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# Central role of betaine–homocysteine S-methyltransferase 3 in chondral ossification and evidence for sub-functionalization in neoteleost fish

Joana Rosa [a,b], Daniel M. Tiago [a], Cátia L. Marques [a,1], Parameswaran Vijayakumar [a,2], Luis Fonseca [a], M. Leonor Cancela [a,c], Vincent Laizé [a,*]

[a] Centre of Marine Sciences (CCMAR), University of Algarve, Faro, Portugal
[b] PhD Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine (DCBM), University of Algarve, Faro, Portugal
[c] Department of Biomedical Sciences and Medicine (DCBM), University of Algarve, Faro, Portugal

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ABSTRACT

*Background:* To better understand the complex mechanisms of bone formation it is fundamental that genes central to signaling/regulatory pathways and matrix formation are identified. Cell systems were used to analyze genes differentially expressed during extracellular matrix mineralization and *bhmt3*, coding for a betaine–homocysteine S-methyltransferase, was shown to be down-regulated in mineralizing gilthead seabream cells.
*Methods:* Levels and sites of *bhmt3* expression were determined by qPCR and *in situ* hybridization throughout seabream development and in adult tissues. Transcriptional regulation of *bhmt3* was assessed from the activity of promoter constructs controlling luciferase gene expression. Molecular phylogeny of vertebrate BHMT was determined from maximum likelihood analysis of available sequences.
*Results: bhmt3* transcript is abundant in calcified tissues and localized in cartilaginous structures undergoing endo/perichondral ossification. Promoter activity is regulated by transcription factors involved in bone and cartilage development, further demonstrating the central role of Bhmt3 in chondrogenesis and/or osteogenesis. Molecular phylogeny revealed the explosive diversity of *bhmt* genes in neoteleost fish, while tissue distribution of *bhmt* genes in seabream suggested that neoteleostean Bhmt may have undergone several steps of sub-functionalization.
*Conclusions:* Data on *bhmt3* gene expression and promoter activity evidences a novel function for betaine–homocysteine S-methyltransferase in bone and cartilage development, while phylogenetic analysis provides new insights into the evolution of vertebrate BHMTs and suggests that multiple gene duplication events occurred in neoteleost fish lineage.
*General significance:* High and specific expression of Bhmt3 in gilthead seabream calcified tissues suggests that bone-specific betaine–homocysteine S-methyltransferases could represent a suitable marker of chondral ossification.



## 1. Introduction

Vertebrate skeleton is a multifunctional organ that provides support and protection for internal organs, storage and balance for minerals (mainly calcium and phosphorus) and, in mammals, plays hematopoietic functions [1,2]. It is mainly composed of bone and cartilage and undergoes constant ossification and remodeling through complex cellular and molecular mechanisms [3]. Although human and mouse genetics have largely expanded our understanding of skeletal development and bone formation during the last decades, current knowledge on mechanisms underlying physiological and pathological processes is still insufficient to develop successful therapies for human skeletal and bone diseases. Thus, the use of alternative non-mammalian animal models, *e.g.* zebrafish, *Xenopus* and chicken, to study skeletal development and bone mineralization has been sought. Fish species – mainly the zebrafish and Japanese medaka – have received a particular attention from the scientific community. Their similarity with mammals regarding biochemical, developmental and physiological mechanisms, in particular those underlying skeletal development and bone mineralization, as well as the presence in fish genome of orthologs for most mammalian genes, and a number of technical advantages (*e.g.* large progeny, external reproduction fast growth and translucent larvae) make fish models a suitable complement or alternative to mammalian models [4–7]. Several *in vitro* cell systems of fish origin have been developed to complement *in vivo* systems, and various bone-derived cell lines capable of extracellular matrix mineralization and representing different bone cell types and fish species have been established in the

* Corresponding author at: Centre of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.
*E-mail address:* vlaize@ualg.pt (V. Laizé).
[1] New address: Instituto Português do Mar e da Atmosfera; EPPO — Estação Piloto de Piscicultura de Olhão, Olhão, Portugal.
[2] New address: Centre for Ocean Research, Sathyabama University, Chennai, Tamilnadu, India.

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last decade [8–11]. Mineralogenic cell lines developed from gilthead seabream vertebra (VSa13 and VSa16) were used to identify genes differentially expressed during *in vitro* mineralization and possibly involved in mechanisms of bone cell differentiation and endochondral ossification in fish [12–14]. In a recent report by Tiago et al. [14], expression of a betaine–homocysteine S-methyltransferase (BHTM) gene was strongly down regulated (>10 fold) during extracellular matrix mineralization and was proposed to play a central role in the mechanisms underlying the mineralogenic capacity of these cells. BHMT is a cytosolic and zinc-dependent enzyme [15] that catalyzes the remethylation of homocysteine (Hcy) into methionine (Met) using betaine as methyl donor [16]. Primarily produced in the liver and kidney [17], BHMT is involved in the branch-point metabolism of Hcy, along with methionine synthase (MS). Hcy metabolism has been the subject of a particular attention since hyper-homocysteinemia (HHcy; high levels of Hcy in plasma) is associated in human with vascular diseases, renal insufficiency, non-insulin dependent diabetes, adverse pregnancy outcomes, Alzheimer's disease and osteoporosis [18–23]. In a bone context, HHCy was reported to hamper bone formation by inhibiting osteoblast differentiation and/or activity, and to trigger bone resorption by stimulating osteoclast formation/differentiation and activity [24,25]. On the basis of existing data, we hypothesize that BHMT could play a role in mineralization through its ability to convert homocysteine into methionine and therefore limit its anti-osteogenic/pro-resorptive properties. The aim of the present study was to characterize sites and levels of *bhmt* gene expression throughout gilthead seabream development, in adult tissues and during *in vitro* mineralization, and to identify transcriptional factors responsible for the regulation of its expression. Taxonomic distribution and molecular phylogeny of BHMT proteins in vertebrates will also be assessed.

## 2. Materials and methods

### 2.1. Larvae, juvenile and adult fish culture

Eggs collected from natural spawning of gilthead seabream *Sparus aurata* (IPMA, Olhão, Portugal) were placed at 16 °C in a closed recirculating system of 32-ppm seawater under a 12:12-h light–dark photoperiod. After mouth opening, larvae were fed with rotifers (enriched with cultured marine microalgae *Tetraselmis suecica* and *Isochrysis galbana*) until 20 days post-hatching (DPH) and with freshly hatched *Artemia* nauplii from 20 to 50 DPH. Juvenile and adult fish were reared at 16–20 °C in 100-L seawater (32-ppm) tanks with a 12:12-h light–dark photoperiod, aeration of 100 ml air/min, and renewal flow of 1 tank/day; they were fed with artificial food (Sorgal).

### 2.2. Cell culture and extracellular matrix mineralization

Gilthead seabream bone-derived cell lines VSa13 and VSa16 were cultured as previously described by Pombinho et al. [8]. Extracellular matrix (ECM) mineralization was induced in confluent cultures by supplementing medium with 50 μg/ml of L-ascorbic acid, 10 mM of β-glycerophosphate and 4 mM of $CaCl_2$ (all from Sigma-Aldrich) for 4 weeks. Culture medium was renewed twice a week. Mineral deposition was revealed through von Kossa staining [8].

### 2.3. RNA preparation

When sampled for RNA preparation, larvae and juvenile fish were given a lethal anesthesia of 500 ppm of 2-phenoxyethanol (Sigma-Aldrich), then washed twice in phosphate-buffered saline solution (PBS) and stored at −80 °C in 5 ml of TRIzol reagent (Sigma-Aldrich). Adult fish (two males and one female) were anesthetized (200 ppm of 2-phenoxyethanol) and sacrificed according to guidelines on animal experimentation available in Portugal. Tissues (pooled, 1:1 gender ratio) were sampled and washed twice in PBS and stored at −80 °C in 10 volumes of TRIzol reagent. Total RNA was extracted from cell cultures (three replicates per condition) using the method described by Chomczynski and Sacchi [26] and from samples stored in TRIzol following the manufacturer's instructions. RNA quantity was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and 5 μg of each RNA sample was fractionated on 1% (*w*/*v*) agarose-formaldehyde gels for quality control.

### 2.4. bhmt3 cDNA and gene cloning

*bhmt3* cDNA was amplified by PCR from a Marathon cDNA library (Clontech) prepared from mRNA of adult gilthead seabream liver, kidney and testis using gene-specific primers (Table 1) designed in available ESTs (GenBank accession nos. FP336052, AM965012, FM151264, AM969436 and CX735005), adapter primers AP1 and AP2 and advantage cDNA polymerase mix (Clontech). *bhmt3* gene was amplified by PCR from genomic DNA prepared from tissues of a single individual using DNeasy Blood & Tissue kit (QIAGEN), advantage *ThT* Polymerase mix (Clontech) and gene-specific primers (Table 1). All PCR products were size-separated by agarose-gel electrophoresis, purified using Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare), cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced on both strands.

### 2.5. Analysis of gene expression levels by quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed using iCycler iQ system (Bio-Rad). Total RNA (1 μg) was treated with RQ1 RNase-free DNase (Promega) then reverse-transcribed at 37 °C for 1 h using M-MLV reverse transcriptase, RNase-out (Invitrogen) and oligo-dT reverse primer. The reaction mixture containing cDNA (1 μl of 1:10 dilution of reverse transcription reaction), 0.4 μM of gene-specific primers (Table 1), and 1× iQ SYBR Green I mix (ABgene) was submitted to the following PCR conditions: an initial denaturation step at 95 °C for 15 min then 50 cycles of amplification (each cycle is 30 s at 95 °C, 45 s at 68 °C). Levels of gene expression were calculated using the ΔΔCt method [27] and normalized using levels of *rpl27a* housekeeping gene expression.

**Table 1**
PCR primers used in this study.

| Name | Sequence (5′-3′)[a] |
|---|---|
| SauBHMT3_Rv2_promoter | TTCTCCAGGGCGAAGACAAAGC |
| SauBHMT3_Rv1_promoter | TCCCGCATCCAGACGCTCC |
| SauBHMT3_promo_Rv_pGL4_HindIII | CACGC**AAGCTT**ATCCTGCTCCCCTTCTTCTGTC CAGC |
| SauBHMT3_promo_Fw_pGL4_XhoI | CCGGAG**CTCGAG**TATCCTCATCCCTCTTCAGT TTGGTCAGC |
| SauBHMT3_promo_Fw2_pGL4_XhoI | CCGGAG**CTCGAG**GTCAAATGTATCAGAGAGAG |
| SauBHMT3_promo_Fw3_pGL4_XhoI | CCGGAG**CTCGAG**GATCAATTACGGTAAGTA CAATT |
| SauBHMT3_promo_Fw4_pGL4_XhoI | CCGGAG**CTCGAG**GTGCGGGAGCAGGCGG |
| BHMT3 qPCR Fw | CCACTGAGCGCCTGCTGACCACGTTA |
| BHMT3 qPCR Rv | CATTTACCATCAACGGGTGCCTGTTTG |
| BHMT4 qPCR Fw | CGTGCTGGAGATGCAGAAGAAGCC |
| BHMT4 qPCR Rv | GACTCTTTTCTCCCTCGTCCCTCTGTTGTAG |
| BHMT5 qPCR Fw | CTGAGTCAGCTCATCGCCTCCAGTAGAG |
| BHMT5 qPCR Rv | CCACAGAACAGTGCCAGTCTCAAGTGAAGG |
| BHMT6 qPCR Fw | GTTGAGCCATGTCACCGGCATCAC |
| BHMT6 qPCR Rv | GACTTCGCATAGCAGGGAGTCATGGAAAC |
| BHMT7 qPCR Fw | GGAGATCAGTGGTAACGTCCCCAATATCAC |
| BHMT7 qPCR Rv | CTGTGGCTCGTCACATAACACGGAGTCTTAG |
| RPLreal-FW | AAGAGGAACACAACTCACTGCCCCAC |
| RPLreal-RV | GCTTGCCTTTGCCCAGAACTTTGTAG |

[a] Bold underlined sequence indicates cutting sites for endonucleases *Xho*I and *Hind*III in forward (FW) and reverse (RV) primers, respectively.