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Osteocrin Is a Specific Ligand of the Natriuretic Peptide Clearance Receptor That Modulates Bone Growth*[S]

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Osteocrin (Ostn) is a recently discovered secreted protein produced by cells of the osteoblast lineage that shows a well conserved homology with members of the natriuretic peptide (NP) family. We hypothesized that Ostn could interact with the NP receptors, thereby modulating NP actions on the skeleton. Ostn binds specifically and saturably to the NP peptide receptor-C (NPR-C) receptor with a K_d of ~5 nM with no binding to the GC-A or GC-B receptors. Deletion of several of the residues deemed important for NP binding to NPR-C led to abolition of Ostn binding, confirming the presence of a "natriuretic motif." Functionally, Ostn was able to augment C-type natriuretic peptide-stimulated cGMP production in both pre-chondrocytic (ATDC5) and osteoblastic (UMR106) cells, suggesting increased NP levels due to attenuation of NPR-C associated NP clearance. Ostn-transgenic mice displayed elongated bones and a marked kyphosis associated with elevated bone cGMP levels, suggesting that elevated natriuretic peptide activity contributed to the increased bone length possibly through an increase in growth plate chondrocyte proliferation. Thus, we have demonstrated that Ostn is a naturally occurring ligand of the NPR-C clearance receptor and may act to locally modulate the actions of the natriuretic system in bone by blocking the clearance action of NPR-C, thus locally elevating levels of C-type natriuretic peptide.

Osteocrin (Ostn)[3] is a recently discovered novel small secreted protein with prohormone-like characteristics (1). To date, the exact role of Ostn has not been elucidated. Limited homology, however, was observed between Ostn and members of the natriuretic peptide family, suggesting a possible functional link. The natriuretic system, key in the maintenance of vascular tone and cardiovascular homeostasis, consists of three related natriuretic peptides (NPs), ANP, BNP, and CNP (2) and three receptors (NPRs) mediating the biological activity of these peptides. The GC-A receptor, which preferentially binds ANP and BNP, and the GC-B receptor, whose cognate ligand is CNP, are coupled to guanylyl cyclase, producing cGMP as a secondary messenger (3, 4). The third receptor, NPR-C, has no guanylyl cyclase activity and binds all three NPs with similar affinity (5). To date, no specific endogenous ligand has been identified for NPR-C, and it is thought to act mainly as a clearance receptor (6). However, other biological functions have been postulated for this receptor (6).

A number of recent reports have demonstrated that the NPs also play a key role in regulation of the skeleton (7). Mice overexpressing either BNP (8) or CNP (9) or lacking NPR-C (10) display elongated bones, whereas mice lacking CNP (11) or a functional GC-B receptor exhibit dwarfism (12). Interestingly, we initially identified Ostn in cells of the osteoblast lineage, the bone-producing cells, which together with its regulatory effects on these cells suggested a role in bone activity. Given the homology of Ostn to the NP family and its putative bone regulatory action, a role for Ostn as a molecule mediating co-operation between the natriuretic and skeletal regulatory systems presented an intriguing possibility.

Investigation of the nature of Ostn interactions with the natriuretic system *in vitro* demonstrated specific binding to the natriuretic clearance receptor (NPR-C) and the ability to augment NP activity. Furthermore, overexpression of Ostn using the collagen type I promoter in transgenic mice resulted in enhanced bone growth associated with elevated cGMP levels. We, therefore, propose a role for Ostn as a specific NPR-C ligand capable of modulating local levels of CNP and its effects on skeletal development.

EXPERIMENTAL PROCEDURES

Reagents

Rat ANP-(1–28) and CNP-(1–22) were purchased from Sigma. The cGMP EIA (Biotrak System) was from Amersham Biosciences. The C-terminal synthetic mouse peptides, Ostn-(107–129) (amidated) (^{106}Y**D**HSKKRFGIPMDRIGRNRL-SNSR^{129}) and Ostn-(117–130) (^{116}C**M**DRIGRNRLSNSRG^{130}), were from Sigma and Affinity BioReagents (Golden, CO), respectively. These peptides are consensus mammalian

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[3] The abbreviations used are: Ostn, osteocrin; Ostn-TG, Ostn-transgenic mouse; rhOstn, recombinant human Ostn; NP, natriuretic peptide; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide; NPR, NP receptor; NPR-C, natriuretic clearance receptor; PLAP-Ostn, placental alkaline phosphatase-osteocrin fusion protein; HEK cells, human embryonic kidney cells; RT, reverse transcriptase.

sequences for these regions of the Ostn protein. The human peptides Ostn-(83–133) and ^{125}I-labeled Ostn-(83–133) were purchased from Phoenix Peptides (Belmont, CA).

Cell Culture

Human embryonic kidney cells (HEK293) and rat osteosarcoma cells (UMR106) were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Invitrogen). Mouse pre-chondrocytic cells, ATDC5, were maintained in 1:1 Ham's/F-12 (Invitrogen) with 10% fetal calf serum (Cansera), 1 mM glutamine (Wisent), 100 μg/ml streptomycin, 100 units/ml penicillin (Wisent), 30 nM sodium selenite (Sigma), and 10 μg/ml transferrin (Sigma). Exact experimental conditions are described below.

Vectors

To generate a secreted placental alkaline phosphatase-Ostn fusion protein (PLAP-Ostn), the mouse Ostn sequence covering amino acids 29–130 was amplified by PCR with forward 5′-tctctgtcgacttagcatcagg-3′ and reverse 5′-ccatcagcctctggaactggagag-3′ primers. The PCR product was digested with SalI (underlined) and cloned into an XhoI/PmeI-digested pAPtag5 vector containing the PLAP sequence (GenHunter, Nashville, TN).

The resulting PLAP-Ostn plasmid and the pAPtag5 were transiently transfected into HEK293 cells (QBiogene, Carlsbad, CA) using Effectene (Qiagen, Mississauga, ON, Canada). The day after transfection, cells were washed and incubated for 48 h in serum-free Dulbecco's modified Eagle's medium. The conditioned medium was collected, cells and debris were spun out, and the supernatant was stored at 4 °C after buffering with 20 mM HEPES (pH 8). Quantification of the PLAP-Ostn fusion protein was assayed by direct enzyme-linked immunosorbent assay using the Ostn-(107–129) peptide as standard curve. Briefly, samples and standards were mixed with carbonate buffer 100 mM (pH 10) and bound onto high protein-binding capacity polystyrene (Corning Costar) 96-well plates for 1 h at 37 °C. Wells were washed, blocked with 5% skim milk in Tris-buffered saline-Tween (TBST) for 1 h at 37 °C, incubated with the anti-Ostn antibody (1:2500 in TBST with 2.5% skim milk) for 1 h at 37 °C, then washed and incubated with an anti-rabbit antibody linked to horseradish peroxidase (Sigma) (1:30,000 in TBST with 2.5% skim milk) for 45 min at 37 °C. Revelation was achieved with *o*-phenylenediamine dihydrochloride Sigma Fast substrate (Sigma) in the dark for 30 min and read on a microplate reader at 492 nm. SDS-PAGE and Western blotting against Ostn was also performed to confirm the presence of the fusion protein.

The expression plasmid for rat GC-A containing the entire coding sequence cloned between the NheI-KpnI sites of cytomegalovirus-driven pBK plasmid (Stratagene, La Jolla, CA) was kindly provided by Dr. A. De Léan (Département de Pharmacologie, Université de Montréal). The human NPR-C and GC-B coding sequences were amplified by RT-PCR from human embryonic kidney poly(A) RNA (Clontech, Palo Alto, CA) with the following primer pairs: NPR-C, 5′-agggcaagctctttcttgcg-3′ (forward) and 5′-gggcttcctttaagctactg-3′ (reverse); GC-B, 5′-ctgctgctttatccccatgg-3′ (forward) and 5′-ggtttacaggagtccaggag-3′ (reverse). The resulting PCR products were then cloned downstream of the cytomegalovirus promoter into the pcDNA1.1 plasmid (Invitrogen). All constructs were validated by DNA sequencing.

Recombinant Human Ostn

To produce a bacterial human Ostn (recombinant human Ostn (rhOstn)), its cDNA encoding amino acids 23–133 was PCR-amplified with oligos 5′-gagggtacccgtagatgtaacaacaacagagg-3′ (forward) and 5′-ctcctgcagttagcctctggaatttgaaagccg-3′ (reverse). The purified PCR fragment was digested with KpnI and PstI and cloned into pQE30 plasmid and transformed into the *Escherichia coli* strain SG13009 (Qiagen). The N-terminal six-histidine-tagged rhOstn was purified from the soluble bacterial extract by sonication followed by chromatography through nickel-nitrilotriacetic acid-Sepharose (Qiagen) and a Sepharose-SP cationic exchanger (GE Healthcare). The final rhOstn preparation was estimated to be ~95% pure by SDS-PAGE and silver staining and was quantified by direct enzyme-linked immunosorbent assay as described above.

Binding Studies

PLAP-Ostn Fusion Protein—Binding studies were performed as described previously (13–15). Briefly, HEK293 cells were transiently transfected with the appropriate expression plasmids (GC-A, GC-B, or NPR-C) or a negative control (cytomegalovirus-based green fluorescent protein expression plasmid (pQBIfc3, Qbiogene)). Forty-eight hours later cells were washed twice with Hanks' balanced salt solution containing 0.1% D-glucose, 0.5% bovine serum albumin, 20 mM HEPES, and 0.05% NaN_3. Binding of the PLAP-Ostn-containing conditioned media with or without the various peptides (500 μl total/well) was performed at 25 °C for 15 min. Cells were washed 6 times with Hanks' balanced salt solution for 5 min each and lysed with 10 mM Tris-HCl (pH 8) containing 0.1% Triton X-100 at 25 °C, and endogenous alkaline phosphatase was inactivated at 65 °C for 10 min. PLAP activity was measured in the linear range by a standard enzymatic assay using *p*-nitrophenyl phosphate as substrate (Sigma).

^{125}I-Labeled Ostn-(83–133)—Intact cell binding studies with radiolabeled Ostn-(83–133) were carried out in confluent ATDC5 cells. Briefly, ATDC5 cells were washed in cold phosphate-buffered saline then incubated in Ham's/F-12 plus 0.1% bovine serum albumin with 50,000 cpm of ^{125}I-labeled Ostn-(83–133) for 90 min at 4 °C with varying concentrations of cold Ostn-(83–133) competitor. Cells were washed in cold phosphate-buffered saline and lysed into 0.5 M NaOH, and the bound cpm were counted on a Wallac Wizard 1470 gamma counter (PerkinElmer Life Sciences). Binding curves were analyzed by nonlinear regression using GraphPad Prism software (GraphPad Software, San Diego, CA).

cGMP Assays

For cGMP assays, cells were washed and incubated for 10 min in culture media containing 0.1% bovine serum albumin and 0.25 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Sigma) to minimize cGMP degradation. Treatments were carried out in the presence of 3-isobutyl-1-methylxanthine for 15 min, and cells were collected in ice-cold 65% ethanol. Cell extracts were assayed in duplicate using

cGMP Direct Biotrak EIA (Amersham Biosciences) according to the manufacturer's protocol.

Generation of Transgenic Mice

Transgenic mice were generated by nuclear microinjection of a 4454-bp DNA fragment containing the rat collagen 1 α1 3.6-kilobase promoter (−3500 to +115) (GenBank™ accession number J04464) and the mouse Ostn coding region. Five hundred copies were microinjected into the pronuclei of C3B6F1 fertilized eggs (C57BL/6J x C3H/HeJ F1 hybrid) which were then transplanted to the oviducts of pseudopregnant foster mothers using standard protocols at the Transgenic Facility at the Institut de Recherches Cliniques de Montréal (16). Three independent mouse lines, 650, 677, and 688, were generated arising from three different founders. Genotyping was carried out by Southern analysis of EcoRI-digested genomic DNA with a mouse Ostn coding region probe or by PCR using inter-exon primers covering the Ostn coding region (1). Long bone lengths were measured using fine calipers on dissected bones. Tail lengths were measured from anus to tail tip using a ruler. All measurements were undertaken by the same operator blinded to the mouse genotype.

For immunohistochemistry, bones were fixed, decalcified, embedded, and cut according to standard protocols (17). An Ostn-specific antibody (1) was used for immunolabeling and visualized with DAKO Envisision + System-HRP (AEC) (DAKO, Carpinteria, CA) as per the manufacturer's protocol. Proliferation of growth plate chondrocytes was assessed using a proliferating cell nuclear antigen staining kit (Zymed Laboratories Inc., San Francisco, CA) on 7-week-old tibiae from Ostn-Tg and wild type littermates. After immunohistochemistry, positively stained cells in the entire growth plate were counted and expressed as the percentage of total cell number.

To measure cGMP levels in the bones of wild type and transgenic mice, 10–14-day-old mice were euthanized, and the femurs and tibia were dissected and cleaned of any adjacent soft tissue. The whole bones including epiphysis, metaphysis, diaphysis, and marrow were then immediately homogenized in 1 ml of cold 65% ethanol using a Polytron homogenizer and stored at −80 °C until assay. For assay, the bone extracts were spun at 12000 rpm at 4 °C for 10 min, and the supernatant was evaporated to dryness and resuspended in 1 ml of cGMP assay buffer. The cGMP assay was then carried out according to the manufacturer's protocol using 25-μl aliquots as for the cellular assays.

Northern Blotting

RNA was isolated from whole bones, isolated tissues, or whole cell lysates using Trizol™ (Invitrogen). Tendons and muscles were obtained from 3-month-old rats and the bone tissue from 4-day-old neonates. Northern blots were generated on nylon membranes (Osmonics, Westborough, MA) by standard methods (18). Filters were prehybridized for 4 h and hybridized overnight in Church buffer (19) at 65 °C. The rat Ostn cDNA probe corresponded to the full coding sequence. A mouse glyceraldehyde-3-phosphate dehydrogenase cDNA probe corresponding to −21 to 956 bp of GenBank™ accession number M32599 was generated by PCR. Probes were labeled with [α-^{32}P]dCTP using a standard random priming protocol (18).

RT-PCR for Natriuretic Peptide Receptors

RNA was extracted from confluent ATDC5 prechondrocytes and UMR106 osteoblasts 7 days post-confluence using Trizol™. For RT-PCR, cDNAs were generated with Superscript II™ reverse transcriptase (Invitrogen) and random hexamer priming from 2 μg of total RNA, and PCR amplification was carried out with gene-specific primers using Taq DNA polymerase (Amersham Biosciences). Gene-specific primers (all 5′-3′) and conditions were as follows: NPR-A, forward tggatctcaagtgggagcacag and reverse gatctgcatagagcacaagc (annealing temperature = 58 °C, 35 cycles, product 261 bp); NPR-B, forward aactgatgctggagaaggagc and reverse gcgagtaagatggttgaactggac (annealing temperature = 58 °C, 35 cycles, product 305 bp); NPR-C, forward ctacatccaaggcagcgagcg and reverse gcaaccacagagaagtcccca (annealing temperature = 56 °C, 35 cycles, product 492 bp); 18 S ribosomal RNA, forward tcaagaacgaaagtcggagg and reverse ccaactaagaacggccatgc (annealing temperature = 62 °C, 20 cycles, product 324 bp).

RESULTS

Ostn Sequence Homology—Initial analysis of Ostn species conservation identified Ostn in humans, cows, mice, rats, chickens, and snakes (1). Further analysis through genomic data mining has identified Ostn in amphibians (*Ambystoma tigrinum tigrinum*, Eastern tiger salamander) and fish (*Danio rerio*, Zebrafish) as well as chimpanzee, pig, and dog. Fig. 1 shows the alignment between Ostn protein sequences from various vertebrate species with strong conservation of the C-terminal half of Ostn (*e.g.* human to amphibian = 81% similarity).

Within the Ostn C-terminal region are two highly conserved putative dibasic motifs (Fig. 1, *shaded*) which may represent active processing sites for proteinases. N-terminal microsequencing of purified Ostn from the conditioned media of HEK293 cells stably expressing mouse Ostn revealed the presence of a fragment starting at Ser^{80}, thus demonstrating processing at the KKKR site. Processing at the KRR site has not as yet been demonstrated.

The two dibasic sites delimit similar sequences (Fig. 1, *boxed*), which contain motifs found in the NPs (NP-like motifs, *NM*). Fig. 2 shows the alignment between the human Ostn motifs (NM1 and NM2) and NPs. Residues shaded in *gray* are well conserved, particularly those marked with *asterisks* (Phe^{7}, Gly^{8}, and Arg^{13}, numbered according to CNP) which are considered important in binding to the NPR-C receptor. Note, however, the absence of the two cysteine residues present in all NPs (Fig. 2, *shaded black*).

Extra-osseous Ostn Expression—Although initially identified as an osteoblast-specific gene, further analyses of non-osseous tissues demonstrated Ostn expression in other stromal-derived tissues. Northern blotting has demonstrated that Ostn was expressed at significant levels in both leg tendons/ligaments and skeletal muscle of young adult rats (Fig. 3). Ostn levels appeared very high in tendons, with weaker expression in muscle. However, direct comparisons are difficult to draw due to differing cell den-

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Human       MLDWRLASAHFILAVTLTLWSSGKVLSVDVTTT-EAFDSGVIDVQSTPTVREEKSATDLTAKLLLLDELV
Chimpanzee  MLDWRLASAHFILAVTLTLWSSGKVLSVDVTTT-EAFDSGVIDVQSTPTVREEKSATDLTAKLLLLDELV
Dog         MLDWRLANAHFILAMTLMLWSSGKVHSVDVAT--EAFDSGVIDVQSPPTVREEKSATNLAAKLLLLNELV
Bovine      MLDWRLASAHFILAMTLMLWSSGKVFSVGVTT--EAFDSGVLGVQSSPTVREAKSATDLAAKLLLLDELV
Pig         MMDWRLASVHFILAVTLMLWSSGKVLSMDVTT--KAFDSELIDVEPPPTMTEEKSATDLAAKLLLLDELV
Mouse       MLDWRLASTHFILAMIVMLWGSGKAFSVDLASQ----EFGTASLQSPPTAREEKSATELSAKLLRLDDLV
Rat         MLDWRLASAHFLLAMILMLWGSGKAFSVDLAS--EASEFGAESLQSPPTTREEKSATELAAKLLLLDDLV
Chicken     MLQFQLVVVHLALVITLLQWHSSSVLLAEAAPEP-LEPSAALGMAAHPTASEEKSASSLAAKLLLLDELV
Salamander  MLESRFLCARFLLAVGLIQWNAGRLLQAGAAPE-SSDSSRLLDTGSHSASSEEKAATDLVAKLLLLDELV
Zebrafish   MLGCGCVLLSCLLTLTLFHCSAESLHIPQGRPE-YVESSVVEGRSVQRGQMEQKTSGALSAKLLLHDQLV
Python      ----------------------------------------------TASEEKSATDLVAKILLLNELV

Human       SLENDVIETKKKRSFSGFGSPLDRLSAGSVD-HKGKQRKVVDHPKRRFGIPMDRIGRNRLSNSRG
Chimp       SLENDVIETKKKRSFSGFGSPLDRLSAGSVD-HKGKQRKVVDHPKRRFGIPVDRIGRNRLSNSRG
Dog         SLENDVIETKKKRSFSGFGSPLDRLSAGSVD-HKGKQRKVIDHPKRRFGVPVDRIGGNRLSNSRG
Bovine      SLENDVIETKKKRSFSGFGSPLDRLSAGSVS-HKGKQRKVVDHPKRRFGIPMDRIGRNRLSNSRG
Pig         SLENDVIETKKKRSFSGFGSPLDRLSAGSPD-HKGKQRKVVDHPKKRFGIPVDRIGRNRLPNSRG
Mouse       SLENDVFETKKKRSFSGFGSPLDRLSAGSVE-HRGKQRKAVDHSKKRFGIPMDRIGRNRLSSSRG
Rat         SLENDVFETKKKRSFSGFGSPLDRLSAGSVE-HRGKQRRVVDHSKKRFGIPMDRIGRNRLSSSRG
Chicken     SLENEVTETKKKRSFPGFGSPIDRISATSVD-AKGKQRKVVELPKRRFGVPLDRIGVSRLGNTKG
Salamander  SLENDVMETKKKRSFPGFGSPLDRLSAASTE-LKTKQRKVVEHPKRRFGVPLDRIGVNRLSNSRG
Zebrafish   RLENDVIETKRKRSFPGSNTPLDRLSISTMDPKSNKQRKAVELPRRRVSVPIDRIGVGRLPSSRG
Python      SLENDVFETKKKRSFSGFGSPLDRLSVG----LKAKQRKAVELPKKRFGIPLDRIGVNRLSGSRG
                                  NM1                              NM2
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FIGURE 1. **Alignment of Ostn amino acid sequences from various species.** Alignment of Ostn amino acid sequences from 11 species. The putative cleavage sites are *shaded*, and the two regions with homology to the NPs, NM1 and NM2, are *boxed*. Sequences were derived as previously described (1) and from GenBank ESTs CF787546 (pig), CN052128 (salamander), and AL918290 (zebrafish) and Ensembl genome release 21.3b.1, 21.1.1, and pre-release for zebrafish, chimpanzee, and dog. The human protein is numbered 1–133 for reference.

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NM1  (87-95)          FGSPLDRLS
NM2  (116-124)        FGIPMDRIG
ANP  (1-28)      SLRRSSCFGGRMDRIGAQSGLGCNSFRY
BNP  (1-32)   SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH
CNP  (1-22)      GLSKGCFGLKLDRIGSMSGLGC
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FIGURE 2. **Ostn homology to the NPs.** Amino acid alignment of human NM1 and NM2 with human ANP, BNP, and CNP. Identical residues are *shaded gray*, and the cysteines conserved in the NPs but absent in Ostn are *shaded black*. The residues important for binding of NPs to NPR-C and conserved in Ostn are marked by *asterisks*.

sities and cell population homogeneity in the various tissues. Preliminary immunohistochemistry localization of Ostn in long bones and teeth confirmed it is present in knee joint and periodontal ligaments (supplemental Fig. 1).

Ostn Binding to Natriuretic Peptide Receptors—Two approaches were used to characterize Ostn binding to NPRs. First, a heterologous expression system (transiently transfected HEK293 cells) in conjunction with an Ostn-fusion reporter (PLAP-Ostn) was utilized to demonstrate receptor-specific binding. Further characterization of the binding of Ostn to the NPR-C receptor was then undertaken using an iodinated synthetic C-terminal Ostn peptide (^{125}I-labeled Ostn-(83–133)) in a pre-chondrocytic cell line (ATDC5) and which endogenously express natriuretic receptors. These cells have the potential to differentiate into matrix producing chondrocytes with insulin treatment. However, for our studies the presence of excessive matrix would greatly complicate binding studies, and the expression pattern of the NPRs was most appropriate in the pre-differentiated confluent state. Functional consequences of Ostn binding to NPR-C were then demonstrated by an

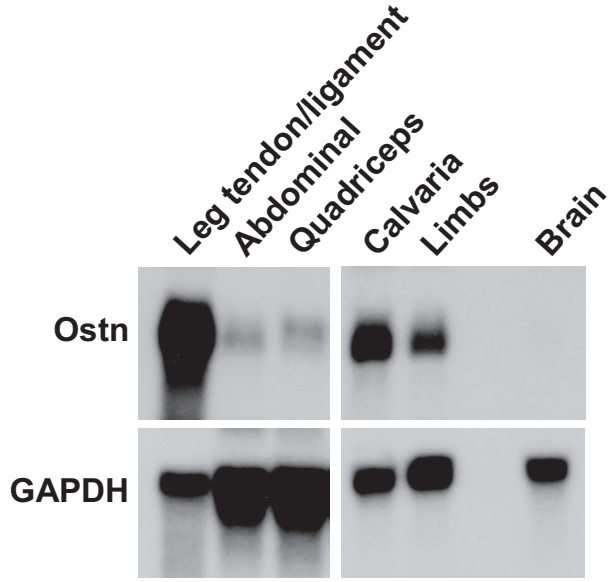

FIGURE 3. **Expression of Ostn in adult rat non-osseous tissues.** Northern blots were generated with total RNA extracted from adult leg tendons/ligaments, abdominal muscles, quadriceps, and brain and neonate calvaria and limbs (whole tibiae and femora). Significant Ostn expression is seen in adult tendon and to a lesser extent in muscle. A high level of Ostn expression is also seen in neonate bone with no expression in adult rat brain. Twenty μg of total RNA were loaded. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown as a loading control.

enhanced cGMP response to CNP treatment in ATDC5 cells and an osteoblastic cell line, UMR106.

The Ostn fusion protein, PLAP-Ostn, comprised an N-terminal secreted PLAP moiety linked to full-length mouse Ostn

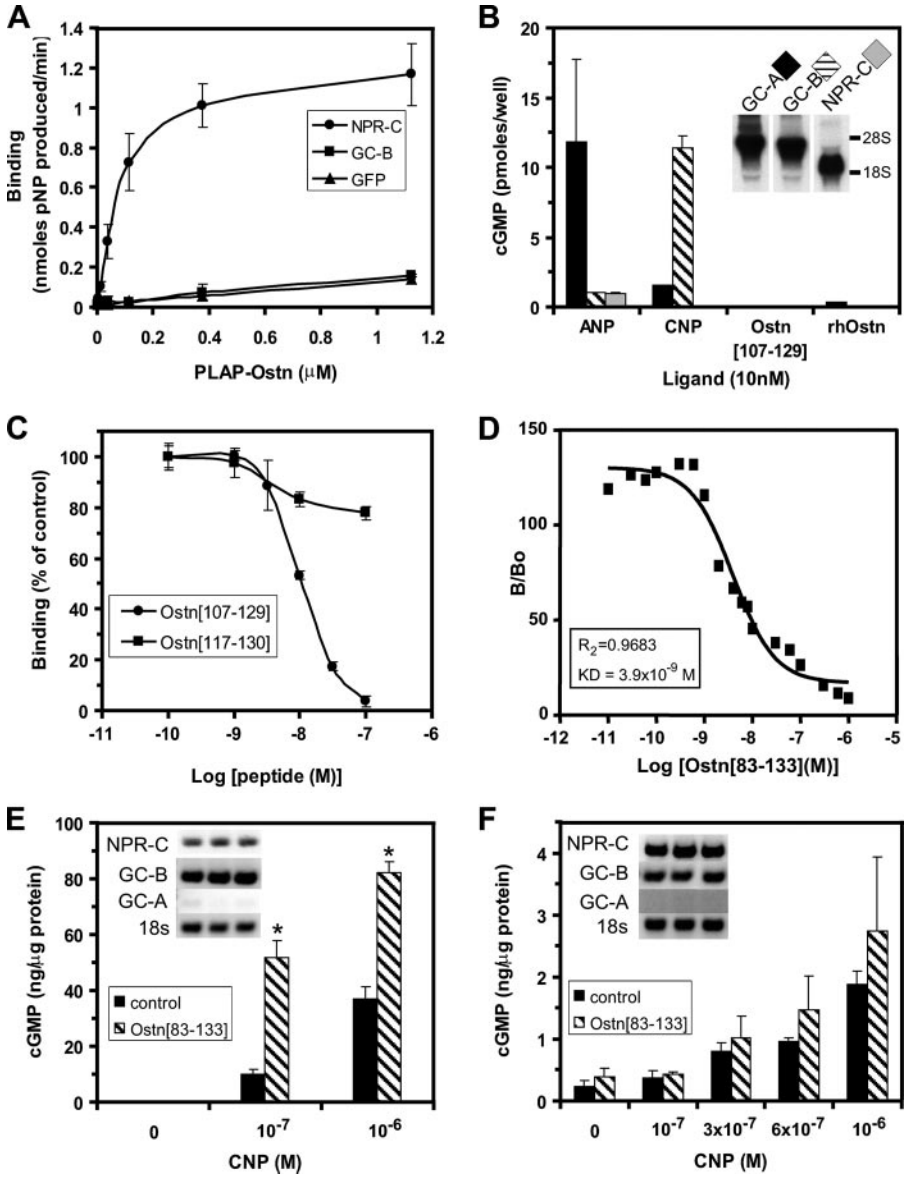

FIGURE 4. **Characterization of binding of Ostn to the NPR-C receptor and its effects on ANP activity.** *A*, PLAP-Ostn binding to NPR-C-is specific and is saturable. Identical nonspecific binding of PLAP-Ostn to GC-A-, GC-B-, or green fluorescent protein-transfected cells was observed. Only results for the green fluorescent protein and GC-B binding are shown for clarity (mean ± S.D.; $n = 4$). *B*, functional validation of the GC-A and GC-B constructs by transient transfection in HEK293 cells. Levels of cellular cGMP were measured after 15 min of incubation with 10 nM ANP, CNP, Ostn-(107–129), or rhOstn. No induction of cGMP was seen in response to Ostn-(107–129) or rhOstn. Northern blotting shows similar levels of expression of the transfected receptors. Data are representative of three independent experiments performed in triplicate. *C*, displacement curve of PLAP-Ostn (30 nM) by Ostn-(107–129) or Ostn-(107–129) peptides in NPR-C-overexpressing HEK293 cells. Data are representative of three independent experiments performed in triplicate. *D*, displacement curve of ^{125}I-labeled Ostn-(83–133) (50,000 cpm) by Ostn-(83–133) in ATDC5 cells. Data are representative of four separate experiments each with three replicates. *E*, augmentation of cGMP induced by 10^{-7} and 10^{-6} M CNP by co-treatment with 10^{-7} M Ostn-(83–133) in ATDC5 prechondrocytes. Expression of NPRs was confirmed by RT-PCR in triplicate (*inset*). *, $p \leq 0.01$, CNP *versus* CNP + Ostn-treated. The figure is representative of two separate experiments each with three replicates. *F*, augmentation of cGMP induced by 10^{-7}–10^{-6} M CNP by co-treatment with 10^{-7} M Ostn-(83–133) in UMR106 osteoblasts. Expression of NPRs was confirmed by RT-PCR (*inset*). The figure is representative of two separate experiments each with three replicates. K_d values for binding data were calculated using GraphPad Prism (GraphPad Software). Comparisons of CNP *versus* CNP/Ostn treatment were made by analysis of variance using Statview (SAS Institute, Cary, NC).

(residues 29–130). Conditioned media containing PLAP-Ostn was used to assess Ostn binding on transiently transfected HEK293 cells. We first wished to establish the specificity of PLAP-Ostn for the different NPRs. Saturable binding of the PLAP-Ostn fusion protein was observed exclusively on NPR-C-overexpressing cells (Fig. 4*A*). No specific binding was seen on cells overexpressing either green fluorescent protein, GC-B (Fig. 4*A*) or GC-A (not shown). To verify functionality of the overexpressed receptors, intracellular cGMP levels were measured in transfected HEK293 cells upon stimulation with the appropriate ligand. As expected, ANP elicited the greatest response via GC-A and CNP through GC-B, indicating the receptors were functionally expressed in this heterologous system (Fig. 4*B*). Because of the presence of endogenous GC-A in HEK293 cells, low levels of cGMP were also induced by ANP after transfection with either GC-B or NPR-C (Fig. 4*B*). Consistent with absence of PLAP-Ostn binding to GC-A or GC-B, both a C-terminal Ostn peptide encompassing NM2 (Ostn-(107–129)) (see Fig. 2) or a recombinant form of human full-length Ostn (rhOstn) failed to activate either GC-A or GC-B (Fig. 4*B*).

To investigate the role of the proposed natriuretic motifs in Ostn binding to NPR-C, two synthetic Ostn peptides, Ostn-(107–129) described above and Ostn-(107–129), lacking part of NM2, were used for competition binding studies. HEK293 cells transfected with NPR-C were co-incubated with ~30 nM PLAP-Ostn and increasing concentrations of Ostn-(107–129) or Ostn-(107–129). Ostn-(107–129) was able to compete off 50% of the binding of PLAP-mouse Ostn in the $\sim 10^{-9}$–10^{-8} M range, in contrast to Ostn-(107–129), which was unable to efficiently compete up to 10^{-7} M (Fig. 4*C*).

Having established that Ostn binds to the NPR-C receptor and that at least the NM2 motif was important for this interaction, we further characterized binding in ATDC5 cells which express both GC-B and NPR-C receptors (Fig. 4*E*, *inset*) (20, 21). Competitive binding experiments with ^{125}I-labeled Ostn-(83–133) and increasing concentrations of cold Ostn-(83–133) gave an average K_d of 4.8 ± 0.9 nM with a curve characteristic of competitive binding for a single receptor site (Fig. 4*D*).

Binding of Ostn to NPR-C could result in attenuation of the clearance action of this receptor, thus increasing NP availability and cGMP production upon stimulation of GC-A or GC-B. We tested this hypothesis in ATDC5 and UMR106 cells whereby NP signaling (cGMP production) was assessed in the presence or absence of Ostn-(83–133). ATDC5 cells express both GC-B and NPR-C at confluence (Fig. 4*E*, *inset*). When treated with 10^{-7} or 10^{-6} M CNP, ATDC5 cells produced 10.1 ± 1.9 and 37.0 ± 4.5 ng of cGMP/μg of protein, respectively. However, if the cells were incubated with the same concentrations of CNP together with 10^{-7} M Ostn-(83–133), cGMP production was augmented 5.2- and 2.2-fold, respectively ($p \leq 0.01$). UMR106 osteoblasts were used at 7 days post-confluence when both GC-B and NPR-C were expressed (Fig. 4*F*, *inset*). Treatment of UMR106 with a range of CNP concentrations from 10^{-7} to 10^{-6} M resulted in a dose-responsive cGMP production. At all CNP concentrations tested, co-treatment with Ostn augmented cGMP production, with an average increase of 1.4-fold ($p \leq 0.05$) (Fig. 4*F*). Using the heterologous HEK293 model, we showed that Ostn could not compete off either ANP or CNP for the endogenous GC-A or the overexpressed GC-B receptors, respectively (see supplemental Figs. 2 and 3). However, in both cases Ostn could effectively compete with either ANP or CNP for the NPR-C receptor, with concomitant activation of their respective cognate receptors and increase in intracellular cGMP accumulation (supplemental Figs. 2 and 3).

Characterization of Ostn Transgenic Mice—Reflecting the initial identification of Ostn in osteoblastic cells, we investigated its role in the skeleton *in vivo* by generating transgenic mice utilizing the rat 3.6-kilobase collagen type I promoter to overexpress mouse Ostn in osteoblast-lineage cells (Ostn-TG) (22). It should be noted, however, that the collagen type I promoter also drives expression in the perichondrium, skin, and tendons (23). Three independent mouse lines were established and analyzed with all three lines showing similar phenotypes. Northern blotting confirmed high levels of transgene expression in bone with no apparent effects on endogenous expression (data not shown). Immunohistochemical staining demonstrated elevated Ostn protein levels in osteoblastic cells of 4-day-old Ostn-TG tibiae *versus* wild type littermates (Fig. 5, *A* and *B*). Clear expression can be seen in the cuboidal osteoblasts in the primary spongiosa and surrounding periosteum, sites rich in osteoblasts. Expression was also seen in the perichondrium surrounding the growth plate (a known site of collagen type I expression). Ostn-TG mice displayed no gross physiological defects, with similar body weight to their wild type littermates. Life spans of the Ostn-TG mice appeared normal with no evidence of early death up until 12 months of age. Bone mineral density as well as lean and fat mass, as measured by dual energy x-ray absorptiometry (DEXA) in 8-month-old mice from the three transgenic lines, showed no significant differences between transgenic and wild type littermates. All three Ostn-TG mice lines did exhibit one significant phenotype, however, that of elongated limbs and tails and a marked kyphosis (Fig. 5, *C* and *D*). The kyphosis and elongated tail length were evident from weaning and were present throughout the adult life of the mouse. The kyphosis was presumably due to elongated vertebrae causing a spinal deformation. Measurements of tail (Fig. 5*G*) and

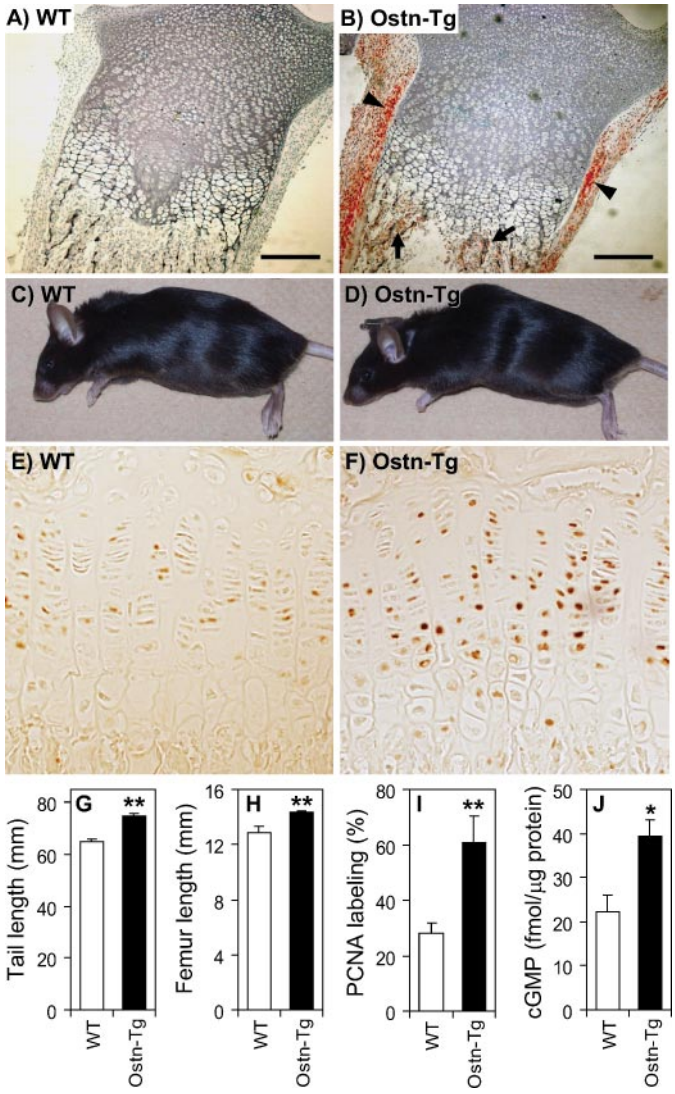

FIGURE 5. **Ostn-transgenic (Ostn-TG) mice have longer bones.** Ostn-TG mice with overexpression of Ostn in osteoblast lineage-specific cells were generated using the 3.6-kilobase collagen type I promoter. Immunohistochemistry using an Ostn-specific antibody on 4-day-old tibia of wild type (*WT*) (*A*) and Ostn-TG (*B*) mice. Ostn expression in WT is non-detectable (*A*), whereas overexpression in Ostn-TG is evident (*red staining*) on cuboidal osteoblasts adjacent to trabecular bone of the primary spongiosa (*arrows*) and within the perichondrium (*arrowheads*) (*B*). Counter stain is methyl green. The *scale bar* is 200 μm. *C* and *D*, gross appearance of WT (*C*) and Ostn-TG (*D*) which have a marked kyphosis presumably due to vertebral overgrowth. *E* and *F*, proliferation in growth plate chondrocytes was measured in tibiae of 7-week-old male mice using proliferating cell nuclear antigen immunohistochemistry. Proliferating chondrocytes are clearly more abundant in Ostn-TG (*F*) mice than WT littermates (*E*). Tail (*G*) and femur (*H*) lengths of 8-week-old Ostn-TG line 650 males are significantly longer than wild type littermates (n = 7–12). *I*, quantification of proliferating cell nuclear antigen immunohistochemistry showed an 80% increase in staining of growth plate chondrocytes (n = 4) (*J*). cGMP levels were significantly higher in 10–14-day-old Ostn-TG mice femurs and tibiae than their WT littermates (n = 11–28). $p < 0.05$ (*) and $p < 0.01$ (**), Ostn-TG *versus* WT. Data are expressed as the mean ± S.E. Comparison were made by analysis of variance using Statview.

femur (Fig. 5*H*) lengths in 8-week-old males in the 650 line showed 15 and 12% increases, respectively, compared with wild type littermates ($p < 0.01$). Overall, across the three transgenic lines, tail-length was increased 14.5 ± 1.2% ($p < 0.01$) and the length of all long-bones by 7.1 ± 0.4% ($p < 0.05$) (n = 8–13). An underlying cause of increased bone length could be changes in the growth

plate. Analysis of the growth plate in 7-week-old male tibiae showed no significant changes in the width of proliferative of hypertrophic zones, but a doubling in proliferation in the growth plate chondrocytes was seen by proliferating cell nuclear antigen labeling ($p < 0.05$; $n = 4$) (Fig. 5, *E–F* and *I*).

To determine whether the increases in bone length in Ostn-TG mice could be due to increased NP signaling, cGMP levels were measured in the femurs and tibias of these animals. Levels of cGMP in 10–14-day-old Ostn-TG bones were 77% higher than in wild type littermates ($p < 0.05$)(Fig. 5*J*), thus confirming Ostn was modulating NP activity in bone.

DISCUSSION

Previously we have reported the identification and initial characterization of Ostn (1). Further analysis of the extensive sequence databases now available provided confirmation of the presence of Ostn across a range of mammalian, bird, and amphibian species. Interestingly, it should be noted, however, that the homology is reduced in zebrafish (54% similarity), and Ostn has not yet been identified in the puffer fishes *Fugu rubripes* and *Tetraodon nigroviridis*. Such departure from the stronger conservation evident in higher vertebrates may represent the differing requirements for salt and water homeostasis in fish. Furthermore, differences in the cellularity of bone, such as osteocyte numbers and, thus, skeletal physiology, may further explain the absence of Ostn in *Fugu* (24, 25).

The most consistent homology between species for Ostn lies in the C-terminal region of the protein, and it is this region that displays homology to the NPs. Most interestingly, the best conserved homology with the NPs includes the residues Phe[7], Gly[8], and Arg[13] (numbering according to CNP) that have been demonstrated to be necessary for peptide binding to the NPR-C receptor (26–28). Furthermore, the lack of the two cysteine residues present in all NPs indicates Ostn does not form the cyclic ring structure that is essential for binding to the receptors signaling through cGMP, GC-A, and GC-B (29, 30). Interestingly, synthetic ring-deleted, linear analogues of the NPs such as des-Cys[105], Cys[121]-(104–126), C-ANF (des-[18]QSGLG[22]-ANP (4–23)), and AP-811 have been shown to be specific ligands of NPR-C (26, 28, 31–33), suggesting that Ostn and its derived proteolytic peptides could represent specific, naturally occurring ligands of NPR-C.

Specific and saturable binding of Ostn to overexpressed NPR-C was demonstrated *in vitro*, and this binding appeared to be mediated through the natriuretic motif that we identified (Fig. 2). Previous studies have estimated the K_d of other NPR-C-specific ligands, such as AP-811 and C-ANF, for the NPR-C receptor to be in the high picomolar/low nanomolar range (26, 28, 33, 34). Thus, our estimate of a K_d of ~5 nM in ATDC5 cells appears to be an appropriate affinity to enable functionality.

More importantly Ostn was capable of attenuating the inhibitory action of NPR-C on the ability of both ANP and CNP to stimulate their cognate receptors GC-A and GC-B. Thus, we demonstrated that full-length Ostn protein or a fragment thereof can bind to NPR-C and partially block its clearance activity toward NPs thereby restoring signaling.

Overexpression of Ostn in the bones of transgenic mice lead to a significant increase in bone length and a marked kyphosis

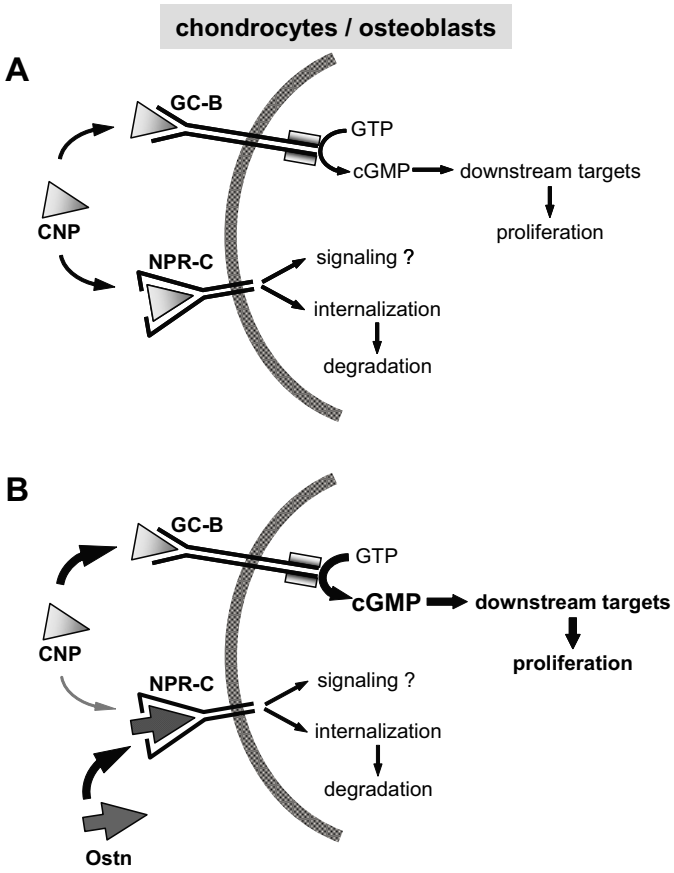

FIGURE 6. **Ostn blocks the clearance action of NPR-C thereby increasing activity of the NPs in the bone compartment.** *A*, both the GC-B and NPR-C receptors are expressed in osteoblasts and chondrocytes; thus, the activity of the NPs (normally CNP in the skeleton) is governed by the distribution of CNP between the signal mediating GC-B receptor and the NPR-C clearance receptor. *B*, in the presence of Ostn, CNP access to NPR-C is blocked leading to increased binding of CNP to the GC-B receptor. This results in an increase in GC-B-mediated cGMP production, magnifying the downstream biological effects of the natriuretic system, which in the skeleton leads to elongated bones.

(presumably due to vertebral overgrowth). This Ostn-TG phenotype was strikingly reminiscent of the NPR-C knock-out mice (10, 35) as well as the BNP- and CNP-overexpressing mice (36, 37). In all cases, bone overgrowth presumably correlated with increased NP bioavailability. The presence of the GC-A and GC-B receptors and production of cGMP in response to NPs has been well established in both osteoblasts (38–43) and chondrocytes (21, 44–47).

Although blocking of the NPR-C receptor may result in increased bioavailability of all three NPs, CNP is considered to be the major effector in bone. Mice lacking CNP displayed severe dwarfism associated with reduced growth plate heights (11), whereas inactivation of either the ANP or BNP locus does not cause skeletal abnormalities (48, 49). Overexpression of CNP in chondrocytes rescued achondroplasia (9), and CNP but not ANP or BNP has been demonstrated to be expressed in cultured tibiae (50). Thus, we propose a mechanism whereby Ostn potentiates the effects of CNP on longitudinal bone growth through binding NPR-C and blocking its clearance action (Fig. 6).

Previous models demonstrating bone length changes mediated by alterations in CNP/GC-B/NPR-C activity appear to be

due to changes in the growth plate, with altered chondrocyte proliferation and differentiation (9, 11, 37). Similarly, we saw an increase in proliferation in growth plate chondrocytes, suggesting Ostn may act to stimulate bone growth through actions on the growth plate chondrocytes in the same fashion as direct manipulation of the natriuretic system. CNP antagonism of the anti-proliferative effects of FGF2/FGFR3 in chondrocytes has been proposed as a mechanism through which such changes could occur (51). Interestingly, endogenous Ostn has been shown to be expressed in mature chondrocytes (52). The secreted soluble nature of Ostn would also allow its activity distal to the point of expression allowing osteoblasts adjacent to the growth plate to exert paracrine effects. Thus, the elevated Ostn expression in the osteoblasts and perichondrium surrounding the growth plate in the Ostn-TG would also allow paracrine actions on the chondrocytes.

There is mounting evidence that NPR-C is more than a clearance receptor (for review, see Ref. 6). NPR-C-specific ligand binding has been demonstrated to inhibit adenylate cyclase (53, 54) and modulate ion channel activity (55, 56). Thus, the discovery of an endogenous and specific ligand for NPR-C may have further significance beyond just quenching of NPR-Cs clearance function. The role of the natriuretic peptides in adult bone has not been studied. Interestingly Ostn is down-regulated in aged bone and by 1,25-dihydroxyvitamin D_3 (1), which is reciprocal to NPR-C (39, 57). The potential thus exists for Ostn to counter the possibly inhibitory effects of increased NPR-C activity in aged bone and potentially even exert anabolic effects.

Of further interest is the fact that we have detected expression of Ostn in other mesenchymal tissues such as muscle fascia and tendons corroborating other studies showing muscle expression (58–61). Expression of Ostn in tendons is of particular interest due to high levels of CNP seen in tendons and muscles,[4] alluding to a role of Ostn as a modulator of CNP action in these tissues as well. These tissues all originate from a common stromal progenitor population presenting an intriguing role for Ostn in mediating interactions between the natriuretic system and tissues of stromal origin.

Thus, we have identified Ostn as the first naturally occurring NPR-C specific ligand, thereby illustrating another level of regulation in the action of the NPs and suggesting a mechanism for direct regulatory interaction between the natriuretic pathway and the skeletal and other mesenchymal systems. The recent identification of a human syndrome, Acromesomelic Dysplasia, type Maroteaux, which is characterized by dwarfism caused by a mutation in the GC-B receptor, further highlights the potential importance of regulation of the natriuretic system in the skeleton (62). Elucidation of the nature of these interactions will provide valuable insight into possible therapeutic interventions such as was recently demonstrated by rescue of achondroplasia using CNP (9).

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[4] E. Espiner and T. Prickett, personal communication.

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