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Sturgeon Osteocalcin Shares Structural Features with Matrix Gla Protein: Evolutionary Relationship and Functional Implications

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Running title: *An ancestral osteocalcin with MGP features*

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Background: Osteocalcin (OC) and matrix Gla protein (MGP) are evolutionarily related proteins present in bone matrix.
Results: Sturgeon OC exhibits structural and expression/accumulation features similar to MGP.
Conclusion: Data supports the hypothesis that OC originated from MGP and sturgeon protein represents an ancestral form of OC.
Significance: New insights into the evolution of bone Gla proteins and vertebrate bone tissue.

SUMMARY

Osteocalcin (OC) and matrix Gla protein (MGP) are considered evolutionary related because they share key structural features although they have been described to exert different functions. In this work, we report the identification and characterization of both OC and MGP from the Adriatic sturgeon, a ray-finned fish characterized by a slow evolution and the retention of many ancestral features. Sturgeon MGP shows a primary structure, post-translation modifications, and patterns of mRNA/protein distribution and accumulation typical of known MGPs, and it contains 7 possible Gla residues that would make the sturgeon protein the most γ-carboxylated among known MGPs. In contrast, sturgeon OC was found to present a hybrid structure. Indeed, while exhibiting protein domains typical of known OCs, it also contains structural features usually found in MGPs (e.g. a putative phosphorylated propeptide). Moreover, patterns of OC gene expression and protein accumulation overlap with those reported for MGP; OC was detected in bone cells and mineralized structures, but also in soft and cartilaginous tissues. We propose that, in a context of a reduced rate of evolution, sturgeon OC has retained structural features of the ancestral protein that emerged millions of year ago from the duplication of an ancient MGP gene and may exhibit intermediate functional features.

Osteocalcin (OC), also known as bone Gla protein and matrix Gla protein (MGP) are characterized by the presence of Gla residues resulting from the post-translational vitamin K-dependent γ-carboxylation of specific glutamates, which confer these proteins their ability to bind calcium and calcium/phosphate crystals such as hydroxyapatite [1, 2, 3, 4]. OC is a small secreted protein of 5-6 kDa synthetized as a pre-propeptide, originally isolated from organic matrix of bovine bone [5] and considered specific for vertebrate calcified structures, where it is secreted mainly by osteoblasts and odontoblasts. Depending on the species, OC contains 3-4 Gla residues located within a conserved domain in the central part of the mature protein [6, 7]. It has been proposed that OC has a pivotal role in controlling tissue mineralization mechanisms [8, 9, 10] and Fourier transform infrared microspectroscopy (FT-IRM) studies have shown that OC is required for the correct maturation of hydroxyapatite in mammalian bone [8]. More recently, it has also been proposed that the undercarboxylated form of OC may function as a hormone and be involved in regulating glucose metabolism and fat mass as well as murine and human fertility [11, 12, 13].

MGP is a 10 kDa secreted protein containing a phosphorylated domain, a γ-glutamyl carboxylase recognition site, and 4-5 Gla residues; it was originally purified from the organic matrix of mammalian bone [2] and later identified in the organic matrix of bone and calcified cartilage from other mammals [14, 15, 16], amphibians [17], and bony and cartilaginous fish [18, 19]. It is produced and secreted mainly by vascular smooth muscle cells [20] and chondrocytes [21] and significantly accumulated in bone, cartilage, tooth cementum and soft tissues such as kidney, respiratory system and heart [2, 14, 17, 19, 22, 23, 24]. Although the molecular mechanism of MGP action remains poorly understood, numerous studies have reported a major role in the inhibition of soft tissue calcification. The spontaneous calcification of arteries and cartilage in mouse lacking MGP [25], and phenotype rescue after restoration of MGP expression [26], together with abnormal cartilage and artery calcification in patients with loss-of-function mutations in MGP gene [27] are evidences towards a role in the prevention of ectopic mineralization. In addition to the 4-5 Gla residues believed to be important in binding Ca^{2+} and calcium crystals, MGP contains phosphorylated serine residues that may further regulate its activity, and it was shown that both γ-carboxylation and serine phosphorylation contribute to MGP function as a calcification inhibitor [3].

Although the two proteins share several structural motifs, they have different functions and play different roles in tissue mineralization [26]. Based on evident similarities in protein structure and gene organization, OC and MGP have been considered to be evolutionarily related; current hypothesis is that OC gene appeared from a duplication of an ancestral MGP gene that may have occurred approximately 380 million years ago (before the branching of cartilaginous fish). The duplicated gene would have diverged throughout vertebrate evolution to give the modern OC gene described in the literature. It was also speculated that the appearance of MGP and OC would be concomitant or followed by the emergence of cartilage and bone structures, respectively [7].

With more than 32,000 species, fish form the most diverse group of vertebrates; they appeared approximately 395 million years ago and lobe-finned fish are considered to be the ancestors of all terrestrial vertebrates. Fish, in particular ray-finned fish, have been shown to be suitable models not only to study OC and MGP proteins but also to provide key insights towards the understanding of how these proteins and calcified tissues have evolved [7, 19, 28, 29]. Among ray-finned fish, the Chondrostei (Acipenseriformes, e.g. sturgeons, and Polypteriformes, e.g. bichirs) occupy a particular position; their endoskeleton is primarily cartilaginous, while exoskeleton is composed of bony plates. They represent one of the oldest groups of bony fish and have undergone an exceptionally slow evolution. It has been proposed that their skeleton would represent the ancestral state of vertebrate skeleton [30]. Based on this proposition, we speculated that mineral-binding Gla proteins from Chondrostei, in particular osteocalcin, may have also undergone a slow rate of evolution and thus resemble the ancestral form of vertebrate proteins. We isolated MGP and osteocalcin from mineralized tissues of the Adriatic sturgeon *Acipenser naccarii* and determined their primary protein structure, patterns of gene expression and sites of protein accumulation.

EXPERIMENTAL PROCEDURES

Biological material – Specimens of *Acipencer naccarii* (Adriatic sturgeon), kindly supplied by aquaculture Rio Frio (Granada, Spain), were kept until used at 20-22ºC in a closed circuit equipped with a biological filter and a natural photoperiod. Frozen branchial arches from adult sturgeons were also obtained from aquaculture Rio Frio.

Collection of mineralized tissues – Branchial arches and ganoid plates were freed from adhering soft tissues, extensively washed in distilled water then acetone (Merck), air dried and ground to powder in a mortar. Powder was washed three times with a 10-fold excess of 6 M guanidine-HCl (Sigma), washed extensively with water and acetone and air dried.

Extraction of mineral-bound proteins – Mineral-bound proteins were extracted from ganoid plates and branchial arches using a 10-fold excess of 10% (v/v) formic acid for 4 h at 4°C as previously described [17]. Extracted proteins were separated from the insoluble collagenous matrix by filtration through filter paper then dialyzed at 4°C against 50 mM HCl using 3,500 molecular weight tubing (Spectra/Por 3, Spectrum). Dialysis solution was changed twice a day for 2 days, then extract was freeze-dried, dissolved in 6 M guanidine-HCl, 0.1 M Tris, pH 9, and further dialyzed against 5 mM ammonium bicarbonate.

Purification of branchial arches soluble proteins – Soluble desalted protein extracts collected after ammonium bicarbonate dialysis were analyzed by SDS-PAGE and the protein profile revealed by staining with Coomassie Brilliant Blue (CBB; Bio-Rad) and a Gla-specific staining (DBS; Sigma-Aldrich) as described [19]. Protein extracts from branchial arches were further purified by ionic exchange in a 1 ml RESOURCE™ Q column (GE Healthcare). Bound proteins were eluted with a continuous gradient of 20 mM Tris-HCl (pH 8) and 0.5 M NaCl. The resulting peak fractions were dialyzed against 5 mM ammonium bicarbonate, analyzed by SDS-PAGE and pure protein identified by N-terminal sequencing analysis using an Applied Biosystems Model 494 sequencer equipped with an on-line high performance liquid chromatographer (HPLC) for separation and detection of phenyl thiohydantoin (PTH) amino acid derivatives.

Purification of branchial arches insoluble proteins – Insoluble protein extracts from branchial arches and ganoid plates, collected after dialysis with 5 mM ammonium bicarbonate, were analyzed by SDS-PAGE and their protein profile was revealed by staining with CBB and DBS. Gla-containing proteins were further purified by reverse phase high performance liquid chromatography (RP-HPLC) from branchial arches preparation [31], transferred to PVDF membranes and identified by N-terminal sequencing as described above.

Western and dot blot analysis – Aliquots of total protein were fractionated by SDS-PAGE on a 4-12% gradient polyacrylamide precast gel containing 0.1% SDS (NuPage, Invitrogen), stained with CBB and DBS as previously described [19, 31], and transferred onto nitrocellulose membranes (Amersham Biosciences). OC and MGP proteins were detected by incubating blots over night with the primary polyclonal antibodies against meagre *Argyrosomus regius* (Ar) OC and MGP or against shark *Galeorhinus galeus* (Gg) MGP [28] diluted 1:100 in 5% (w/v) non-fat dried milk powder in TBST (15 mM NaCl, 10 mM Tris-HCl, 0.05% Tween 20, pH 8). Proteins phosphorylated on serine residues were detected using a specific anti-phosphoserine (anti-pSer) rabbit polyclonal antibody (Invitrogen). Alkaline phosphatase-labeled goat anti-rabbit IgG or peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma) diluted 1:30,000 in TBST were used as secondary antibodies. Visualization of immunoreactive bands was achieved using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphatase (NBT/BCIP) substrate solution (Calbiochem) as described [19, 32], or Western Lightning Chemiluminescence Plus kit (Perkin Elmer).

RNA and DNA preparation – Total RNA was extracted from adult sturgeon tissues (including bone, cartilage and major soft tissues) as previously described [31]. RNA integrity was checked by agarose-formaldehyde gel electrophoresis, and concentration was determined by spectrophotometry at 260 nm. Sturgeon genomic DNA was extracted from muscle as previously described [31].

cDNA cloning – Based on alignments of known OC sequences and on N-terminal sequence of sturgeon MGP (AnMGP) protein, degenerated forward primers OC_1F and MGP_1F were designed and used in combination with the universal adapter primer to PCR amplify the 3' end of sturgeon OC and MGP cDNAs from ganoid plates and branchial arches reverse-transcribed RNA (using MMLV-RT, Invitrogen), respectively. The 5' ends of those cDNAs were amplified by RACE-PCR using specific reverse primers OC_1R, and MGP_1R, and a sturgeon Marathon cDNA library (Clontech) previously prepared [31]. Sequences of all PCR primers used in this study are presented in **Table 1**. All PCR products were cloned into pCR^{II}TOPO (Invitrogen) and sequenced on both strands.

In silico analysis – Deduced amino acid sequences were aligned using M-Coffee multiple sequence alignment software [33] and manual adjustments were made to improve alignments. Signal peptides and phosphorylation sites were predicted using SignalP 3.0 [34] and NetPhos 2.0 [35], respectively.

Gene cloning – Sturgeon OC gene was PCR amplified from GenomeWalker libraries prepared as suggested by the manufacturer (Clontech) or from genomic DNA using gene-specific primers designed based on cDNA sequence and intronic sequences as they became available. Fragments including partial exon 1 and intron 1, and partial intron 3 and exon 4 were amplified from GenomeWalker libraries *Stu*I and *Pvu*II using OC_2F and OC_2R specific primers and AP1, respectively. The overlapping fragment between exon 1 and intron 3 was obtained using genomic DNA as template and primers OC_3F and OC_4R.

Measurement of relative gene expression by quantitative real-time PCR – One microgram of total RNA was treated with RQ1 RNase-free DNase (Promega) and reverse-transcribed at 37ºC with MMLV-RT (Invitrogen) using either gene-specific reverse primers or universal dT-adapter. Quantitative real-time PCR (qPCR) was performed using iCycler iQ apparatus (Bio-Rad) and primer sets OC_RT1F / OC_RT1R to amplify sturgeon OC, GAPDH_RT1F / GAPDH_RT1R to amplify sturgeon GAPDH, HPRTI_RT1F / HPRTI_RT1R to amplify sturgeon HPRTI, and MGP_RT1F / MGP_RT1R to amplify sturgeon MGP. PCR reactions, set up in duplicates, were as follows: 2 µl of RT diluted 1:10, 8 µl of primer mix (final concentration of each primer is 0.2 µM) and 10 µl of Absolute QPCR SYBR Green Fluorescein mix (ABgene). PCR reactions were submitted to an initial denaturation step at 95ºC for 15 min and 55 cycles of amplification (one cycle is 30 s at 95ºC and 30 s at 68ºC). Fluorescence was measured at the end of each extension cycle in the FAM-490 channel. Levels of gene expression were calculated using the comparative method (ΔΔCt) and normalized using gene expression levels of GAPDH or HPRTI housekeeping genes. qPCR values are presented as mean of triplicates ± standard deviation. Sequences of all qPCR primers used in this study are presented in **Table 1**.

Histological sample preparation – Samples were collected as previously described [31] and included either in paraffin or in Historesin Plus (Leica Microsystems). Routine staining methods using Harris hematoxylin-eosin (CI 75290, Sigma) and Alcian Blue 8GX (CI 74240, Sigma), pH 2.5 were carried out in adjacent sections. Mineral deposits were detected through von Kossa's and Alizarin red S staining [23].

In situ hybridization - A 725-bp fragment of sturgeon OC cDNA (spanning from nucleotide 468 to the 3' end) cloned into pCR^{II}-TOPO was either linearized with *Apa*I and transcribed with SP6 RNA polymerase to generate antisense riboprobe, or linearized with *Hin*dIII and transcribed with T7 RNA polymerase to generate sense riboprobe. Both probes were labelled with digoxigenin using RNA labelling kit (Roche). Sections were digested with 40 µg/ml of proteinase K (Sigma) in 1×PBS (phosphate-buffered saline) containing 0.1% of TWEEN 20 (Sigma) for 45 min and hybridized at 68°C overnight. Hybridization and signal detection were performed as previously described [31, 32]. Negative controls were performed with sense probes.

Immunolocalization – Immunohistochemical staining experiments were performed using paraffin- and Histo-resin-plus-embedded tissue sections as previously described [19, 32]. Briefly, the endogenous peroxidase activity was blocked with 3% H_2O_2 in Coons buffer (CBT: 0.1 M Veronal, 0.15 M NaCl, 0.1% Triton X-100) for 15 min, and non-specific antibody binding was blocked with 0.5% (w/v) BSA. Incubation with affinity-purified polyclonal ArOC primary antibody [28], diluted 1:100, and anti-serum GgMGP antibody [28], diluted 1:250, was performed overnight in a humidified chamber at room temperature. Peroxidase activity was detected using peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma) and 0.025% 3,3-diaminobenzidine (Sigma) as previously described [19, 32]. Immuno fluorescence was detected using a procedure similar to that described above, where incubation with FITC-conjugated goat anti-rabbit IgG secondary antibody (Sigma) was done in a humidified dark chamber. For negative controls, primary antibody was replaced with both normal rabbit serum and BSA in CBT.

RESULTS

Identification of an osteocalcin with MGP features in Adriatic sturgeon – The sturgeon osteocalcin (AnOC) cDNA was obtained through a combination of standard PCR and RACE-PCR amplifications. Longest transcript spanned 1,192 bp (accession number EF413584) and contained a 303-bp open reading frame encoding a 100-aa peptide with high similarity with OC already characterized in other species (**Figure 1A**). Analysis of deduced amino acid sequence revealed (i) a 20-aa signal peptide, (ii) a 34-aa propeptide containing a putative recognition site for γ-glutamyl carboxylase (GGCX) and a furin-like cleavage motif (RKKR), and (iii) a 46-aa mature protein containing 3 glutamic acid at sites known to be γ-carboxylated in other OCs and 2 cysteine residues essential to the formation of a disulfide bond (**Figure 1A** and **Figure 2A**). Interestingly, N terminus of sturgeon OC did not align very well with that of other OCs and numerous (11) serine residues occurred within the pro- and mature peptides; nine of them were predicted to be phosphorylated, in particular those at positions 25, 27, 31, 33 and 67, which had a score above 0.96 (**Figure 1A**). Serine phosphorylation is a key feature of MGP and we decided to further investigate this striking similarity. Alignment of sturgeon OC propeptide with the N-terminus of vertebrate MGP (**Figure 1B**), revealed that 4 of these putative phosphoserines aligned with serines previously shown to be phosphorylated in MGP proteins [14, 17]. A motif sharing some homology with MGP-specific ANXF cleavage site was also identified within the propeptide of sturgeon OC, upstream of OC-specific RKKR furin cleavage site (**Figure 1B** and **Figure 2A**). Altogether, these observations show that the protein deduced from sturgeon OC cDNA combines domains and motifs present in both MGP and OC proteins (**Figure 2A**), which can eventually originate (i) a typical OC if the propeptide is cleaved by the furin-like enzyme or (ii) a MGP/OC hybrid protein if protein precursor is only cleaved at the signal peptide cleavage site.

Sturgeon OC gene was later cloned through a combination of gene walking and genomic PCRs (accession number EF413587). It exhibited a structure identical to other OC genes with 4 coding exons and 3 introns inserted in phase 1 (introns 1 and 2) and phase 2 (intron 3). Following the typical pattern of OC genes, (i) exon 1 encodes the signal peptide and its cleavage site; (ii) exon 2 encodes part of the propeptide; (iii) exon 3 encodes the remaining propeptide containing the GGCX recognition and furin-like cleavage sites, and part of the mature protein containing one Gla residue; and (iv) exon 4 encodes the remaining mature protein containing two Gla residues and the two conserved cysteines (**Figure 2B**). Although this structural data is indicative of an OC gene, this is not conclusive since OC and MGP genes exhibit a very similar molecular structure, e.g. similar phase of introns and length of exons [7], only differing in the presence of an extra intron and exon positioned in the 5' UTR of fish and amphibian MGP genes [36, 37]. The absence, in the sturgeon OC gene, of these extra sequences typical of all fish MGP genes analyzed to date, further indicate that this is indeed sturgeon OC gene.

Identification of sturgeon calcified skeletal structures – Histomorphological characterization of ganoid plates (GP) (**Figure 3A**), branchial arches (BA) (**Figure 3B**) and vertebra (Vt) (**Figure 3C**) tissues was performed through routine haematoxylin-eosin (HE), von Kossa, Alizarin red and Alcian blue staining procedures. HE staining of GP sections revealed the presence of osteocyte-like cells (Oc) (**Figure 3 A1**) entrapped within a calcified matrix, as demonstrated by positive staining with von Kossa (**Figure 3 A2**) and Alizarin red (**Figure 3 A3**), and the periosteum (Pt) surrounding the mineralized matrix (**Figure 3 A1**), which was positively stained with Alcian blue (**Figure 3 A4**) and negatively stained with von Kossa (**Figure 3 A2**). In BA, calcification was only detected associated with the gill rakers (**Figure 3 B1 and B2**), while mature chondrocytes (MC) were found enclosed in a cell-rich hyaline cartilage at the basis of BA filaments (**Figure 3 B3**) positively stained with Alcian blue (**Figure 3 B4**), and negatively stained with both von Kossa and Alizarin red (results not shown). Identified sturgeon Vt structures were dorsal and ventral cartilages (HC), a thick notochord sheath (NS), the spinal cord (SC) and the perichondrium (Pc) (**Figure 3 C1**). HC was found to be composed by mature chondrocytes immersed in a homogeneously hyaline cartilage, positively stained with Alcian blue (**Figure 3 C2**), surrounded by immature chondrocytes and the perichondrium (results not shown). The vertebral centra were found to be entirely cartilaginous without positive staining with either von Kossa or Alizarin red (results not shown).

Identification of sturgeon OC protein in the mineral phase of skeletal tissues – To further determine which protein(s) related to sturgeon OC gene would accumulate in the mineral phase of sturgeon skeletal tissues, matrix proteins of sturgeon calcified cartilage and bone were acid-extracted from branchial arches and ganoid plates, respectively. Dialysis of acid extracts against ammonium bicarbonate (pH 8) originated a soluble and an insoluble protein extract. While OC-related peptides were expected to be present in the ammonium bicarbonate soluble extract (OC is highly soluble in water at neutral pH), MGP-related peptides usually precipitate from the ammonium bicarbonate solution since they are poorly soluble in aqueous solutions at pH 7-8. Protein extracts were analyzed by SDS-PAGE and presence of Gla-containing proteins was evaluated through DBS staining. Migration profile of soluble extracts from branchial arches (BA) and ganoid plates (GP) were similar and exhibited a major protein band positive for DBS and with an apparent molecular mass of approximately 10 kDa (**Figure 4A**). In order to remove high and low molecular weight contaminants, branchial arches soluble extract was further purified through ionic exchange chromatography over a RESOURCE Q column. Chromatogram profile revealed the presence of a major peak eluting at fractions 39-49 (**Figure 4B**), which were dialyzed against ammonium bicarbonate and analyzed by SDS-PAGE. A single protein band of 10 kDa positive for DBS was detected in all fractions (**Figure 4C**), confirming the successful purification of a Gla-containing protein. N-terminal sequence of the protein present in fraction 44 was determined and the first 19 aa were identified as NADLTLSPQKLXSLSXVXX (no phenyl-thiohydantoin (PTH)-derivatives were detected at positions 12, 16, 18 and 19). This sequence was in total agreement with the protein sequence deduced from sturgeon OC cDNA and indicated a proteolytic cleavage at the furin-like site (**Figure 1A**). Furthermore, DBS staining of purified protein and the absence of identifiable PTH derivatives at positions 12, 16 and 19, which are Glu residues in the cDNA-deduced protein, strongly suggested the presence of Gla residues at these positions in sturgeon osteocalcin. The absence of an identifiable PTH derivative at position 18 further confirmed the presence of the cysteine residue previously evidenced at this position in cDNA-deduced protein.

Polyclonal antibodies against meagre (*Argyrosomus regius*; Ar) OC and against shark (*Galeorhinus galeus;* Gg) MGP [28], were tested for cross-reactivity with sturgeon OC through western blotting of soluble extracts. An immunoreactive band with an apparent molecular mass of approximately 10 kDa was detected in branchial arch soluble extract by anti-ArOC antibody (**Figure 4D**), while anti-GgMGP antibody did not recognize any band (**Figure 4D**), indicating the absence of MGP in this soluble extract, and confirming antibody specificity. In addition, dot blot analysis of the ionic exchange chromatography fraction 44, previously identified as OC by N-terminal sequence analysis, showed a positive signal with anti-ArOC (results not shown), further confirming the osteocalcin identity of sturgeon soluble protein.

Identification of sturgeon MGP characterized by a high Gla content – The insoluble fraction of branchial arch acid extracts, shown previously to contain the Gla-rich protein (GRP) [31], was further purified through a combination of RP-HPLC and gel filtration chromatography and analyzed by SDS-PAGE. Although GRP represents the major insoluble protein and exhibits the strongest DBS staining, a second protein with a reddish-like coloration by DBS and an apparent molecular mass of 14 kDa was also detected [31]. N-terminal sequence of the protein present in RP-HPLC fraction 54, according to the protein profile previously published [31], was determined as YXSDESFDSGEDVFMNPYSANSFMN (no PTH derivative was detected at position 2) and found to be similar to known MGP sequences (**Figure 5**). A 14-kDa protein with a similar behavior was also detected in the insoluble extract from ganoid plates (**Figure 6A**). It was more abundant in this bone-derived extract than in the previously characterized cartilage-derived extract (i.e. from branchial arches). The MGP nature of this protein was further confirmed by western blot using anti-ArMGP and anti-GgMGP polyclonal antibodies, and MGP-containing RP-HPLC fraction 54 as positive control. Although anti-ArMGP antibody was able to recognize MGP from the RP-HPLC fraction 54, immunoreactive signal was faint and almost undetectable in insoluble crude extracts of both branchial arches (**Figure 6B**) and ganoid plates (results not shown). On the contrary, anti-GgMGP antibody strongly immunoreacted with the 14-kDa protein (**Figure 6C**), thus confirming the presence of MGP also in the ganoid plates. These results also showed that sturgeon MGP epitopes were better recognized by GgMGP antibody. Anti-ArOC antibody failed to detect MGP protein in branchial arches insoluble extract and RP-HPLC fraction 54 (results not shown), again showing the absence of cross-reactivity among the different Gla-containing proteins with the antibodies used and confirming their specificity. Phosphorylation status of sturgeon MGP was investigated by western blot using an anti-phosphoSer antibody. A strong immunoreactive band corresponding to a protein of approximately 14 kDa was observed in branchial arch insoluble extract (**Figure 6D**), suggesting that sturgeon MGP is indeed phosphorylated. However, because serine residues at positions 3, 6 and 9, known to be phosphorylated in other species, did not give blanks in N-terminal sequence of sturgeon MGP protein, they may be only partially phosphorylated, as previously determined for meagre MGP [19]. The cDNA of sturgeon MGP was later cloned through a combination of RT- and RACE-PCR amplifications using degenerated primers designed from the N-terminal sequence previously determined. Longest transcript spanned 663 bp and contained an open reading frame of 294 bp encoding a polypeptide of 97 aa (accession number HM182000). Deduced protein sequence was in full agreement with the N-terminal sequence of the mature protein purified through RP-HPLC, and its comparison with sequences in GenBank database confirmed its identity as sturgeon MGP (**Figure 5**). *In silico* analysis of MGP precursor revealed a signal peptide of 19 aa and a mature protein of 78 aa, which contains (i) five putative phosphorylated serine residues, (ii) a putative GGCX docking site, (iii) a ANSF motif, and (iv) seven possible Gla and two Cys residues (**Figure 5**). Phosphorylation sites were predicted based on homology with other MGP proteins and using NetPhos 2.0 (**Figure 5**). Putative phospho-serines located in the N-terminus correspond to the three serine residues previously shown to be phosphorylated in meagre MGP and conserved in most known MGP. However, the phosphorylation of serine residues in the core of Gla domain (positions 46 and 54 in sturgeon mature MGP) has never been reported before. The stronger immunoreaction of sturgeon MGP to anti-phosphoSer antibodies (**Figure 6D**), when compared to the signal obtained for meagre MGP (using a comparable amount of protein, results not shown), could indicate that these two serine residues are also phosphorylated in sturgeon protein. The absence of a PTH derivative at position 2 of the N-terminal sequence where a Glu residue is present in the cDNA-deduced protein sequence, may indicate the presence of a Gla residue at this position in sturgeon MGP. Furthermore, 6 more Glu residues were found in sturgeon MGP at the place of confirmed Gla residues in other vertebrate MGPs (e.g. *Homo sapiens*, *Gallus gallus*, *Xenopus laevis*, *Argyrosomus regius* and *Galeorhinus galeus;* **Figure 5**). These results combined with the atypical intense coloration obtained for sturgeon MGP after DBS staining (reddish-like coloration), suggests a high γ-carboxylation status with 7 possible Gla residues, which, if confirmed, would make sturgeon protein the most γ-carboxylated MGP.

Overlapping patterns of expression for sturgeon OC and MGP genes – Levels of OC and MGP gene expression were determined by qPCR in a variety of adult sturgeon tissues, including soft, cartilaginous and bony tissues. MGP presented a typical tissue distribution, with highest levels of gene expression found in cartilaginous tissues and a significant expression in heart, although it also showed an atypical expression in bony tissues possibly due to the presence of bone-associated cartilage tissue (**Figure 7**). OC transcript was detected in all 17 tissues analyzed, with highest levels in bony tissues, as previously observed in mammals [38, 39, 10], amphibians [40] and teleost fishes [19, 23, 29], but also with significant levels in cartilaginous and soft tissues (**Figure 8A**). Since OC is considered a bone-specific protein, produced by osteoblasts in bone and odontoblasts in teeth [6, 9, 38, 19, 23, 29], the presence of sturgeon transcript in soft and cartilage-containing tissues was surprising, although in accordance with the high quantity of protein extracted from branchial arches. A similar tissue distribution was obtained when a second housekeeping gene, i.e. GAPDH, was used to normalize OC gene expression (results not shown). To further investigate the atypical pattern of OC gene expression in adult tissues and establish the identity of OC-expressing cells in sturgeon tissues, *in situ* hybridization was performed in sections from soft, cartilage and bone sturgeon tissues (**Figure 8B**). In mineralized structures OC mRNA was strongly detected in the osteoblast-like cells in the periosteum (arrows), forming the surrounding lining cells of bone, of ganoid plates (**Figure 8 B1**), segmented ray fins (**Figure 8 B2**), and gill rakers (**Figure 8 B3**). Although with less intensity OC mRNA was also detected in osteocyte-like cells of sturgeon cellular bone, i.e. ganoid plates (results not shown) and ray fins (**Figure 8 B2**, asterisk). In cartilage-containing tissues, e.g. branchial arches and vertebra, OC mRNA was found essentially in mature chondrocytes of the cartilaginous matrix forming the branchial arches (**Figure 8 B4**), and sporadically in some immature chondrocytes adjacent to the perichondrium of vertebra, which surrounds the cartilaginous matrix (results not shown). In both branchial arches and vertebra, OC mRNA was detected in the vascular smooth muscle cells of small blood vessels and capillars (results not shown). In heart sections OC mRNA was strongly detected in the cardiac muscle fibers of the ventricle (**Figure 8 B5**) and in the vascular smooth muscle cells of arteries (**Figure 8 B6**). No signal was observed in negative controls performed with sense riboprobe (results not shown).

Distinct patterns of accumulation for sturgeon OC and MGP proteins – Anti-ArOC and anti-GgMGP antibodies, previously validated by western blot, were used to determine sites of OC and MGP protein accumulation, respectively, by immunohistochemistry in bone, cartilage-containing tissues, and also in heart (**Figure 9**). While OC was found highly accumulated in the mineralized extracellular bone matrix of ganoid plates (**Figure 9 A1**), it was weakly accumulated in the lining cells of the periosteum surrounding the bone mineralized matrix (**Figure 9 A2**). In contrast, MGP was preferably accumulated in the lining cells of the periosteum (**Figure 9 B2**), although positive staining was also detected in the mineralized matrix (**Figure 9 B1**). Similar results were obtained by immunofluorescence, using FITC-conjugated goat anti-rabbit IgG secondary antibody and ArMGP as primary antibody for MGP detection (data not shown). A similar staining pattern was obtained in the gill rakers, where the mineralized matrix, preferentially accumulate OC (**Figure 9 A3**), while MGP is mainly detected associated with the cells forming the mineralization front surrounding the bone matrix (**Figure 9 B3**), which showed no staining for OC (**Figure 9 A3**). In the cartilage of branchial filaments, OC was barely detected (**Figure 9 A4**), while MGP was strongly associated with the mature and proliferative chondrocytes and with the undifferentiated chondrocytes of the perichondrium (**Figure 9 B4**). In sturgeon vertebra OC was barely detected in the fibroblast-like cells of the cartilage ligament region, and in the differentiated chondrocytes surrounding the cartilage (**Figure 9 A5**) and adjacent to the perichondrium (results not shown). In contrast, MGP was preferentially and strongly detected in the perichondrium region and in the chondrocytes surrounding the cartilage and adjacent to the perichondrium, while the fibroblast cells of the ligaments were barely stained (**Figure 9 B5**). Interestingly, cardiomyocytes of the ventricle cardiac fibers were positive for both OC (**Figure 9 A6**) and MGP (**Figure 9 B6**), although MGP exhibited a higher accumulation. Finally, both proteins were strongly accumulated in blood vessels of vertebra (**Figure 9 A7, B7**) and branchial arches (**Figure 9 B8**). Negative controls were performed by omitting the primary antibody in the case of the affinity purified anti-ArOC, and by substituting the primary antibodies with normal rabbit serum when anti-GgMGP was used.

DISCUSSION

We report here the identification and characterization of the mineral-binding Gla-containing proteins, OC and MGP, in the calcified cartilage and bone of Adriatic sturgeon, and present biological data corroborating the previously proposed hypothesis of an evolutionary relationship between both proteins.

Sturgeon MGP behaves as other MGP proteins, exhibiting similar primary structure, post-translation modifications (phosphorylation and γ-carboxylation), and patterns of mRNA and protein distribution. Evidence was collected towards the presence of 7 Gla residues in sturgeon MGP, which, if confirmed, will be the most densely γ-carboxylated MGP in vertebrates. Similarly, sturgeon GRP was recently shown to be the most densely γ-carboxylated GRP in vertebrate, with 16 Gla residues [31]. These findings suggest the presence of a highly efficient γ-carboxylation system in sturgeon, that could be related to the cartilaginous nature of its internal skeleton and the need for more efficient mechanisms to control mineralization. In this aspect, it would be interesting to determine whether the activity of sturgeon γ-glutamyl carboxylase is also above the levels usually observed in similar tissues in other organisms.

On the contrary, sturgeon OC does not behave as other OC proteins, accumulating in both the mineral phase of bone and calcified cartilage. Evidence was collected towards the presence of motifs and residues characteristic of MGP proteins in sturgeon OC propeptide, in particular the presence of several putative phosphorylated serines arranged in a domain similar to that observed in MGP but not to that reported in the propeptide of fish-specific osteocalcin isoform (OC2) [41]. The furin-cleaved peptide, and not the propeptide, was purified from sturgeon calcified matrix, suggesting that the mineral-binding sturgeon OC is a typical post-translationally processed OC protein lacking the putative phosphorylated domain. Although furin cleavage preferentially occurs intracellularly in the *trans*-Golgi network within the secretory pathway [42, 43, 44], it can also take place (i) at the cell surface [42] or (ii) within the extracellular compartment [42], as it happens in the activation of the BMP antagonist nodal during mammalian antero-posterior axis formation [45]. The fate of OC propeptide is controversial. While some studies have suggested that it remains in the cell surface and is not co-secreted with the mature peptide [46], others have shown that OC propeptide is present in blood serum and could be used as a marker for osteoblastic function [47]. Although both OC and GRP were shown to be processed at the polybasic RXXR furin cleavage site, showing that this enzyme is present and active in sturgeon, the possibility that the propeptide, which is likely to be phosphorylated, does exists in the extracellular matrix is conceivable and in concordance with what was observed for the human OC propeptide, shown to be co-secreted with the mature protein [47]. The existence of a proteolytic processing in MGP at the highly conserved ANSF motif [7] has been suggested based on the detection of corresponding fragments in MGP preparations from mammalian, shark (Price, unpublished results) and a teleost fish [19]. The propeptide of sturgeon OC would be very similar to the N-terminal fragment of MGP processed at the ANSF site, which must be present in the extracellular matrix since the complete mature MGP protein is detected bound to the mineral phase. The fate of this phosphorylated MGP fragment is unknown as well as its putative function, but we propose a similar fate for sturgeon OC propeptide, although further studies aiming at understanding the localization and biochemical characteristics of this peptide are required. In addition, three putative phosphorylated serine residues are predicted within the mature OC protein, which would confer a new embodiment to the protein and a new dimension to its putative function. It has been demonstrated that OC and MGP, despite structural similarities, do not share the same function, and that OC, despite a Gla domain, cannot act as a calcification inhibitor of extracellular matrix calcification *in vivo* as does MGP [26]. It was also demonstrated that a phosphorylated domain is necessary to MGP anti-mineralization activity and that this domain, absent in OC, functions in synergy with the Gla domain to contribute to MGP function [3]. It is conceivable that an osteocalcin exhibiting a phosphorylated domain, as sturgeon OC, would function as a calcification inhibitor, overlapping with MGP function, and thus also found at sites of MGP gene expression and protein accumulation. Indeed sturgeon OC was not only expressed by osteoblasts, as expected for an osteocalcin, but also by chondrocytes, cardio myocytes and vascular smooth muscle cells, which are cells normally expressing MGP mRNA [14, 23, 48, 49]. Similarly, mature protein accumulated not only in the mineralized matrix of bone but also in cartilaginous cells, heart and blood vessels, which are typical sites of MGP accumulation [14, 24]. We have previously developed vertebra and branchial arches derived cell cultures from sturgeon [50] and have observed that these cells do not produce a mineralized extracellular matrix when treated with a mineralogenic culture medium. Interestingly, the levels of osteocalcin significantly increase upon mineralogenic treatment in vertebra derived cells, suggesting a response to mineralization stimuli, possibly associated to a need to control mineralization events (our unpublished data).

Sturgeons are considered as being among the most ancient actinopterygian fishes [51] and to have undergone remarkably little morphological changes, indicating that their evolution has been exceptionally slow with a significant slower rate of molecular change than observed for most fish [52]. The discovery, in Adriatic sturgeon, of a typical OC protein that has retained MGP features is consistent with the current theory that both OC and MGP share a common ancestral entity, with OC originating, through gene duplication and subsequent sequence divergence, from an ancestral MGP gene [7]. We propose that sturgeon hybrid protein represents an ancestral form of OC, with features close to the protein that originated from the gene duplication event that reportedly occurred 380 million years ago. For the same reasons, we also propose that sturgeon MGP may be very close to the protein ancestral to most ray-finned fish and the presence of numerous putative γ-carboxylation sites in sturgeon MGP at positions known to be carboxylated in other fish MGPs further supports this hypothesis. The presence of more carboxylation sites in sturgeon proteins (i.e. MGP and GRP) also suggests that overall carboxylation of Gla proteins has probably decreased throughout evolution of ray-finned fish. This is, to the best of our knowledge, the first work providing clear biological evidence pointing towards the evolutionary relationship between OC and MGP proteins.

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FOOTNOTES

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The nucleotide sequences reported in this paper have been submitted to the GenBank™ sequence database with accession numbers EF413584 (Adriatic sturgeon OC cDNA), EF413587 (Adriatic sturgeon OC gene) and HM182000 (Adriatic sturgeon MGP cDNA).

FIGURE LEGENDS

FIGURE 1. **Comparison of Adriatic sturgeon OC sequence with other known OCs (A) and with the N-terminus of known MGPs (B).** *A*, OC sequences from ray-finned fish (sturgeon, meagre and zebrafish), amphibian (xenopus), mammals (human, rat and bovine) and bird (chicken) were aligned using M-Coffee. First amino acid of predicted mature peptide is numbered as 1. Residues 100% conserved are highlighted in *gray* and serine residues in sturgeon OC are highlighted in *black*. *Asterisks* indicate predicted phosphorylation sites and those marked with *double asterisks* have a probability score above 0.96. *Black dots* indicate 100% conserved Gla residues. *Vertical arrows* indicate cleavage sites after signal peptide and propeptide. GenBank accession numbers for OC sequences: AF459030 (meagre *Argyrosomus regius*); AY178836 (zebrafish *Danio rerio*); AF055576 (xenopus *Xenopus laevis*); NP954642 (human *Homo sapiens*); NP038200 (Norway rat *Rattus norvegicus*); NP776674 (bovine *Bos taurus*); NM205387 (chicken *Gallus gallus*). *B*, partial propeptide sequence of sturgeon OC (*rectangle* in panel A) was aligned with N-terminal sequences of known MGPs using M-Coffee. Residues 100% conserved are highlighted in *gray* and serine residues in sturgeon and partially conserved in other species are highlighted in *black*. *Asterisks* indicate phosphorylation sites reported in MGP. *ANXF* indicates MGP proteolytic cleavage site. *Black arrowheads* indicate amino acids known to be essential for γ-glutamyl carboxylase binding (GGCX). GenBank accession numbers for MGP sequences: AF334473 (meagre *Argyrosomus regius*); P56620 (blue shark *Galeorhinus galeus*); AF055588 (xenopus *Xenopus laevis*); Y13903 (chicken *Gallus gallus*); BC005272 (human *Homo sapiens*).

FIGURE 2. **Structural organization of Adriatic sturgeon OC protein (A) and gene (B)**. *A*, cDNA-deduced OC sequence. SP, signal peptide; PP, propeptide; MP, mature protein. *Circled P*, phosphorylation sites predicted by NetPhos 2.0; *Circled Gla*, putative γ-carboxyglutamic acid residues;

Dashed line, intramolecular disulfide bond; *Circled C*, conserved cysteine residues; *GGCX,* γ-glutamyl carboxylase recognition site; *RKKR*, furin-like proteolytic cleavage site; *APPF*, motif similar to MGP-specific proteolytic cleavage site ANXF, where *PP* correspond to putative phosphorylated serine residues in sturgeon OC. *B*, Organization of OC gene (between initiation (ATG) and termination (TAG) codons). Exons (E) 1 to 4 are represented by *solid lines* and introns (I1 to I3) by *gray triangles* (intron size (bp) is indicated inside each triangle).

FIGURE 3. **Detection of mineralized/non-mineralized structures in skeletal tissues of a young adult sturgeon.** *A*, Histological characterization of ganoid plate sections through staining with hematoxylin-eosin (panel 1), von Kossa (panel 2), alizarin red (panel 3) and alcian blue (panel 4). *Oc*, osteocytes; *Pt*, periosteum. *B*, Histological characterization of branchial arches sections through staining with hematoxylin-eosin (panels 1 and 3), alizarin red (panel 2), and alcian blue (panel 4). *MC*, mature chondrocytes; *Asterisk* indicate mineralized matrix of gill rakers. *C*, Histological characterization of vertebra sections through staining with hematoxylin-eosin (panel 1) and alcian blue (panel 2). *HC*, hyaline cartilage; *Pc*, perichondrium; *SC*, spinal cord; *NS*, notochord sheath. Magnification is ×10 in panels A1, B3 and B4, ×20 in panels A2, A3, A4, B1, B2 and C2, and ×5 in panel C1.

FIGURE 4. **Extraction (A), purification (B, C) and characterization (D) of Adriatic sturgeon OC from the mineral phase of adult calcified tissues.** *A*, Protein profile of crude soluble extracts of the mineral phase of sturgeon branchial arches (BA) and ganoid plates (GP) and detection of Gla-containing proteins revealed through CBB and DBS staining, respectively. *B*, Purification of proteins present in BA soluble extract through ionic exchange chromatography (IEC) performed over a RESOURCE Q column. Protein profile was obtained by measuring absorbance (Abs) of effluent fractions at 280 nm (*black triangles*) and 230 nm (*black circles*). *C*, Protein profile of IEC fractions revealed through CBB and DBS staining. *D*, Western blot analysis of proteins present in branchial arches soluble extract using anti-ArOC and anti-GgMGP antibodies. *Arrows* indicate OC migration position. Profile of SeeBlue pre-stained molecular weight marker (MW) is indicated beside gel pictures.

FIGURE 5. **Comparison of sturgeon MGP sequence with other known MGPs.** MGP sequences from cartilaginous fish (blue shark), teleost fish (meagre), mammal (human), bird (chicken), and amphibian (xenopus) were aligned using M-Coffee. Numbering is indicated according to the first amino acid of the mature peptide. Serine residues known to be phosphorylated in other species are highlighted in *black*; Serine residues predicted to be phosphorylated in sturgeon MGP are highlighted in *dark gray*. Conserved cysteines are highlighted in *light gray*. *Vertical arrow* indicates cleavage site after signal peptide. *White box* indicates ANXF sequence; *Black arrowheads* indicate amino acids known to be essential for γ-glutamyl carboxylase binding (GGCX). *Black dots* indicate Glu residues known to be γ-carboxylated. GenBank accession numbers for MGP sequences: P56620 (blue shark *Galeorhinus galeus*); AF334473 (meagre *Argyrosomus regius*); BC005272 (human *Homo sapiens*); Y13903 (chicken *Gallus gallus*); AF055588 (xenopus *Xenopus laevis*),.

FIGURE 6. **Identification (A), detection (B, C) and characterization (D) of Adriatic sturgeon MGP from insoluble protein extracts.** *A*, Protein profile of the insoluble extract of the mineral phase of sturgeon ganoid plates (GP) revealed by CBB staining. *B-D*, Western blot analysis of proteins present in branchial arches (BA) and ganoid plates (GP) insoluble extracts using anti-ArMGP (B), anti-GgMGP (C) and anti-phospho-serine (anti-PSer; D) antibodies. F54, RP-HPLC MGP containing fraction 54 used as positive control. *Arrows* indicate migration position of identified MGP and OC. Profile of SeeBlue pre-stained molecular weight marker (MW) is indicated beside gel pictures.

FIGURE 7. **Levels of MGP gene expression in adult Adriatic sturgeon tissues.** Levels of gene expression were determined by real-time qPCR in adult soft, bone and cartilage-containing tissues, and normalized using HPRTI. Level in kidney (Kd) was used as reference and set to 1. Lv, liver; Sl, spleen;

Gn, gonads; Br, brain; Ht, heart; Sp, spine; GP, ganoid plate; Md, mandibula; HP, head plate; Op, operculum; BA, branchial arches; Sk, skull; PV, posterior vertebra; AV, anterior vertebra. Values are the mean of 3 replicates and are indicated with standard deviation.

FIGURE 8. **Levels (A) and sites (B) of OC gene expression in adult Adriatic sturgeon tissues.** *A*, Levels of gene expression were determined by real-time qPCR in adult soft, bone and cartilage-containing tissues, and normalized using HPRTI. Level in liver (Lv) was used as reference and set to 1. Br, brain; Ht, heart; Ms, muscle; Kd, kidney; Gn, gonads; Sl, spleen; PV, posterior vertebra; AV, anterior vertebra; BA, branchial arches; Sk, skull; Sp, spine; Md, mandibula; HP, head plate; Ct, cleithrum; GP, ganoid plate; Op, operculum. Values are the mean of 3 replicates and are indicated with standard deviation. *B*, Sites of gene expression determined by ISH in ganoid plate (*panel 1*), ray fin (*panel 2*), branchial arches (*panels 3 and 4*) and heart (*panels 5 and 6*). *Arrows* indicate the periosteum surrounding the mineralized matrix in ganoid plates, ray fin and gill-rakers. *Asterisk* indicates osteocyte-like cell. Magnification is ×10 in panels 1-5 and ×20 in panel 6.

FIGURE 9. **Sites of OC (A) and MGP (B) protein accumulation in Adriatic sturgeon skeletal tissues.** Protein immunolocalization with anti-ArOC and anti-GgMGP antibodies was performed in tissue sections prepared from non-demineralized (*panels A1 and B1*) and demineralized (*panels A2 and B2*) ganoid plates, branchial arches (*panels A3, A4, B3 and B4*), vertebra (*panels A5, B5*), heart (*panels A6 and B6*), and blood vessels from vertebra (*panels A7 and B7*) and branchial arches (*panel B8*). *Asterisks* indicate mineralized bone matrix and *arrows* indicate the periosteum surrounding bone matrix. *LR*, ligament region of cartilages in vertebra; *Pc*, perichondrium. Magnification is ×10 in panels A5, A6, B5 and B6 and ×20 in panels A1, A2, A3, A4, A7, B1, B2, B3, B4, B7 and B8.

Table 1. Oligonucleotides used for PCR and qPCR amplifications.

Primer name	Sequence (5' to 3')
cDNA cloning	
OC_1F	GATGGAYGCYGCCTACACSRCCTACTA
OC_1R	CGGGGATTGGTCCGAAGTGTTTTTGATA
MGP_1F	GAYGARTCNTTYGAYTCNGGNGARGAY
MGP_1R	GCTCTTGTACCGTTCCATTAACCTCTCAT
Gene cloning	
OC_2F	CGAGAGAGACAGAGAAGACACTAGA
OC_2R	TGCGAGATTTACTGGAGCAAAAGTC
OC_3F	CTCCTCTCCCTCATCACCCTCGCTCTG
OC_4R	CAGCCACTGACTCACTGTGTGACCCTG
qPCR	
OC_RT1F	TCTGACGCTGTTTTGCTCCAGTAAATCTCG
OC_RT1R	CGTTTCAGGGAAAATACCCAAAAGCAATA
MGP_RT1F	TTTATGAATCCCTACAGTGCGAACTCCT
MGP_RT1R	GTAGCGACGGGCGAAGCGGTCA
GAPDH_RT1F	TCTGACTTCAATGGAGACACCCGCT
GAPDH_RT1R	CACGAGGTCCACGACTCTGTTGCTGTA
HPRTI_RT1F	TGAAGAGCCCTGACCACGCCGA
HPRTI_RT1R	CGTGCCACCAAGAAACAGCAAATACAA

FIGURE 1

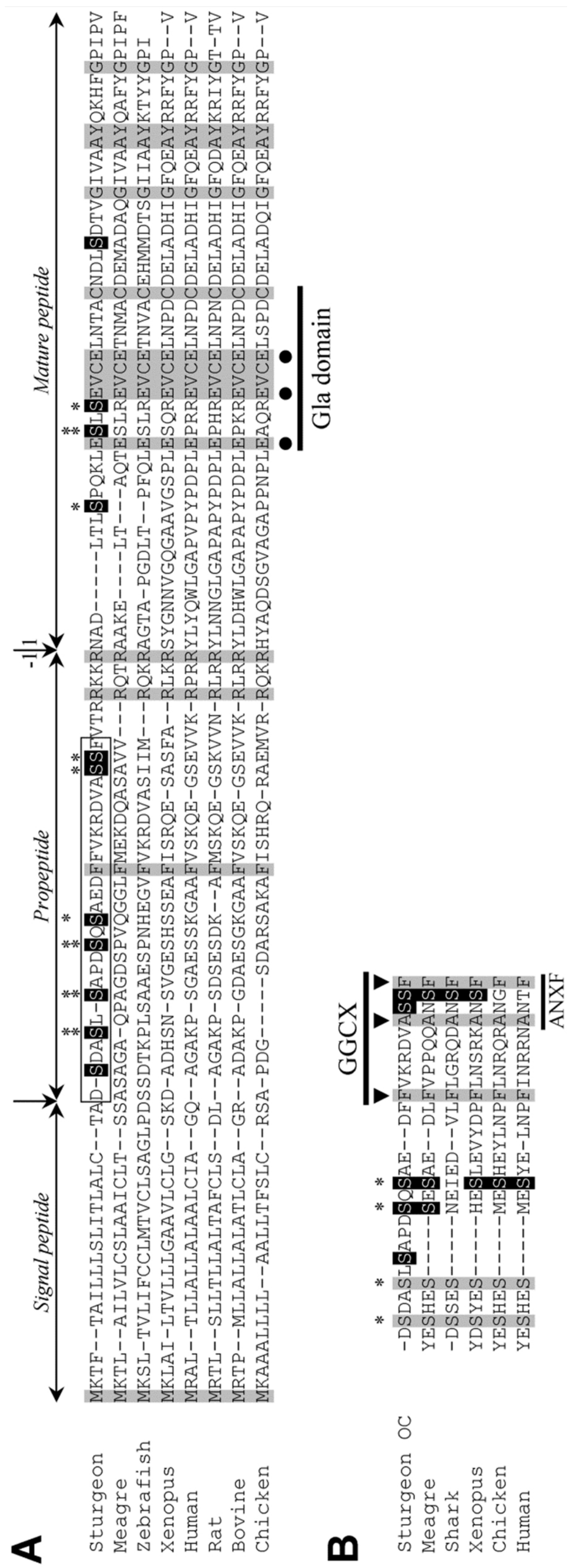

A
B
Signal peptide
Propeptide
Mature peptide
Gla domain
GGCX
ANXF
Sturgeon
Meagre
Zebrafish
Xenopus
Human
Rat
Bovine
Chicken
Sturgeon OC
Shark

FIGURE 2

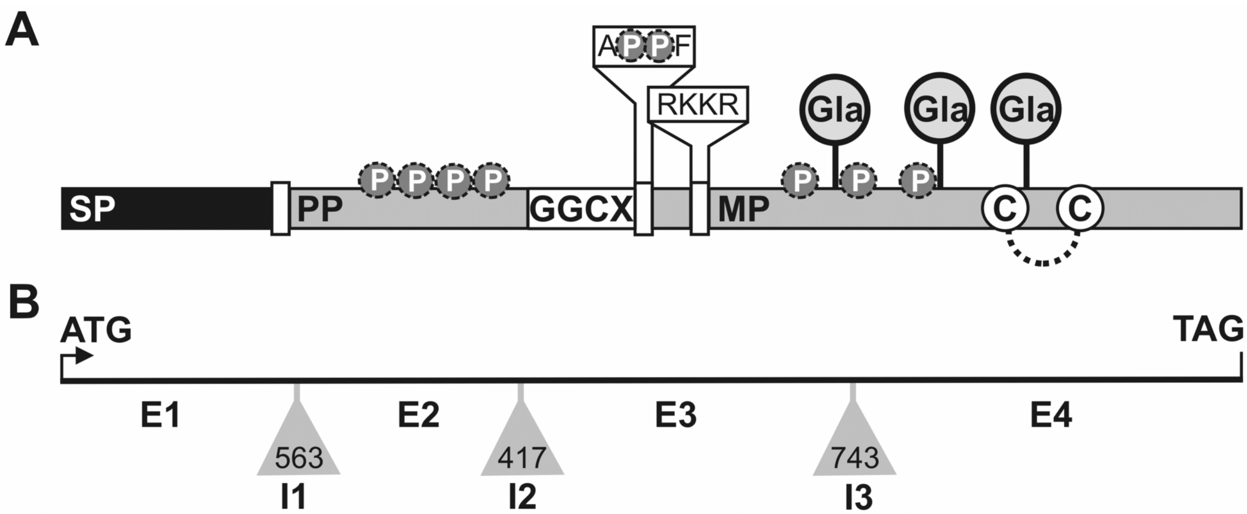

A
SP
PP
GGCX
MP
RKKR
Gla
Gla
Gla
P
C
C
B
ATG
TAG
E1
E2
E3
E4
563
417
743
I1
I2
I3

FIGURE 3

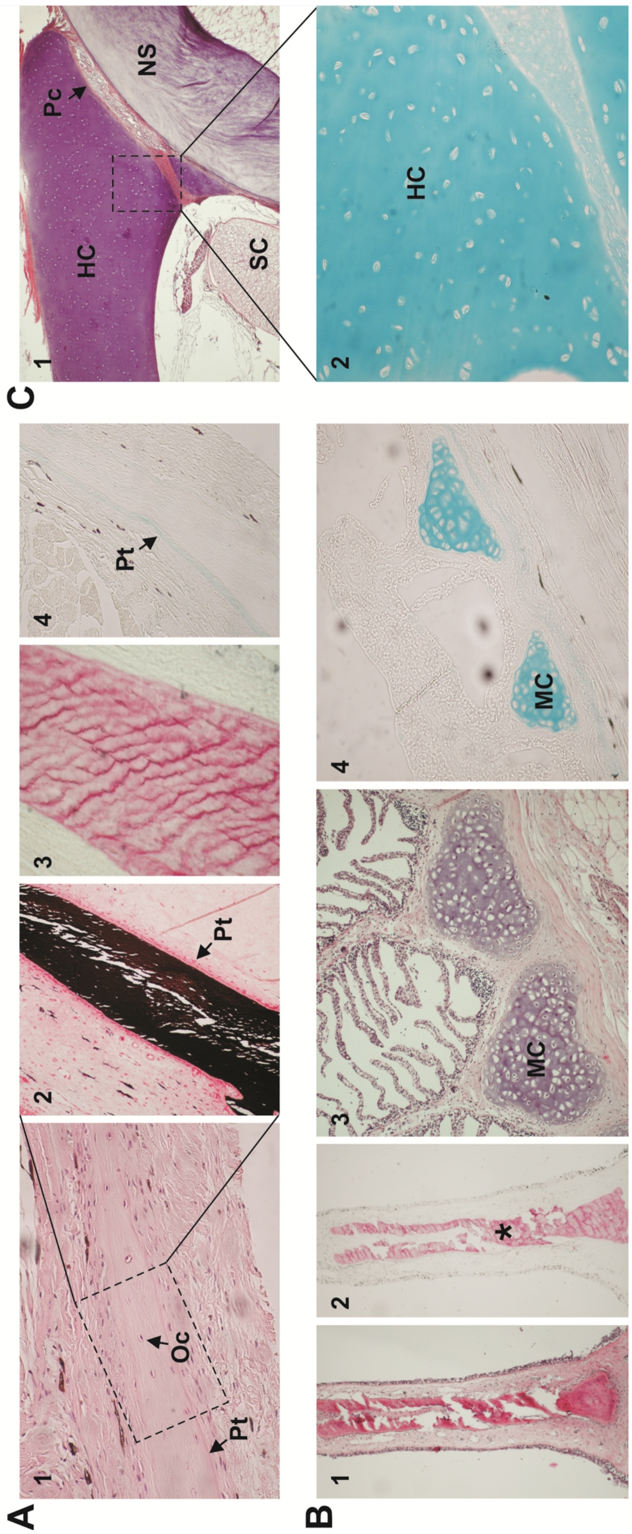


FIGURE 4

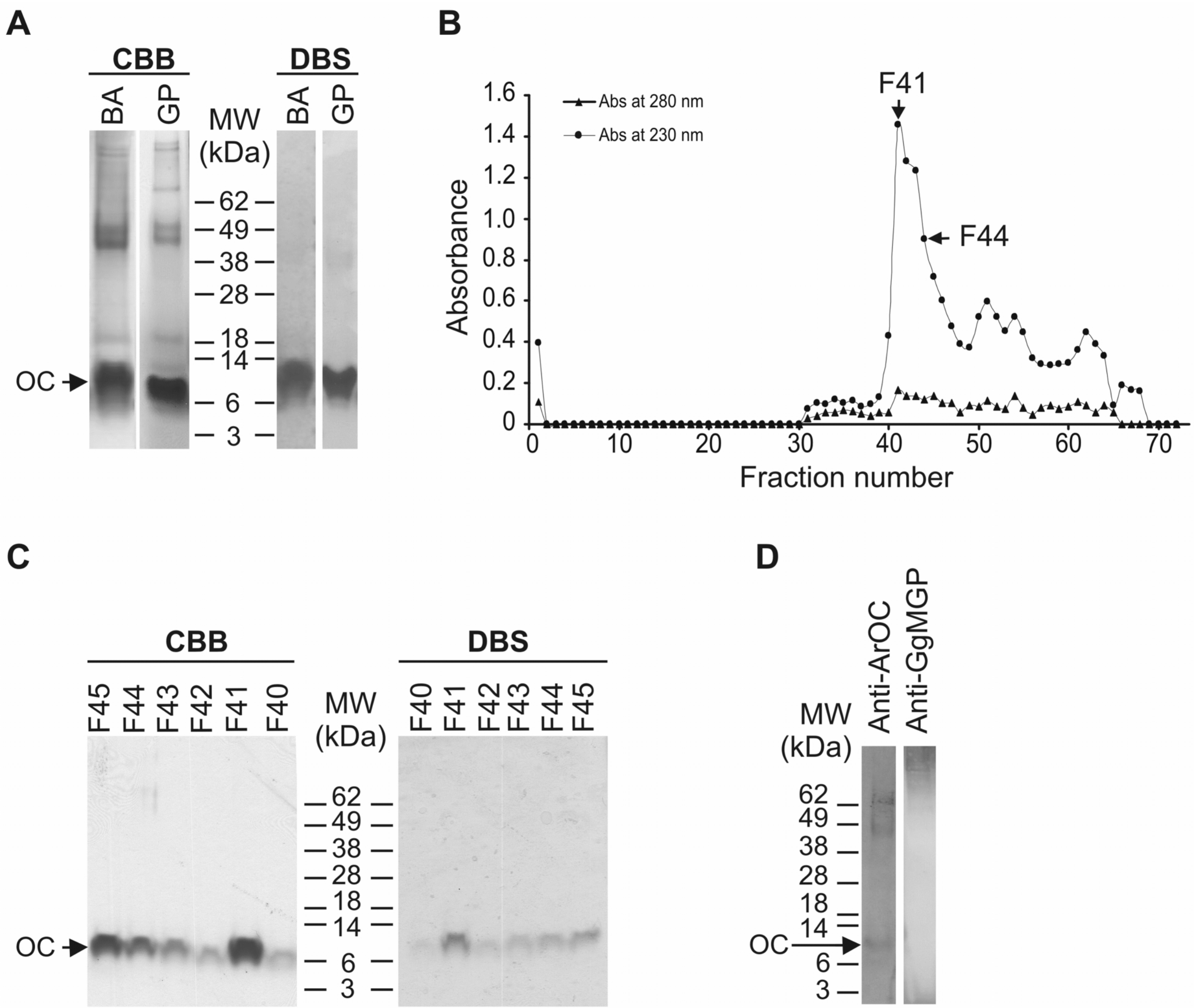

A
CBB
DBS
BA
GP
MW (kDa)
62
49
38
28
18
14
6
3
OC
B
F41
F44
Abs at 280 nm
Abs at 230 nm
Absorbance
Fraction number
C
F45
F44
F43
F42
F41
F40
D
Anti-ArOC
Anti-GgMGP

FIGURE 5

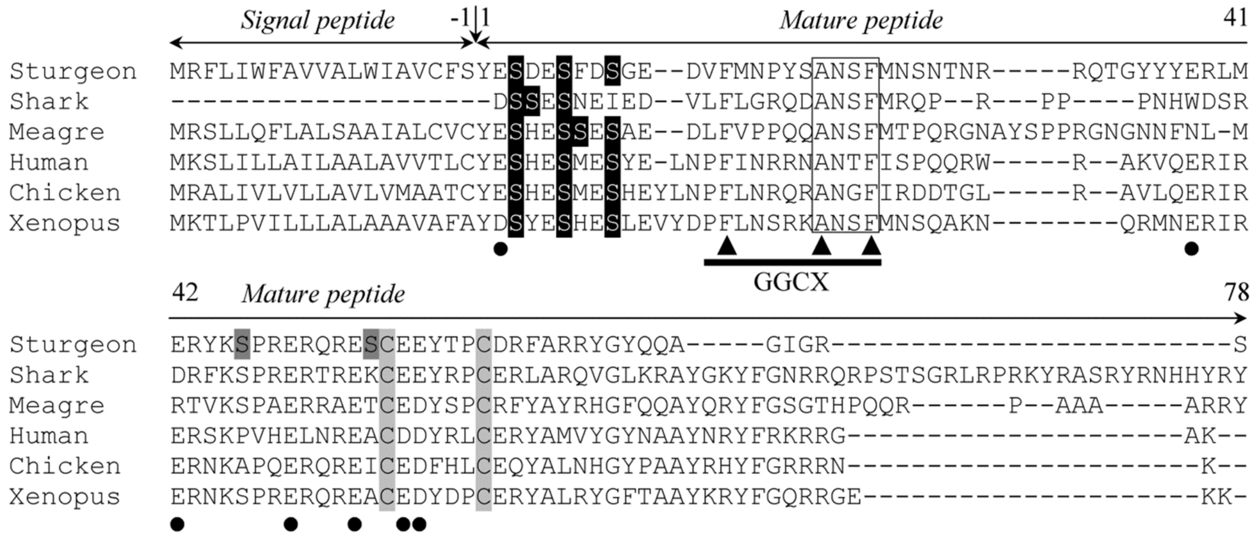

Signal peptide
-1
1
Mature peptide
41
Sturgeon MRFLIWFAVVALWIAVCFSYESDESFDSGE--DVFMNPYSANSFMNSNTNR-----RQTGYYYERLM
Shark --------------------DSSESNEIED--VLFLGRQDANSFMRQP--R---PP----PNHWDSR
Meagre MRSLLQFLALSAAIALCVCYESHESSESAE--DLFVPPQQANSFMTPQRGNAYSPPRGNGNNFNL-M
Human MKSLILLAILAALAVVTLCYESHESMESYE-LNPFINRRNANTFISPQQRW-----R--AKVQERIR
Chicken MRALIVLVLLAVLVMAATCYESHESMESHEYLNPFLNRQRANGFIRDDTGL-----R--AVLQERIR
Xenopus MKTLPVILLLALAAAVAFAYDSYESHESLEVYDPFLNSRKANSFMNSQAKN--------QRMNERIR
GGCX
42
Mature peptide
78
Sturgeon ERYKSPRERQRESCEEYTPCDRFARRYGYQQA-----GIGR-------------------------S
Shark DRFKSPRERTREKCEEYRPCERLARQVGLKRAYGKYFGNRRQRPSTSGRLRPRKYRASRYRNHHYRY
Meagre RTVKSPAERRAETCEDYSPCRFYAYRHGFQQAYQRYFGSGTHPQQR------P--AAA-----ARRY
Human ERSKPVHELNREACDDYRLCERYAMVYGYNAAYNRYFRKRRG--------------------AK--
Chicken ERNKAPQERQREICEDFHLCEQYALNHGYPAAYRHYFGRRRN---------------------K--
Xenopus ERNKSPRERQREACEDYDPCERYALRYGFTAAYKRYFGQRRGE--------------------KK-

FIGURE 6

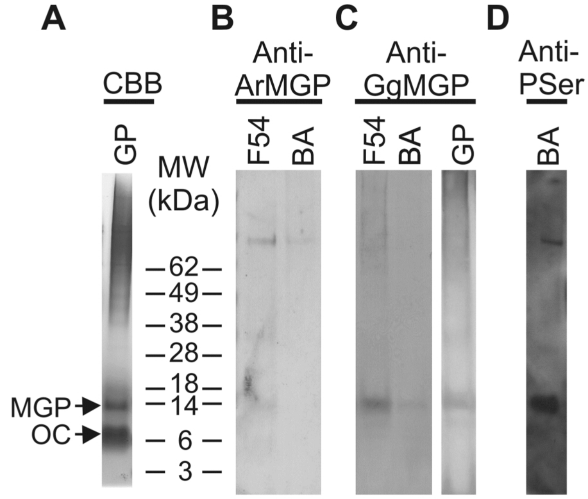

A
B
C
D
CBB
Anti-
ArMGP
Anti-
GgMGP
Anti-
PSer
GP
MW
(kDa)
F54
BA
F54
BA
GP
BA
62
49
38
28
18
14
6
3
MGP
OC

FIGURE 7

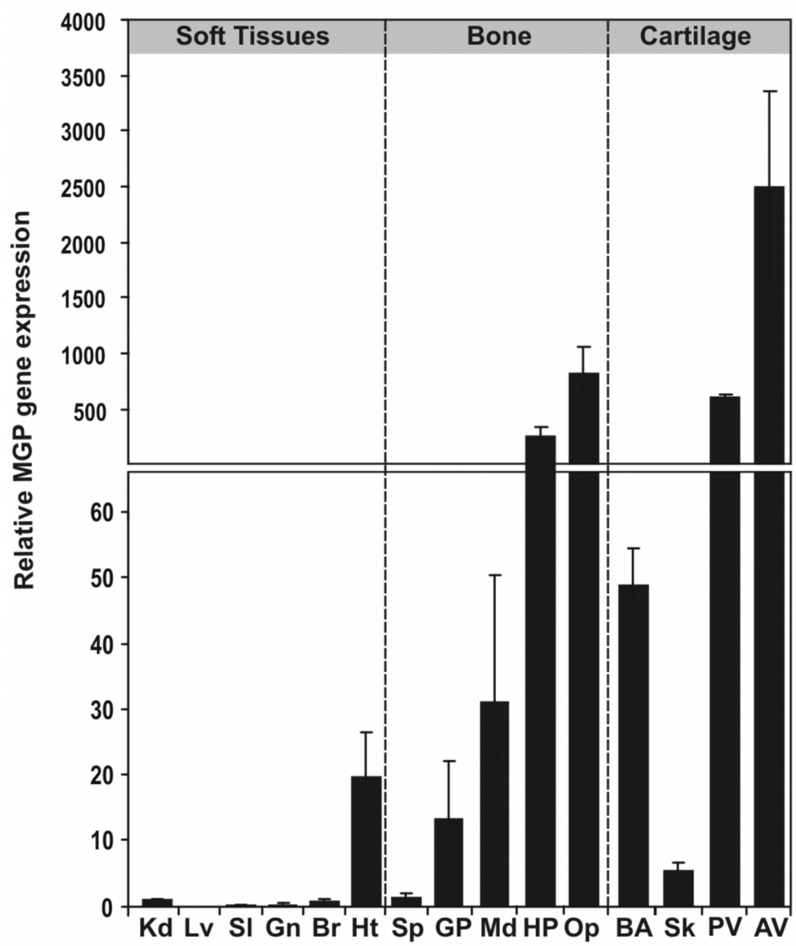

Soft Tissues
Bone
Cartilage
Relative MGP gene expression
4000
3500
3000
2500
2000
1500
1000
500
60
50
40
30
20
10
0
Kd
Lv
Sl
Gn
Br
Ht
Sp
GP
Md
HP
Op
BA
Sk
PV
AV

FIGURE 8

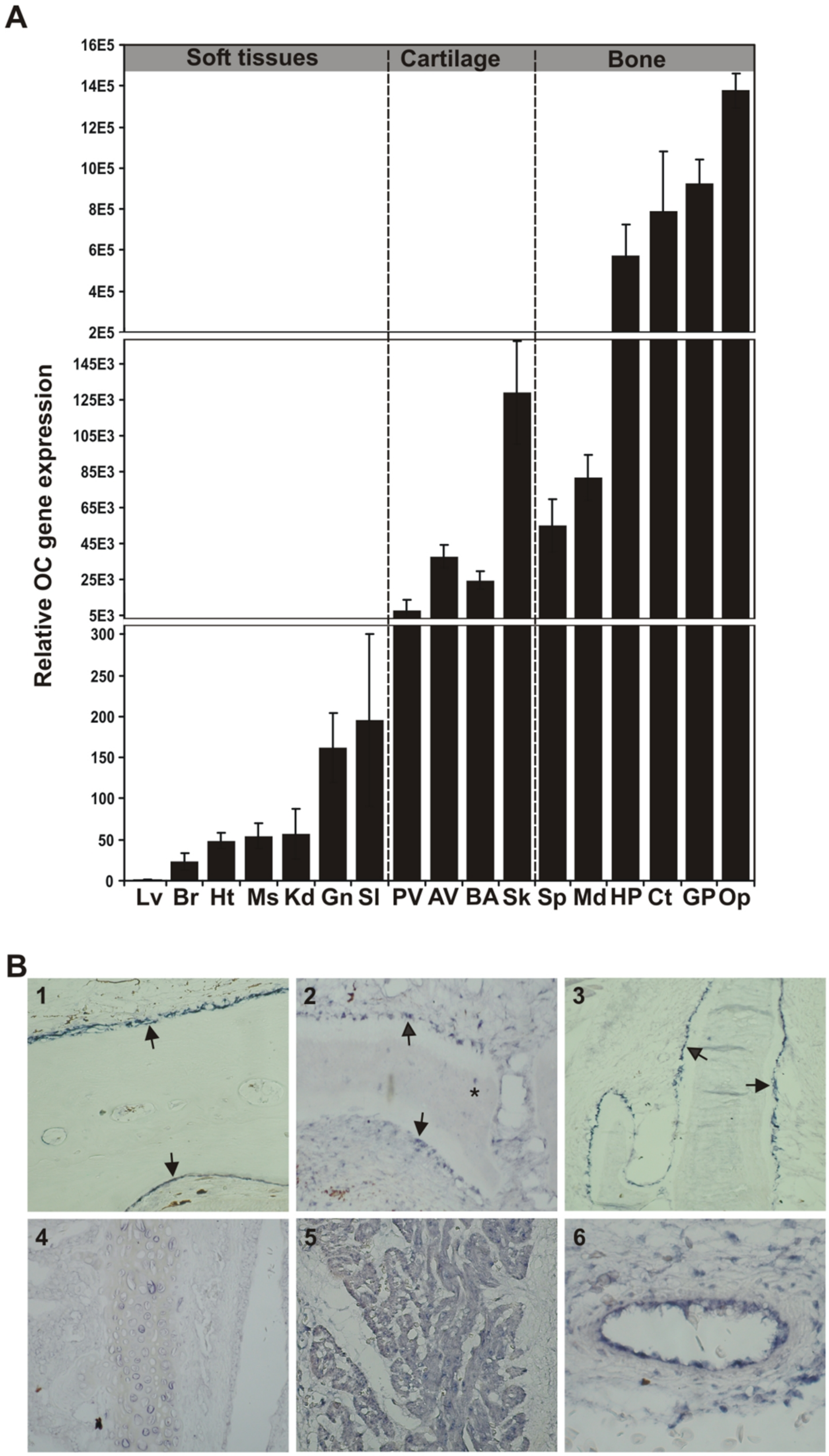
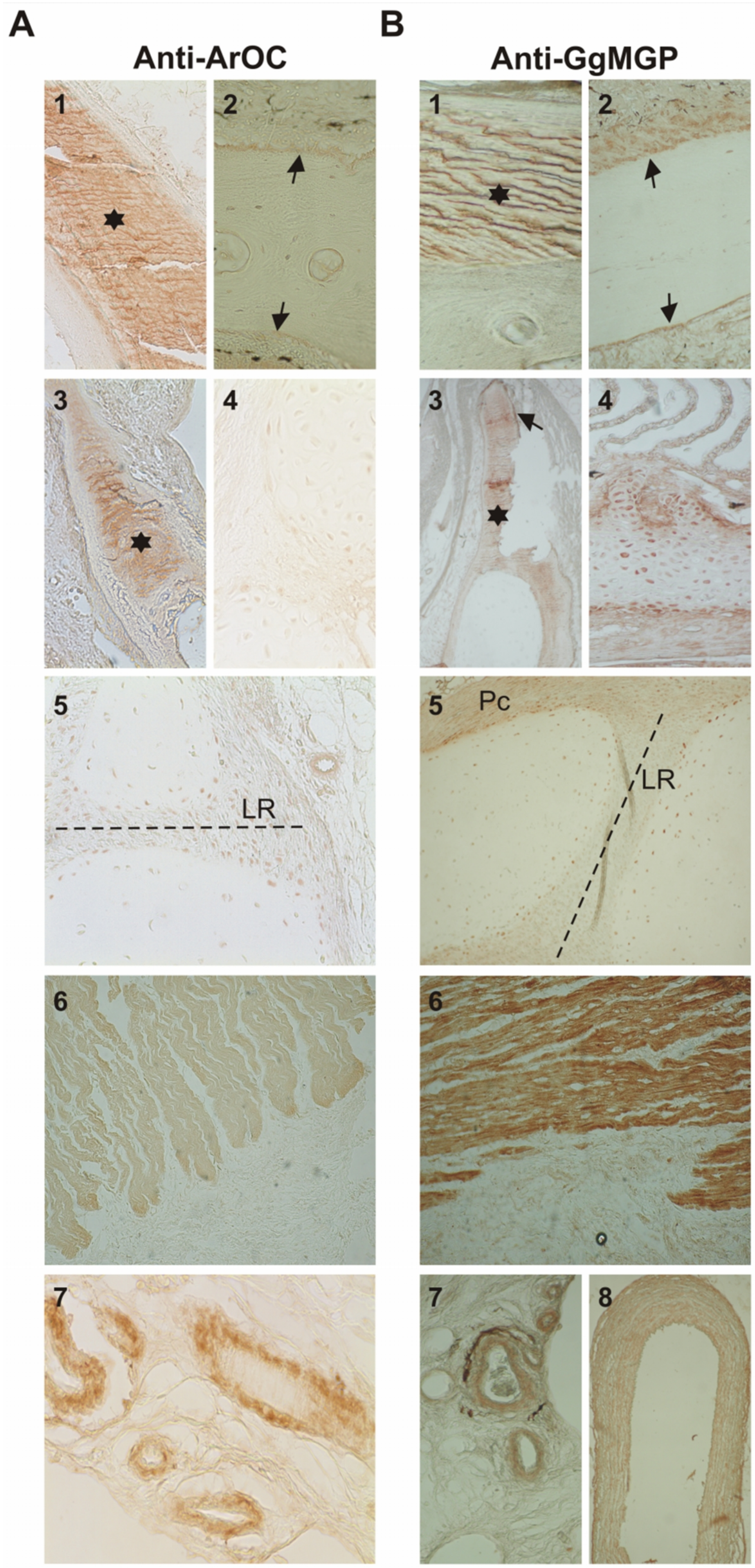

A
Soft tissues
Cartilage
Bone
Relative OC gene expression
16E5
14E5
12E5
10E5
8E5
6E5
4E5
2E5
145E3
125E3
105E3
85E3
65E3
45E3
25E3
5E3
300
250
200
150
100
50
0
Lv
Br
Ht
Ms
Kd
Gn
Sl
PV
AV
BA
Sk
Sp
Md
HP
Ct
GP
Op
B
1
2
3
4
5
6

FIGURE 9



Sturgeon Osteocalcin Shares Structural Features with Matrix Gla Protein: Evolutionary Relationship and Functional Implications

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