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Faculdade de Ciências do Mar e do Ambiente

Endocrine regulation of extracellular matrix proteins in calcified tissue in teleost fish

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Dissertação apresentada à Universidade do Algarve para obtenção do grau de Doutor em Biologia, especialidade de Biologia Molecular I hereby declare that my dissertation contains material that as not been submitted for a degree or diploma or any other qualification at any other university. The contents of this dissertation are of exclusive responsibility of the author.

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Skeletal tissue structure and cellular organization are poorly studied in teleost fish and the aim of the present thesis is their characterization in order to study how they may contribute to calcium homeostasis. The morphology of cartilage and bone observed using general histological techniques during sea bream (*Sparus auratus*) skeletogenesis allowed the tissue and cellular transformations which occur during endochondral and dermal ossification to be characterized. Alternative ossification processes were observed in skeletal structures such as the gill arches, where skeletal tissue other than typical cartilage and bone were observed. A characteristic of teleost fish is the presence of an external covering of calcified scales and their morphology is described in a marine (sea bream) and a euryhaline species (tilapia, *Oreochromis mossambicus*). *S. auratus* and *O. mossambicus* scales were identified as elasmoid scales and characteristic features of this scale type, such as the focus, the *circuli* and the *radii* were observed in both species.

The bone matrix probably plays a fundamental role in mobilization and release of calcium from skeletal tissue and for this reason one of the major non-collagenous proteins of the bone matrix, osteonectin (OSN), was isolated from a sea bream intervertebral tissue library and characterized. Sea bream OSN cDNA was shown to be homologous to other vertebrate OSN, and the deduced amino acid sequence shares identical structure with its vertebrate counterparts. Both RT-PCR and ISH analysis showed that OSN mRNA is most abundant in sea bream calcified tissues although a weak signal was detected occasionally in soft tissues. During ontogeny, OSN mRNA was first detected by RT-PCR at early gastrulation and its expression profile presented a series of maximum and minimum points during larvae development. Its expression was first detected by in situ hybridization (ISH) in 6 days post hatch larvae and the localization and intensity of the signal varied with age. Both dermal and endochondral skeletal elements were shown to express OSN and results suggests that OSN may play a role during both chondrogenesis and osteogenesis. OSN mRNA is also very abundant in scales of juvenile and adult sea bream and tilapia suggesting it may also participate in the regulation of scale mineralization. The expression pattern of several additional extracellular matrix proteins, type I collagen, $\alpha 1$ (Col1A1), type V collagen, $\alpha 2$ (Col5A2), fibronectin (FN), tartrate-resistant acid phosphatase (TRACP) and acidic secreted protein in cartilage (ASPIC), was studied by ISH in sea bream and tilapia scales. Col1A1 is very abundant in scales from both juvenile and adult sea bream and tilapia. TRACP is also expressed in scales from juveniles and adults of both species although in relatively few cells. Col5A2 and FN are only detected in sea bream scales and ASPIC is not expressed in scales from either species. The ISH of ECM proteins in scales together with the general histological methods, permitted the identification of putative osteoblasts and osteoclasts in the scale matrix indicating that this tissue is actively metabolized.

In order to establish if the scales in fish can contribute to the rise in calcium associated with estrogen, the presence of estrogen receptors was studied. Estrogen receptor isoforms (α , β 1 and β 2) expression was characterized by immunohistochemistry using sea bream specific polyclonal antisera and in the scales of juvenile and adult sea bream and tilapia. Estrogen receptors are expressed in both sea bream and tilapia scales in the putative osteoclasts although signal intensity varies with the species and the age of the animals. These results suggest that one of the mechanism by which estrogen may influence scale turnover is through binding to estrogen receptors expressed in osteoclasts and modulating their activity. The way in which hormones act on calcified tissue in fish to bring about calcium mobilization is totally unstudied. In order to establish if this process involves ECM turnover, the effect of the only hypercalcaemic hormone so far identified in fish, parathyroid hormone related protein (PTHrP), on sea bream OSN mRNA expression, was evaluated using an *in vitro* scale bioassay. The results of the bioassay showed that PTHrP downregulates OSN expression in sea bream scales and suggest that the action of PTHrP in calcium balance may include regulation of ECM proteins involved in bone and/or scale matrix formation and mineralization.

A estrutura e a organização celular dos tecidos esqueléticos estão pouco estudadas nos teleósteos e o objectivo da presente tese consistiu na sua caracterização de forma a estudar como é que esses tecidos podem contribuir para a homeostase do cálcio. A morfologia da cartilagem e do osso observada usando técnicas histológicas gerais durante a formação do esqueleto em dourada (*Sparus auratus*) permitiu a caracterização dos tecidos e das transformações celulares que ocorrem durante a ossificação endocondral e dermal. Foram observados processos de ossificação alternativos em estruturas esqueléticas como os arcos branquiais, onde foram observados outros tecidos esqueléticos, diferentes da cartilagem e do osso. Uma característica dos peixes teleósteos é a presença de uma cobertura externa de escamas calcificadas. A sua morfologia foi descrita numa espécie marinha (dourada) e numa espécie eurihalina (tilápia, *Oreochromis mossambicus*). As escamas de *S. auratus* e *O. mossambicus* foram identificadas como escamas elasmóides e características deste tipo de escama como o foco, os *circuli* e os *radii* foram observadas em ambas as espécies.

A matriz óssea desempenha, provavelmente, um papel fundamental na mobilização e na libertação de cálcio a partir dos tecidos esqueléticos e, por esse motivo, uma das principais proteínas da matriz óssea, a osteonectina (OSN), foi isolada e caracterizada a partir de um banco de tecido intervertebral de dourada. Foi demonstrado que o cDNA da OSN de dourada é homólogo ao de outras OSNs de vertebrados, e que a sequência de aminoácidos partilha estruturas idênticas à das moléculas equivalentes noutras espécies de vertebrados. Os resultados obtidos por RT-PCR e por ISH mostraram que o mRNA de OSN é mais abundante nos tecidos calcificados de dourada apesar de ocasionalmente ter sido detectado um sinal fraco em tecido moles. Durante a ontogenia, o mRNA de OSN foi detectado pela primeira vez no início da gastrulação e o perfil de expressão apresenta uma série de máximos e minímos durante o desenvolvimento larvar. A sua expressão foi detectada por hibridação in situ (ISH) em larvas a partir do 6º dia após eclosão e a localização e intensidade do sinal variaram com a idade. Elementos esqueléticos de origem endocondral e dermal expressam OSN e os resultados sugerem que a OSN pode desempenhar funções durante a condrogénese e a osteogénese. O mRNA da OSN é também muito abundante nas escamas de douradas e tilápias, juvenis e adultas, sugerindo que a OSN pode participar nas regulação da mineralização das escamas. O padrão de expressão de várias outras proteínas da matriz extracelular como, colagénio tipo I, cadeia α1 (Col1A1), colagénio tipo V, cadeia α2 (Col5A2), fibronectina (FN), fosfatase ácida resistente ao tartarato (TRACP) e a proteína acídica, secretada pela cartilagem (ASPIC) foi estudado por ISH em escamas de dourada e de tilápia. O Col1A1 é muito abundante nas escamas de douradas e tilápias, quer juvenis, quer adultas. A TRACP também é expressa em escamas de juvenis e adultos de ambas as espécies embora em relativamente poucas células. O Col5A2 e a FN foram detectadas apenas em escamas de douradas e a ASPIC não é expressa em escamas de nenhuma das duas espécies. A ISH das proteínas da matriz extracelular nas escamas, em conjunto com métodos histológicos permitiu a identificação de células, supostamente osteoblastos e osteoclastos, na matriz das escamas indicando que este tecido é activamente metabolizado.

Para estabelecer se as escamas podem contribuir, nos peixes, para o aumento dos níveis de cálcio associado ao estrogénio, foi estudada a presença de receptores de estrogénio (ER). A expressão das isoformas dos ER (α, β1 and β2) foi caracterizada por imunohistoquímica usando anticorpos policlonais específicos para dourada, em escamas de dourada e tilápia, juvenis e adultas. Os ER são expressos nas escamas de ambas as espécies nas células identificadas como osteoclastos embora a intensidade do sinal varie com a espécie e com a idade dos animais. Estes resultados sugerem que um dos mecanismos pelos quais o estrogénio pode influenciar a renovação das escamas consiste na ligação aos ER e na modulação da sua actividade. O modo como as hormonas actuam nos tecidos calcificados em peixe para promover a mobilização do cálcio não está estudado. O efeito da única hormona hipercalcémica identificada em peixe, a proteína relacionada com a hormona da paratiróide (PTHrP), na expressão do mRNA de OSN de dourada foi avaliado usando escamas num bio-ensaio *in vitro*. Os resultados mostraram que a PTHrP reduz a expressão de OSN em escamas de dourada e sugere que a acção da PTHrP no balanço de cálcio pode incluir a regulação da matriz extracelular envolvidas na formação e mineralização da matriz do osso e/ou das escamas.

Chapter 1 – General introduction

1.1 General overview	2
1.2 Vertebrate skeletal tissue	3
1.2.1 Cartilage	4
1.2.2 Bone	5
1.2.3 Fish scales	9
1.3 Extracellular matrix of vertebrate skeletal tissue	13
1.3.1 Composition of the extracellular matrix - terrestrial vertebrates	13
1.3.1.1 Collagens	14
1.3.1.2 Non collagenous proteins	14
1.3.1.3 Other components of the extracellular matrix	16
1.3.2 The extracellular matrix of cartilage and bone	17
1.4 Skeletal formation, growth and remodelling in vertebrates	19
1.5 Regulation of skeletal development and remodelling	23
1.6 Regulation of scale development and regeneration	27
1.7 Endocrine regulation of calcium homeostasis	28
1.8 Fish models used in the present work	35
1.8.1 Gilthead sea bream	35
1.8.2 Tilapia	36
Objectives of the thesis	38
Chapter 2 – General materials and methods	
2.1 Experimental animals	40
2.2 Tissue fixation and processing	41
2.3 General histology	41
2.3.1 Haematoxylin-eosin staining	42
2.3.2. Toluidine blue staining	42
2.3.3 Masson's Trichrome staining	42
2.3.4 Alcian-haematoxylin-van Gieson staining	43
2.3.5 Whole-mount cartilage - bone double staining	44
2.4 mRNA tissue distribution by <i>in situ</i> hybridization	44

2.4.1 Riboprobe synthesis	45
2.4.2 In situ hybridization	49
2.5 Immunohistochemistry	51
2.6 Semi-quantitative RT- PCR	53
2.7 Northern blot	54

Chapter 3 – Cellular organization of skeletal tissue in sea bream (*Sparus auratus*) larvae

3.1 Introduction	56
3.2 Material and Methods	57
3.2.1 Sampling	57
3.2.2 Whole-mount cartilage - bone double staining	57
3.2.3 Processing	57
3.2.4 General histology - gills	58
3.3 Results	58
3.4 Discussion	78

Chapter 4 – Isolation and characterization of piscine osteonectin and downregulation of its expression by parathyroid hormone-related protein

4.1 Introduction	85
4.2 Material and Methods	87
4.2.1 Fish maintenance and tissue sampling	87
4.2.2 Construction of an intervertebral tissue cDNA library from S. auratus	87
4.2.3 Cloning and characterization of OSN cDNA from S. auratus	88
4.2.4 <i>In silico</i> analysis	88
4.2.5 Semi-quantitative RT-PCR and Northern Blot	89
4.2.6 <i>In Situ</i> Hybridization (ISH)	90
4.2.7 In vitro sea bream scales bioassay	90
4.2.8 Statistics	91
4.3 Results	91
4.3.1 Characterization and phylogenetic analysis of sea bream OSN cDNA .	91
4.3.2 sbOSN mRNA tissue expression	96
4.3.3 Effect of PTHrP on sbOSN expression in fish scales	99
4.4 Discussion	101

Chapter 5 – Ontogeny of osteonectin expression in embryos and larvae of sea bream (*Sparus auratus*)

5.1 Introduction	
5.2 Material and Methods	110
5.2.1 Sampling, tissues fixation and preservation	110
5.2.2 RNA extraction and semi-quantitative RT-PCR	110
5.2.3 Histology	111
5.2.4 Tissue distribution by in situ hybridization (ISH)	112
5.2.5 Statistical analysis	113
5.3 Results	113
5.4 Discussion	

Chapter 6 – Extracellular matrix protein expression in sea bream (*Sparus auratus*) and tilapia (*Oreochromis mossambicus*) scales

6.1 Introduction	124
6.2 Material and Methods	126
6.2.1 Sampling of scales	126
6.2.2 General histology of scale	127
6.2.3 RT-PCR of target ECM genes in scales	127
6.2.4 <i>In situ</i> hybridization	128
6.2.5 Production of antibodies	133
6.2.6 immunohistochemistry	135
6.3 Results	135
6.4 Discussion	152
Chapter 7 – General discussion	160
References	169
Appendix I	
Appendix II	

Chapter 1

Figure 1.1 - Chondrocyte and osteoblast lineage	4
Figure 1.2 - Schematic representation of the growth plate in mammals	5
Figure 1.3 - Schematic representation of bone in mammals	6
Figure 1.4 - Osteoclast lineage	7
Figure 1.5 - Schematic representation of an osteoclast	8
Figure 1.6 - Various types of scales	11
Figure 1.7 - Elasmoid scale	12
Figure 1.8 - Schematic representation of mammalian endochondral ossification	20
Figure 1.9 - Bone remodelling cycle	22
Figure 1.10 - RANKL/RANK/OPG system	26
Figure 1.11 - A scheme presenting the interaction between the principle organs and hormones involved in calcium homeostasis in mammals	29
Figure 1.12 - Scheme of gilthead sea bream (Sparus auratus)	
Figure 1.13 - Image of a Mozambique tilapia (Oreochromis mossambicus)	37

Chapter 2

Figure 2.1	- Schematic representation of the cloning vectors	48

Chapter 3

Figure 3.1 - Sagittal sections of alcian blue/alizarin red stained, whole mount larval sea bream 8 days post hatch (dph)	.60
Figure 3.2 - Sagittal sections of whole mount alcian blue/alizarin red stained larval sea bream 8 and 20dph, counterstained with haematoxylin and eosin	.61
Figure 3.3 - Schematic representation of the initial and final stages of the development of the pelvic fin supports in <i>Sparus auratus</i>	.62
Figure 3.4 - Sagittal section of the jaw region of whole mount alcian blue/alizarin red stained larval sea bream at 20 and 45dph and counterstained with haematoxylin and eosin.	.65
Figure 3.5 - Sagittal sections of whole mount alcian blue/alizarin red stained larval sea bream 45 and 61dph showing the ocular cavity, and counterstained with haematoxylin and eosin	.66
Figure 3.6 - Sagittal sections of the haemal arch in sea bream larvae 45 and 61dph stained using the whole mount alcian blue/alizarin red method	68
Figure 3.7 - Sagittal sections of the vertebral centrum in sea bream larvae of 61 and 83dph (C and D) stained by the whole mount alcian blue/alizarin red staining method	.70

Figure 3.8 - Schematic representation of different phases of the development of the caudal skeleton in <i>Sparus auratus</i>	72
Figure 3.9 - Sagittal sections of the soft rays of the caudal fin sea bream larvae of 61 and 83dph stained using the alcian blue/alizarin red whole mount staining technique	73
Figure 3.10 - Sagittal sections of the gill region of sea bream larvae 8, 20 and 45dph stained by the whole mount alcian blue/alizarin red technique	75
Figure 3.11 - Sagittal sections of gill arches of juvenile sea bream stained with alcian blue and haematoxylin and with Masson's trichrome staining	77

Chapter 4

Figure 4.1 - Nucleotide and deduced amino acid sequence of sbOSN cDNA	93
Figure 4.2 - Multiple sequence alignment of OSNs from tetrapods and fishes	95
Figure 4.3 - Phylogenetic rooted tree of known OSNs obtained using the Neighbour Joining method and with rat hevin protein as outgroup	96
Figure 4.4 - sbOSN expression in adult tissues	97
Figure 4.5 - In situ hybridization of sbOSN in adult kidney and mid gut	98
Figure 4.6 - <i>In situ</i> hybridization of sbOSN in adult operculum, scales, gill lamellae and gill arches	100
Figure 4.7 - In situ hybridization of sbOSN in adult vertebrae and neurocranium	102
Figure 4.8 - Effect of PTHrP on sea bream scales in vitro	104

Chapter 5

Figure 5.1 - RT-PCR analysis of osteonectin expression during embryo and larval development of sea bream	.114
Figure 5.2 - Schematic representation of the onset and relative intensity of sbOSN expression during sea bream larval development determined using ISH	.115
Figure 5.3 - Masson's trichrome staining of sections of a 47dph sea bream larvae and ISH with a sbOSN riboprobe of adjacent sections	.117
Figure 5.4 - Alcian blue/alizarin red whole mount staining of 20dph and 46dph sea bream larvae and ISH with a sbOSN riboprobe	.118
Figure 5.5 - sbOSN mRNA expression in transverse sections of sea bream larvae of 15 and 25dph	.119

Chapter 6

Figure 6.1 - Schematic representation of the full-length cDNAs of the sea bream genes	129
Figure 6.2 -Multiple alignment of regions of the sea bream (sb) and tilapia (nt) sequences of estrogen receptors showing the peptides used to produce the antibodies	134
Figure 6.3 - Juvenile and adult scales of sea bream and tilapia	137
Figure 6.4 - Adult sea bream scale stained with alcian blue and alizarin red (double staining for cartilage and bone)	138

Figure 6.5 - Saggital section from the lower jaw of a juvenile sea bream	
(80 days post hatch (dph)) stained with Masson's trichrome showing scales in situ	140
Figure 6.6 - Schematic representation of a juvenile sea bream scale	142
Figure 6.7 - Schematic representation of a juvenile tilapia scale	143
Figure 6.8 - General histology and in situ hybridization of Col1A1 in juvenile sea bream?	145
Figure 6.9 - In situ hybridization of OSN in juvenile sea bream and tilapia	146
Figure 6.10 - In situ hybridization of fibronectin in juvenile and adult sea bream scales?	148
Figure 6.11 - In situ hybridization of TRACP in juvenile sea bream	149
Figure 6.12 - RT-PCR amplification of Col1A1, Col5A2, FN, OSN and TRACP in adult sea bream kidney, opercular bone, cranial bone and scales	150
Figure 6.13 - Whole mount immunohistochemistry of estrogen receptors α , β 1 and β 2 in sea bream and tilapia scales	152

Chapter 1

Table 1.1 - The collagen family and subfamilies	15
Table 1.2 - Examples of non-collagenous proteins present in the extracellular matrix	16
Table 1.3 - Other components of the extracellular matrix	17
Table 1.4 - Examples of genes stimulated or inhibited by PTH in mammals	31
Table 1.5 - Actions of $1,25(OH)_2D_3$ on its target organs and respective effect on calcium plasmatic concentration	32

Chapter 2

Table 2.1 - Restriction enzymes	6
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Chapter 4

Table 4.1 - Characteristics of known osteonectin cDNAs and corresponding proteins	92
Table 4.2 - Comparison of amino acid sequence similarity for osteonectin proteins	94

Chapter 6

Table 6.1 - Specific primers used to amplify each of the target genes by RT-PCR and respective annealing temperature	128
Table 6.2 - Characteristics of cDNA clones used to generate riboprobes for in situ hybridization	130
Table 6.3 - Examples of the most significant Blast hits obtained for each of the gene sequences used for riboprobe production	131
Table 6.4 - Rabbit anti-sea bream antibodies produced and their origin, notation used and respective peptide sequences	133
Table 6.5 - Antibodies used and their respective final concentration	135
Table 6.6 - Relative expression intensity of the target genes analysed by whole mount <i>in situ</i> hybridization	141
Table 6.7 - Relative expression intensity of the estrogen receptors (α , β 1 and β 2) analysed by whole mount immunohistochemistry	151

- APES aminopropyltriethoxysilane
- BCIP 5-bromo-4-chloro 3-indolylphosphate
- BMP bone morphogenetic protein
- Ca²⁺ ionic calcium
- cAMP cyclic adenosine monophosphate
- cDNA complementary deoxyribonucleic acid
- CFU colony forming units
- DEPC diethyl pyrocarbonate
- DNA deoxyribonucleic acid
- DNase deoxyribonuclease
- dph days post hatch
- ECM extracellular matrix
- EDTA ethylenediaminetetraacetic acid
- ER estrogen receptor
- hpf hours post fertilization
- IHC immunohystochemistry
- ISH in situ hybridization
- mRNA messenger ribonucleic acid
- NBT nitroblue tetrazolium chloride
- ORF open reading frame
- PCR polymerase chain reaction
- pfu plaque forming units
- PTH parathyroid hormone
- PTHrP parathyroid hormone related protein
- RNA ribonucleic acid
- RNase ribonuclease
- RT-PCR reverse transcriptase polymerase chain reaction
- SEM standard error of measurement
- Tm anneling temperature
- UTR untranslated region