Osx expression. BMP-2 and IGF-1 induced a selective phosphorylation of PKD, and PKD was required for mineralization. PKC/D and MAPK signaling also mediate Runx2 activity. Therefore, to document the implication for Runx2 in Osx regulation, we blocked Runx2 activity using a dominant negative Runx2 construct and an ubiquitination mediator for Runx2 degradation. We showed that blocking Runx2 activity inhibited the BMP-2-mediated induction of Osx. These studies implicated that multiple signaling pathways mediate Osx, a critical gene for osteoblast differentiation and bone formation. In addition to Runx2, other signaling components may be necessary to regulate Osx during osteoblast lineage progression.
implicate that alternative signaling pathways may act independent of, or in parallel to, Runx2 during osteoblast lineage progression (4, 21–23). Therefore, we investigated whether Otx expression in mesenchymal progenitor cells required Runx2 in the absence or presence of the BMP-2 stimuli.

Previous studies implicate that during osteoblast differentiation, the MAPK-induced signal was downstream of Ras, a substrate of growth factor signaling, which cross-talks to PKC/D signaling (5, 10, 24). As a common mediator of BMP-2 and IGF-I signaling, we wanted to delineate the role of PKC/D signaling in the transcriptional regulation of Otx. Stress-activated MAPK component JNK was also studied as a mediator of the growth factor-induced effect on osteoblast lineage progression. Our results suggested that BMP-2- and IGF-I-induced Otx up-regulation proceeds via PKD. Further, osteoblast differentiation was arrested due to PKD inhibition as the cells did not mineralize and Otx expression was abolished. The synergistic interactions displayed by BMP-2 and IGF-I were also disrupted due to the inhibition of PKD. Additionally, Runx2 was required but not sufficient for the BMP-2-mediated Otx induction. This is the first study to show that Otx regulation by BMP-2 and IGF-I proceeds via PKD and MAPK signaling in hMSC. Multiple signaling pathways converged on Otx regulation to mediate osteoblast lineage progression.

**EXPERIMENTAL PROCEDURES**

**Materials—**Bone marrow derived hMSC were obtained from BioWhittaker, Inc. (Walkersville, MD) and maintained according to the manufacturer’s instructions. hMSC were certified positive for adipogenic, chondrogenic, and osteogenic potential assays by BioWhittaker, Inc. Mouse progenitor C3H10T1/2 cells were from American Tissue Culture Collection (ATCC) (Manassas, VA). All MAPK and PKC inhibitors were obtained from Calbiochem-Novabiochem. BMP-2 was from R&D Systems (Minneapolis, MN), and IGF-I was from Chiron (Emeryville, CA). Monoclonal anti-pJNK, polyclonal anti-JNK, anti-pPKD (Ser916 and Ser854), anti-PKD, anti-phosphop38/44, anti-p38 and Western protein assay kits and protease inhibitors were from Pierce. Effectene transfection reagents according to manufacturer’s instructions and exposed to Biomax Kodak film.

**Cell Culture—**hMSC and C3H10T1/2 cells were plated in 35-mm culture wells and grown in basal media approximately to 70% confluency. The basal medium for C3H10T1/2 cells was Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Basal medium for hMSC was complete medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Go6976 (PKD inhibitor), or Go6983 (PKC inhibitor) as indicated under the Materials and Methods section. Our results suggested that BMP-2- and IGF-I-induced Otx up-regulation proceeds via PKD. Further, osteoblast differentiation was arrested due to PKD inhibition as the cells did not mineralize and Otx expression was abolished. The synergistic interactions displayed by BMP-2 and IGF-I were also disrupted due to the inhibition of PKD. Additionally, Runx2 was required but not sufficient for the BMP-2-mediated Otx induction. This is the first study to show that Otx regulation by BMP-2 and IGF-I proceeds via PKD and MAPK signaling in hMSC. Multiple signaling pathways converged on Otx regulation to mediate osteoblast lineage progression.

**RESULTS**

**RNA Extraction and Quantification—**Total RNA was extracted using the RNeasy kit and DNase I treatment according to the manufacturer’s protocol. The amount of extracted RNA was quantified using the Ribogreen kit and the manufacturer’s instructions and exposed to Biomax Kodak film.

**Cell Transfections—**C3H10T1/2 cells were plated in 12-well plates at an initial density of 4 × 10⁵ cells/well in complete medium the day before transfection. The cells were transfected with 1 μg of the pCMV5B(FLAG)Smurf1 plasmid pRL-Luc along with empty vector or AMLI/ETO or Smurf1 plasmid at various doses (as indicated under “Results”) using Effectene transfection reagent according to manufacturer’s instructions. In each case, 10 ng of pRL-Luc co-transfected to provide a means of normalizing the assays for transfection efficiency. Cell lysates were collected in lysis buffer 48 h after transfection, and Renilla and luciferase activities were measured with the use of a Dual-reporter luciferase assay system in a Tecan SpectraFluoPlus plate reader (Tecan, Switzerland), and standardized for transfection efficiency.

**Statistical Analysis—**For quantitative assays, the coefficient of variation was calculated from three assay replicates and did not exceed 3% for all treatment groups. Intra-day variation did not exceed 5%. Treatment groups within experiments were performed in triplicate and reported as means ± S.E. Each experiment was repeated three times. Statistical analysis was performed using Statview software (Cary, NC) to determine significance among treatment groups. Analysis of variance followed by Tukey-Kramer post hoc test was performed for the experiments. Statistical significance was established at p < 0.05.

**RESULTS**

Within 1 h of treatment, rhBMP-2 activated PKD and JNK in hMSC (Fig. 1, A and B). For PKD activation, we used two different PKD antibodies to detect Ser⁷⁴⁷/⁷⁴⁸ and Ser ⁹¹⁶ phosphorylation in hMSC. In response to BMP-2, Ser⁹¹⁶ was phosphorylated; however, no change in phosphorylation of Ser⁷⁴⁷/⁷⁴⁸ was detected. We previously reported that MAPK components Erk and p38 were also activated in response to BMP-2 stimuli (5). To study the implication of stress-activated MAPK component JNK and protein kinase D (PKC/D), we used selective inhibitors. PKC inhibitors Go6976 and Go6983 have different PKC inhibitor specificities; although Go6976 inhibits Ca²⁺-dependent isoforms and PKD, Go6983 does not inhibit PKD and serves as a general PKC inhibitor (6, 29).
with Runx2 and mediate its degradation via the 26S proteasome (27, 31). Therefore, to determine whether Smurf1-mediated Runx2 degradation causes functional changes in Runx2, we examined the ability of Runx2 to activate a specific reporter gene, 6XOSE2-Luc, in C3H10T1/2 cells. The pGL3-6XOSE2-Luc construct was transiently co-transfected into C3H10T1/2 cells with increasing amounts of Smurf1 expression plasmid. Reporter activity that was induced with endogenous Runx2 activity was reduced (Fig. 5A), and degradation of Runx2 protein was confirmed with Western blot analysis (Fig. 5B). Smurf1 mediated Runx2 degradation and caused a decrease in Runx2 activity.

We confirmed our results using a dominant negative Runx2 construct to block Runx2 activity. AML1/ETO does not contain the transactivation domain and the C-terminal domain of Runx2 (26, 32). It contains only the DNA binding domain of Runx2 and acts in a dominant negative manner. Runx2 activity was also reduced due to overexpression of AML1/ETO in C3H10T1/2 cells (Fig. 5A).

To determine the negative effects of AML1/ETO and Smurf1 on Runx2 activity, we transfected cells with empty vector or AML1/ETO or Smurf1 constructs in the presence or absence of BMP-2. Gene expression analysis indicated that Runx2 plays an important role for both basal and BMP-2-mediated-Osx expression in C3H10T1/2 cells (Fig. 6A). Both Smurf1 and AML1/ETO overexpressing cells exhibited Osx expression in the presence of BMP-2 treatment. This outcome indicated that Runx2 activity is not entirely blocked (leaky expression) or that additional signaling components were still active to induce Osx expression downstream of BMP-2 signaling. Alp expression was also inhibited due to blocking Runx2 activity with AML1/ETO or Smurf1 (Fig. 6B).

**DISCUSSION**

Skeletal development requires the integration of multiple signaling pathways, and understanding the multitude of cell signals remains as a challenge. Bone formation and remodeling require the careful orchestration of a number of signaling components, which in turn regulate the activities of osteoblast lineage-specific master genes and their cellular substrates. Runx2 and Osx are key transcription factors that control the osteoblast fate. Although current literature provides a broad assessment of the regulatory mechanisms that control Runx2, our knowledge of Osx regulation is limited. We previously reported that BMP-2 and IGF-I induce Osx in hMSC (5). The MAPK component p38 is required for BMP-2 induced effect, whereas Ras and one of its downstream effectors Erk were required for mineralization (5).

Ras proteins act as molecular switches downstream of tyrosine kinases (33). Ras-GTP-binding proteins activate Raf kinase activity (34, 35), in addition to phosphatidylinositol 3-kinase (36), ralGDS (37), and ATP6 (38, 39). Activated Raf initiates a signal transduction cascade that involves components of the MAPK family members. In addition to Ras, Raf protein kinase has also been reported to be activated via diacylglycerol-regulated PKC (24); PKC induces cross-talk between PKC, Raf, and MEK/ERK signaling pathways (40–42). The signaling cascades downstream of growth factors, mediated by PKC and MAPK, affect cellular substrates that regulate osteoblast proliferation and differentiation. Here, we presented our studies to elucidate the possible involvement of PKC and MAPK components in eliciting the BMP-2- and IGF-I-mediated effect on Osx transcriptional regulation in mesenchymal stem cells.

We showed that BMP-2 requires PKD, but not the conventional PKCs, to stimulate Osx expression during osteoblast lineage progression (Fig. 2A). As an upstream regulator of p38...
and JNK signaling (6), inhibition of PKC/D blocked BMP-2-mediated Alp expression (Fig. 2B). PKD was also required for mineralization of hMSC (Fig. 2C). Within 1 h of BMP-2 treatment, we detected phosphorylation of the Ser$^{916}$ residue of PKD (Fig. 1A); however, we were unable to detect any changes in the phosphorylation of Ser$^{744/748}$. A study by Lemonnier et al. (6) presented similar results in MC3T3.E1 cells. It has been reported that Ser$^{916}$ is activated in response to autophosphoryl-
ation of PKD and serves to monitor PKD catalytic activity (43). Additionally, Ser744 and Ser748 are the activating residues of the PKD catalytic domain (44). The same group of researchers have also reported that PKC phosphorylates these residues, leading to activation of PKD (45). In addition to PKD, the requirement for JNK in Osx expression is evident, as indicated by gene expression analysis (Fig. 2B). As an activator of Smad signaling, which cooperates with AP-1, BMP-2 signaling may require JNK to activate the AP-1 complex. AP-1-dependent transcription of target genes can be achieved by phosphorylation of the c-Jun transactivation by JNK (46–48). BMP-mediated activation of JNK and p38 proceeds via PKD in MC3T3.E1 cells (6), and a similar scenario may also be valid in hMSC.

MAPK and PKD signaling components may also mediate the IGF-I-induced effects on cell proliferation and differentiation. Although the IGF-I-mediated stimulation of Osx is not as dramatic as BMP-2, combined delivery of these growth factors gave a synergistic response in hMSC. Evidence from ST2 cells indicates that a decline of IGF-I may be necessary to permit terminal differentiation of mouse stromal cells;2 however, at an early step in proliferation-differentiation, IGF-I may still have a role as a positive regulator, perhaps via cross-talk with BMP-2 signaling. IGF-I activates MAPK and PKD within 1 h of treatment in hMSC (Fig. 3, A and B). We detected a decrease in Osx stimulation when hMSC were treated with inhibitors of MAPK (Erk, p38, and JNK) as well as PKC/D components (Fig. 4A). MAPK and PKC/D also mediate the IGF-I-induced effect in other cellular systems. PKC is thought to bind the CCAAT/enhancer-binding protein site within the promoter region of human IGF-I to mediate its expression (49). Further, IGF-I is reported to activate JNK in promoting the survival of T lymphocytes (50) and Erk1/2 and JNK in promyelocytic cell lines (18). IGF-I also activates PKC isoforms in several different processes (17, 51, 52); however, our study is a first in showing the activation of PKD in response to IGF-I stimulation during osteoblast differentiation of hMSC. The PKD activation mediated by IGF-I may not necessarily proceed independent of PKC activity (Fig. 4A), a point that will be the focus of future studies.

2 Personal communications, Dr. E. Gazzerro, Saint Francis Hospital, Hartford, CT.
In addition to MAPK and PKD, Runx2 mediates the BMP-2 signal and is regulated at different levels via MAPK and PKC. Runx2 is a critical gene for progression of osteoblast differentiation (28), and its association with Osx has been presented in genetic studies (1). Several studies implicate the presence of Runx2-independent mechanisms for ossification (21–23). These studies implicate that additional signaling pathways may act in parallel to, or independent of, Runx2 during osteoblast differentiation. Our studies in hMSC with the use of a protein translation inhibitor (cycloheximide) indicate that the BMP-2-mediated induction of Osx requires de novo protein synthesis even in the presence of Runx2 expression.3

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Here, we have also presented evidence for the requirement for Runx2 in mediating the BMP-2-induced effect on Osx expression. In agreement with studies by Zhao et al. (27, 53), we showed that Smurf1 blocks Runx2 and that Runx2 was required for BMP-2-mediated Osx expression (Fig. 6A). Smurf1 exhibits inhibitory effects on BMP signaling in cooperation with Smad6/7 in vivo (54). We confirmed our results with the use of a dominant negative Runx2 construct, which is reported to exhibit strong DNA binding activity but lack transactivation ability (32, 55). Interestingly, overexpression of the Runx2 inhibitory constructs significantly decreased, but did not abolish, Osx expression (Fig. 6A). This result indicated either that there was still leaky expression of Runx2 despite the presence of inhibitory factors or that additional factors (e.g. Dlx5) acting downstream of BMP-2 could induce Osx independent of the levels of Runx2 activity.

MAPK and PKC/D components may regulate Osx directly or indirectly via their effects on signaling components acting downstream of the growth factors. It remains to be determined whether the signaling molecules such as Dlx5, MAPK, and PKC/D regulate the Osx promoter. Milona et al. (56) have reported that there is an OSE2 element in the Sp7 regulatory region, so the Osx promoter may be a direct target of Runx2. Further analysis on the relationship between Runx2 and Osx will be the focus of future studies.

Growth factors and downstream signaling components may also exert their effects at the post-translational level. Although we and others have been unable to detect changes in Runx2 expression levels in response to IGF-I (5, 57), IGF-I may affect Runx2 or Osx activity at the post-translational level. It is possible that the BMP-2 mediated effect on Osx may require not only an adequate amount of Runx2 protein but also post-trans-
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