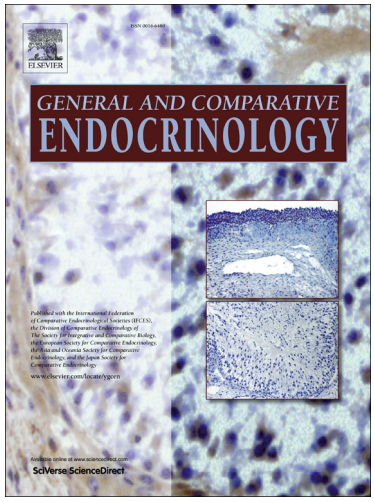

Comparative analysis of a teleost skeleton transcriptome provides insight into its regulation

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Comparative analysis of a teleost skeleton transcriptome provides insight into its regulation

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Abstract

An articulated endoskeleton that is calcified is a unifying innovation of the vertebrates, however the molecular basis of the structural divergence between terrestrial and aquatic vertebrates, such as teleost fish, has not been determined. In the present study long-read next generation sequencing (NGS, Roche 454 platform) was used to characterise acellular perichondral bone (vertebrae) and chondroid bone (gill arch) in the gilthead sea bream (*Sparus auratus*). A total of 15.97Mb and 14.53 Mb were produced, respectively from vertebrae and gill arch cDNA libraries and yielded 32,374 and 28,371 contigs (consensus sequences) respectively. 10,455 contigs from vertebrae and 10,625 contigs from gill arches were annotated with Gene Ontology terms. Comparative analysis of the global transcriptome revealed 4249 unique transcripts in vertebrae, 4201 unique transcripts in the gill arches and 3700 common transcripts. Several core gene networks were conserved between the gilthead sea bream and mammalian skeleton. Transcripts for putative endocrine factors were identified in acellular gilthead sea bream bone suggesting that in common with mammalian bone it can act as an endocrine tissue. The acellular bone of the vertebra, in contrast to current opinion based on histological analysis, was responsive to a short fast and significant ($p<0.05$) down-regulation of several transcripts identified by NGS, osteonectin, osteocalcin, cathepsin K and IGFI occurred. In gill arches fasting caused a significant ($p<0.05$) down-regulation of osteocalcin and up-regulation of MMP9.

Keywords:

Advanced teleost; Endocrine; NGS; Tissue responsiveness; Vertebrae and gill arch Transcriptomes

1. Introduction

An articulated endoskeleton that is calcified is a unifying innovation of the vertebrates and its evolution was accompanied by species-specific specialisation. For example, the sharks and rays developed a cartilaginous skeleton that is light and flexible [15], whilst in bony fishes, the skeleton is mineralized but it is largely avascular, and a lightweight vascular skeleton only developed in terrestrial vertebrates. In advanced bony fishes, like the gilthead sea bream (*Sparus auratus*), the skeleton is produced and maintained by chondrocytes, osteoblasts and osteoclasts but is considered to be acellular as it lacks osteocytes within the calcified extracellular matrix (ECM), although they occur in some basal bony fishes (e.g. salmon) [135]. The vertebrate skeleton consists of endochondral bone formed by mineralization of a cartilaginous template secreted by chondrocytes and dermal bone formed by mesenchyme cells that differentiate directly into osteoblasts. Compared to mammals additional types of skeletal tissue have been identified in teleost fish and there is a richer diversity of cartilage types (see [10, 11, 141]). One "intermediate skeletal" tissue which is well characterized is chondroid bone, which has features of both bone and cartilage and develops from osteogenic precursors.

Skeletal bone is in slow but continuous turnover with osteoclasts derived from the monocyte/macrophage lineage resorbing bone while osteoblasts build bone. The balance between bone formation and resorption is achieved by cross-talk between transcription factors, receptors and hormones, which have been intensely studied in the last decades in mammals (see [15, 23, 44, 87]). In contrast, knowledge about bone turnover in acellular teleost bone is still rudimentary, although recent studies have started to elucidate this process [32, 35, 135], and several genes and proteins of calcified

74 tissue have been characterized [71, 85, 93, 101]. However, histological studies indicate
75 that bone turnover is a slow process and the majority of studies of this process have
76 focused on a modest number of genes and proteins and no large-scale study representing
77 a comparative analysis of the composition and regulation of cartilage and bone ECM
78 exists.

79 The skeleton in vertebrates protects, supports and permits movement, and the
80 mobilisation or deposition of calcium (Ca) and phosphorus (P) from this tissue
81 contributes to calcium homeostasis [13, 24, 46, 134]. The structural difference in the
82 skeleton between aquatic and terrestrial vertebrates is presumably derived from the
83 effects of gravity and the erratic supply of Ca and P from the diet in the latter [45, 135].
84 The maintenance of Ca homeostasis in terrestrial vertebrates involves hypercalcaemic
85 factors, that promote Ca uptake, such as parathyroid hormone (PTH) and prolactin
86 (PRL), and hypocalcaemic factors that inhibit Ca uptake like calcitonin (CT) and
87 somatostatin (SS). In fish, PTH related protein (PTHrP) rather than PTH seems to be the
88 hypercalcaemic factor [39] and the role of PTH remains unresolved [19, 45]. The role of
89 calcitonin in calcium homeostasis in fish is still controversial [89] and stanniocalcin is
90 an anti-hypercalcaemic hormone and prevents the uptake of Ca via the gills and
91 intestine [56, 134].

92 The skeleton is known to be a recipient of hormonal inputs and in mammals the
93 pituitary endocrine axis stimulates bone formation via the growth hormone/IGF1 axis
94 and regulates bone resorption, via follicle stimulating hormone (FSH) and thyroid
95 stimulating hormone (TSH) [15]. A recent shift in this paradigm has occurred as it has
96 been proposed that bone also acts as an endocrine organ capable of influencing
97 functions that have nothing to do with its own integrity [62]. Skeletal remodelling

consumes large amounts of energy and bone regulates energy and whole-glucose metabolism via factors it liberates (e.g. osteocalcin; [62, 77]).

The present study focuses on the skeleton of a Sparidae, the gilthead sea bream (*Sparus auratus, Linnaeus 1758*), an important Mediterranean aquaculture species that had a global production greater than 120,000 metric tons in 2008 [34]. Guided by Krogh's principal "*for a large number of problems there will be an animal of choice*" the gilthead sea bream was selected as an experimental model because it is a marine teleost, eurythermic, is medium sized and a protandrous hermaphrodite. Moreover, the gilthead sea bream is a representative of the Perciformes (>7000 members), one of the largest vertebrate orders and it lives longer and is larger than other model fish species, which facilitates sampling and manipulation of both juveniles and adults. In addition, although the genome of gilthead sea bream is unsequenced, numerous molecular resources exist [1,476 nucleotide sequences; 74,877 Expressed sequence tags (ESTs); 92,468 Genome Survey sequences (GSS)], and recently deep sequencing studies have been reported from whole larval [140] and skeletal tissues [42], and inclusive *de novo* construction of a gilthead sea bream transcriptome database had become available [18]. The aim of the present study was to use long-read NGS to generate and compare the bone (vertebrae) and chondroid bone (gill arch) transcriptome in the teleost gilthead sea bream. Insight into skeletal evolution was gained by comparison of the molecular fingerprint of fish bone and chondroid bone with that of terrestrial vertebrates. Moreover, the responsiveness of bone (vertebrae) and chondroid bone (gill arch) was evaluated by quantitative PCR using fasted gilthead sea bream bone since this challenge has previously been shown to modify the structure and activity of bone in rainbow trout and tilapia [94, 122, 123]. Overall the study provides new insights into the skeleton of teleosts and its potential endocrine function.

123

124 2. Material and methods

125 2.1. Fish

126 Juvenile gilthead sea bream (weighing 88.1 ± 7.3 g (mean ± SD); n =35), were
127 reared and maintained at the Institute de Recerca i Tecnologia Agroalimentaries (IRTA)
128 at St Carles de la Rapita (IRTA-SCR, Spain) according to the standard production
129 procedures. Two hundred fish were maintained in two 400 litre tanks (22.5 $kg.m^{-3}$) in a
130 temperature-controlled seawater re-circulation system (IRTAmar^{TM}) at a mean
131 temperature of 21°C (20.7 – 21.4^{o}C) and natural photoperiod (13L:11D). Fish were fed
132 a commercial diet (OptiBreamTM, Skretting; pellet size: 2.6 mm) once daily at a ration
133 level of 3% (mass food/mass fish in tank).

134 Food was withheld from gilthead sea bream for 5 days prior to sampling in order
135 to stimulate skeletal turnover as previously observed in a study of the skin and scales
136 [129]. Five individuals were sacrificed with an overdose of bicarbonate-buffered
137 tricaine methanesulphonate (1:5,000 mass/volume; MS222, Sigma, Madrid, Spain) in
138 seawater followed by spinal cord transection. Gill arches and vertebrae samples were
139 dissected out and placed in RNAlater (Sigma-Aldrich, Spain), before freezing tissue
140 was cleaned of adhering muscle, spinal cord and blood vessels and then flash frozen in
141 liquid nitrogen and stored at -80°C until further analysis. The experiment was conducted
142 in September 2009 in accordance with the Guidelines of the European Union Council
143 (86/609/EU) for the use of laboratory animals and the recommendations of the
144 Association of Animal Behaviour [3].

145

2.2. RNA extraction

Total RNA was extracted from samples of vertebrae (V) and gill arches (GA) using a Maxwell®16 System (Promega, USA) following the manufacturer's instructions. Concentration and quality of the extracted RNA was determined by spectrophotometry (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific, USA) and electrophoresis on 1.5% agarose gels. RNA samples for each tissue were stored in absolute ethanol and sent to the Max Planck Institute (Cologne, Germany) and RNA quality accessed with a LabChipGX (Caliper Life Sciences, USA). Only RNA samples with a quality score higher than seven were pooled for sequencing.

2.3.cDNA library production, 454 sequencing and assembly

2.3.1. cDNA library production and sequencing

Pools of RNA from vertebrae or gill arches of 5 individuals were used for cDNA library preparation and RNA sequencing. Ribosomal (rRNA) was depleted using a RiboMinus™ Eukaryote Kit (Invitrogen, Germany) and the resulting polyadenylated (polyA) mRNA was used to construct two cDNA libraries (Vertebrae and Gill Arches) using a cDNA Rapid Library Preparation Kit (Roche, Germany) according to the manufacturer's instructions. Each library had a unique barcode and was amplified by emulsion PCR and sequenced using a GS-FLX platform (Roche).

2.3.2 Transcriptome assembly

Sequencing reads were edited by screening for adaptor sequences and other artefacts of the pyrosequencing procedure [26] and then assembled into contiguous consensus sequences (contigs) using MIRA 3 [25]. Files containing the edited sequencing reads have been submitted to the Sequence Read Archive (SRA, accession number ERP002185, [117]).

2.4 Annotation and mapping of assembled contiguous sequences

2.4.1 Sequence annotation

Annotation of contigs longer than 99bp was achieved by submitting the data to GenBank non-redundant database of proteins [12, 43] and the Swissprot database [120] and using the BLASTx algorithm [2] to search for sequence similarity. Sequence similarity searches were limited to the vertebrate taxa and only matches with an E-value lower than 1e-10 were considered significant. The vertebrae and gill arch contigs were also blast searched against the *Sparus auratus* ESTs database in NCBI and only the matches with an E-value below the threshold of 1e-50 were considered.

2.4.2 Mapping and functional annotation

The annotated sequences were mapped to Gene Ontology (GO) [4] terms using Blast2GO program v2.5.1. [27]. Specific GO terms were selected from the pool of mapped GO terms for each sequence applying an annotation score with a GO weighting of 5 and Annotation Cut-off of 55 combined with an E-value Hit Filter of 10^{-6}. Level 2 GO pie charts for Biological Process, Molecular Function and Cellular Component were produced using sequence filters of 1% of the total sequences.

To enrich the data further and focus the analysis, specific filters were used to identify genes with GO classifications associated with: calcium; ECM; bone; cartilage; ossification and chondrocytes, osteoblasts, osteoclasts and osteocytes. Candidate genes with a previously demonstrated association with the vertebrate skeleton or involved in established signaling pathways were also identified.

2.5 Quantitative real time RT-PCR (qPCR) of selected candidate genes in vertebrae and gill arches

196 *2.5.1 Experiments*

197 An experiment with juvenile gilthead sea bream was performed to assess the
198 responsiveness to fasting of bone and cartilage using some of the genes identified by
199 NGS. Fish were weighed (body weight 32.6 ± 4.5 g) and randomly divided between two
200 experimental tanks of 110L (8 individuals/tank). The treatment group (fasted) were
201 deprived of food for 5 days and the control group was fed twice daily (3% wet fish
202 weight; Balance 3, Sorgal). The fish were housed in a through-flow seawater system,
203 maintained at 20.5°C, with constant aeration and a photoperiod of 12h light: 12h dark.
204 For sampling fish (n = 8) were anaesthetized in 2-phenoxyethanol (diluted 1:10,000 in
205 seawater, Sigma–Aldrich, Madrid, Spain), weighed and sacrificed by sectioning the
206 spinal cord. Gill arches and vertebrae were dissected out and cleaned of muscle, nervous
207 tissue and blood vessels before freezing in liquid nitrogen and storing at -80^{o}C.

208 *2.5.2 Quantitative qRT-PCR*

209 For total RNA extraction the frozen gill arches (n = 8) and vertebrae (n = 8)
210 were pulverized using a mortar and pestle in the presence of liquid nitrogen before using
211 the Maxwell®16 System (Promega, USA) following the manufacturer's instructions.
212 Concentration and quality of the extracted RNA was determined by spectrophotometry
213 (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific, USA) and
214 electrophoresis on 1.5% agarose gels. Total RNA (2 – 3 μg) was treated with DNase
215 using the DNA-free kit (Ambion, UK) and cDNA synthesis was carried out as described
216 in Vieira *et al.* [130].

217 Quantitative RT-PCR (qPCR) was used to analyse the abundance of some of the
218 transcripts detected in the 454 transcriptome, ALP, TRAP, MMP9, Cathepsin K,
219 Osteonectin, Osteocalcin and IGF1 in juvenile gilthead sea bream gill arches and
220 vertebra. Specific primers were designed for each transcript (Table 1) based on the full

sequence available in the Genbank database. The three estrogen receptors, ERα, ERa and ERβb previously identified [130] in skeletal tissue were also quantified. Transcripts were quantified by qPCR in duplicate 10 µl reactions that contained 2 µl of each cDNA diluted 1:10 and 300 nM of each specific primer (Table 1) and 5 µl EvaGreen (Sso Fast EvaGreensupermix, Bio-Rad Laboratories, USA) using a StepOnePlus qPCR thermocycler with StepOne software v2.0 (Applied Biosystems, UK). PCR cycling conditions were 30 sec at 95 °C, 45 cycles of 5 sec at 95°C and 10 sec at 60°C followed by a final melt curve between 60 and 95°C, which gave single products/ dissociation curves in all reactions.

The relative standard curve method was used for quantification [76]. Standard curves relating amplification cycle to initial template quantity (in ng) were generated using serial dilutions of template (isolated by RT-PCR and quantified). qPCR efficiency ranged between 88 – 100% with $R^2 > 0.985$. Amplicons were sequenced to confirm qPCR specificity. Absence of genomic DNA contamination was confirmed by qPCR using cDNA reactions from which reverse transcriptase was omitted. Candidate reference genes tested included beta actin (β-actin) and ribosomal protein S18 (rps18). β-actin was chosen as the reference gene as it had low variation between cDNA samples and no significant differences in transcript expression existed between tissue and groups. Relative expression levels were calculated by dividing the relative quantity (ng) between the target and reference gene in each cDNA sample.

2.5.3 Statistical Analysis

Statistical significance of qPCR experiments was assessed by two-way analysis of variance (ANOVA) using SigmaStat v. 3.5 (SPSS Inc, USA), with statistical significance set at $p<0.05$. If the data deviated from normality, log10 transformations

were performed. The dataset for ERβa and MMP9 failed equality of variance and so a non-parametric ANOVA on ranks test was used.

3. Results and discussion

In spite of its central importance, relatively few studies have analysed the molecular differences underlying skeletal evolution. There are currently two viewpoints: 1) that the general structural conservation of the skeleton during chordate evolution means that core gene networks have probably also been conserved [35]; or 2) from a comparative study of the gar and zebrafish skeleton that the molecular fingerprints of chondrocytes, osteoblast and osteoclast, were not fixed during early vertebrate evolution [32]. The results of the present study using NGS contribute to the debate on skeletal evolution and homologues of many skeleton related transcripts in mammals were identified in the teleost, gilthead sea bream. Moreover, molecular differences in the transcriptome of the perichondral bone that ossifies from the perichondrium of cartilage (eg. vertebrae) and chondroid bone (eg. gill arch) containing chondrocyte-like cells were similar to those in mammals [33]. The molecular fingerprint of skeletal tissue in gilthead sea bream was compared to other vertebrates and used to test the hypothesis that advanced teleost bone may act as an endocrine tissue.

3.1 Outcome of Roche 454 sequencing of gilthead sea bream vertebrae and gill arches

Three 454 (Roche) sequencing runs with non-normalised cDNA libraries from vertebrae and gill arches were generated (Table 2). After removing adaptor sequences and small reads (<99 bp), 215,030 and 195,661 reads representing 79.7% and 75.8% of raw reads from the vertebra and gill arch libraries, respectively, were entered into

MIRA for assembly. A total of 15.97Mb were produced from the vertebrae cDNA library and resulted in 32,374 contigs (consensus sequences) with an average length of 493bp (Table 2 and Sup. file 1A). The largest contig comprised 4,044bp (261 reads) and was putatively identified as myosin heavy chain (E value 0.0). For the gill arch cDNA library a total of 14.53 Mb was produced and assembled into 28,371 contigs with an average length of 514bp (Table 2 and Sup. file 1B). The largest contig was 5,049bp (787 reads) and was putatively identified as Collagen typeIα2.

Recently, several gilthead sea bream transcriptomes have been published that were generated by NGS and include whole larvae (68,289 contigs, [140]), skeletal muscle (43,461 contigs, [42]) and also intestine (9,475 contigs), head-kidney (14,008 and 12,474 contigs), skeletal muscle (7,808 contigs) and blood (12,003 contigs) have been published [18]. However, none of the studies have generated skeletal tissue specific transcriptomes and the present study is unique as it releases a large volume of bone and cartilage specific transcriptome data, which contributes new knowledge about the teleost skeleton transcriptome and its potential regulation.

3.2 Transcriptome annotation and gene ontology analysis

Sequence similarity searches (Blastn) with the assembled sequences against the *Sparus auratus* ESTs deposited in NCBI revealed that only 41% and 42.6% of the vertebrae and gill arch contigs respectively, had previously been isolated. Contig (>99bp) annotation carried out using sequence similarity searches against the GenBank non-redundant protein and Swissprot databases identified 40.9% of the contigs from vertebrae and 48.3% of the contigs from gill arches (Table 3). Contigs were annotated with the best Blast match. The putative identification of the most abundant contigs from both libraries is represented in the Supplementary file 2. Gene Ontology (GO)

information retrieved with Blast2GO, annotated 10,455 and 10,625 contigs from vertebrae and gill arches, respectively (Table 3 and Sup. file 3).

3.3 Calcium and skeleton related transcripts in vertebrae and gill arches

7,949 contigs in vertebrae and 7,901 contigs in the gill arches libraries with GO annotation corresponded to unique genes. Comparison of these unique genes revealed 3,700 common to both tissues (Fig. 1A). The characteristics of the unique annotated contigs in vertebrae and gill arches were further established by extracting those that could be linked to bone, cartilage, mineralization and calcium (Fig. 1B and C). Genes associated with specific bone cell types: chondrocytes, osteoblasts, osteoclasts and osteocytes were also identified. In the following subsections a brief description of genes linked with calcium and the skeleton is given, the number of reads for these genes in vertebrae and gill arch libraries are given in the Supplementary file 4.

3.3.1 Calcium

Calcium ions act as important second messengers for many intracellular processes, including bone homeostasis, and calcium signalling and is mediated by specific calcium-binding proteins (CaBP). In the present study 28% of the GO terms that were related to Ca were common in both the vertebrae and gill arch libraries and 40% and 32% were only found in vertebrae or gill arches, respectively. However, caution is required with the interpretation of the NGS results as the method used was not quantitative.

The next section gives a brief overview of some of the transcripts identified that encoded CaBP. Parvalbumin, calmodulin, S100 proteins and calcineurin are intracellular CaBP that have been described in mammalian and teleost bone [38, 91,

109, 110, 118, 127] and were identified in both gilthead sea bream vertebrae and gill arches. In addition, four annexins, A1, A2, A5 and A6 (the latter only in vertebrae) were also identified and these CaBP have previously been identified in rat osteoblasts and are involved in bone mineralisation [7, 69]. Transcripts for calreticulin, a multifunctional protein involved in the immune system and in bone mineralization [115], which also plays a role in cellular repair in teleost was also identified [22]. However, the identification in both vertebra and gill arch of the CaBP calsequestrin, which is typical of the sarcoplasmic reticulum of skeletal and cardiac muscle [8], suggests that muscle was not totally eliminated from the bone samples utilised.

Calcium-related genes that were only identified in vertebrae included the extracellular matrix proteins Secretagogin, Asporin and Scinderin (SCIN) a Ca^{2+} dependent actin-severing protein. Secretagogin is a secreted CaBP present in the cytoplasm and associated with cell-cycle regulation in both mammals and teleosts [16, 55]. Interestingly asporin is expressed in human osteoblastic cell lines and induces collagen mineralization [59]. SCIN regulates chondrocyte proliferation and differentiation in mammals [90], and in zebrafish it is duplicated, Scinla is abundant in the adult cornea and Scinlb has a widespread expression including cartilage [58]. In gilthead sea bream Scinla was present in both vertebrae and gill arches and Scinlb was in gill arches, and further studies will be required to characterise their function in these tissue. Likely contamination of the vertebra with nervous tissue was indicated by the presence of the postsynaptic membrane proteins, calsyntenin 1, 2 and 3 in the transcriptome [49]. Hemicentin1 and Grancalcin were two of the calcium related genes only identified in gill arches. Hemicentin is an extracellular adhesive protein in zebrafish and *C. elegans*, that anchors and holds cells together to maintain tissue integrity [20, 131]. Grancalcin is a calcium-binding protein abundant in neutrophils and

macrophages [17] and associated with the innate immune response in teleosts [78]. The presence of Grancalcin transcripts in the gill arches may come from the presence in the extracted samples of extensive vascular tissue and this needs to be further explored.

3.4 Genes encoding proteins of the skeletal extracellular matrix (ECM)

A number of transcripts encoding proteins associated with skeletal tissue in terrestrial vertebrates were readily identified in the transcriptome of both vertebrae and gill arches (Fig. 1C). Skeletal ECM is comprised of basic structural proteins, collagens, proteoglycans, and glycoproteins, that occur as large families of matrix macromolecules [68]. Collagens are abundant proteins, and at least 30 isoforms have been identified and the most abundant in bone ECM is Collagen type I [63]. Transcripts for Col I were identified in both vertebrae and gill arches and transcripts encoding collagen types IX, X and XI were also identified. Collagen type II, which is more characteristic of cartilaginous ECM was only identified in the gill arch transcriptome.

The cartilaginous ECM from both terrestrial and aquatic vertebrates contains a variety of regulatory proteins that include small leucine-rich repeat proteins and proteoglycans (SLRPs, [93]). These proteins ensure correct assembly of collagen fibrils and regulate mineral deposition in bone. Members of the small leucine-rich repeat protein family were identified in both gilthead sea bream transcriptomes and included biglycan, lumican, epiphycan, decorin, osteoglycin and keratocan (only in gill arches). Aggrecan, an abundant non-collagenous glycoprotein of cartilage that is a hallmark of chondrogenesis and has been conserved during evolution [50] was restricted to the gilthead sea bream gill transcriptome. Other non-collagenous extracellular matrix proteins expressed in the gilthead sea bream transcriptomes were members of the matrilin family. Matrilin-1 and -4 were detected in gilthead sea bream gill arches and

matrilin-2 in vertebrae. In mouse and zebrafish matrilins are differentially distributed and matrilin-1 and -3 are expressed in all cartilage regions, matrilin-2 in proliferative and the upper hypertrophic zones and matrilin-4 in the epiphyseal cartilage [70, 72].

Non-collagenous ECM proteins, more characteristic of terrestrial vertebrate bone were identified in gilthead sea bream vertebrae and gill arches transcriptomes, and included Osteonectin (OSN) and Osteocalcin. Osteopontin (Spp1) required for mineralization of bone in both terrestrial vertebrates and fish [35, 37, 66]. A range of other transcripts corresponding to putative ECM proteins of the vertebrate skeleton were also identified and are presented in Fig. 1C. The level of conservation between terrestrial vertebrates and the gilthead sea bream (see figure 1C) provides support for the notion of general structural conservation of the skeleton during chordate evolution (hypothesis 1).

3.5 Molecular fingerprints of the skeletal tissue

3.5.1 Chondrocyte related genes

In teleosts, such as zebrafish and gar, although the structure of hyaline cartilage differs from that in mammals [136], Col2a1, ColXa1, Sox9 and runt-related transcription factor (runx2) transcripts are expressed by chondrocytes and suggests their molecular fingerprint may be conserved in vertebrates [32]. Many factors previously described in mammals as specifically associated with chondrocytes [63, 137] were also present in the gilthead sea bream vertebrae and gill arch transcriptomes (Fig. 2). These included Sox9 a regulator of chondrogenesis, which prevents chondrocyte hypertrophy, Sox6 that is co-expressed with Sox9 in all pre-cartilaginous condensations in mammals [54, 137] and Sox8, a negative regulator of osteoblast differentiation in mice [108] (Fig. 3). Further homologues of the Sox family identified in the gilthead sea bream

transcriptomes, Sox3, Sox17 and 18, have not previously been reported in the vertebrate skeleton. Bone morphogenetic proteins (BMPs), which play multiple roles in chondrocyte differentiation and proliferation [88] were also represented and included homologues of BMP1, BMP2, BMP3, BMP4 and BMP8 (Fig. 2). Runx2 previously identified in the zebrafish and gar and involved in chondrocyte hypertrophy and induction of endochondral bone formation [36, 61] was present along with runx1 and 3.

Sonic hedgehog (Shh), a signaling molecule, associated with bone patterning and scale formation in teleosts [53, 74] was identified in the gilthead sea bream gill arch transcriptome. In contrast, Indian hedgehog (Ihh) that regulates growth plate chondrocyte maturation and differentiation in terrestrial vertebrates [44, 132] and which in teleosts is present in hypertrophic chondrocytes of cartilaginous elements of the craniofacial and fin endoskeleton in zebrafish [5] was not identified (Fig. 2), although it is not possible to exclude the possibility that the depth of sequencing was insufficient to capture rare transcripts. Nonetheless, it is clear from a cursory consideration of Fig. 2 that many of the factors identified in mammalian bone are also present in the gilthead sea bream vertebrae and gill arches transcriptomes. The results support the general notion that gene networks have been conserved in the skeleton during chordate evolution (hypothesis 1). Future work should aim to map these factors to specific cell types, but also focus on novel transcripts described for the first time in a teleost skeleton.

3.5.2 Common gene networks regulating chondrocytes and osteoblasts

In general, common pathways regulate cartilage and bone formation and these involve members of fibroblast growth factors (FGFs), the Wnt family, and the

419 Transforming growth factor beta (TGFβ) superfamily that includes the BMPs and their
420 associated transcription factors.

421 In mammals, FGF family members are associated with cartilage and bone [30],
422 for example FGF9 regulates chondrocyte hypertrophy and FGF18 is expressed in the
423 perichondrium and signals to chondrocytes through FGF receptor 3 (FGFR3) expressed
424 in proliferating zones [44, 81, 141]. In the gilthead sea bream transcriptomes FGF9, 18
425 and 3 were not identified although homologues of FGF2, FGF10 and the FGF1 and 2
426 receptors were identified (Fig. 2) suggesting the FGF family also regulates cartilage and
427 bone in teleosts. The signal transducer STAT1 is proposed to be one mediator of
428 FGFR3 actions in chondrocyte differentiation [107] and may act as a negative regulator
429 of proliferation [57, 141]. In salmon and zebrafish, STAT1 has been associated with the
430 immune system and hematopoiesis [113, 116] and in the gilthead sea bream it was
431 identified in both vertebrae and gill arches, although its function remains to be
432 established.

433 The canonical Wnt/β-catenin signalling pathway in mammals acts at two stages,
434 first promoting chondroprogenitor differentiation and later to promote chondrocyte
435 hypertrophic differentiation and subsequent endochondral ossification [44, 124]. In
436 teleosts Wnt/β-catenin genes are expressed in fin regeneration and tail development [1,
437 65], only Wnt4a was identified in gill arches, but it is possible that other Wnt members
438 were also expressed, but at such low abundance that they were not detected. In relation
439 to β-catenin, a protein that controls osteoprogenitor cell differentiation into osteoblasts
440 in mammals [86, 141], a homologue was identified in both vertebrae and gill arches
441 (Fig. 2 and 3).

442

443 TGFβ/BMP signalling is involved in many cellular processes and has recognized
444 roles in bone formation during mammalian development. The TGFβ superfamily is
445 comprised of over forty members, such as TGFβs, BMPs and Activins. Smads are a
446 family of intracellular proteins that mediate signalling by members of the TGFβ
447 superfamily and members of this family have been identified in several organisms of the
448 animal kingdom [51]. Smads 2, 3, 4 and 7 expression has been reported in the gill
449 filaments of rainbow trout [41] and a homologue of the Smad4 gene was detected in the
450 gilthead sea bream gill arches and Smad1 in vertebrae. BMP signalling in teleosts was
451 shown to be involved in fin growth and scleroblast differentiation in zebrafish [74, 114]
452 and BMP2 has been identified in gilthead sea bream calcified tissues [99] and in the
453 present study BMP1 and 3 were identified in vertebrae and BMP2 and BMP1 receptor
454 A (BMP1RA) were only identified in the gill arch library and BMP4 and 8 were
455 detected in both vertebrae and gill arches and supports a role for BMP signalling in
456 teleosts cartilage and bone. In mammals there are three TGFβ ligands and their
457 homologues have already been identified in teleost fish [75, 121]. Nevertheless, little
458 information is available about the receptors and their interactions with the ligands, but
459 recently a study suggested that TGFβ type I and II receptors may modulate immune
460 responses in teleost fish [83]. In gilthead sea bream, three TGFβ receptors were
461 identified: TGFβR1 and 3 in gill arches and TGFβR2 in both vertebrae and gill arches;
462 as well the three ligands: TGFβ1 in vertebrae and TGFβ2 and 3 in both vertebrae and
463 gill arches, which suggests that TGFβ ligands and their receptors play a role in bone and
464 cartilage development (Fig 2 and Fig 3).
465
466 *3.5.3 Osteoblast related genes*

The osteoblasts have a central role on bone and secrete a number of bone-related extracellular matrix proteins (like Osteocalcin, Spp1 and bone sialoprotein) and express high levels of alkaline phosphatase (ALP), which generates inorganic phosphate for mineralization. The osteoblasts also express and respond to osteotropic hormones and cytokines [64].

Comparisons of terrestrial vertebrate skeletal tissues indicate that the molecular fingerprints of chondrocytes or osteoblasts do not vary greatly [32]. Previous small scale comparisons of molecular factors regulating the skeleton in terrestrial vertebrates and teleosts suggested that both conservation [32] and divergence occur [5, 32, 80]. However, analysis of the global transcriptomes in the present study, which does not determine cellular localization or association with specific processes, suggests overall significant conservation of the molecular fingerprint between skeletal tissue of mammals and the gilthead sea bream (Fig. 3).

Some of the key factors important in the regulation of osteoblasts in mammals and identified in the gilthead sea bream transcriptomes include Runx2, which is expressed during development of the skeletal tissue in zebrafish [36] and controls osteoblast differentiation in mammals (see [61, 66, 86]). Osterix/Sp7, a zinc finger protein regulating osteoblast differentiation and previously identified in medaka [102], was identified in both vertebrae and gill arches transcriptomes. In mammals, Msh homeobox (Msx) 1 and 2 and Distal less homeobox (Dlx) 3 and 5 proteins, are involved in osteoblast differentiation and proliferation [9, 52, 73, 86]. In zebrafish Dlx5 is present in the developing visceral skeleton and during scale regeneration [126, 128] and Msx genes are involved in fin regeneration [96]. In the gilthead sea bream transcriptomes, homologues of Msx1 (only in gill arches) and Dlx3 were identified along with several other regulatory factors including Twist 2 (Fig. 3), a helix-loop-helix protein, that

492 regulates chondrogenesis during viscerocranial development in zebrafish [138]. The
493 presence of a putative Twist2 paralogue in the gilthead sea bream vertebrae
494 transcriptome indicates a putative function in perichondral bone.
495
496 *3.5.4 Osteocyte related genes*
497 Osteocytes are star-shaped cells with a reduced capacity to produce ECM and
498 are proposed to orchestrate bone remodelling in terrestrial vertebrates by regulating
499 osteoblast and osteoclast activities [125]. Tetrapods and basal bony fish possess
500 "cellular" bone that contains osteocytes while the bone in advanced teleosts such as
501 gilthead sea bream lacks osteocytes and is considered to be "acellular" [33]. Osteocytes
502 produce PHEX (phosphate regulating endopeptidase homolog, X-linked), FGF23 and
503 KLOTHO, which are regulators of P homeostasis and bone mineralization [67, 105]. In
504 teleosts FGF23 is expressed in the corpuscles of Stannius and contribute to calcium and
505 phosphate homeostasis and KLOTHO is expressed in adult kidney [14, 84]. Neither
506 PHEX nor FGF23 homologues were identified in the gilthead sea bream and a putative
507 KLOTHO transcript was identified in the vertebrae (Fig. 5). It will be of interest in the
508 future to establish in teleosts with acellular bone the origin of osteocyte type factors.
509
510 *3.5.5 Osteoclast related genes*
511 Osteoclasts differentiate from myeloid precursor cells and in basal teleosts with
512 cellular bone, like zebrafish and carp, remodeling is proposed to resemble that of
513 mammals [135]. Molecular data supports this notion and key factors such as PU.1,
514 TRAF6, macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear
515 □ B ligand (RANKL) and Osteoprotegerin (OPG), which control monocyte
516 differentiation into osteoblasts and activated osteoclasts in mammals also occurs in

basal teleosts [47, 103, 135]. None of the genes involved in osteoclast differentiation were identified in the gilthead sea bream transcriptomes (Fig. 4).

In advanced teleosts, like gilthead sea bream, with acellular bones, osteoclasts are mononucleated although occasional multinucleated osteoclasts have been described [112]. One of the basic features of osteoclasts is secretion of TRAP, which acts at the bone surface [28, 106] and mononucleated osteoclasts in both mammals and teleosts participate in minor and smooth bone resorption. Activated osteoclasts dissolve hydroxyapatite by secreting hydrocloridric acid and then Cathepsin K and matrix metalloproteinase 9 (MMP9) degrade the organic bone matrix [87]. TRAP and vitronectin are also characteristic markers with abundant expression in activated osteoclasts in mammals [97]. Cathepsin K has recently been implicated in the resorption of teleost bone and scale matrix [6, 126], although MMP9 has been linked with immune function [21, 139]. Orthologues of cathepsin K, MMP9 and TRAP (marker of osteoclasts) were abundant in both gilthead sea bream vertebrae and gill arches. The absence of transcripts associated with osteoclast differentiation but abundance of those typical of activated osteoclasts is coherent with the skeletal mobilization that accompanies fasting.

Summarising this section (see figures 2, 3 and 4), many transcripts characteristic of mammalian bone cells (chondrocytes, osteoblasts and osteoclasts) were identified in gilthead sea bream vertebrae and gill arches transcriptomes. The results support the view that "*general structural conservation of the skeleton occurred during chordate evolution"* and this is presumably the reason core gene networks have been conserved [35]. Nonetheless, the specific tissue localization of the transcripts identified in the present work will be of interest to assess if the molecular fingerprints of bone cell types were maintained in the teleost fish gilthead sea bream. An caveat of the present NGS

approach is that non identification of a transcript in the gilthead sea bream vertebrae or gill arch transcriptomes does not mean that it is not expressed, as transcripts of very low abundance, not annotated because of poor sequence conservation, or that are expressed at specific stages or in response to a challenge may not be represented/identified in the present transcriptomes.

3.6 Expression analysis in gilthead sea bream vertebrae and gill arches by quantitative RT-PCR

The skeleton has a slow turnover relative to many other tissue and overt changes in the structure of bone generally only arise after prolonged treatments. Nonetheless, the cells responsible for bone homeostasis should respond rapidly to any challenge and this was one of the reasons fish were fasted prior to tissue sampling for transcriptome analysis. To assess the impact of fasting on transcript abundance in vertebrae and gill arches, real-time PCR analysis was performed using transcripts detected by NGS (read numbers Sup. file 4) and associated with specific processes or cell types (Fig. 5). A cursory comparison was made of NGS transcript read number and qPCR abundance. Unsurprisingly, the transcript abundance obtained from non-quantitative NGS (read numbers Sup. file 4) relative to qPCR was dissimilar as libraries were not normalized, thus differential expression analysis should not be carried out [92].

The expression of ALP and TRAP type 5, which are mammalian and teleost osteoblast and osteoclast markers, respectively were analysed (Fig. 5A and B). ALP mRNA transcript expression did not change significantly between vertebrae and gill arches. In contrast, TRAP transcripts were significantly more abundant in vertebrae compared to gill arches ($p<0.001$). Fasting did not alter the expression of these transcripts.

The transcript abundance of ECM proteins characteristic of bone, Osteonectin (OSN) and Osteocalcin, were also analysed (Fig. 5C and D). OSN was significantly ($p<0.001$) down-regulated in vertebrae from fasted fish relative to control fish. OSN was significantly ($p<0.001$) more abundant in vertebrae than in gill arch samples. Osteocalcin gene expression is restricted to osteoblasts [48, 60, 97] and presumably this explains the significantly ($p<0.001$) higher transcript abundance in vertebrae relative to gill arches where levels are at the limit of detection. As observed for OSN, there is a significant ($p<0.01$) reduction in osteocalcin transcripts in vertebrae and also in gill arches ($p<0.05$) from fasted fish relative to control fish.

Expression of MMP9 and Cathepsin K, genes characteristic of mature osteoclasts and associated with matrix degradation [87] were also analysed (Fig. 5E and F). MMP9 and Cathepsin K were both significantly ($p<0.001$) more abundant in vertebrae than in gill arches and a significant ($p<0.05$) increase in MMP9 occurred in the gill arches of fasted fish relative to the control. In relation to mRNA transcripts of Cathepsin K, fasting caused a significant decrease ($p<0.05$) in vertebrae.

Insulin-like Growth Factor 1 (IGF1) and Growth-hormone (GH) are major regulators of linear bone growth and body size in mammals [82] and teleosts [31, 133]. IGF1 mRNA levels despite being of low abundance were significantly ($p<0.001$) higher in gilthead sea bream vertebrae compared to gill arches (Fig. 5G). A comparison of IGF1 mRNA levels revealed significant ($p<0.001$) down-regulation in the vertebrae of fasted fish relative to control fish.

In gilthead sea bream three ERs receptors have previously been isolated and are expressed in skeletal tissue, such as the scales, vertebrae and dentary bone and are associated with calcium mobilisation [95, 130]. The results of the present qPCR study confirmed the low to undetectable levels of sbERα mRNA in skeletal tissue in the

592 gilthead sea bream. The duplicate ERβ transcripts (sbERβa and sbERβb), had a similar
593 low abundance in the vertebrae and gill arches, although sbERβa had more expression
594 ($p<0.01$) in the vertebrae than in the gill arches. Fasting caused a significant ($p<0.01$)
595 increase in sbERβa transcript abundance in both vertebrae and gill arches relative to the
596 respective control animals, presumably linked to the need to mobilize calcium.

597 In summary, transcripts characteristic of bone cells, osteoblasts and osteoclasts,
598 were more abundant in the vertebrae (perichondral bone) than in the gill arches.
599 Moreover, a short-term fast (5 days) depressed transcripts associated with bone
600 formation (OSN, osteocalcin, IGF1) but did not cause up-regulation of transcripts
601 associated with bone mobilization with the exception of ERs and MMP9 in gill arches.
602 Future studies deploying a fasting time course and analyzing other biochemical and
603 metabolic indicators and other key transcripts identified in the present NGS study will
604 contribute to our understanding of the dynamics of skeletal turnover.

605

606 **3.7 Endocrine regulation of bone**

607 The development and homeostasis of the skeleton is under the control of the
608 endocrine system and factors, such as Parathyroid Hormone (PTH), estrogens, GH and
609 Vitamin D3, and other growth factors, like IGF1, TGFβ and FGF2 (for review see [86]).
610 The sequences of several hormones and their receptors were used in searches of the
611 gilthead sea bream vertebrae and gill transcriptomes and the results are summarized in
612 Table 4.

613 Members of the GH and IGF1 receptor signaling pathways exert their actions on
614 bone and several members belonging to this pathway were identified in the gilthead sea
615 bream transcriptomes (Table 4), including duplicate GH receptors (GHR1 and GHR2),
616 Insulin receptor (INSR) and Insulin-like growth factor binding proteins (IGFBPs). In

relation to the IGFBPs, in mammals they have been related with the skeleton, in particular IGFBP2 is required for osteoclasts differention and IGFBP6 is expressed in osteoblasts [29, 98]. IGFBPs have also been isolated in teleosts and IGFBP4 is suggested to inhibit growth and development [79]. IGFBP1, 2 and 3 were identified only in gilthead sea bream vertebrae whilst IGFBP6 and 7 were only identified in gill arches (Table 4), which may indicate they have specific roles in gilthead sea bream skeletal tissue, although further studies will be required to consolidate this hypothesis.

The regulation of skeletal calcium metabolism in mammals involves the hypercalcaemic hormones PTH, Vitamin D3 and calcitonin (CT). PTH binds to osteoblasts and induces the production of M-CSF and RANKL which stimulate osteoclast maturation [97, 100]; Vitamin D promotes the differentiation of osteoclasts by stimulating RANKL production by osteoblasts [97]; and CT inhibits bone resorption by inhibiting osteoclasts [97, 100]. The role of the skeleton on Ca balance in teleosts is not straightforward, because in teleosts the intestine, gills and skin/scales epithelia can take up Ca from the bathing water [104]. Moreover, bone in many teleosts is acellular leading to the proposal that Ca is probably not mobilized from the endoskeleton [111, 135] but instead the scales act as a source of readily mobilized Ca [123, 135]. In fish PTHrP increases osteoclast activity *in vitro* in gilthead sea bream [46, 104]. Studies in the rainbow trout suggested that Vitamin D exerts a physiological regulation in relation to environmental calcium concentrations [119]. To assess the regulation of the teleost skeleton, the gilthead sea bream vertebrae and gill arches transcriptomes were analysed to identify calcitropic hormones and their receptors (Table 4). Neither vitamin D, its receptor, PTH nor PTHrP was identified, although the PTH receptor 1 was identified in gilthead sea bream vertebrae. Two calcitonin-like receptors (CLRL1 and CLR2) and the ligand, calcitonin 1, were also identified in the same library. No orthologue of

642 stanniocalcin, an anti-hypercalcaemic hormone of the Corpuscles of Stannious [56,
643 134], or it receptors, were identified in gilthead sea bream vertebrae or gill arches. The
644 results suggest that the turnover of bone to regulate Ca and Pi levels in gilthead sea
645 bream may occur via the endocrine action of known calcitropic hormones.

646 The text book view of the skeleton is as a protective and supportive organ.
647 However, recent studies of bone have changed this paradigm, particularly those
648 revealing that the skeleton can act as an endocrine organ, regulating energy metabolism
649 and reproduction in mammals [40, 62]. Bone physiology and energy metabolism are
650 linked by hormones, such as leptin and pro-opiomelanocortin-α (POMC) and
651 melanocortin receptor 4 (MC4R) [62]. So far a similar role for the skeleton in teleosts
652 remains to be established. However, a short fast (5 days) caused significant down-
653 regulation of transcripts for OSN, Osteocalcin and IGF1 (Fig. 5C, D and G), and
654 although the proteins were not analyzed, the results suggest a link between these factors
655 and energy balance in the gilthead sea bream. Moreover, the identification in the present
656 study of POMC in gilthead sea bream vertebrae and Osteocalcin, an osteoblast-specific
657 protein that regulates energy metabolism in mammals [62, 77] makes this a promising
658 avenue for future research.

659

660 ## 4. Conclusions

661 This study reports for the first time the global transcriptome of perichondral
662 bone (vertebrae) and chondroid bone (gill arch) of a teleost fish, the gilthead sea bream.
663 The results unveil the molecular fingerprint and reveal many novel transcripts identified
664 for the first time in the vertebrae and gill arches of an advanced teleost. Transcripts with
665 very low abundance, with poor sequence conservation or expressed at specific stages or
666 in response to specific challenges may not be represented/identified in the

transcriptome. Nonetheless the large volume of bone and gill arch specific transcripts contribute new knowledge about the teleost skeleton transcriptome and its potential regulation, and will be valuable for future studies of skeletal regulation and turnover in teleosts. The conservation observed between transcripts present in the bone and cartilage of gilthead sea bream compared to mammals suggests that during skeletal evolution in chordates, core molecular fingerprints were retained. Future studies will be required to confirm the tissue and cell specific localisation of transcripts.

The gilthead sea bream vertebrae and gill arches differ at a morphological and cellular level [33] and also respond differently to a selective estrogen receptor modulator (SERM), raloxifene ([130]; personal observations). The present study of the vertebrae and gill arches transcriptomes identifies both common and very different gene complements in the tissues, which may explain the basis for the different tissue responsiveness to SERMs, but also to short term fasting and presumably also to other challenges. Tissue expression analysis by qPCR revealed that in gilthead sea bream significant changes in transcript abundance occurred even during a short fast and the modifications detected may indicate that, in common with mammals, the teleost skeleton acts both as a calcium reservoir and also as an endocrine tissue and is involved in energy balance regulation [62, 77].

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Figure legends

Figure 1 –**A**) Venn diagram with the distribution of the annotated transcripts between vertebrae, gill arches or both libraries; **B**) proportion of the calcium related genes present only in vertebrae, gill arches or both libraries; **C**) proportion of the skeletal tissue related genes present only in vertebrae, gill arches or both libraries.

Figure 2 – Schematic representation of the key events occurring during chondrogenesis leading up to ossification. The diagram is based on results from studies of mammalian skeletal tissue and includes the principal ECM proteins and transcripts characteristic of different differentiation states: proteins [β-catenin; Gli2/3 – GLI family zinc finger 2/3; IHH – Indian hedgehog; IGF1 – Insulin-like growth factor 1; Msx2 – Msh homeobox 2; PTHrP – Parathyroid hormone related peptide; SHH – Sonic hedgehog; TGFβ1/2/3 – Transforming Growth factor beta 1/2/3; Wnt3A/7A/14 – Wingless-type MMTV integration site family, member 3A/7A/14], growth factors [BMP 2/4/7 – Bone morphogenetic protein 2/4/7; FGF 2/4/8/10/18 – Fibroblast growth factor 2/4/8/10/18; FGFR 1/2/3 – Fibroblast growth factor receptor 1/2/3], signal transducers [Stat1 – Signal transducer and activator of transcription 1; VEGF - Vascular endothelial growth factor] and transcription factors [ATF4 – Activating transcription factor 4; Osterix; Runx2 – Runt-related transcription factor 2; Sox5/6/9 – SRY (sex determining region Y)-box 5/6/9]. Genes identified in both the gilthead sea bream vertebrae and gill arches transcriptomes are not highlighted; genes highlighted in grey were only identified in the gill arch transcriptome; genes highlighted in black with white letters were only identified in the vertebrae transcriptome; genes struck-out were not identified in either gilthead sea bream transcriptomes. [Adapted from [44]] ECM proteins gene symbols: HAPLN1 – Hyaluronan and proteoglycan link protein 1; COMP – Cartilage oligomeric matrix protein. (*) indicates that N-Cadherin is not an ECM protein but a transmembrane protein.

Figure 3 – Schematic representation of the key events in osteoblastogenesis. The diagram is based on results from studies of mammalian skeletal tissue and includes the principal ECM proteins and transcripts characteristic of different differentiation states: proteins [β-catenin; Cyr61– Cysteine-rich angiogenic inducer 61; Dlx3/5 – Distal-less homeobox 3/5; FGF23 – Fibroblast growth factor 23; LEF1 – Lymphoid enhancer-binding factor 1; Msx1/2 – Msh homeobox 1/2], enzymes [ALP – Alkaline phosphatase; PHEX – Phosphate regulating endopeptidase homolog X-linked], signal transducers [STAT1– Signal transducer and activator of transcription 1] and transcription factors [c-Fos – Cellular oncogene c-fos; Hoxa2/10 – Homeobox A2/10; JunD – Jun D proto-oncogene; Runx2– Runt-related transcription factor 2; Sox8 – SRY-box 8; Twist 1/2 – Twist homolog 1/2]. Genes identified in both the gilthead sea bream vertebrae and gill arch transcriptomes are not highlighted; genes highlighted in grey were only identified in the gill arch transcriptome; genes highlighted in black with white letters were only identified in the vertebrae transcriptome; genes struck-out were not identified in either gilthead sea bream transcriptomes. (*) indicates that CDH11 (Cadherin-11) is not an ECM protein but a transmembrane protein.

Figure 4 – Schematic representation of the events leading to osteoclastogenesis. The diagram is based on results from studies of mammalian skeletal tissue and includes transcripts characteristic of differentiation state. Genes identified in both the gilthead sea bream vertebrae and gill arches transcriptomes are not highlighted; genes highlighted in grey were only identified in the gill arches transcriptome; genes highlighted in black with white letters were only identified in the vertebrae transcriptome; genes struck-out were not identified in either gilthead sea bream transcriptomes. Gene Symbols: PU.1 – Hematopoietic transcription factor PU.1; M-CSF – Macrophage colony-stimulating factor 1; RANK – Receptor activator of nuclear factor kappa-B; RANKL – Receptor activator of nuclear factor kappa-B ligand; OPG – Osteoprotegerin; IL-1 – Interleukin 1; TNF-α – Tumor necrosis factor-alpha; PGE_2 – Prostaglandin E2; TRAF6 – TNF receptor-associated factor 6; ERα/β – Estrogen Receptor alpha/beta; CRLR 1/2 – Calcitonin receptor-like receptor 1/2; TGFβ1 – Transforming Growth

factor beta 1; MMP 9/13 – Matrix metallopeptidase 9/13; TRAP – Tartrate-resistant acid phosphatase type 5; PTHR1 – Parathyroid hormone receptor 1; MITF – Microphthalmia-associated transcription factor; PIK3R1 – Phosphatidylinositol 3-kinase regulatory subunit alpha; NFATC1 – Nuclear factor of activated T-cells cytoplasmic 1.

Figure 5 – Quantitative RT-PCR of the relative expression of transcript mRNA in vertebrae and gill arches from control and 5 days fasted juvenile gilthead sea bream (n = 7 or 8): TRAP; ALP; OSN; Osteocalcin; MMP9; Cathepsin K; Estrogen receptor (ER)βa; ERβb; IGF1. Data is presented as the Mean ± SEM of the ratio of target template:β-actin (reference gene). Different letters for a given gene indicate groups that are significantly different ($p<0.05$; Two-way ANOVA).

Supporting information

Supplementary file 1 – Contig length distribution for gilthead sea bream vertebrae (A) and gill arches (B) 454 sequence assembly with MIRA3 [25].

Supplementary file 2 – Tables with the top 30 most expressed contigs for gilthead sea bream vertebrae and gill arches.

Supplementary file 3 – Gene Ontology (level 2) terms describing the biological processes, molecular functions and cellular components represented by the contigs generated by assembly of 454 sequences from gilthead sea bream vertebrae (A) and gill arches (B).

Supplementary file 4 – Table listing the read number in the vertebrae and gill arch transcriptomes of all the transcripts mentioned in the manuscript. The percentage of representation of those genes in each library is given, as well their gene symbol and full name.

1171 **Table 1–** List of primers used for gene expression analysis by quantitative RT-PCR. Gene
1172 name, accession number/reference, primer sequence, amplicon length (bp), annealing
1173 temperature (Ta, ºC), qPCR efficiency (%) and R^2 are indicated for each primer pair. F=
1174 forward and R= reverse primer.

Gene Name	Genbank Accession No	Primer sequence (5'→ 3')	Amplicon (bp)	Ta (ºC)	Efficiency	R^2
Osteonectin	AJ564190	F: GCAAGAAGGGCAAAGTGTG R:GTGGCAGGAGGTGTCGTAGG	143	60	89%	0.99
Osteocalcin	AF289506	F:TCCGCAGTGGTGAGACAGAAG R:CGGTCCGTAGTAGGCCGTGTAG	150	64	96%	0.99
MMP9	AM905938	F: ATTCAGAAGGTGGAGGGAGCG R: CATTGGGGACACCACCGAAGA	151	60	90%	0.99
Cathepsin K	DQ875329	F: AGCGAGCAGAACCTGGTGGAC R: GCAGAGTTGTAGTTGGGGTCGTAG	179	60	89%	0.99
IGF1	AY996779	F: TGTCTAGCGCTCTTTCCTTTCA R: AGAGGGTGTGGCTACAGGAGATAC	84	60	98%	0.99
ALP	AY266359	F: CTGCCGTCCGTTCCCAGTGTA R: CTCATTGTCGGAGTACCAGT	176	60	100%	0.99
TRAP	FM147928	F: CTTAATCGTTGCCATCCCTGTG R: CTCCCATCTGCTCTGCTACTTTG	194	60	88%	0.99
ER □	AJ006039	F:AAACCACCTCAACACCCATCTACAG R:GCACACGGCACAGAAACGCATC	173	60	93%	0.99
ERβa	AF136980.1	F: TGTCATCGGGCGGGAAGG R: GCTCTTACGGCGGTTCTTGTCT	188	60	92%	0.99
ERβb	AJ580048	F: ACAAACCCTTCACCGAGTCCAG R: AACTCTACGAAGCCAGGTATCTTT	109	60	98%	0.99
RPS18	AM490061	F: AGGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACC	164	60	97%	0.99
Beta-Actin	X89920	F: CCCTGCCCCACGCCATCC R: TCTCGGCTGTGGTGGTGAAGG	94	60	95%	0.99

1175

1176 **Table 2** – Summary statistics of 454 sequencing and assembly.

454 sequencing	Vertebrae	Gill arch
Total number of raw reads	271,613	258,102

Total number of assembled reads	215,030	195,661
MIRA assembly		
Number of contigs	32,374	28,371
Total number of bases in contigs (Mb)	15.97	14.53
Number of contigs >500bp	10,865	10,592
Average length of contigs (bp)	493	514
Largest contig (bp)	4,044	5,049
Number of contigs <100bp	521	632

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1179 **Table 3** – Blast, mapping and annotation results for contigs ≥100bp.

	Vertebrae	**Gill Arch**
Total number of contigs ≥100bp	31,853	27,739
Number of contigs with/without a Blast hit	13,031/18,822	13,401/14,338
Number of contigs with/without GO mapping	11,287/1,744	11,537/1864
Number of contigs with/without GO annotation	10,455/832	10,625/912

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1182 **Table 4** – Search results for endocrine hormones and receptors in the sea bream
1183 vertebrae and gill arches transcriptomes. Accession number and Expectation (E) value
1184 of the best match is given. *N/A* – Not Applicable.

	Vertebrae			**Gill Arches**		
Hormone/Receptor	**Contig ID**	**Acession #**	**E value**	**Contig ID**	**Acession #**	**E value**
PTH	*N/A*			*N/A*		
PTHrP	*N/A*			*N/A*		
PTHR1	0096_c8020	NM_131357.1	4,00E-10	*N/A*		
Calcitonin	0096_rep_c14862	AJ309015.1	1,00E-54	*N/A*		
CGRP	*N/A*			*N/A*		
CTR	*N/A*			*N/A*		
CRLR1	0096_c27415	AB219835.1	5,00E-119	*N/A*		
CRLR2	0096_rep_c20571	AB219837.1	5,00E-111	*N/A*		
Staniocalcin	*N/A*			*N/A*		
Vitamin D receptor	*N/A*			*N/A*		
Somatostatin (SS)	*N/A*			*N/A*		

SSR2	0096_c18575	XP_002665650.1	4,04E-54	*N/A*		
GH	*N/A*			*N/A*		
GHR1	0096_rep_c7168	AF438176.2	2,00E-180	0097_rep_c1450	AF438176.2	0.0
				0097_c13122	AF438176.2	0.0
				0097_c16752	ACT20710.1	5,33E-49
GHR2	0096_rep_c12585	AAT76436.1	3,28E-78	0097_rep_c17649	AAT76436.1	1,13E-92
Insulin Receptor	0096_c17184	XP_003448585.1	2,60E-93	0097_c11133	XP_003448585.1	1,79E-113
IGF1	0096_rep_c5448	GQ924783.1	6 E -05	0097_rep_c4535	GQ924783.1	5 E -05
IGF2	0096_c14720	AAY46224.1	3,17E-54	0097_rep_c3994	AAY46224.1	1,22E-54
IGFBP1	0096_c14455	XP_002192074.1	7,57E-68	*N/A*		
IGFBP2	0096_c15837	XP_003453223.1	1.49E-89	*N/A*		
IGFBP3	0096_c23286	ACD11356.1	9,19E-55	*N/A*		
IGFBP6	*N/A*			0097_c21530	ABV58580.1	3,46E-36
IGFBP7	*N/A*			0097_rep_c2906	XP_003458710.1	7,60E-25
				0097_rep_c17981	XP_003458710.1	2,15E-16
Er□	*N/A*			*N/A*		
ER□1	*N/A*			0097_rep_c16411	AJ489523.1	0.0
ER□2	*N/A*			*N/A*		
Prolactin	*N/A*			*N/A*		
Prolactin receptor	*N/A*			*N/A*		
Somatolactin (SL)	*N/A*			*N/A*		
SLR	*N/A*			*N/A*		
Neuropeptide Y (NPY)	0096_c23123	NP_001116379.1	9,55E-18	0097_c18703	BAB62409.1	1,21E-31
NPY receptor	*N/A*			*N/A*		
Leptin	*N/A*			*N/A*		
Leptin receptor	0096_rep_c20191	NM_001130869.1	3,00E-91	0097_rep_c25365	NM_001130869.1	5,00E-100
	0096_rep_c3995	NM_001130869.1	4,00E-39	0097_rep_c16559	NM_001130869.1	2,00E-60
TSH	*N/A*			*N/A*		
TSHR	*N/A*			0097_c16473	DQ386646.1	0.0
FSH	*N/A*			*N/A*		

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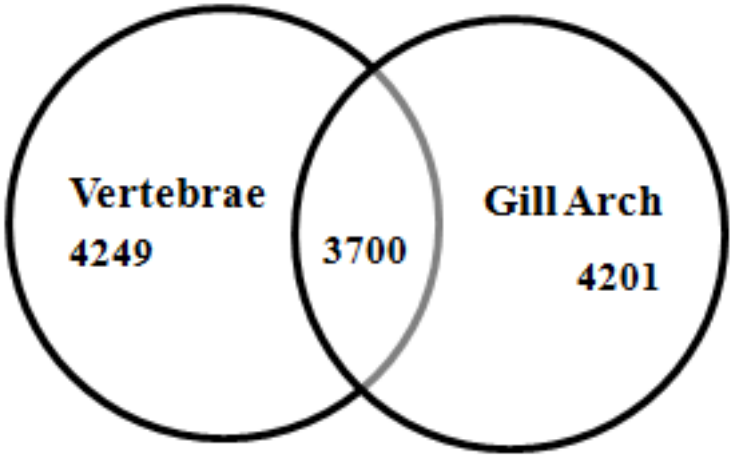

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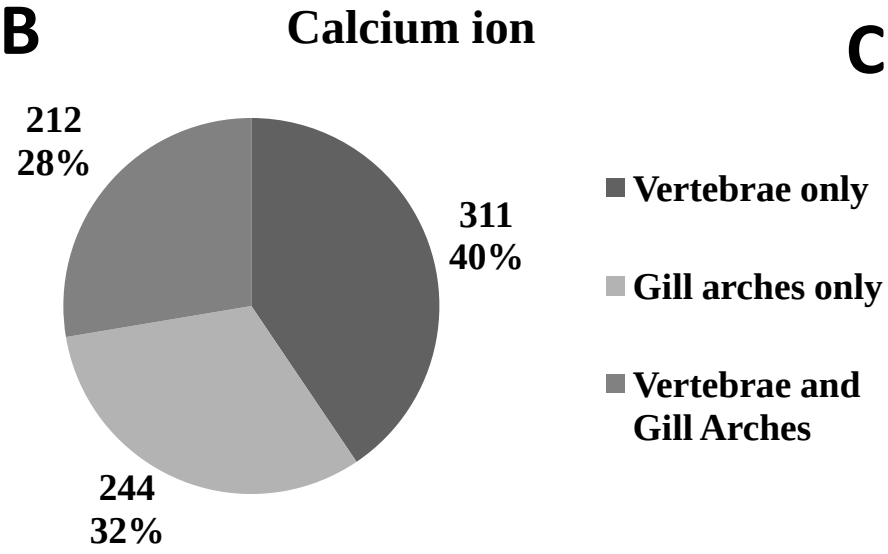

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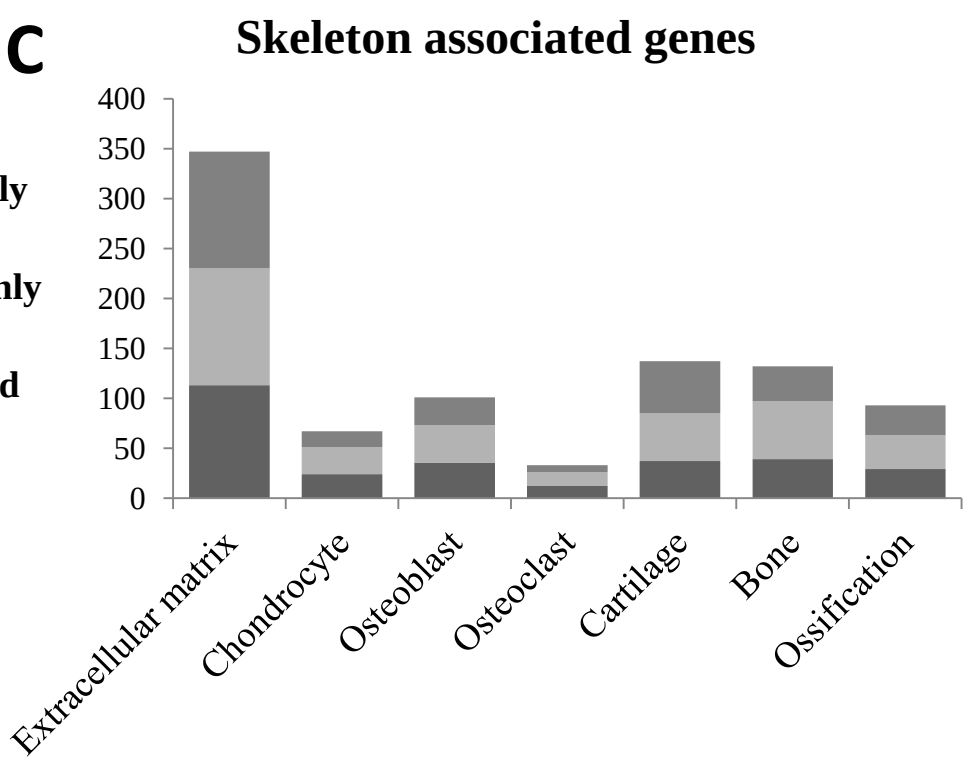
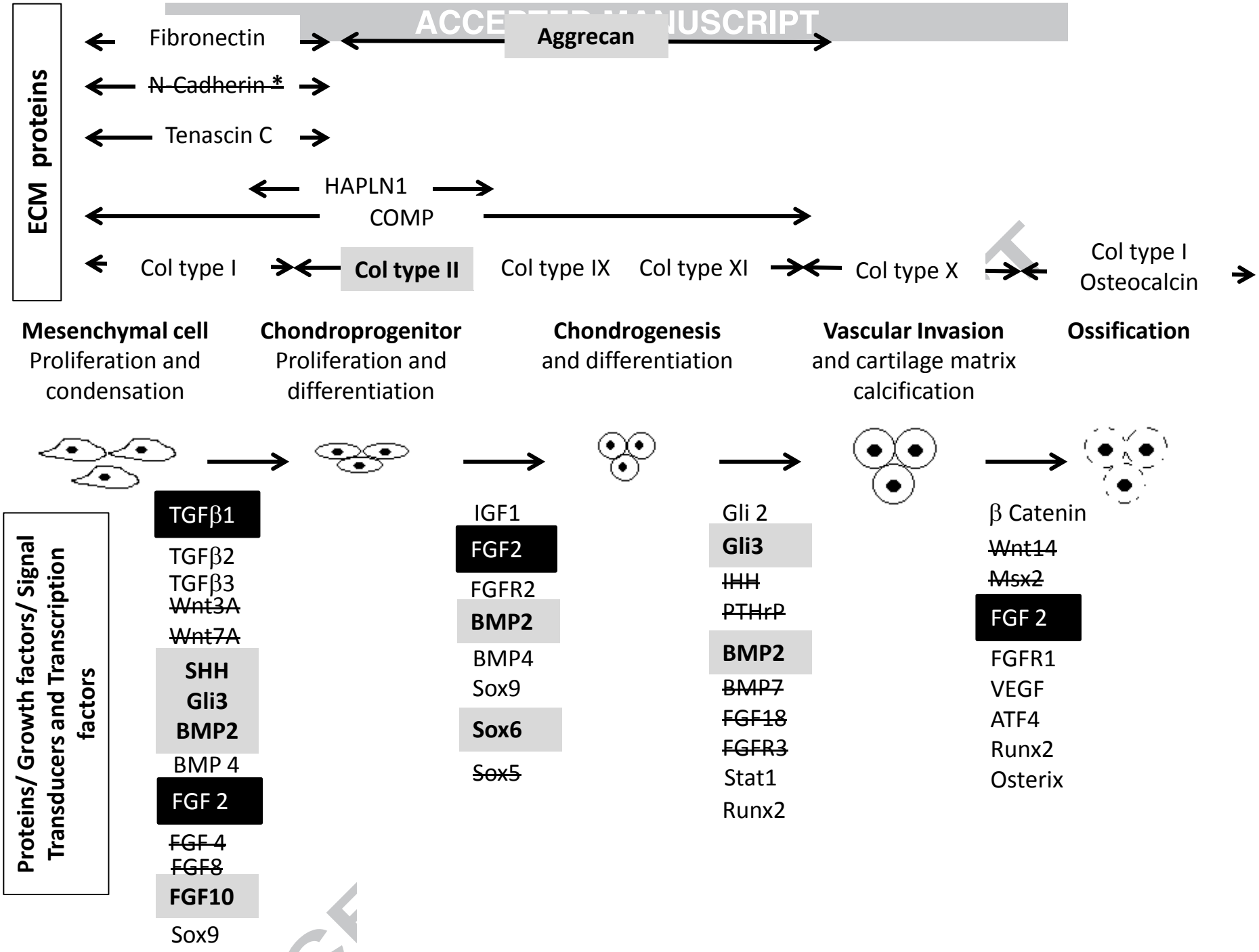
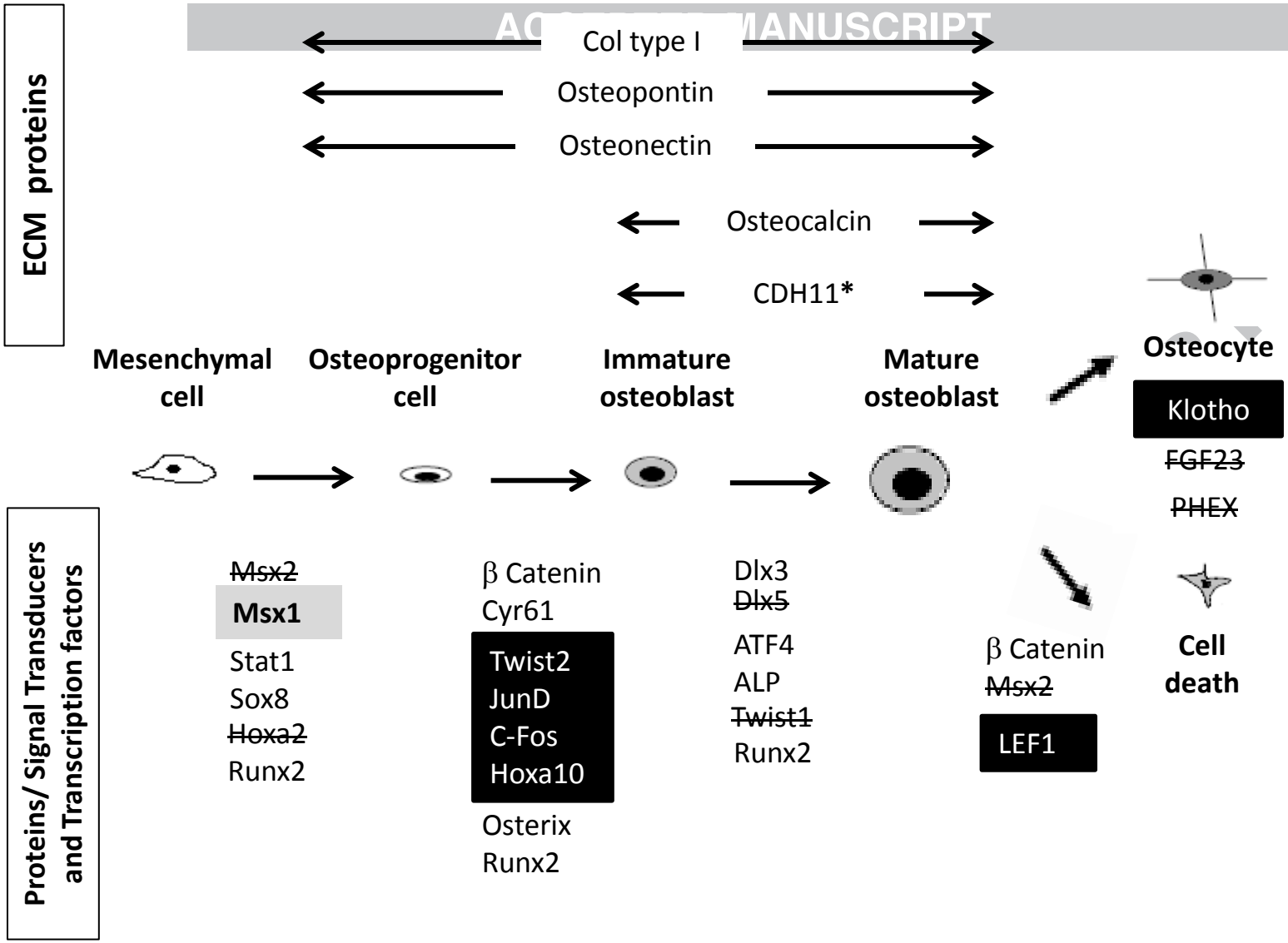
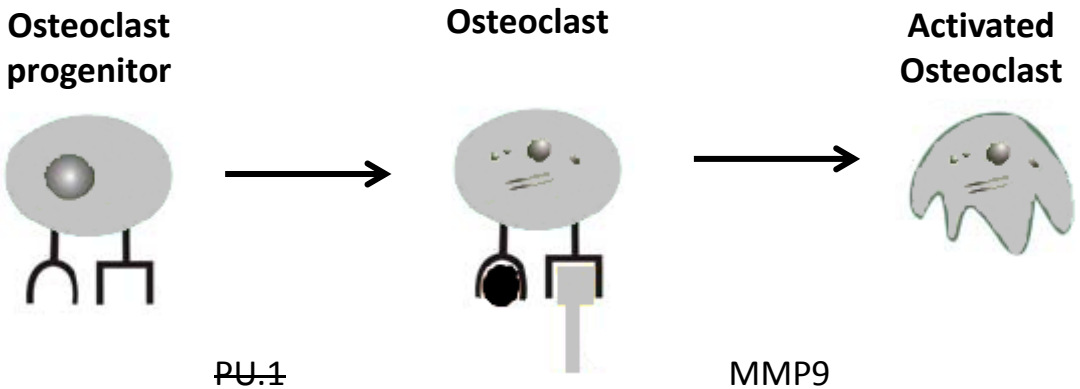






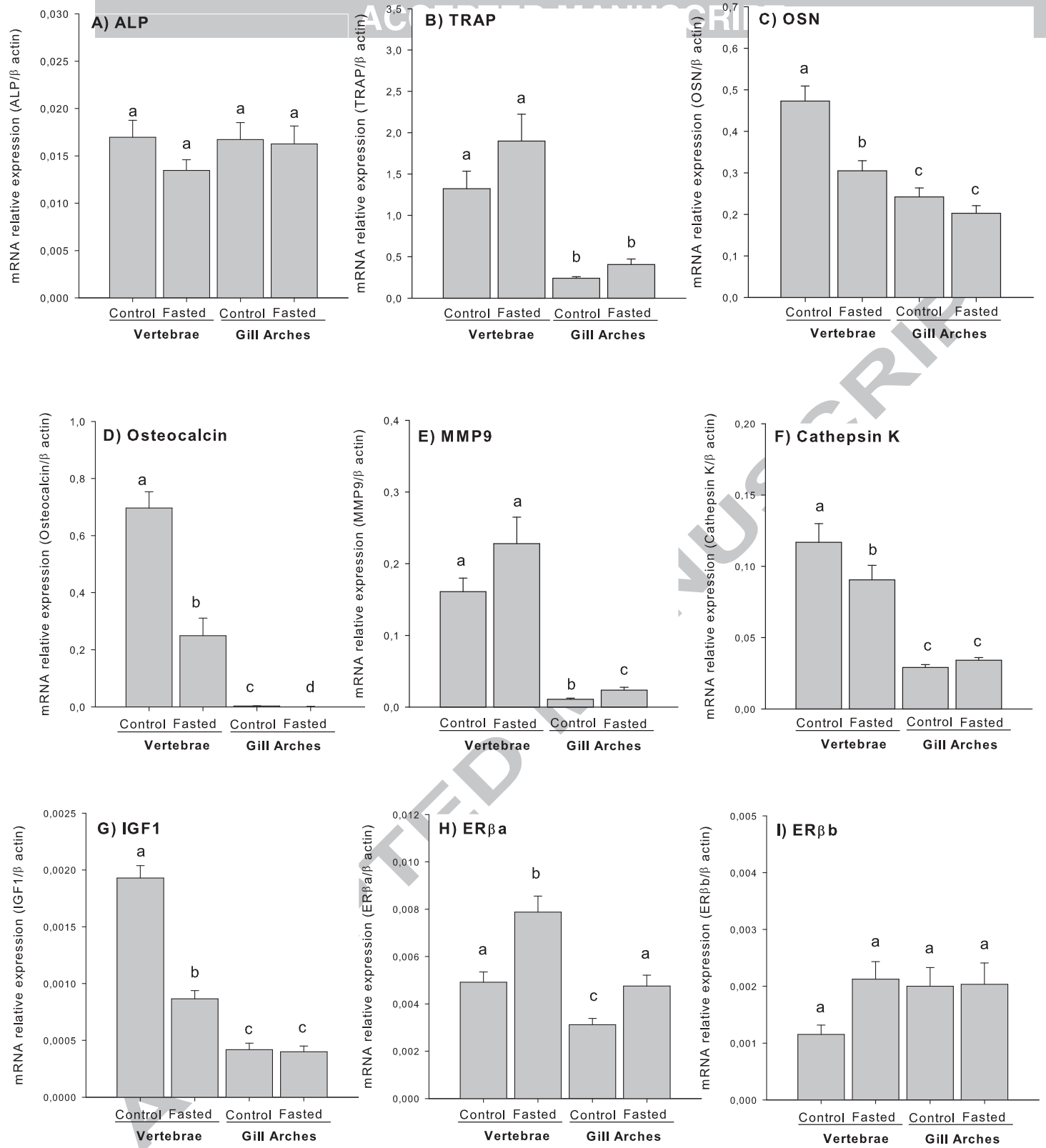


~~PU.1~~
~~M-CSF~~
~~RANKL~~
~~RANK~~
~~OPG~~
~~IL-1~~
~~TNF-α~~
~~PGE_2~~
~~TRAF6~~
~~ERα~~

ERβ
CRLR1
CRLR2
TGFβ1

MMP9
MMP13
Cathepsin K
TRAP
PIK3R1
NFATC1
~~PTHR1~~
~~Vitronectin~~

CLR1
CLR2
MITF



Highlights

- Comparison of sea bream vertebrae and gill arches global transcriptomes.
- Large scale conservation of core gene networks between teleost and mammalian skeletal tissue.
- Paracrine and endocrine transcripts in skeletal tissue.
- Responsiveness of vertebrae and gill arches transcripts to fasting.