Cells have ingenious mechanisms for interpreting complex signals from their external microenvironment. Previously, we have shown that phosphophoryn (PP) regulates the expression of bone/dentin marker genes via the integrin/MAPK signaling pathway. This directs proliferation, differentiation, and apoptosis for proper tissue development and remodeling. During formation of mineralized tissues such as bone and dentin, the cell utilizes several intracellular signaling pathways representing an interconnected network of proteins with the goal to decipher cues from the ECM. We focused our current work on the role of the non-collagenous proteins of dentin such as phosphophoryn (PP) in cell signaling and differentiation. We have determined the involvement of the MAPK pathway in PP’s signaling (1). In the present study, our hypothesis is that the Smad pathway might be involved since many of the genes up-regulated by PP are critical to bone differentiation.

The Smad pathway is one such pathway important in signal interpretation from the ECM during osteogenesis/dentinogenesis. Smad proteins are intracellular signaling molecules that are activated by members of the transforming growth factor (TGF-β) family of growth factors which includes the bone morphogenetic proteins (BMPs). Smads are the initial responders to receptor activation of the TGF-β family and have been studied as transcriptional activators of cell differentiation. There are three types of Smads: receptor-regulated (R-Smads), common mediator (Co-Smads), and inhibitory (I-Smads) (2). Smad signaling initiates with ligand binding to type I and type II receptor serine/threonine kinases on the cell surface. The type II receptor phosphorylates the type I receptor which then phosphorylates the R-Smads (Smad1, Smad2, Smad3, Smad5, and Smad8) in the C-terminal MH2 domain of Smad1/2. Following phosphorylation of the R-Smads (Smad1, Smad5, and Smad8 primarily by BMPs and Smad2 and Smad3 primarily by TGF-β), heteromeric complexes with Smad4 are formed and translocate to the nucleus where they regulate transcription of target genes together with other nuclear cofactors. Several excellent reviews on Smad signaling are available in the literature and discuss the caveats and specifics of Smad signaling (2–7).

BMPs are potent morphogens that exhibit a variety of roles across tissue and cell types (8–15). Their functions have been studied in many organisms from Drosophila to humans and their role in development is highly specialized and critical. For example, during limb development, BMP-2, BMP-4, and BMP-7 are essential signals for apoptosis (16–18). BMPs are also important in centralization of the mesoderm in Xenopus (14). Conversely, neural differentiation and dorsal mesoderm formation is favored when BMP signals are attenuated by inhibitors such as Noggin (13, 14). BMPs are also potent osteoinductive factors (19, 20). BMP-2, which signals via Smad1, also activates MAPK components, Erk and p38 (21–23). The mode of receptor oligomerization has been shown to dictate which pathway BMP-2 activates (24). The Smad pathway can be influenced by factors other than TGF-β/BMP via the MAPK pathway (25, 26). Smad activity may be a point of convergence from complex interconnected signaling networks and could help interpret the diverse messages from the ECM into cell fate decisions (26).

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**Extracellular Matrix-mediated Signaling by Dentin Phosphophoryn Involves Activation of the Smad Pathway Independent of Bone Morphogenetic Protein**

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The ECM relays complex signals from the microenvironment to cells. This directs proliferation, differentiation, and apoptosis for proper tissue development and remodeling. Smads are intracellular signaling molecules that are activated by members of the transforming growth factor (TGF-β) family of growth factors which includes the bone morphogenetic proteins (BMPs). Smads are the initial responders to receptor activation of the TGF-β family and have been studied as transcriptional activators of cell differentiation. There are three types of Smads: receptor-regulated (R-Smads), common mediator (Co-Smads), and inhibitory (I-Smads) (2). Smad signaling initiates with ligand binding to type I and type II receptor serine/threonine kinases on the cell surface. The type II receptor phosphorylates the type I receptor which then phosphorylates the R-Smads (Smad1, Smad2, Smad3, Smad5, and Smad8) in the C-terminal MH2 domain of Smad1/2. Following phosphorylation of the R-Smads (Smad1, Smad5, and Smad8 primarily by BMPs and Smad2 and Smad3 primarily by TGF-β), heteromeric complexes with Smad4 are formed and translocate to the nucleus where they regulate transcription of target genes together with other nuclear cofactors. Several excellent reviews on Smad signaling are available in the literature and discuss the caveats and specifics of Smad signaling (2–7).

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We have recently reported that a non-collagenous protein, PP, has a role of the Smad pathway in human adult mesenchymal stem cells following treatment with recombinant PP (rPP). We observed that PP enhanced phosphorylation of Smad1 within 30 min and Smad1 translocation to the nucleus within 1 h. PP up-regulated the expression of Smad1 target genes, Smad6, Dlx5, and Runx2. The timing of PP activation of Smad1 implies this is a direct effect; however, we also investigated the possible involvement of bone morphogenetic proteins in PP stimulation of the Smad pathway. PP was shown to up-regulate Bmp-2 gene expression 12 h post-treatment with PP, which is much later than initial detection of Smad1 phosphorylation at 30 min. Furthermore, addition of Noggin did not block Smad1 phosphorylation by PP. We propose that PP could signal via the Smad pathway by either directly stimulating the phosphorylation of Smad1 via integrins or other mechanisms. These might include integrin/bone morphogenetic protein receptor interactions or involvement of PP with other growth factors leading to the modulation of intracellular signaling. It is noteworthy that a non-transforming growth factor-β family member activates the Smad pathway. The role of PP in regulating the Smad pathway raises very interesting questions regarding the role of PP during bone and tooth development.
Phosphophoryn Activates Smads

TABLE 1
Sequences for qPCR primers and probes

| Gene   | Accession number | Forward primer          | Reverse primer          | Taqman® probe          |
|--------|------------------|-------------------------|-------------------------|------------------------|
| Human  | NM005585         | GCCACTTGATCTGCTGGATT    | CACCCGGAGCAGTGATGAG     | CACATTGCTCTACAGTGAAGCAGGCTACCA |
| Smads6 |                  |                         |                         |                        |
| Human  | NM005522         | CAGACCTGCGACGCACTGGC   | GCTGAAAGCTCCGCAAGAAGT   | CCACTGCTCTACAGGAGATGCC |
| Dlk1   |                  |                         |                         |                        |
| Human  | NM004348         | AACCCAGAAGTCGACTATCAA   | GGGCACTACCGAGGGCATG     | CTTTTACTTACACCCCGCAGTCACCTC |
| Runx2  |                  |                         |                         |                        |
| Human  | NM001200         | GGGCATCCTCTCACCACAAAAG  | TTACAGCTGGACTTAAGGCGTT  | AAAACGTCAAGCAAAAAACAGGCC |
| BMP-2  |                  |                         |                         |                        |

signaling role in mesenchymal stem cells, osteoblasts (MC3T3-E1), and fibroblasts (NIH3T3) cells (1). PP is localized in dentin and bone and is a regulator of matrix mineralization (27). Our recent work has demonstrated that PP activates many of the same target genes as BMP-2, including Runx2, Osteocalcin, and osteocalcin (Ocn) (1). We further showed that PP triggers activation of the MAPK pathway (1). We suspect that BMP-2 and PP might be functioning cooperatively to stimulate progression of the osteogenic pathway and this could involve multiple signaling pathways. In the present study, we hypothesized that the Smad pathway is mediated by PP. To test our hypothesis, we exposed human adult mesenchymal stem cells (hMSC) to recombinant PP (rPP) and determined the levels of Smad phosphorylation, its subsequent nuclear translocation, and transcriptional activity. We found that PP regulates the Smad pathway and this may be independent of BMPs.

EXPERIMENTAL PROCEDURES

Materials

hMSC were obtained from BioWhittaker, Inc. (Walkersville, MD). Mesenchymal stem cell medium, mesenchymal cell growth supplement, 1-glutamine, penicillin, and streptomycin were obtained from BioWhittaker, Inc. and added to the medium according to the manufacturer’s specifications to prepare complete mesenchymal stem cell medium (basal medium). rhBMP-2 and BMP-2 enzyme-linked immunosorbent assay was obtained from R&D Systems. Noggin-Fc was purchased from Sigma. Total protein assay kits and protease inhibitors were obtained from Pierce. RNeasy Kit and DNase I were obtained from Qiagen (Valencia, CA). Quantitative real-time PCR master mix reagents and 18S primers and probe were purchased from Applied Biosystems (Foster City, CA). All target gene primers and probes were purchased from Integrated DNA Technologies, Inc (Coraille, IA). Anti-phosphorylated Smad1 (Ser463/Ser465) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit-HRP was purchased from Cell Signaling Inc. (Beverly, MA). Western lightening chemiluminescence reagents were purchased from PerkinElmer Life Sciences. Goat anti-rabbit-Cy3 was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All other chemicals were obtained from Sigma.

Methods

Cell Culture—hMSC were seeded and maintained in basal medium at 37 °C, 5% CO2, with humidity for Western blotting and immunocytochemical assays. Cells were cultured in medium containing 0.5% serum for 12–16 h prior to treatment with either rhBMP-2 or rPP. For all experiments, the dose of rhBMP-2 and rPP was 250 ng/ml. In our previous studies, this was optimal for activation of signal transduction pathways (1). Non-phosphorylated rPP was produced in Escherichia coli (BL21) in our laboratory using standard glutathione S-transferase fusion protein methodology where the glutathione S-transferase was cleaved and pure rPP was purified as described previously (1) and is maintained in solution with double distilled H2O.

Detection of Smad1 Activation—Cells were cultured in 35-mm plates as described above. Cells were treated in triplicate with either 250 ng/ml rhBMP-2 or rPP. Basal medium (0.5% serum) was the negative control. Cells were treated for 30 min and 1, 2, 6, 12, 18, and 24 h. Cells were lysed on ice in RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) in the presence of protease inhibitors. The lysed cells were then pooled and protein concentration determined. The lysates were stored at −80 °C at least 2 h prior to use. Sixty micrograms of total protein were loaded onto 10% SDS gels and subjected to SDS-PAGE. Gels were blotted onto polyvinylidene difluoride membranes and probed with anti-phosphorylated Smad1 (1:200) by Western blotting. Bands were detected by chemiluminescence of HRP and exposure to X-Omat Kodak film. Where noted, quantification of band intensities was performed using Kodak 1D Image Analysis Software.

Immunocytochemistry—Cells were cultured as described above on 12-mm round glass coverslips. Cells were then treated with either rhBMP-2 or rPP for 1, 6, and 12 h. All subsequent steps were carried out at room temperature. Cells were rinsed twice with PBS and fixed in 4% paraformaldehyde for 10 min. Fixed cells were rinsed twice in wash buffer (PBS, 0.05% Tween 20) and then permealized with 0.1% Triton X-100 in PBS for 5 min. Cells were then rinsed twice with wash buffer and incubated in a blocking buffer containing 5% goat serum in PBS for 30 min. Cells were then incubated for 1 h in primary antibody (antiSmad1, 1:200 in blocking buffer). Cells were then washed three times in wash buffer and incubated with a Cy3-conjugated secondary antibody (goat anti-rabbit) for 1 h. Cells were washed three times with wash buffer and a final wash with double distilled H2O. Coverslips were mounted inverted on glass microscope slides using Gelvatol mounting medium and visualized using epi-fluorescence and a Cy3 filter set.

Quantitative Real-time PCR Analysis—Cells were cultured in complete basal medium in 35-mm plates to ~70% confluence. Cells were then treated in triplicate with rhBMP-2 or rPP for 30 min and 1, 2, 6, 12, 18, and 24 h. Total RNA was extracted using RNeasy kit with DNase I digestion. After RNA extraction, quantitative real-time PCR (qPCR) analysis was carried out as described previously (1). Sequences for all target gene primers and probes are shown in Table 1. Gene expression levels were calculated as fold changes compared with basal medium controls for each time point based on the comparative ΔΔCt method (separate tubes) (29). Fold differences were calculated for each treatment group using normalized Ct values for the negative control at the appropriate time point as the calibrator. All target genes were normalized to the reference housekeeping gene, 18S.

Inhibition of BMP-2 Signaling—Cells were cultured as described above. To determine the dose of Noggin required to inhibit BMP-2 activity in hMSC, cells were treated with 250 or 500 ng/ml Noggin-Fc for 1 h in the presence of rhBMP-2. Noggin had been added to the
rhBMP-2-containing media and preincubated 30 min at 37 °C prior to addition to the cells. To determine whether Smad1 signaling by PP involved BMP-2, cells were incubated with PP and Noggin (also preincubated) for 30 min and 1, 6, 12, 18, and 24 h. Cell lysates were harvested and analyzed for pSmad1 by Western blotting as described above.

Statistical Analyses—All experiments were performed at least three times, and one representative experiment is reported as the mean of three treatment triplicates ± S.E. For qPCR assays, the coefficient of variation was calculated from three assay replicates. For all treatment groups and target genes analyzed, the coefficient of variation did not exceed 3%. One-way analysis of variance followed by Tukey-Kramer’s post-hoc test using SYSTAT 9 software (Richmond, CA) was performed to determine significance among treatment groups. A p value <0.05 was considered statistically significant.

RESULTS

PP Activates pSmad1—We hypothesized that the Smad pathway, a key regulatory pathway of osteogenic lineage progression, was involved in PP-mediated signaling, as PP was previously shown to up-regulate several key bone/dentin marker genes (1). Therefore, we examined the activation of the Smad pathway by analysis of the phosphorylation of the BMP-2 site on Smad1 (Ser463/Ser465), nuclear translocation, and transcriptional activity following treatment of hMSC with PP. We detected an increase in phosphorylated Smad1 (pSmad1) within 30 min of treatment (Fig. 1A). Our positive control, BMP-2, is a known stimulator of Smad1. BMP-2-treated cells maintained enhanced pSmad1 for at least 24 h compared with control. The optimal dose of rPP for pSmad1 activation appeared to be 250 ng/ml, which was comparable with stimulation with rhBMP-2 (Fig. 1B). By immunocytochemical analysis, pSmad1 was primarily cytoplasmic in cells maintained in basal medium (Fig. 2, A–C). pSmad1 nuclear translocation occurred within 1 h of treatment with either rhBMP-2 (Fig. 2, D–F) or rPP (Fig. 2, G–I). A higher magnification of Fig. 2G is shown in Fig. 2K, exhibiting the intense nuclear pSmad1 in hMSC treated with rPP for 1 h.

PP Activates pSmad1 Target Genes—To further demonstrate the activation of Smad pathway during PP signaling we quantified expression of direct target genes of the Smad transcription complex Smad6, Dlx5, and Runx2 using real-time PCR. In hMSC, Smad6 and Dlx5 gene expression was enhanced following treatment with rPP (Fig. 3). A 3.2-fold increase in Smad6 gene expression was observed 12 h following stimulation with rPP (Fig. 3A). This increased to 4.5-fold through 18 h and then declined to basal levels by 24 h. Similarly, Dlx5 gene expression was elevated 3-fold at 18 h following rPP treatment and returned to basal levels at 24 h (Fig. 3B). For both genes, PP-stimulated gene expression followed a similar pattern to BMP-2; however, in both cases, BMP-2-stimulated expression occurred earlier and induced higher fold changes for both Smad6 (up to 21.5-fold at 18 h) and Dlx5 (up to 13-fold at 18 h). We have previously reported that Runx2 is up-regulated in hMSC by PP after 48 h of culture (1). In the present study, we have found that Runx2 is up-regulated also earlier at 12 h (2.6-fold) and is maintained at this level up to 18 h (Fig. 3C) using a higher dose of rPP (250 ng/ml versus 50 ng/ml). Runx2 declined to basal level at 24 h. Osx was not affected by treatment with rPP over this time course (levels were below threshold of detection, data not shown). BMP-2 treatment groups induced Osx within 18 h (data not shown), which is consistent with our previous reports (1, 21). In addition, it has been shown that induction of Runx2 gene expression can occur without subsequent expression of Osx (30, 31).
BMP-2 Gene Expression Is Enhanced by PP—To determine whether PP activates the Smad pathway via BMP-2, qPCR analysis for Bmp-2 gene expression was performed on hMSC treated with PP (Fig. 4A). In PP-treated hMSC, Bmp-2 gene expression remained at basal levels up to 6 h following treatment. Then, Bmp-2 gene expression was significantly enhanced at 12 h (8.0-fold) and 18 h (4.6-fold) following treatment with rPP. BMP-2 gene expression returned to basal levels 24 h post-PP treatment. This suggests that PP directly activates the Smad pathway independent of BMP during earlier time points (prior to 12 h).

PP Activation of pSmad1 Does Not Require BMP-2 Signaling—Following an analysis of PP-stimulated Smad1 activation in hMSC, we did not expect that BMP would be directly involved in initial Smad1 activation due to timing of activation (within 30 min) and that BMP-2 gene expression is not up-regulated until at least 6–12 h later. However, we pursued the possibility that BMP binding to its receptor could be required for Smad activation by PP. Therefore, we inhibited BMP binding to its receptor by addition of Noggin, a competitive receptor antagonist of BMP signaling. Thus, if PP-mediated activation of the Smad pathway involved BMP-2 receptor binding, we hypothesized that Noggin would interfere with such a mechanism. We demonstrated the dose dependence of Noggin on inhibition of BMP-2-stimulated pSmad1 in hMSC (Fig. 5A). 500 ng/ml of Noggin significantly reduced the level of pSmad1 to basal levels (Fig. 5B). In the presence of PP, the pSmad1 activation was not inhibited in the presence of Noggin (Fig. 5C). This suggests that PP activates the Smad pathway in a mechanism that is independent of BMP-2/receptor binding.

DISCUSSION

Our finding that a protein distinct from the TGF-β family stimulated the Smad pathway was quite intriguing. We have previously determined the role of PP in signaling in human mesenchymal stem cells via the MAPK pathway. In the present study, we have demonstrated the involvement of the Smad pathway in PP-mediated signaling. The current study provides the necessary data to validate the proposed theories of others, which suggest direct involvement of non-collagenous proteins of the ECM during key stages of tissue development, morphogenesis, and regeneration (32).

PP-treated hMSC showed enhanced phosphorylation and nuclear
translocation of Smad1 with increased expression of Smad target genes. Noggin, an inhibitor of BMP-2-mediated signaling, did not block phosphorylation of Smad1 in hMSC treated with PP. BMP-2 receptor binding does not appear to be required for induction of Smad1 by PP. This suggested to us that PP does not stimulate Smad1 by first stimulating additional autocrine or paracrine BMP-2 production or by increasing BMP-2 bioavailability. PP activated Smad1 within 30 min and was maintained at least for 24 h. Furthermore, PP did not up-regulate Bmp-2 gene expression significantly over control until 6–12 h later. Rapid activation of pSmad1 and failure of Noggin to inhibit PP signaling indicate a direct

FIGURE 5. Inhibition of BMP-2 by Noggin. Cells were serum-derived for 16 h prior to treatment with 250 ng/ml rhBMP-2 or rPP for 30 min and 1, 6, 12, and 18 h. Prior to addition to cells, medium was preincubated for 30 min at 37 °C in the absence or presence of Noggin (250 or 500 ng/ml). Sixty micrograms of total protein were analyzed by SDS-PAGE and Western blotting for pSmad (1:200). pSmad1 was visualized using chemiluminescence of HRP. A, 500 ng/ml was required to inhibit rhBMP-2 stimulated pSmad1. B, quantification of band intensities as mean percent of control, mean ± S.E., n = 3. *, significant from control, p < 0.05; **, significant from BMP-2, p < 0.05. C, 500 ng/ml Noggin did not inhibit PP-stimulated pSmad1.

FIGURE 6. Model of PP signaling pathways via MAPK and Smad pathways. Dashed lines indicate unanswered connections between MAPK and Smad pathways during PP signaling.
Phosphophoryn Activates Smads

effect. Taken together, the data collectively indicate that PP activates the Smad pathway independently of BMP-2. Based on the data presented previously (1) and in this manuscript, we propose a model for PP signaling depicted in Fig. 6.

Not only is the phosphorylation of Smad1 important for transduction of the pathway, but it must also translocate into the nucleus as a complex with the Co-Smads. Phosphorylated Smad1 exhibited nuclear translocation following PP treatment. Once in the nucleus, the Smad complex was transcriptionally active and stimulated the expression of Smad1 target genes Smad6, Dlx5, and Runx2. Although this correlates with induction of Bmp-2 gene expression, failure of Noggin to prevent pSmad1 activation by PP suggests this occurs independently of BMP2/4/7. Smad6 is an inhibitory Smad and a direct target of BMP/Smad signaling. Smad6 provides feedback inhibition of BMP-receptor activation by blocking continued Smad1 phosphorylation by the BMP receptors. Here, we have shown that PP also up-regulates Smad6. Perhaps, PP activates Smad6 as a feedback mechanism to block further stimulation of Smad and MAPK pathways. Smad6 has been shown to inhibit p38 signaling by directly interacting with TAK-1 (33). Runx2 was activated by PP, but not Osx, providing further evidence that BMP-2 is likely not the primary mechanism of action for induction of the Smad pathway by PP. It appears that PP activation of the Smad pathway follows a similar profile as BMP-2, with aspects of the pathway becoming activated from initial phosphorylation by PP at the same site as BMPs (Ser463/465) followed by nuclear translocation over a similar time course and activation of the same Smad target genes. Thus, it appears the Smad pathway is intact, although not stemming from a BMP stimulus but rather PP.

It is unclear at this time what additional mediators upstream of the MAPKs and Smad1 may be involved with PP signaling and activation of Smad target genes and what other pathways might be involved. In our previous work, we have demonstrated that PP activates the integrin/MAPK signaling (1), and this could give clues to possible mechanisms by which PP affects the Smad pathway. Integrin signaling could modulate Smad transcriptional activity. For example, when focal adhesion kinase is inhibited by antisense, Smad transcriptional activity is reduced in the presence of BMP (34). Thus a link between integrins and growth factors pathways (Smads) could be influenced by additional ECM signals. Could PP activate the Smad pathway via integrins? Does this involve an interaction between integrins and BMP receptors or other growth factor receptor(s)? These questions are pertinent in light of the many opportunities for cross-talk between the intracellular signaling pathways triggered by ECM stimuli (25, 35–37), which are relevant to mineralized tissue morphogenesis.

The MAPK pathway can influence the Smad pathway by modulating Smad nuclear translocation. A link between the Smad and MAPK pathways was described by Kretzschmar et al. (26). The authors reported that BMP-2 and EGF trigger opposing signals which converge on Smad1 (26). Activation of Erk kinases by fibroblast growth factor 2, hepatocyte growth factor, and insulin-like growth factor II were shown to prevent nuclear translocation of Smads and thus inhibit BMP signaling in Xenopus (25). From this work, it was postulated that Smad1 receives opposing phosphorylation signals at different sites within the protein and the net effect determines the distribution of Smad1 in the cytoplasm and nucleus. Since Smad1 is important in the transcriptional activity of many bone-related genes such as Runx2 (38), the signaling network of Smads and MAPK and their interconnectivity should be further investigated to understand progression of the osteogenic lineage. Multiple opportunities for cross-talk between pathways exist in both the cytoplasm and nucleus. As mentioned above for focal adhesion kinase and Smads, it is possible that signals from integrins and growth factor receptors could merge in the nucleus to control osteoblast differentiation (34). This is evident in a recent study demonstrating the importance of the growth factor-regulated kinase RSK2, which was shown to be required for osteoblast differentiation (39). RSK2 phosphorylates and therefore regulates the activity of ATF4, an important transcription factor during osteoblast differentiation (40). In particular, it was recently shown that the transcription factors Runx2 and ATF4 co-regulate osteoblast differentiation through activation of Ocn (41). MAPK is also important for phosphorylation and activation of Runx2 (42), which could involve signaling from PP. The role of PP-mediated activation of MAPK and the connection to the Smad and other pathways and related additional effects are not yet completely understood and require further investigation. Since MAPK and Smad signaling pathways are dually activated by PP and BMP-2, it is possible that PP might contribute to the compensatory signaling networks that are triggered by the ECM and regulate bone/dentin formation.

These data raise interesting questions pertaining to the involvement of PP/Smad signaling with respect to the MAPK pathway, BMPs, and other signaling factors within the ECM. Cells use multiple pathways to decipher signals from the ECM into changes in cell function. BMP and PP each activate the MAPK pathway and Smad pathway. What is the connection, if any, between these events? Furthermore, what is the biological significance of Smad pathway activation by PP in the context of tissue morphogenesis? For example, during tooth development many epithelial-to-mesenchymal interactions are carried out by BMP signaling through the Smad pathway. We could now speculate a role of the matrix in the development of the tissue and a possible role in epithelial-mesenchymal interactions. This involvement of non-collagenous matrix proteins in tissue morphogenesis is worthy of further investigation. During tooth development, PP is secreted by odontoblasts and is localized along the mineralization front to regulated mineral deposition (43–45). One can envision a role for PP during the early phases of tooth development where PP signals odontoblast precursor cells in the microenvironment to differentiate into more mature odontoblasts. PP might also play a later role not only in the regulation of mineral deposition, crystal formation, and growth but also in the maintenance of the odontoblastic phenotype throughout tooth morphogenesis. PP might contribute to positive signaling related to the induction of key ECM proteins during dentin formation and mineralization. In addition, subsequent feedback inhibition of convergent signaling pathways such as the Smad and MAPK pathways can occur, both of which are dually activated by PP or other growth factors within the niche such as BMPs. In this fashion, PP might regulate cessation of signaling once optimal differentiation or mineral deposition is achieved. This could be relevant in the context of tissue homeostasis as well as morphogenesis, since the nature of dentin is generally non-resorbing. Given that native PP is highly phosphorylated (46–49), the role of the phosphoserines related to PP’s signaling function remain unknown. We are actively pursuing the role of phosphorylation of PP on its signaling function. Another interesting question is how are the signaling pathways, which are activated by numerous diverse stimuli, channeled into the appropriate pathway? What is the net effect of a combination of signals? These questions undoubtedly become increasingly important when defining the necessary and sufficient signals required for tissue morphogenesis.

Our ongoing work is focused on the potential cross-talk that might occur between MAPKs and Smads during PP signaling and how PP could participate in signaling of complex combinatorial cues from the microenvironment transduced to the cell during tissue development and morphogenesis.

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VOLUME 281 • NUMBER 9 • MARCH 3, 2006
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