Osteocrin, a Novel Bone-specific Secreted Protein That Modulates the Osteoblast Phenotype*

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Gethin Thomas‡§, Pierre Moffatt†‡, Patrick Salois‡, Marie-Hélène Gaumond‡, Rock Gingras‡, Èric Godin‡, Dengshun Miao§, David Goltzman¶, Patrick Salois§, Pierre Moffatt‡, Pierre Salois‡, and Christian Lantcót‡

From ‡Phenogene Therapeutics Inc., 416 de Maisonneuve West, Suite 1020, Montreal, Quebec H3A 1L2, Canada, and the ¶Calcium Research Laboratory, McGill University Health Centre, McGill University, Montreal, Quebec H3A 1A1, Canada

Although a number of secreted factors have been demonstrated to be bone regulators, none of these are unique to bone. Using a viral-based signal-trap strategy, we have identified a novel gene we have termed "osteocrin." A 1280-bp mRNA encodes osteocrin producing a mature protein of 103 amino acids with a molecular mass of 11.4 kDa. Osteocrin shows no homology with any known gene except for two conserved sequence motifs reminiscent of dibasic cleavage sites found in peptide hormone precursors. Immunofluorescence and Western blot analysis confirmed the secretory nature of osteocrin. Two protein species were identified in the medium of cells overexpressing osteocrin, a full-length 11.4 kDa species and a processed ~5 kDa species. Mutation of the 76KKKR dibasic cleavage site abolished the appearance of this smaller osteocrin fragment. By in situ hybridization in mouse embryos, osteocrin was expressed specifically in Cbfa1-positive, osteocalcin-negative osteoblasts. Immunohistochemistry on adult mouse bone showed osteocrin localization in osteoblasts and young osteocytes. By Northern blot analysis, osteocrin expression was only detected in bone, expression peaking just after birth and decreasing markedly with age. In primary osteoblastic cell cultures osteocrin expression coincided with matrix formation then decreased in very mature cultures. Treatment of cultures with 1,25-dihydroxyvitamin D₃ resulted in a rapid dose-dependent down-regulation of osteocrin expression, suggesting direct regulation. Chronic treatment of primary cultures with osteocrin-conditioned media inhibited mineralization and reduced osteocalcin and alkaline phosphatase expression. These results suggest that osteocrin represents a novel, unique vitamin D-regulated bone-specific protein that appears to act as a soluble osteoblast regulator.

Bone is a dynamic tissue that is continually being modeled and remodeled through the coordinated actions of the bone forming osteoblasts and bone resorbing osteoclasts. Such remodeling is necessary to respond to the continually changing mechanical and regulatory demands placed upon the skeleton (1–4). The balance of the activity of the osteoblasts and osteoclasts is tightly regulated at both the systemic and local levels through the actions of a number of secreted molecules. Systemically, calcitropic hormones such as 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)¹ and parathyroid hormone (PTH) can regulate both bone formation and resorption, acting on both osteoclastic and osteoblastic cell lineages (5–8), whereas 1,25(OH)₂D₃ also acts via the intestine and kidney to regulate systemic calcium and phosphate availability (9, 10). Other hormones, such as growth hormone (11), estrogens (12), and thyroid hormone (13) also influence bone mass. More recently, it has been reported that the peptide hormone leptin exerts a strong systemic antiosteogenic effect when activating the sympathetic nervous system through a hypothalamic relay (14, 15).

Remodeling occurs in discrete units, termed basic multicellular units, consisting of a cone of cutting osteoclasts followed by osteoblasts laying down a new collagenous matrix (16, 17). Osteoblasts also secrete abundant amounts of non-collagenous proteins, which play key roles in the maturation and mineralization of the bone matrix (18, 19). In addition to building bone, the osteoblasts are directly and indirectly involved in regulating bone metabolism. Osteoblasts secrete molecules that regulate osteoclastogenesis and/or osteoclast activity, such as receptor activator of NFκB ligand and osteoprotegerin (20) and also release growth factors into the bone matrix, such as members of the insulin-like growth factor, transforming growth factor, and fibroblast growth factor families (21). These factors are thought to be stored and released during successive remodeling cycles to control local bone formation and resorption (21–23). Insulin-like growth factor-1 and basic fibroblast growth factor, for example, act in a paracrine fashion stimulating osteoblast proliferation and activity (24–27).

It should be noted that none of the secreted bone regulatory proteins described to date are specifically expressed by bone cells. In fact, currently, only four highly bone-specific genes have been identified, two key transcription factors (Cbfa-1 (28) and osterix (29)) and two structural proteins (bone sialoprotein (30) and osteocalcin (31, 32)). To identify novel potential bone-specific regulatory molecules, we undertook to screen cDNA libraries from bone using a viral-based signal trap technology (33). We report here the identification of a novel gene specifically expressed in osteoblasts. The protein contains dibasic cleavage sites conserved in vertebrate homologues and reminiscent of those

¹ The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; PTH, parathyroid hormone; nt, nucleotide; RACE, rapid amplification of cDNA ends; RT, reverse transcription; aa, amino acids; βGP, β-glycerophosphate; PB, phosphate buffer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Cbfa-1, core binding factor alpha-1.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AY399681, AY399682, and AY399683.

‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed: Phenogene Therapeutics Inc., 416 de Maisonneuve West, Suite 1020, Montreal, Quebec, H3A 1L2, Canada. Tel.: 514-288-9099; Fax: 514-288-1111; E-mail: gthomas@phenogene.com.

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found in hormonal precursors. Functionally, this novel protein appears able to modulate osteoblastic differentiation.

**MATERIALS AND METHODS**

**Cloning of Full-length Mouse Osteocrin cDNA**—We have previously developed a screening system that allows the rapid identification of nucleic acids encoding signal peptides from complex libraries of cDNA fragments (33). A library enriched in 5’ fragments of cDNAs derived from developing calvaria (33) was obtained using a protocol adapted from the "oligo-capping" method (34).

The original osteocrin fragment retrieved from this screening protocol comprised nt 1–430 of the corresponding full-length clone (Fig. 1), which was subsequently obtained using a modified 3’-RACE strategy as follows. Five µg of e15.5 mouse calvaria total RNA was reverse-transcribed with Superscript II™ (Invitrogen) using a di15 primer-linker. cDNA was subjected to 25 cycles of PCR with the Titan™ RT-PCR kit (Boehringer Mannheim) using forward primer 25–10G (see Table I for primer sequences) and reverse primer 18–s (see Table I for primer sequences) and reverse primer 18–s (see Table I for primer sequences) and reverse primer 18–s (see Table I for primer sequences). The PCR products were cloned in place of the PCR products were cloned in place of the...
saline and accumulation in serum-free Dulbecco’s modified Eagle’s medium for 48 h for analysis of secreted products. For conditioned media treatment of primary osteoblast cultures, media from osteocrin or mock-transfected HEK293 cells were diluted 1:6 in normal culture media and replenished every 2–3 days from day 2 until the termination of the experiment at day 18. To measure 45Ca uptake, 45CaCl2 (1 mCi/ml) (Amersham Biosciences) was added to the medium for the last 48 h of the experiment. Medium was collected and the cells scraped into 5% (v/v) trichloroacetic acid. Cell/matrix-associated 45Ca incorporation was calculated as a percentage of the total input in the cellular and medium fraction modified for gene expression analysis. Cells were then incubated for 1 h with a mix of goat anti-rabbit coupled to AlexaFluor 594 and goat anti-mouse AlexaFluor 488 (both Molecular Probes, Eugene, OR) in PB for 1 h. Cells were washed twice and observed by fluorescence microscopy. All steps were performed at room temperature.

For Western blotting, protein in the conditioned medium was precipitated by adding trichloroacetic acid to a final concentration of 10% (w/v) and incubated on ice for 1 h. After centrifugation at 12000 × g for 20 min at 4°C, the pellet was washed once with chilled acetone, dried briefly, and resuspended directly in 2× Laemli loading buffer (Bio-Rad, Mississauga, Ontario, Canada) and boiled. For cell extracts, cells were rinsed with phosphate-buffered saline and solubilized in lysis buffer (50 mM Tris-HCL, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% IGEPAI-630™, and 1% (v/v) protease inhibitor mixture (Sigma)). Cell debris and insoluble material were pelleted by centrifugation at 12000 × g for 5 min at 4°C. The soluble proteins were mixed with 1 volume of 2× Laemli loading buffer and boiled. Proteins were electrophoresed on a 16.5% denaturing Tris-Tricine polyacrylamide gel (Bio-Rad) and transferred onto 0.2 µm nitrocellulose (Protran, Schleicher, and Schuell, Keene, NH) according to standard Western blot protocols. All subsequent incubations were performed at room temperature. The membrane was incubated in a 1/800 dilution (v/v) of osteocrin-antiserum in Tris-buffered saline, 0.1% Tween 20 with 2.5% (w/v) dried milk followed by goat anti-rabbit horseradish peroxidase (Sigma) diluted 1/30000 in TBST with 2.5% (w/v) dried milk and the signal visualized with ECL™ reagent (Amersham Biosciences).

**In Situ Hybridization—In situ hybridization was performed on mouse embryonic tissue sections from e16.5 to e17.5 as described previously (36). The mouse cRNA antisense probes used were as follows: an 815-bp cbfa-1 fragment (nt 891–1746 of GenBank™ accession number AF102844), an 187-bp osteocalcin fragment (nt 1–187 of GenBank™ accession number U11542), and a 431-bp osteocrin fragment (nt 1–431 of sequence shown in Fig. 1A). Cbfa-1 and osteocalcin were exposed for 21 days and osteocrin for 30 days.

**Northern and RT-PCR Analysis—**RNA was isolated from whole bones or osteoblastic cell cultures using Trizol™ with glycogen (5 µg/ml) as carrier according to the manufacturer’s instructions. For RT-PCR, cDNAs were generated with Superscript II™ reverse transcriptase and oligo-dT<sub>18</sub> priming, and PCR amplification carried out with gene-specific primers using Taq DNA polymerase (New England Biolabs). For sequencing, PCR products were purified using the QIAquick™ PCR purification kit (QIAGEN). Products were subjected to automated sequencing using ABI BigDye terminator cycle sequencing reagents (Applied Biosystems) and analysed on an ABI 3730xl. Sequences were aligned with ClustalX (37) and edited with GeneDoc (38). For RNA and cDNA samples from osteocrin or mock-transfected HEK293 cells, the same number of copies of each cell line were added to the samples.
Genetic-specific primers and conditions were as follows: osteocrin, forward 21–14G and reverse 21–15G (annealing temp = 52 °C, 28 cycles, product 378-bp); GAPDH, forward 18–9G and reverse 19–12G (annealing temp = 56 °C, 33 cycles, product 398-bp). Northern blots were generated on nylon membranes (Osmonics, Westborough, MA) by standard methods (37). Filters were prehybridized for 4 h and hybridized overnight in Church buffer (38) at 65 °C. The rat osteocrin cDNA probe corresponded to the full coding sequence (Fig. 1A). For rat osteocalcin, a cDNA corresponding to the full coding sequence (GenBank™ accession number M25490) was generated by PCR. A 681-bp mouse fragment corresponding to GenBank™ accession number J04806 was cloned for the osteopontin cDNA probe. For alkaline phosphatase, a 341-bp fragment corresponding to GenBank™ accession number NM013059 was generated by PCR. Probes were labeled with [α-32P]dCTP using a standard random priming protocol (37).

Immunohistochemistry—Immunohistochemistry was as described previously (39). Briefly, decalcified tibial sections were stained with primary osteocrin antibody or preimmune rabbit serum overnight at room temperature followed by biotinylated goat anti-rabbit IgG (Sigma) and stained using the avidin-biotin-peroxidase complex technique.

RESULTS

Identification and Cloning of Osteocrin—We previously reported the application of a novel technology, based on a signal-trap, to identify cDNAs encoding secreted and membrane-bound proteins (33). Screening of a mouse e15.5 calvarial bone library identified a 433-bp fragment encoding a putative translation product containing a signal peptide deriving from a novel unannotated gene. A modified 3′-RACE strategy was used to obtain the full-length cDNA. Sequences from at least five different clones for reconstitute the full-length cDNA sequence shown in Fig. 1A. This mouse cDNA is 1280-bp containing an open reading frame of 393 bp flanked by 61 bp and 826 bp of untranslated sequences at the 5′ and 3′ ends, respectively. A polyadenylation signal is found 14 bp upstream of a poly(A) stretch. The putative initiator ATG codon is found in an adequate Kozak context (AA-GATGC). Delineation of the corresponding genomic locus was enabled by BLAST searches of the mouse genome sequence (www.ensembl.org). The gene is located at chromosome 16B2 (syntenic to human chromosome region 3q28). Covering ~44 kb, the mouse gene locus is unusually large for a 1280 mRNA derived from only 5 exons (Fig. 1B).

Protein Structure and Secretion of Osteocrin—The novel mouse cDNA encoded a protein of 130 residues containing a signal peptide (Fig. 1A, underlined). The SignalP v2.0 server (40) predicted two possible signal peptidase cleavage sites, at Ala-25 and Ser-27 (Fig. 1A, arrowheads). Initial mass spectrometric analyses on a semi-purified preparation gave a size of 11371 Daltons favoring Ser-27 as the predominant cleavage site. Such a size also matched exactly the predicted molecular weight from the sequence suggesting no post-translational modifications. The mature 11.4-kDa protein has an isoelectric point of 10.02. Extensive in silico analysis suggested that the protein presented no strong homology with any known protein or protein domains. However, two sequence motifs reminiscent of dibasic cleavage sites found in peptide hormone precursors were evident: KKKR at position 76–79 and KKR at position 110–112 (Fig. 1A, boxed). Such putative cleavage sites could create peptides of 48, 30, and 18 aa, respectively. Because of these features and its bone-specific expression (see “Restricted Expression of Osteocrin”), we tentatively named this novel gene osteocrin.

To further investigate the significance of the putative processing sites in mouse osteocrin, we determined whether the dibasic sites were conserved in other species. Rat and human full-length cDNAs and a partial cDNA from snake were cloned using RT-PCR. In addition, in silico searches (GenBank™)
identified a single bovine EST (accession number BF045261) and two chicken ESTs (accession numbers ChEST861c21 and ChEST83i24) (41). Overall amino acid homology between different species is high (Fig. 2A), for example human and mouse sequences are 74% identical (84% similar) and human and chicken sequences are 59% identical (71% similar). Further, the dibasic cleavage sites are conserved across species (Fig. 2A) with the C-terminal half of the protein from the KKKR dibasic site being the most conserved. For instance, the C-terminal portion of the human protein (residues 116–133) differs by only

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3 amino acids from that of the snake protein (C-terminal 90% similarity, full-length similarity of 79%).

Functionality of the predicted signal peptide in osteocrin was confirmed by immunofluorescence in HEK293 human fibroblasts and UMR106 rat osteosarcoma cells transfected with a vector expressing osteocrin. Labeling was clearly localized in the secretory apparatus of transfected cells, confirmed by colocalization with the Golgi apparatus-specific Golgi 58 kDa protein (Fig. 2B). Western blot analysis of transfected HEK293 cells demonstrated that osteocrin was constitutively secreted and accumulated in the culture medium of transfected cells (Fig. 2C, lane 1). Similar accumulation (data not shown) and localization (Fig. 2B) was seen in UMR106 osteoblasts transfected with the same vector. Some osteocrin was also detected in the cellular extract, most likely residual from the secretory apparatus (Fig. 2C, lane 3). No signal was detected in the mock-transfected HEK293 cells (Fig. 2C, lane 3). Three bands were visible in the media. Two closely separated bands can be seen at ~11.4 kDa, which might arise from alternate or incomplete processing of the signal peptide, as described above. The third band, at ~5 kDa, could represent the 51-aa fragment generated by processing at the 76KKKR79 dibasic cleavage site (Fig. 2C, lane 1). To test this hypothesis, we substituted an alanine and a serine for the 76KKKR79 site. Due to the replacement of 4 charged residues (KKKR) with two uncharged residues (AS) in the mutated osteocrin, the mutated molecule migrated at a slightly lower position than the native molecule (Fig. 2C, lanes 1 versus 2 and 4 versus 5). Interestingly, the band at ~5 kDa was no longer detected in the medium from cells transfected with the mutated osteocrin cDNA (Fig. 2C, lane 2), suggesting this dibasic cleavage site is functional. Difficulties of visualization of small protein fragments may result in this band being underrepresented. However, longer exposure demonstrated the smaller band could still not be detected in the mutant osteocrin-transfected cells (Fig. 2C, lane 2, lower panel).

Restricted Expression of Osteocrin—In situ hybridization using a probe corresponding to the coding sequence of mouse osteocrin revealed a very defined and restricted expression pattern in embryonic mice. Expression first appears at e13.5 in the bone rudiments (data not shown). Osteocrin expression highlighted the periosteum in a subset of Cbfa1-positive cells of the developing e16.5 ribs (Fig. 3A) and the e17.5 fore limb (Fig. 3B). Closer examination of e16.5 tibiae demonstrated that osteocrin was expressed in periosteal osteoblasts that are Cbfa1-positive but osteocalcin-negative (Fig. 3C).

By Northern blot analysis, osteocrin message could not be detected in tissues other than bone (Fig. 4A) except for a smaller sized transcript detected in rat spleen (Fig. 4B). Cloning and sequencing of this isoform suggested it is transcribed from an alternate promoter upstream of the third exon of the gene. The significance of this rat isoform is unclear because it does not contain an open reading frame. Furthermore, no such transcript was detected in mouse spleen.

By high cycle number RT-PCR (40 cycles), we could detect expression of osteocrin in muscle, kidney, testes, and heart, albeit at very low levels (data not shown). Osteocrin expression was detected in the UMR106 rat osteosarcoma cell line but not in undifferentiated and mineralizing mouse MC3T3 osteoblasts or human MG-63 and SaOS-2 osteosarcoma cell lines (Fig. 4C).

In bone, expression of osteocrin was highest in embryos and neonates, peaking at 4 days of age in both calvaria and long bones and decreasing steadily with age to very low levels in 8-month-old long bones (Fig. 4D). The age-related decrease was
less marked in calvaria by 8 months. Partitioning of long bones into midshaft (diaphysis and metaphysis) and epiphysial regions demonstrated predominant expression of osteocrin in the diaphysis and metaphysis (data not shown). Maximal expression of osteocrin is reached earlier than that seen for osteocalcin, a marker of osteoblast activity but coincided more with that of tissue-nonspecific alkaline phosphatase. The relative expression levels of osteocrin were very low, as shown by a significantly longer exposure time (72 h) compared with osteocalcin (3 h).

Immunohistochemical localization using antibodies raised against the C-terminal region of osteocrin indicated that the protein was primarily synthesized by cuboidal osteoblasts on the endosteal surface of adult mouse long bones (Fig. 4E). Flattened preosteoblasts and newly formed osteocytes were also labeled, whereas mature enlombed osteocytes were not.

**Regulation of Osteocrin Expression in Calvarial Cultures**—As expected from the in situ data, expression of osteocrin was evident in Northern blot analysis of primary calvarial osteoblastic cells (Fig. 5). Expression was first observed in conjunction with matrix formation between confluence and 5 days post-confluence. This point coincided with the onset of osteocalcin expression and significant up-regulation of osteopontin expression. Osteocrin expression was maintained through the mineralization phase 10 days post-confluence. Interestingly, primary osteoblasts cultured with 10 mM βGP for 10 days post-confluence exhibited decreased osteocrin expression, corresponding with the development of a very differentiated phenotype in these cultures. This down-regulation was, however, not observed for osteocalcin and osteopontin.

The bone-specific nature of the expression of this novel gene led us to investigate whether it was regulated by any of the major calciotropic hormones. Both PTH (10⁻⁸ M) and dexamethasone (10⁻⁸ M), a synthetic glucocorticoid, had no effect on osteocrin expression (data not shown). However treatment of mature cultures of rat primary osteoblasts with 10⁻⁸ M 1,25(OH)₂D₃ in the absence of βGP showed significant reduction after only 6 h and completely abolition of osteocrin expression by 48 h (Fig. 6A). Osteocalcin expression was up-regulated in a similar time frame after 2 h of 1,25(OH)₂D₃ treatment (Fig. 6A). Dose response showed that expression was repressed by 10⁻⁸ M and 10⁻⁶ M, to a lesser extent by 10⁻⁸ M but not by 10⁻¹⁰ M 1,25(OH)₂D₃ (Fig. 6B). Such time and dose response characteristics suggest direct regulation of osteocrin by 1,25(OH)₂D₃.

**Osteocrin Blunts Terminal Differentiation of Osteoblasts**—The biological activity of osteocrin products was assessed using conditioned medium from HEK293 fibroblasts transiently transfected with an expression vector for osteocrin. Primary osteoblasts were chronically treated with conditioned media from osteocrin- or empty vector-transfected cells from day 2 in culture (pre-confluence) until 10 days post-confluence (the mineralization phase). We estimated by direct enzyme-linked immunosorbent assay that the concentration of osteocrin products in conditioned media was in the order of 5 μg/ml. Treatment with osteocrin-containing medium resulted in a 60% decrease in mineralization as measured by ⁴⁵Ca uptake relative to empty vector (3.67% versus 1.61%, p < 0.01) (Fig. 7A). Further, osteocalcin expression was almost completely abolished and alkaline phosphatase expression markedly reduced by treatment with osteocrin-containing medium (Fig. 7B).

**DISCUSSION**

This report describes a novel secreted bone-specific protein that we have termed osteocrin. A cleavable signal peptide is found at the N terminus of the 130 aa mouse protein. Accordingly, the bulk of osteocrin is secreted in the culture medium by transiently transfected fibroblasts and osteoblast-like cells. Osteocrin presents no homology to any known protein or protein domain. However, there are two dibasic cleavage sites for members of the mammalian subtilisin/Kex20-like endoprotease family (42, 43). These putative processing sites are conserved in the osteocrin molecule from terrestrial vertebrates, from snake to human. Further, homology across species is particularly high in the C-terminal half of the molecule, between the putative cleavage sites. The presence and evolutionary conservation of the dibasic sites found in osteocrin are suggestive of processing similar to that seen for hormones such as proopiomelanocortin (44), PTH (45), and parathyroid hormone-related protein (46, 47). In addition, it is noteworthy that all the species of cloned osteocrin have a glycine residue at the C terminus. This raises the possibility that osteocrin products may be amidated, another feature found in many peptide hormones (48, 49). We have shown that processing of osteocrin occurs in transfected fibroblasts producing an immunoreactive fragment of ~5 kDa. Importantly, this fragment is not produced when the dibasic site at position 76–79 is mutated, indicating that it arises from cleavage at this site. Processing of the overexpressed osteocrin is not complete with significant amounts of the 11.4-kDa signal peptide-cleaved form of the protein being released. However, previous studies (50, 51) have indicated that prohormone overexpression can lead to a saturation of processing.

Osteocrin appears to be expressed in a subset of osteoblasts. It is expressed in bone from an early developmental stage...
Expression of osteocrin is rapidly and dose-responsively down-regulated by 1,25(OH)2D3. Changes in RNA levels occur within 6 h of treatment, similar to other directly up-regulated (osteocalcin (65) and osteopontin (68)) and down-regulated (bone sialoprotein (69) and Chbα-1 (70)) vitamin D responsive genes. All these genes are regulated by 1,25(OH)2D3 through well defined vitamin D response elements (71, 72). Preliminary in silico analysis of the 5′ upstream regions of the human and mouse osteocrin genes revealed putative VDREs −4.6 and 5.6 kb upstream of Exon 1, respectively. Whether these motifs are functional or modulate the 1,25(OH)2D3 repression of osteocrin has yet to be confirmed.

In very mature primary osteoblastic cultures, terminal differentiation occurs (in this case induced by long-term culture in conditioned media). Mineralization was reduced together with expression of the mineralization-associated genes, alkaline phosphatase and osteocalcin. The concentration of osteocrin in the conditioned medium may represent supraphysiologic levels, but it has been suggested that local concentrations of other bioactive molecules such as insulin-like growth factor-1 and fibroblast growth factor 2 could be similarly high in the bone microenvironment (73). Osteocrin may therefore act directly on osteoblasts via autocrine or paracrine pathways.

In summary, osteocrin is a novel bone-active molecule that has no homology to any known protein family. The bone-specific expression pattern, direct-regulation by vitamin D and ability to modulate in vitro osteoblast function suggests that osteocrin represents a novel soluble regulator of bone metabolism. Further elucidation of the function and activity of osteocrin may provide new avenues for bone therapeutic approaches.

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