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

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Abstract

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 , $\beta 1$ and $\beta 2$) 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.

Resumo

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 eurialina (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 às 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 mole. 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 mínimos 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 na regulação da mineralização das escamas. O padrão de expressão de várias outras proteínas da matriz extracelular como, colagénio tipo I, cadeia $\alpha 1$ (Col1A1), colagénio tipo V, cadeia $\alpha 2$ (Col5A2), fibronectina (FN), fosfatase ácida resistente ao tartarato (TRACP) e a proteína ácida, 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 , $\beta 1$ and $\beta 2$) 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 de proteínas da matriz extracelular envolvidas na formação e mineralização da matriz do osso e/ou das escamas.

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List of abbreviations

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 - immunohistochemistry
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
T_m - annealing temperature
UTR - untranslated region