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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 characterize acellular perichondral bone (vertebrae) and chondroid bone (gill arch) in the gilthead sea bream (*Sparus auratus*). A total of 15.97 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.

1. Introduction

An articulated endoskeleton that is calcified is a unifying innovation of the vertebrates and its evolution was accompanied by species-specific specialization. For example, the sharks and rays developed a cartilaginous skeleton that is light and flexible (Blair et al., 2008), 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) Witten and Huysseune, 2009. 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 Benjamin, 1989; Benjamin, 1990; Zhang et al., 2009). 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 Blair et al., 2008; Chen et al., 2012; Goldring et al., 2006; Mellis et al., 2011). In contrast, knowledge about bone turnover in acellular teleost bone is still rudimentary, although recent studies have started to elucidate

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this process (Eames et al., 2012; Fisher and Franz-Odendaal, 2012; Witten and Huysseune, 2009), and several genes and proteins of calcified tissue have been characterized (Kluver et al., 2005; Marcellini et al., 2010; Park et al., 2008; Redruello et al., 2005). However, histological studies indicate that bone turnover is a slow process and the majority of studies of this process have focused on a modest number of genes and proteins and no large-scale study representing a comparative analysis of the composition and regulation of cartilage and bone ECM exists.

The skeleton in vertebrates protects, supports and permits movement, and the mobilization or deposition of calcium (Ca) and phosphorus (P) from this tissue contributes to calcium homeostasis (Bentley, 1998; Chester-Jones, 1987; Guerreiro et al., 2007; Wendelaar Bonga and Pang, 1991). The structural difference in the skeleton between aquatic and terrestrial vertebrates is presumably derived from the effects of gravity and the erratic supply of Ca and P from the diet in the latter (Guerreiro et al., 2002; Witten and Huysseune, 2009). The maintenance of Ca homeostasis in terrestrial vertebrates involves hypercalcaemic factors, that promote Ca uptake, such as parathyroid hormone (PTH) and prolactin (PRL), and hypocalcaemic factors that inhibit Ca uptake like calcitonin (CT) and somatostatin (SS). In fish, PTH related protein (PTHrP) rather than PTH seems to be the hypercalcaemic factor (Fuentes et al., 2006) and the role of PTH remains unresolved (Canario et al., 2006; Guerreiro et al., 2002). The role of calcitonin in calcium homeostasis in fish is still controversial (Mukherjee et al., 2004) and stanniocalcin is an anti-hypercalcaemic hormone and prevents the uptake of Ca via the gills and intestine (Ishibashi and Imai, 2002; Wendelaar Bonga and Pang, 1991).

The skeleton is known to be a recipient of hormonal inputs and in mammals the pituitary endocrine axis stimulates bone formation via the growth hormone/IGF1 axis and regulates bone resorption, via follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) Blair et al., 2008. A recent shift in this paradigm has occurred as it has been proposed that bone also acts as an endocrine organ capable of influencing functions that have nothing to do with its own integrity (Karsenty and Oury, 2012). Skeletal remodeling consumes large amounts of energy and bone regulates energy and whole-glucose metabolism via factors it liberates (e.g. osteocalcin; Karsenty and Oury, 2012; Lee et al., 2007).

The present study focuses on the skeleton of a Sparidae, the gilthead sea bream (*S. auratus, Linnaeus 1758*), an important Mediterranean aquaculture species that had a global production greater than 120,000 metric tons in 2008 (FAO). 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 [1476 nucleotide sequences; 74,877 expressed sequencetags (ESTs); 92,468 genome survey sequences (GSS)], and recently deep sequencing studies have been reported from whole larval (Yufera et al., 2012) and skeletal tissues (Garcia de la Serrana et al., 2012), and inclusive *de novo* construction of a gilthead sea bream transcriptome database had become available (Calduch-Giner et al., 2013). 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 (Persson et al., 1997; Takagi, 2001; Takagi and Yamada, 1992). Overall the study provides new insights into the skeleton of teleosts and its potential endocrine function.

2. Materials and methods

2.1. Fish

Juvenile gilthead sea bream (weighing 88.1 ± 7.3 g (mean ± SD); $n = 35$), were reared and maintained at the Institute de Recerca i Tecnologia Agroalimentaries (IRTA) at St. Carles de la Rapita (IRTA-SCR, Spain) according to the standard production procedures. 200 fish were maintained in two 400 L tanks (22.5 kg m^{-3}) in a temperature-controlled seawater re-circulation system (IRTA-mar™) at a mean temperature of 21 °C (20.7–21.4 °C) and natural photoperiod (13L:11D). Fish were fed a commercial diet (Opti-BreamTM, Skretting; pellet size: 2.6 mm) once daily at a ration level of 3% (mass food/mass fish in tank).

Food was withheld from gilthead sea bream for 5 days prior to sampling in order to stimulate skeletal turnover as previously observed in a study of the skin and scales (Vieira et al., 2011). Five individuals were sacrificed with an overdose of bicarbonate-buffered tricaine methanesulphonate (1:5000 mass/volume; MS222, Sigma, Madrid, Spain) in seawater followed by spinal cord transection. Gill arches and vertebrae samples were dissected out and placed in RNAlater (Sigma–Aldrich, Spain), before freezing tissue was cleaned of adhering muscle, spinal cord and blood vessels and then flash frozen in liquid nitrogen and stored at −80 °C until further analysis. The experiment was conducted in September 2009 in accordance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals and the recommendations of the Association of Animal behavior (ASAB, 2003).

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 five 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 artifacts of the pyrosequencing procedure (Chou

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