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FGF2 induced expression of the pyrophosphate generating enzyme, PC-1, is mediated by Runx2 and Msx2

N.E. Hatch[1] and R.T. Franceschi[2]

[1]Department of Orthodontics and Pediatric Dentistry, University of Michigan, Ann Arbor, MI, USA;
[2]Department of Periodontics and Oral Medicine, University of Michigan, Ann Arbor, MI, USA

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Fibroblast growth factor (FGF) signaling plays a critical role in skeletal development, yet the mechanism by which FGF's affect bone mineralization is not well understood. Close review of the literature investigating the effects of FGF's on bone mineralization yields a complex and superficially paradoxical story. FGF2 knockout mice exhibit significantly diminished bone mass, bone formation rate and trabecular bone volume, suggesting that FGF2 is a positive regulator of bone formation, yet FGF2 over expression in mice also results in significantly diminished bone density and trabecular bone volume[1,2]. Furthermore, while systemic FGF1 and FGF2 have a bone anabolic effect *in vivo*, this anabolic effect does not occur until after cessation of FGF treatment. Furthermore, bone mineralization is inhibited during the course of FGF treatment[3,4]. Taken together these results indicate that FGF signaling has conflicting direct and indirect effects on bone mineralization and that FGF's stimulate expression of factors that prevent bone mineralization in the short term, and enhance bone mineralization in the long term. Pyrophosphate is an ideal example of such a bi-functional factor in that, dependent upon levels and tissue environment, pyrophosphate can inhibit or enhance tissue mineralization. To date, the factors involved in the mineralization effects of FGF signaling are unknown. *It is our hypothesis that the mineralization effects of FGF's result from increased pre-osteoblastic pyrophosphate generation via the pyrophosphate generating enzyme, PC-1.*

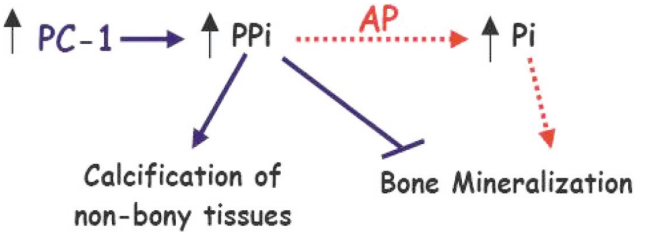

Figure 1. PC-1 influences bone mineralization and the calcification of non-bone tissues. Pre-osteoblastic PC-1 enzyme activity increases extracellular pyrophosphate (PPi). Pyrophosphate inhibits bone mineralization, yet serves as a source of phosphate (Pi) to enhance bone mineralization when it is hydrolyzed by the osteoblastic enzyme, alkaline phosphatase (AP). Dependent upon tissue composition and levels, pyrophosphate can also lead to the pathologic calcification of normally non-mineralized tissues.

PC-1 is a nucleoside triphosphate pyrophosphohydrolase (NTPPPH) that generates pyrophosphate from the hydrolysis of ATP[5]. PC-1 is the primary enzymatic generator of pyrophosphate in osteoblastic cells[6]. The influence of PC-1 on tissue mineralization is complex (Figure 1). PC-1 activity increases extracellular levels of pyrophosphate. Pyrophosphate inhibits hydroxyapapatite crystal formation and propagation[7], yet PC-1 generated pyrophosphate also serves as an essential source of phosphate to enhance bone mineralization when it is hydrolyzed by the osteoblastic enzyme, alkaline phosphatase (AP)[8]. Additionally, while low pyrophosphate levels inhibit non-bone tissue calcification, dependent upon tissue composition, high pyrophosphate levels can lead to pathologic calcification of non-bone tissues[9].

We previously showed that FGF2 specifically up-regulates PC-1 expression in MC3T3E1(C4) calvarial pre-osteoblasts[10],

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Corresponding author: Nan E. Hatch, University of Michigan, Department of Orthodontics and Pediatric Dentistry, School of Dentistry, 1011 N University Avenue, Ann Arbor, MI 48109-1078, USA
E-mail: nhatch@umich.edu

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and that this phenomenon is osteoblast differentiation stage specific. We now find that FGF2 also stimulates expression of PC-1 in primary calvarial pre-osteoblasts (data not shown). Additionally, we find that while other cell types (fibroblastic, pre-myeloblastic and pre-adipocytic cells) express basal levels of PC-1, FGF2 only induces PC-1 expression in osteoblast related cells (data not shown). This indicates that PC-1 induction by FGF2 is cell type specific.

PC-1 expression increases with osteoblast differentiation, but FGF2 does not enhance PC-1 expression in differentiated osteoblasts[10]. This implies that PC-1 expression is tightly regulated, and that the mechanism of PC1 induction during osteoblast differentiation is distinct from the mechanism of induction that occurs with FGF2 treatment of pre-osteoblasts. To more directly investigate the regulation of PC-1, we cloned a 2864 base pair region of the proximal PC-1 gene promoter and constructed a PC-1 gene promoter/firefly luciferase reporter construct (PGL3/PC1). Initial sequence analysis of the PC-1 promoter revealed four consensus Runx2 binding sites. The transcription factor, Runx2, is a master regulator of osteoblastic differentiation, and is required for normal skeletal development and ossification[11]. Discovery of multiple putative Runx2 binding sites within the PC-1 gene promoter, combined with the knowledge that PC-1 expression increases with osteoblast differentiation and that the induction of PC-1 by FGF2 is limited to pre-osteoblasts, led us to hypothesize that Runx2 mediates transcription of this gene. Our results indicate that Runx2 is required for FGF2 responsiveness (data not shown). To establish that Runx2 mediates PC-1 gene expression by binding to the PC-1 gene promoter, we mutated each of the four putative Runx2 binding sites found within the proximal 2.8 kb fragment of this promoter. Mutation of each site resulted in a significant drop in FGF2-dependent PC-1 promoter activity, demonstrating that each site contributes to maximal FGF2 responsiveness. However, mutation of all four binding sites did not further attenuate the FGF2 response, indicating that factors in addition to Runx2 are involved in the regulation of PC-1 expression by FGF2.

To explore the possibility that factors other than Runx2 mediate PC-1 expression downstream of FGF2, the PC1 gene promoter was re-examined for transcription factor binding sites, and one consensus Msx2 binding site was identified. Because Msx2 can inhibit the ability of Runx2 to drive transcription of target genes, we hypothesized that mutation of the Msx2 site might uncover a greater dependence of PC-1 promoter activity on Runx2. Surprisingly, mutation of the Msx2 binding site resulted in diminished promoter responsiveness to FGF2. Furthermore, over-expression of Msx2 dramatically increased PC-1 promoter activity and mRNA expression in response to FGF2, demonstrating that Msx2 stimulates transcription of the PC-1 gene. Notably, co-expression of Runx2 with Msx2 had a synergistic effect, indicating that Runx2 and Msx2 may function together in this process (data not shown).

The finding that Msx2 enhances transcription of PC-1 downstream of FGF2 is particularly striking for two reasons. First, to our knowledge this is the first study to show Msx2 functioning as a transcriptional enhancer downstream of FGF2 in calvarial pre-osteoblastic cells. Understanding how Msx2 can enhance Runx2 transcriptional activity downstream of FGF2 in some cases and inhibit it in others, should increase our ability to control activity of these factors for the development of biologic therapeutics in guided bony tissue regeneration. Second, the finding that Msx2 enhances the effects of FGF2 on expression of PC-1 in calvarial pre-osteoblastic cells supports the idea that FGF receptor and Msx2 activity influence cranial osteogenesis via the same molecular mechanism. Activating mutations in FGF receptors and Msx2 are both associated human malformation syndromes that involve aberrant mineralization of the craniofacial complex and premature cranial suture fusion[12,13]. While the genetics of these syndromes has been established, the precise biologic mechanism by which the disease phenotype occurs is unknown. Of note, FGF receptors and Msx2 are both expressed in the osteogenic front of developing cranial bones, sites of early precursor cell proliferation and differentiation[14,15]. That the bone anabolic effects of FGF's include a transient suppression of bone mineralization has already been discussed. Notably, the bone anabolic effects of Msx2 also appear to transiently inhibit terminal osteogenic cell differentiation, and thus mineralization[14]. While the idea that FGF signaling and Msx2 activity promote precursor cell proliferation while inhibiting terminal osteoblast differentiation is not a novel concept, this is the first report of a direct link between Msx2 and FGF signaling in promotion of a calvarial osteoblast precursor cell phenotype. Our results suggest that FGF's and Msx2 function in the same pathway to promote a pre-osteoblastic cell phenotype that includes the elaboration of factors that will transiently inhibit mineralization. Future studies will be needed to establish the significance of this phenomenon in the overall effects of FGF signaling and Msx2 activity on bone mineralization and craniofacial osteogenesis.

References

1. Sobue T, Naganawa T, Xiao L, Okada Y, Tanaka Y, Ito M, Okimoto N, Nakamura T, Coffin JD, Hurley MM. Over-expression of fibroblast growth factor-2 causes defective bone mineralization and osteopenia in transgenic mice. J Cell Biochem 2005;95:83-94.
2. Montero A, Okada Y, Tomita M, Ito M, Tsurukami H, Nakamura T, Doetschman T, Coffin JD, Hurley MM. Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. J Clin Invest 2000;105:1085-93.
3. Liang H, Pun S, Wronski TJ. Bone anabolic effects of basic fibroblast growth factor in ovariectomized rats. Endocrinology 1999;140:5780-8.
4. Dunstan CR, Boyce R, Boyce BF, Garrett IR, Izbicka

E, Burgess WH, Mundy GR. Systemic administration of acidic fibroblast growth factor (FGF-1) prevents bone loss and increases new bone formation in ovariectomized rats. J Bone Miner Res 1999;14:953-9.
5. Terkeltaub R, Rosenbach M, Fong F, Goding J. Causal link between nucleotide pyrophosphohydrolase over activity and increased intracellular inorganic pyrophosphate generation demonstrated by transfection of cultured fibroblasts and osteoblasts with plasma cell membrane glyoprotein-1. Arthrit Rheum 1994;37:934-41.
6. Johnson K, Moffa A, Chen Y, Prizker K, Goding J, Terkeltaub R. Matrix vesicle plasma cell membrane glycoprotein-1 regulates mineralization by murine osteoblastic MC3T3 cells. J Bone Min Res 1999;14:883-92.
7. Fleisch H, Russell RG, Straumann F. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. Nature 1996;212:901-3.
8. Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R, Millan JL. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. Proc Natl Acad Sci 2002;99:9445-9.
9. Johnson K, Pritzker K, Goding J, Terkeltaub R. The nucleoside triphosphate pyrophosphohydrolase isozyme PC-1 directly promotes cartilage calcification through chondrocyte apoptosis and increased calcium precipitation by mineralizing vesicles. J Rheumatol 2001;28:2681-91.
10. Hatch NE, Nociti F, Swanson E, Bothwell M, Somerman M. FGF2 alters expression of the pyrophosphate/phosphate regulating proteins, PC-1, ANK and TNAP, in the calvarial osteoblastic cell line, MC3T3E1(C4). Connect Tissue Res 2005;46:184-92.
11. Schroeder TM, Jensen ED, Westendorf JJ. Runx2: a master organizer of gene transcription in developing and maturing osteoblasts. Birth Defects Res C Embryo Today 2005;75:213-25.
12. Reardon W, Winter RM, Rutland P, Pulleyn LJ, Jones BM, Malcolm S. Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome. Nat Genet 1994;8:98-103.
13. Lui YH, Kundu R, Wu L, Luo W, Ignelzi MA, Snead ML, Maxson RE. Premature suture closure and ectopic cranial bone in mice expressing Msx2 transgenes in the developing skull. Proc Natl Acad Sci 1995;92:6137-41.
14. Liu Y, Tang Z, Kundu RK, Wu L, Luo W, Zhu D, Sangiorgi F, Snead ML, Maxson, RE Jr. Msx2 gene dosage influences the number of proliferative osteogenic cells in growth centers of the developing murine skull: a possible mechanism for MSX2-mediated craniosynostosis in humans. Dev Biol 1999;205:260-74.
15. Kim H-J, Rice DPC, Kettunen PJ, Thesleff I. FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. Development 1998;125:1241-51.