UNIVERSIDADE DO ALGARVE
FACULDADE DE ENGENHERIA DE RECURSOS NATURAIS

UP-REGULATED GENES DURING IN VITRO MINERALIZATION OF Sparus aurata OSTEOBLAST-LIKE CELLS

(dissertação para a obtenção do grau de mestre em Biotecnologia)

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FARO, 2004

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DATA: 30 de Janeiro de 2005

TÍTULO DA DISSERTAÇÃO: Up-regulated genes during *in vitro* mineralization of *Sparus aurata* osteoblast-like cells.

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Acknowledgments

I am grateful to Professor Leonor Cancela for supervising and supporting this thesis. I also would like to thank her for critically reading the manuscript and for helpful discussions.

I would like to thank my co-supervisor Dr. Vincent Laizé that supervised directly this thesis. Thank you for critically reading the manuscript, for helpful discussions and above all for helping me with lab work and with long sequence analysis.

Thank you so much Asuncion Lago for always being there to help me with everything, including of course for the crucial help on the subtractive libraries.

Thank you Sandrinha for the long scientific discussions, for helping in the lab and most of all for being really my friend.

I would like to thank my colleague António Pombinho for helping me in the lab and for always being cool in the weirdest situations.

Thank you Carla Viegas for helping in the lab and for helpful discussions.

Thanks Natércia Conceição for all the articles and the imaginary "cappuccino mit zhäne" talks.

Thank you Paulo Gavaia and Nuno Henriques for lending me some space on your bench…it was not easy!!!

I would also like to thank the rest of the colleagues for all the attention and friendship shown: Dina Simes, Juan Bosco, Pedro Rodrigues, Rita Ascenso, Jorge Pinto, Ricardo Afonso, João Fidalgo, Marta Rafael, Marta Valente, Ricardo Leite, Sofia Cavaco, Susana Domingos, Brigitte Simões e Daniel Tiago.

Special thanks to my husband Fred for being an amazing person … thanks for making me laugh and push me up when things look a bit grey.

Special thanks also for my parents for the love and support…

Abstract

Vertebrate bone formation involves numerous and complex mechanisms and despite many studies, essentially performed in mammals, most of them remain largely unclear. Few *in vitro* studies, all in mammalian systems, have identified various genes differentially expressed during mineralization but no data is available yet from lower vertebrates. In the present study, it was used the recently developed *Sparus aurata* osteoblast-like cell line VSa16 to construct a cDNA subtractive library (control vs. mineralizing conditions) aimed at the identification of genes associated with fish *in vitro* mineralization. Suppression Subtractive Hybridization (SSH) combined with Mirror Orientation Selection (MOS) identified 194 cDNA clones representing 20 different up-regulated osteoblast-related genes. Among them, four were related to calcium mechanisms: osteopontin-like (SaOP-like), S100-like (SaS100-like), mucin-like (SaMUC) and transgelin2 (SaTGL2). The full length cDNA of SaOP-like (2138 bp) and SaS100-like (8648 bp) was obtained by RACE-PCR. SaOP-like and SaS100-like relative gene expression was analysed in 1) larvae and juvenile fish representing different developmental stages, 2) a broad selection of adult tissues, and 3) cell lines derived from bone and cartilage recently developed in our laboratory. Op-like was shown to be expressed essentially in calcified tissues and to be important for late stages of mineralization. In contrary, S100-like was ubiquitously expressed and found to be important at early stage of larval development. The role of the proteins encoded by these two genes in the process of mineralization is likely to be related to their capacity of binding calcium and hydroxyapatite crystals.

Key words: subtractive libraries, gene differential expression during mineralization, osteoblast and chondrocyte-like cells, *S. aurata* osteopontin-like, *S. aurata* calcium-binding like S100

Identificação de genes expressos em condições de mineralização em células derivadas de osso de *Sparus aurata* e estudo da sua expressão em culturas *in vitro*

Resumo

A formação do osso em vertebrados envolve mecanismos numerosos e complexos e embora se tenham desenvolvido muitos estudos essencialmente em mamíferos, a maioria deles permanece ainda por esclarecer. Poucos estudos *in vitro*, todos em mamíferos, identificaram vários genes diferencialmente expressos durante a mineralização contudo não existem dados disponíveis em vertebrados inferiores. No presente estudo, utilizou-se um linha celular desenvolvida recentemente com células de *Sparus aurata* semelhantes a osteoblastos VSa16 de forma a construir um biblioteca subtractiva de cDNA (condições control vs. mineralizantes), com o objectivo de identificar genes associados à mineralização *in vitro* de peixes. A hibridação subtractiva de supressão (SSH) combinada com a selecção orientada tipo espelho (MOS) permitiu a identificação de 194 clones de cDNA que representava 20 genes differencialmente expressos relacionados com osteoblastos. Quatro candidatos desta biblioteca subtractiva estão relacionados com mecanismos de regulação de cálcio, osteopontina-like (SaOP-like), S100-like (SaS100-like), proteína mucin-like (SaMUC) e transgelin2 (SaTGL2). Clonou-se 2138-pb e 648-pb correspondentes ao comprimento total da SaOP-like e SaS100-like, respectivamente. Analisou-se a expressão relativa da SaOP-like e SaS100-like em 1) diferentes estádios de desenvolvimento de larvas e juvenis de peixe, 2) vários tecidos de dourada adulta e 3) linhas celulares de origem óssea e cartilagínea. Estes dois genes podem estar envolvidos em mecanismos de regulação de cálcio e deposição de minerais durante o processo de mineralização, devido à sua expressão especialmente em tecidos ósseos e estádios de desenvolvimento específicos na dourada.

Palavras-chave: bibliotecas subtractivas, expressão diferencial de genes durante a mineralização, osteoblastos e condrócitos, *S. aurata* osteopontin-like, *S. aurata* calcium-binding like S100.

Abbreviations

$1,25\text{-}(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
^{32}P	phosphorus isotope 32
aa	amino acid
bp	base pairs(s)
cDNA	complementary DNA to RNA
cds	complete coding sequence
dCTP	deoxycytidine 5'-triphosphate
ddH_2O	double-distilled water
DEPC	diethylpyrocarbonate
DMEM	Dulbecco´s modified Eagle medium
DMSO	dimethyl sulfoxide
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECM	extracellular matrix
EDTA	ethylenediaminotetraacetate
FBS	Fetal bovine serum
Gla	γ- carboxyglutamic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*´-2-ethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
MCS	Multiple cloning site
MOPS	3-[*N*-morpholino] propanesulfonic acid
mRNA	messenger RNA

ORF	Open reading frame
PBS	phosphate-buffered saline
Rpm	rotations per minute
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SDS	sodium *n*-dodecyl sulfate
SSC	sodium-chloride sodium-citrate
TAE	Tris-acetate-EDTA
UTR	untranslated region
U	enzyme unit(s)
U.V.	ultra-violet
vol	volume(s)
X-GAL	5-bromo-4-chloro-3-indolyl-β-Dgalactopyranoside

ERRATUM

Page	Where is…	Should be…
iv	8648 bp	648 bp
iv	found to be important	could be important
8	cell differentiation.	cell differentiation (Chang *et al.*, 2000).
12	using recently	using a recently
13	(Pombinho et al., 2004)	(Pombinho *et al.*, 2004)
13, 14	(see Appendix I for composition)	(Appendix I)
15	1 µl cDNA	1 µl of cDNA
16, 22	(see Appendix III)	(Appendix III)
21	3-4 min under UV light.	3-4 min under UV light (Fonseca *et al.*, in press).
22	(accession number not yet attributed)	(accession number AY787209)
25	PolyA+ RNA were	PolyA+ RNA was
27	SHH	SSH
27	The search for up-regulated genes…	All positive clones were selected, giving a total number of 672 clones from single SSH and 960 clones from SSH+MOS. The search for up-regulated genes…
30	by 83 clones	by 123 clones
30	115	114
30	encodes a 86-aa	encondes an 86-aa
31	accession number has not been attributed yet.	with the accession number AY787209.
31	did not identified	did not identify
34	aligned sequences	from aligned sequences
34	located in	located at
34	sites where identified	sites were identified

38	in.brain, gall bladder	in brain, gall bladder
40	efficient subtraction	subtraction efficiency
41	Altogether, results	Altogether, the results
42	mechanisms of related	mechanisms related
50	given not its only to bone specificity	given not only its bone specificity
51	(Adapted from (Beck *et al.*, 2000; Maeda *et al.*, 2004)).	(Adapted from Beck *et al.*, 2000; Maeda *et al.*, 2004).
52	calcified tissues and in mineralized osteoblasts (OP-like) and to be	calcified tissues, in mineralized osteoblasts (OP-like) and during larval

References cited in text but missing in Bibliographic References:

Agüero, F., Campo, V., Cremona, L., Jäger, A., Noia, J.D., Overath, P., Sánchez, D. and Frasch, A. (2002). Gene Discovery in the Freshwater Fish Parasite Trypanosoma carassii:Identification of trans-Sialidase-Like and Mucin-Like Genes. *Infection and Immunity*. 70: 7140–7144.

Bellows, C., Heersche, J. and Aubin, J. (1992). Inorganic phosphate added exogenously or released from betaglycerophosphate initiates mineralization of osteoid nodules *in vitro*. *Bone Miner* 17: 15-29.

Fonseca, V.G., A. Lago-Lestón, V. Laizé and M.L. Cancela. Rapid identification of differentially expressed genes by *in situ* screening of bacteria. *Mol Biotech* (in press).

Kawasaki, K., Suzuki, T. and Weiss, K. (2004). Genetic basis for the evolution of vertebrate mineralized tissue. *Proc Natl Acad Sci U S A* 101: 11356-61.

Kerr, J., Fisher, L., Termine, J. and Young, M. (1991). The cDNA cloning and RNA distribution of bovine osteopontin. *Gene* 108: 237-42.

Nomura, S., Wills, A., Edwards, D., Heath, J. and Hogan, B. (1988). Developmental Expression of 2ar (Osteopontin) and SPARC (Osteonectin) RNA as Revealed by In Situ Hybridization. *J Cell Biol* 106: 441-450.

Young, M., Kerr, J., Termine, J., Wewer, U., Wang, M., McBride, O. and Fisher, L. (1990). cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). *Genomics*. 7: 491-502.

INDEX

I- INTRODUCTION

The vertebrate skeleton has specific developmental and functional characteristics that define its identity in biologic and pathologic terms. Skeleton is composed of multiple elements of various shapes and origins spread throughout the body (Karsenty, 1999; Karsenty, 2003). Two different tissues form most of these skeletal elements: cartilage and bone (Karsenty, 1999; Karsenty & Wagner, 2002). Bone tissue and mechanisms of bone formation will be the focus of this study.

I.1- Bone function and structure

Bone is a highly specialized form of connective tissue that is nature's provision for an internal support system in all higher vertebrates. It is a complex living tissue in which the extracellular matrix is mineralized, conferring marked rigidity and strength to the skeleton while still maintaining some degree of elasticity (Marks & Odgren, 2002).

Bone has mesenchymal and hematopoietic origin and provides mechanical protection for internal organs, allows direction of motion, and facilitates the locomotion process (Lian & Stein, 1999; Tate *et al.*, 2004). In addition, bone provides a protective housing for blood-forming marrow and serves as a reservoir (70-90%) for mineral ions (Ca^{2+}, Mg^{2+}, PO_4^{3-}) (Lian & Stein, 1999; Young, 2003).

There are two histologically defined bone types: dense or compact bone and spongy or cancellous bone (also known as trabecular bone) (Boskey, 1999; Loveridge, 1999; Sommerfeldt & Rubin, 2001). The general features of both compact and trabecular bones are similar. Both are solid mineralized matrices with small canals (canaliculi), spaces (lacunae), and bone cells. In cancellous bone, the matrix lacunae and mineral-encased cells (osteocytes) are organized in the form of thin interconnecting spicules.

In cortical bone, the tissue is organized in Harvesian systems, or osteons, consisting of blood vessels surrounded by concentric and interstitial lamellae (Fig. 1) (Boskey, 1999; Sommerfeldt & Rubin, 2001).

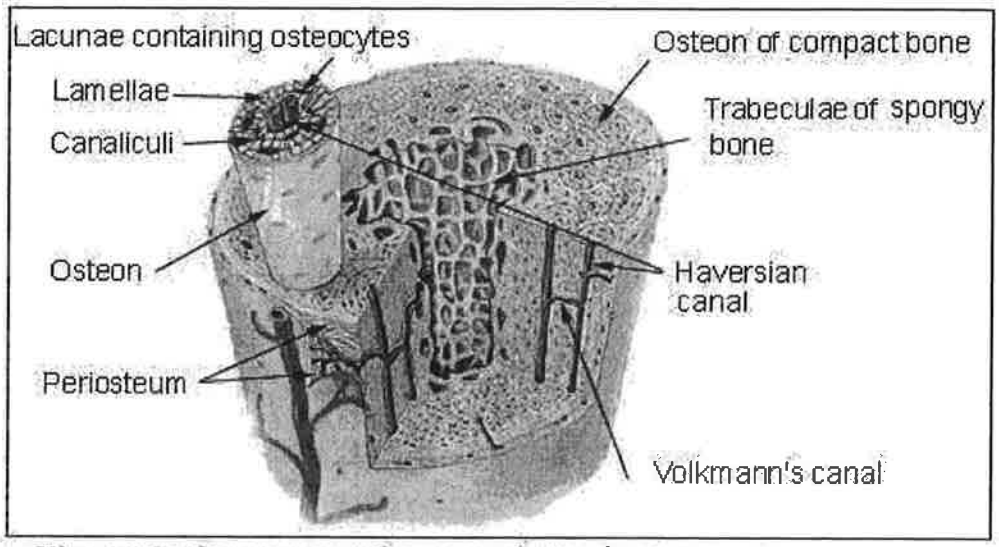


Figure 1: Compact and spongy bone features.
(www.training.seer.cancer.gov)

Bone may also be described based on its mechanism of formation during development. Bones that are developed by the replacement of a cartilage structure (endochondral ossification) are often distinguished from those that form directly (intramembranous ossification) (Boskey, 1999). In intramembranous ossification, the bone matrix mineralizes directly from mesenchymal condensations. In contrast, during endochondral ossification, intermediate steps are involved in which cartilaginous templates prefigure future skeletal elements and play a major role in regulating the developing skeletal elements (Ducy *et al.*, 2000; Mackie, 2003; Nakashima & Combrugghe, 2003).

I.2- Bone cells

Bone has its own specific cell types: osteoblasts, osteocytes and osteoclasts (Karsenty, 1999; Karsenty & Wagner, 2002). Osteoblasts and osteocytes are of mesenchymal origin and share a common progenitor, whereas osteoclasts derive from the myelomonocytic lineage (Karsenty & Wagner, 2002).

Osteoblasts are bone-forming cells and osteoclasts are bone-resorbing cells. Osteocytes, support bone structure and are organized throughout the mineralized bone matrix (Fig. 2).

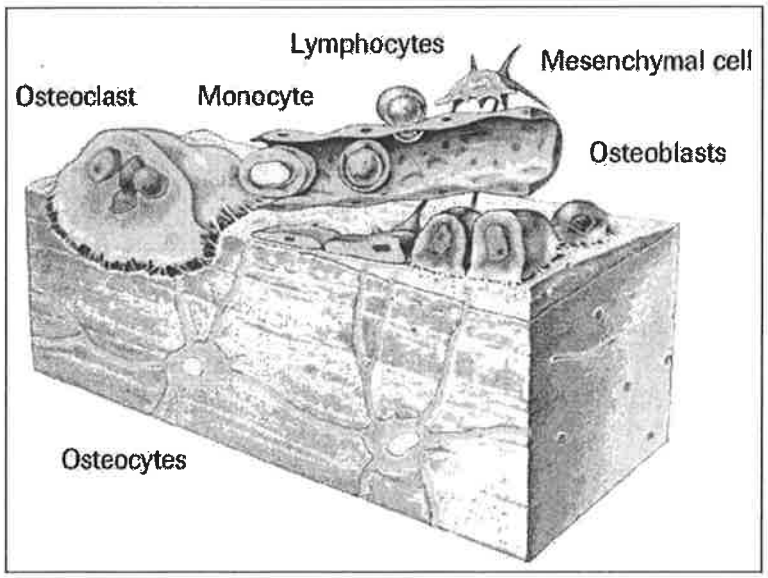


Figure 2: Bone cells.
(www.roche.com/pages/)

The main function of osteoblasts is the secretion of a complex mixture of matrix proteins (known as osteoid); active osteoblasts have a prominent Golgi complex and abundant rough endoplasmic reticulum (Bowtell, 1999; Loveridge, 1999; Sommerfeldt & Rubin, 2001; Mackie, 2003).

Initially, pre-osteoblasts start to grow and proliferate while accumulating glycogen and acid phosphatase. This will decrease with the onset of calcification and the differentiation in mature osteoblasts (Lian & Stein, 1999). On completion of their bone-forming activity most mature osteoblasts become embedded within bone matrix to form the osteocytes.

Some osteocytes will gradually stop secreting the osteoid while others undergo apoptosis (Marie, 2001; Mackie, 2003). Osteoclast degrades mineralized tissues (Karsenty & Wagner, 2002) by dissolving bone after isolating a region of the matrix and secreting HCl and proteinases at that site. Synergy between osteoblast and osteoclast function leads to successive cycles of removal and replacement of bone matrix and allow bone growth, repair and remodeling (Lian & Stein, 1999; Lerner, 2000; Blair *et al.*, 2002).

Proliferation and differentiation of all cells of the osteoblast lineage occur under the influence of a number of transcription factors, growth factors and hormones. As osteoblasts differentiate from their precursors they begin to secrete bone matrix proteins (Lian & Stein, 1999; Ducy *et al.*, 2000; Karsenty, 2000; Mundy *et al.*, 2001). Type I collagen is the major protein in bone matrix, representing about 90% of the organic matrix. Osteoblasts also secrete non-collagenous proteins, including proteoglycans, glycoproteins and γ-carboxylated (Gla) proteins, known to be involved primarily in regulation of bone cell differentiation, migration, proliferation and differentiation (von DER Mark, 1999; Yamaguchi *et al.*, 2000). Osteopontin and bone sialoprotein are adhesive proteins, and appear to be important for cell adhesion of both osteoclasts and osteoblasts while osteocalcin plays an important role in preventing excessive mineralization (Yamaguchi *et al.*, 2000; Marie, 2003; Nakashima & Combrugghe, 2003; Maeda *et al.*, 2004).

In teleost fish, two types of bone have been observed: the cellular bone (osteocytic) and the acellular bone (anosteocytic). In osteocytic bone, osteoblasts secrete a collagenous pre-osseous matrix in which they become enmeshed and become pre-osteocyte (Weiss & Watabe, 1979; Witten *et al.*, 2001). When the pre-osseous matrix mineralizes, pre-osteocytes differentiate in osteocytes that are then completely surrounded by bone. In anosteocytic bone, osteoblasts recede from the mineralizing front and never become trapped as osteocytes (Weiss & Watabe, 1979).

I.3- Bone mineralization

The bulk of the mineral of bone is a crystalline substance, hydroxyapatite (HA: Ca_{10} $[PO_4]_6$ $[OH]_2$), composed mainly of calcium, phosphate and hydroxyl ions (Boskey, 1999) that deposits normally in the skeletal tissues (bone tissue, calcified cartilage, tooth) (Boivin & Meunier, 2003). It is thought that, during the initial phase of mineralization, calcium is deposited in the form of amorphous calcium phosphate precipitate. It is then transformed to hydroxyapatite by the addition of hydroxyl ions to form a crystal lattice into which, by substitution and inclusion, carbonate, citrate and fluoride ions as well as magnesium, potassium and strontium may be introduced (Boskey, 1999).

Physiological bone mineralization in mammals refers to the ordered deposition of hydroxyapatite on a type I collagen matrix (Boskey, 1999), and it is restricted to bones, teeth and hypertrophic growth plate cartilage (Schinke *et al.*, 1999). Several molecules were found to be involved in this process (Schinke *et al.*, 1999) and their sequential expression depends on the location and stage of differentiation of the producing cells (Sommer *et al.*, 1996).

I.4- Bone matrix proteins

An important component of the cellular environment of bone is the extracellular matrix (ECM) (Heinegard *et al.*, 1999), which is a complex of versatile proteins and polysaccharides that are secreted and assembled by cells residing on or in its interstices (Nakajima *et al.*, 2002). The composition and organization of the matrix, and spatial relationships provide specific environmental information to cells (Bidwell *et al.*, 1998; Nakajima *et al.*, 2002). In most vertebrates, bone ECM is composed essentially of collagens, adhesion molecules, enzymes such as acid and alkaline phosphatases, glycoproteins and various noncollagenous proteins such as vitamin K-dependent proteins (Bidwell *et al.*, 1998; Lian & Stein, 1999; Gundberg, 2003).

I.4.1- Collagen

Collagen provides the structural framework of bone and cartilage and corresponds to 90% of the dry mass in bone and 60% in cartilage. There are numerous types of collagen: the fibril-forming collagens are dominant in both tissues. Collagen types I and V can be found only in bone while collagen types II, X and XI are typical of cartilage (Hardingham, 1999; von DER Mark, 1999; Young, 2003).

I.4.2- Adhesion molecules

Cell-matrix and cell-cell interactions are mediated by adhesion molecules. These molecules not only play an important role in the function of fully differentiated cells in the skeleton, but are also increasingly implicated in their developmental pathways (Helfrich & Horton, 1999; Karsenty & Wagner, 2002). The main classes of adhesion molecules implicated in bone and cartilage turnover are integrins (matrix ligand receptors), cadherins (calcium dependent), syndecans (surface proteoglycans) and selectins (leukocyte extravation) (Helfrich & Horton, 1999).

I.4.3- Acid and alkaline phosphatases

Acid and alkaline phosphatases are enzymes expressed in a variety of tissues, but are also widely used as markers for bone cell types. Acid phosphatase is an iron-binding glycoprotein that appears to be secreted by osteoclasts and implicated in bone resorption while alkaline phosphatase is secreted by osteoblasts and involved in cell differentiation and mineralization (Henthorn *et al.*, 1999).

I.4.4- Glycoproteins

Glycoproteins play an important role in the regulation of both cartilage and bone development. They include: osteonectin (ON), growth factors, small integrin-binding ligands with N-linked glycosylation (SIBLINGS), osteopontin (OP), bone sialoproteins (BSPs), dentin sialoprotein (DSP) and matrix extracellular protein (MEPE) (Croucher & Russel, 1999; Gundberg, 2003). Growth factors are primarily responsible for the initial events involved in bone remodeling and comprise various family including the insulin-like growth factor (IGF) family, transforming growth factor-beta (TGF-β) superfamily, fibroblast growth factor (FGF) family and members of the epidermal growth factor family (EGF) (Miyazono, 2000; Mundy *et al.*, 2001).

Bone morphogenetic proteins (BMPs) form a large family of proteins that share common structural features with the TGF-β proteins, and are the most potent inducers of bone formation (Croucher & Russel, 1999; Ducy & Karsenty, 2000; Mundy *et al.*, 2001). OP, MEPE and BSPs are members of the family of small integrin binding ligands with N-linked glycosylation (SIBLING) and together with osteonectin, represent acidic proteins with calcium and hydroxyapatite-binding properties (Hunter *et al.*, 1996; Gundberg, 2003; Young, 2003; Murshed *et al.*, 2004).

I.4.5- Vitamin K-dependent proteins

The members of vitamin K-dependent proteins family play an important role in various tissues and cellular functions (Furie *et al.*, 1999). These proteins contain a variable number of gamma-carboxyglutamic acid (Gla) residues that confer to the protein a high affinity for calcium and hydrohyapatite crystals (Ferland, 1998; Furie *et al.*, 1999; Seibel & Robins, 1999). Bone and cartilage were among the first tissues where Gla proteins were identified and characterized (Gundberg, 2003; Young, 2003). These include: (i) osteocalcin (OC, also named bone Gla protein or BGP), that is mainly produced by osteoblasts and odontoblasts and accumulated in bone and dentin tissues, and (ii) matrix Gla protein (MGP) that is produced mainly by chondrocytes and vascular smooth muscle cells and is accumulated in a variety of tissues. Both proteins are clearly related to the control of the mineralization process (Hunter *et al.*, 1996; Burgoyne & Weiss, 2001; Gundberg, 2003; Murshed *et al.*, 2004).

Members of the family of vitamin K-dependent proteins, namely osteocalcin and matrix Gla protein (MGP), exhibit high affinity to mineral ions and have therefore the capacity to regulate and/or inhibit mineral deposition in normal conditions (Schinke *et al.*, 1999; Gundberg, 2003). Additionally, osteopontin promotes osteoclast attachment to mineralized surfaces, and osteonectin binds collagen, hydroxyapatite, and growth factors (Gundberg, 2003).

I.5- Bone cell cultures

A common goal for cell biologists is the establishment of *in vitro* model systems that faithfully recapitulate a particular biological process that occurs *in vivo*. Cell cultures represent an important tool to understand many biological processes, as gene activity, cell division, protein expression and cell differentiation.

Numerous studies using bone-derived cell cultures to understand mechanisms of bone cell differentiation and function in matrix mineralization have been published. To provide useful information regarding the *in vitro* process of mineralization, a bone cell culture must be able to form an extracellular matrix that can mineralize (Chang *et al.*, 2000) and for that several mineralizing agents are added like β-glicerophosphate (β-GP), vitamin C (Standford *et al.*, 1995; Chang *et al.*, 2000; Sugawara *et al.*, 2002; Beck *et al.*, 2003; Rochet *et al.*, 2003) and calcium (Pombinho *et al.*, 2004). Bone-derived cell lines have been developed successfully in mammals (Standford *et al.*, 1995; Kato *et al.*, 1997; Declercq *et al.*, 2004) and more recently in fish (Pombinho *et al.*, 2004). These fish bone-derived cells lines (named VSa13 and VSa16) were obtained from *Sparus aurata* vertebra and are capable of mineralizing *in vitro* their ECM and express genes found in chondrocyte (VSa13) and osteoblast (VSa16) cell lineages (Pombinho *et al.*, 2004).

I.6- Gene expression and detection

Despite having different bone morphology, fish and mammalian bone-specific cell types exhibit similar mechanisms of gene regulation (Wagner *et al.*, 2003). These mechanisms are still not completely understood (Karsenty & Wagner, 2002; Marie, 2003; Safadi *et al.*, 2003) but many genes (essentially in mammals) have been identified (Kobori *et al.*, 1998; Haudenschild *et al.*, 2001; Doi *et al.*, 2002; Raouf & Seth, 2002; Jong *et al.*, 2004).

There are characteristic changes in gene expression at each stage of bone cells formation (Yamaguchi *et al.*, 2000; Aubin, 2001) in response to systemic and local signaling factors, like fibroblast growth factors (Marie, 2003), bone morphogenetic proteins (Jong *et al.*, 2004) and insulin growth factors (Maeda *et al.*, 2004). Only few genes corresponding to proteins involved in fish bone and cartilage formation have been cloned in seabream, including osteocalcin and, matrix Gla protein (Cancela *et al.*, 1995; Pinto *et al.*, 2001; Pinto *et al.*, 2003; Simes *et al.*, 2003).

Identification of genes associated with normal growth and differentiation during osteogenesis may help elucidating the molecular mechanisms underlying mineralization (Doi *et al.*, 2002). Mineralization is a major characteristic of vertebrates and lower vertebrates represent biological simple study models. In the case of bony fish, they are considered to be an evolutionary success since they represent the largest class of vertebrates with thousands of species with high improvements at the skeletal development. Furthermore, the identification of genes responsible for mineralization in fish can help to find and interpret differences at the evolutionary level of genes and proteins, when comparing to mammals.

The global investigation of changes in gene expression in a biological system can be carried out using several tools that lead to the identification of differentially expressed transcripts between two populations of mRNA (Munir *et al.*, 2004). Various techniques such as differential display, subtractive hybridization (Kobori *et al.*, 1998; Haudenschild *et al.*, 2001) and, more recently, gene array analysis (Doi *et al.*, 2002; Raouf & Seth, 2002; Jong *et al.*, 2004) have led to the identification of novel factors that regulate bone cell development and function in mammals (Safadi *et al.*, 2002).

Despite the fact that these methods have proven their suitability in isolating of differentially expressed genes, sometimes they are incapable of isolating rare transcripts (Munir *et al.*, 2004).

A novel technique called suppression subtractive hybridization (SSH) generates an equalized representation of differentially expressed genes irrespective of their relative abundance (Diatchenko *et al.*, 1996; Boengler *et al.*, 2003). SSH provides high-fold enrichments of differentially expressed mRNAs. This method has been successfully used in numerous works to identify differentially expressed genes in two transcriptomes (Rebrikov *et al.*, 2000; Kiss *et al.*, 2003). Furthermore, since it allows the isolation of differentially expressed cDNAs without a prior knowledge of their sequence, it is highly desirable for studying differential gene expression in systems where information on the genomic sequence is scarce (Munir *et al.*, 2004).

Once isolated by SSH, libraries are enriched with both differentially low and high expressed transcripts through Mirror Orientation Selection (MOS) and selection of different transcripts takes place by differential screening. In differential screening, dot blot arrays of clones from the subtracted library are hybridized with labelled probes from both control and mineralized populations of cDNA.

I.7- Objectives

The main objective of this study is to identify up-regulated genes during tissue mineralization using recently developed *Sparus aurata* osteoblast-like cell line and a combination of suppression subtractive hybridization and differential screening methods. A secondary objective is to clone the full-length cDNA of potentially important genes for tissue mineralization and characterize their expression pattern in various cell lines and tissues as well as during fish development.

II- MATERIALS AND METHODS

II.1- Cell culture

II.1.1- Cell lines and culture conditions

The cell line used in this work, named VSa16, has been recently developed and characterized in the laboratory (Pombinho et al., 2004) and is derived from *Sparus aurata* vertebra. Cells were routinely grown in D-MEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin, 1% fungizone and 2 mM L-glutamine (Invitrogen), and incubated at 33°C in a 10% CO_2 humidified atmosphere. When confluent, cells were trypsinized using solution T (see Appendix I for composition) and diluted 1:2.

II.1.2- Extracellular matrix mineralization and nodule detection

Confluent VSa16 cells were grown for 3 weeks in D-MEM medium under mineralizing or normal conditions. Mineralization of the extracellular matrix (ECM) was induced in confluent cultures by supplementing medium with 50 μg/ml of L-ascorbic acid (vitamin C), 10 mM of β-glycerophosphate and 4 mM of $CaCl_2$ to give, according to medium composition supplied by the manufacturer, a final phosphate concentration of 10.9 mM, and a final calcium concentration of 5.8 mM. Medium was changed and supplements were added twice a week until mineral deposits were revealed by von Kossa staining. For this, cells were washed 3 times with PBS (see Appendix I for composition) at room temperature, fixed with 10% formaldehyde (in PBS) for 1 h at 4°C, washed 3 times with distilled water and then stained with 5% silver nitrate for 30 min under UV light. Staining solution was discarded and cells were washed 3 times with distilled water.

Formation of mineralized nodules in the ECM was observed under an Axiovert 25 inverted light microscope (Zeiss) equipped with phase contrast and linked to a C-3030 digital camera (Olympus).

II.2- Total RNA extraction and polyA+ RNA preparation

Total RNA was extracted from confluent VSa16 cell cultures grown in D-MEM using the acid guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski & Sacchi (1987). Briefly, 5 ml of solution D (see Appendix I for composition) containing a strong denaturating agent (guanidinium thiocyanate) were added to cultures grown in 100-mm culture dishes then cells were detached using a cell scrapper. At this step the genomic DNA was sheared by pipetting up and down the cell suspension 4-5 times with a 5 ml syringe fitted with a 19 gauge needle. Then, 0.1 vol. of 2 M sodium acetate (pH 4.0), 1 vol. of acid phenol (pH 4.3) and 0.2 vol. of chloroform:isoamyl alcohol (49:1 v/v) were added and the mixture was homogenized for 10 s, incubated on ice for 15 min and centrifuged at 10,000×g for 15 min at 4°C. The aqueous phase was transferred into a clean tube and 1 vol. of 100% isopropanol was added. RNA solution was inverted several times and placed at -30°C for at least 1 h. The mixture was centrifuged at 10,000×g for 15 min at 4°C and the pellet resuspended in 500 µl of solution D. One vol. of 100% isopropanol was added and the mixture was placed at -30°C for at least 1 h. Total RNA was recovered at 10,000×g for 15 min at 4°C, washed with 75% ethanol, air-dried and re-suspended in 100 µl of DEPC-treated water. RNA quantity was estimated from absorbance at 260 nm and RNA quality from the 260 nm/280 nm ratio.

Starting with 1 mg of total RNA, polyA$^+$ mRNAs were purified using oligo-d(T)-cellulose chromatography kit (QIAGEN), following manufacturer's protocol.

II.3- Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization (SSH) was performed using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA), following manufacturer's protocol.

II.3.1- cDNA synthesis and *Rsa*I digestion

First strand cDNA was synthesized with 1 µl cDNA synthesis primer (10 µM) in a mixture containing 2 µg of mineralized (prepared from mineralized cultures) and normal (prepared from non-mineralized cultures) mRNA, 1 µl of dNTP mix (10 mM each nucleotide), 1 µl of AMV reverse transcriptase (20 U/µl), 2 µl of 5× first-strand buffer in a final volume of 10 µl at 42°C for 1.5 h. Then, second-strand cDNA synthesis was carried out by adding 10 µl of first-strand reaction with 4 µl of 20× second-strand enzyme cocktail, 48.4 µl of DEPC-treated H_2O, 1.6 µl of dNTP mix (10 µM), 16 µl of 5× second-strand buffer and by incubating this mixture for 2 h at 16°C. Polymerization reaction was performed at 16°C for 0.5 h after addition of 12 U of T4 DNA polymerase and stopped with 4 µl of 20× EDTA/glycogen mix. cDNA solution was cleaned from proteins by 2 rounds of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and chloroform:isoamyl alcohol (24:1 v/v) extractions then precipitated in the presence of 4 M NH_4OAc and 95% ethanol. cDNAs were recovered by centrifugation at 14,000×g for 20 min, and washed with 80% ethanol, air-dried and dissolved in 50 µl of sterile H_2O. A 6-µl aliquot of this solution was kept for later analysis by agarose gel electrophoresis (Appendix I) and the remaining solution was digested with 15 U of *Rsa*I in a final volume of 50 µl at 37°C for 1.5 h. Digestion was stopped by adding 2.5 µl of 20× EDTA/glycogen mix.

After standard extraction and precipitation, digested cDNA was recovered by centrifugation at 14,000×g for 10 min and washed with 80% ethanol, air-dried and dissolved in 5.5 µl of sterile H_2O.

II.3.2- SSH: adaptor ligation and hybridization

Note: Forward and reverse subtractions will represent specific cDNAs up- and down-regulated during mineralization, respectively.

The forward subtracted cDNA was prepared as follows: 1 µl of the mineralized digested cDNA was diluted into 5 µl of sterile water and splitted into 2 aliquots (2 µl each). Each aliquot was ligated with 2 µl of different adaptor (NP1 or NP2, see appendix III) in the presence of 400 U of T4 DNA ligase, 3 µl of DEPC-treated H_20, 2 µl of 5× ligation buffer, in a final volume of 10 µl. In parallel, 2 µl of each adaptor-linked mineralized cDNA were mixed, and this mixture was used as an unsubtracted cDNA control sample. The two pools of cDNA-subtracted samples bearing the different adaptors and the unsubtracted cDNA sample with both adaptors were incubated at 16°C overnight for adaptor ligation. The reaction was stopped by adding 1 µl of EDTA/glycogen mix and heated for 5 min at 72°C to inactivate the enzyme.

Subtractive hybridization was performed in two rounds. First, 1.5 µl of each adaptor-linked cDNA population was separately mixed with an excess of normal cDNA in 1.0 µl of 4× hybridization buffer solution at 68°C for 8 h, after denaturation for 90 s at 98°C. This first round enriches cDNA population in cDNAs specifically up-regulated during mineralization. Then, the two reaction products were mixed in the presence of a 5-fold excess of denatured normal cDNA, placed at 68°C for 16 h and finally supplemented with 200 µl of dilution buffer. During this second round, single-stranded cDNAs specific for the mineralized

conditions, bearing different adaptors, formed hybrids that will be subsequently amplified by two rounds of PCR.

The reverse subtracted cDNA was prepared by using the same protocol but switching the mineralized and normal cDNA samples.

II.3.3- SSH: PCR amplification of subtracted products

Hybridization step was followed by two rounds of PCR amplification: First PCR amplification was conducted for both forward and reverse subtractions using diluted subtracted products obtained from the second hybridization and the diluted unsubtracted cDNA as control. One microliter of sample was added to 24 µl of PCR master mix and PCR amplification was performed as follows: 75°C for 5 min to extend the adaptors; 94°C for 25 s to denature DNA; 25 cycles [94°C for 10 s, 66°C for 30 s, and 72°C for 1.5 min]. Amplified products were diluted 10-fold in sterile water and 1 µl of diluted primary PCR products were added to 24 µl of secondary PCR master mix containing nested primers, NP1 and NP2R (Appendix III) to ensure specific amplification of double-stranded templates containing both adaptors. Considering the amount of amplification of the first PCR, only 10 to 12 cycles for second PCR amplification were performed at 94°C for 10 s, 68°C for 30 s and 72°C for 1.5 min. Primary and secondary PCR products were analyzed on a 2% ethidium bromide agarose gel.

All PCRs were done on a GeneAmp PCR System 2400 thermocycler from Applied Biosystems.

II.4- Mirror orientation selection

To decrease the number of false positive clones after the second PCR amplification, the method "mirror orientation selection" (MOS) described by Rebrikov *et al.* (2000) was used.

Briefly, secondary PCR products from SSH were purified using the DNA and Gel Band Purification kit (Amersham Biosciences) and dissolved in DEPC-treated water to give a cDNA concentration of 20-30 ng/µl. Adaptor 1 was removed by digesting 5 µl of cDNA sample at 37°C for 1 h with 10 units of *Xma*I (New England Biolabs) in a final volume of 20 µl. The enzyme was inactivated by adding 2 µl of 200 mM EDTA and incubated at 70°C for 10 min. One microliter of *Xma*I-digested cDNA was mixed with 1 µl of 4x hybridization buffer (2 M NaCl, 200 mM HEPES pH 8.3, 0.8 mM EDTA) and 2 µl of H_2O and incubated at 98°C for 1.5 min then at 68°C for 4 h. Then, 200 µl of dilution buffer (50 mM NaCl, 20 mM HEPES pH 8.3, 0.2 mM EDTA) was added and the resulting solution was placed at 70°C for 7 min. One microliter of diluted cDNA was added to 20 µl of PCR mix (1x Advantage *KlenTaq* Polymerase Mix, 200 µM dNTPs, 0.6 µM of NP2Rs). The primer used for this amplification was NP2Rs (Appendix III), which is shorter than NP2R to reduce the strong suppression PCR effect that occurs for short DNA fragments. The PCR mixture was incubated at 72°C for 2 min then submitted to a 25 cycle amplification at 95°C for 7 s, 62°C for 20 s and 72°C for 2 min).

II.5- Cloning DNA fragments

Ligation reaction is performed at room temperature for at least 1 h, using 50 ng of pGEM-T Easy vector (Promega) (Appendix II), 1× pGEM-T easy buffer, 1 U of T4 ligase enzyme and 3 µl of PCR product. A tube of DH5α competent cells (ca. 100 µl) was thawed on ice, supplemented with 1-5 µl of either the plasmid solution or the ligation reaction, placed on ice for 30 min and heat-shocked at 42°C for 45 s. Bacteria were then placed on ice for 30 min and 500 µl of SOC (Appendix I) were added. Bacteria were incubated at 37°C at 200 rpm for 45 minutes then 5 µl of a 1 M IPTG were added. Bacteria were plated onto LB-agar plate supplemented with 50 µg/ml of ampicillin and 0.8 mg/plate of X-Gal (Appendix I). Plates were incubated overnight at 37°C and positive clones were identified according to the white/blue colour selection [interruption of the β-galactosidade gene by DNA insert prevents its expression and the formation of the enzyme that would degrade the X-Gal present in the medium and give the colonies a blue colour; the positive (containing the insert) colonies should be, therefore, the white ones].

II.5.1- Cloning of subtracted cDNA templates

After the second step of PCR amplification, only forward-subtracted products with and without MOS (forward MOS/ forward) from each cDNA population were cloned into the pGEM-T Easy vector (Promega) and transformed into competent DH5α *Escherichia coli* cells as referred previously. Colonies were grown overnight at 37°C in Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml), X-gal (0.8 mg/plate) and IPTG (1 M) were added for blue/white colony screening. All positive clones were grown in 96 well plates (each well supplemented with 100 µl of LB with ampicillin) for 16 hours at 37°C.

II.6- Preparation of subtracted probes

Four tubes of either forward or reverse subtracted secondary PCR were pooled and supplemented with 5 µl of 0.2 M EDTA. DNA was cleaned using one round of phenol-chloroform-isoamyl alcohol (25:24:1 v/v) and chloroform-isoamyl alcohol (24:1 v/v) extractions were then precipitated in the presence of 4 M NH_4OAc and 95% ethanol. The DNA pellet was washed in 80% ethanol, air-dried and dissolved in 24 µl of sterile H_2O. Adaptors were finally removed from the ends of cDNAs to avoid hybridization with themselves during differential screening, by digestion with 10 U of *Rsa*I and 10 U of *Eae*I for 2 h at 37°C then with 10 U of *Sma*I for 2 h at 25°C, using adequate restriction enzyme buffers supplied by the manufacturer.

Adaptors were separated from cDNAs by electrophoresis on a 2% low-melting point agarose/ethidium bromide gel. When well separated, the region containing the adaptors was cut off and discarded, and gel was run backwards until cDNAs were concentrated in a small region. DNA was purified from gel using DNA and Gel Band Purification kit. Adaptor-free cDNAs from forward and reverse subtraction were radiolabeled (Appendix III) with [α-^{32}P]dCTP (3000 Ci/ml; Amersham Biosciences) using the random priming Rediprime II kit (Amersham Biosciences) and purified by spin filtration (Microspin S-200 HK columns, Amersham Biosciences).

II.7- Differential screening

To identify differentially expressed cDNAs in the forward-subtracted products, clones were subjected to differential screening. For that, 3 µl of each forward MOS and forward positive clones, grown at 37°C for 16 h in 96-well plates supplemented with LB and ampicillin, were spotted onto nylon membranes in duplicates (Hybond-XL, Amersham Biosciences). Membranes were air-dried and cells were lyzed by placing the membrane on top of 2 ml of lysis solution (0.5 N NaOH) with the colony facing up and incubated at room temperature for 10 min. Afterwards, membranes were neutralized twice using 2 ml of Tris-Cl 1 M pH 5.4, air-dried and the DNA cross-linked for 3-4 min under UV light. Membranes were washed twice for 5 min in boiling 0.1% SDS (Appendix I) and pre-hybridized for 2 h at 42°C in 40 ml of UltraHyb hybridization solution (Ambion). Each duplicate membrane was hybridized with the forward or reverse radiolabeled DNA probes overnight at 42°C. Membranes were washed twice in 2× SSC/ 0.1% SDS at 42°C for 5 min, twice in 0.1× SSC/ 0.1% SDS at 42 °C for 20 min (Appendix I), air-dried and exposed to a Kodak XAR film (Amersham Biosciences) overnight. Films were revealed using standard methods and Kodak developer and fixative.

Differentially expressed clones, identified manually, were sequenced (Macrogen, Korea) and identified by homology searches in public databases using BLAST facilities at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

II.8- Northern blot

Total RNA was extracted as described by Chomczynki & Sacchi (1987). Ten micrograms of total RNA were fractionated on 1.2% agarose formaldehyde gel and transferred to Hybond-XL nylon membrane (Amersham biosciences) by capillary blotting with 10× SSC (Appendix VI). Membranes were probed, as described in paragraph II.7, using either the 838-bp osteopontin-like cDNA fragment (accession number n°AY651247) or the 648-bp S100-like cDNA (accession number not yet attributed) obtained during this study. Relative levels of mRNA were determined by densitometry methods using the Quantity One (Bio-Rad) software and normalized using the house keeping cDNA of L27a ribosomal protein (accession number n°AY188520) of *S. aurata*.

II.9- Cloning of *S. aurata* osteopontin-like full length cDNA

Osteopontin-like full-length cDNA was cloned using Marathon™ cDNA Amplification Kit (Clontech) following the manufacturer's instructions. Briefly, purified mRNAs from VSa16 cells were used to construct an adaptor-ligated cDNA library. A complete osteopontin-like cDNA was obtained through 5'- and 3'-RACE PCRs using AP1 or AP2 oligonucleotides (Clontech, see appendix III), and specific primers designed based on the osteopontin-like partial cDNA previously obtained. PCR fragments were size fractionated by agarose gel electrophoresis, purified and subcloned into pGEM-T Easy vector.

II.10- Protein sequence analysis

Several Internet tools were used to analyze OP-like and S100-like protein sequence. These include: SignalP for the prediction of signal peptide (www.cbs.dtu.dk/services/SignalP); NetPhos for the prediction of serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins (www.cbs.dtu.dk/services/NetPhos); Sequence Manipulation Suite to analyse and format DNA and protein sequences (http://www.bioinformatics.org/sms/); Prosite for the prediction of protein domains (au.expasy.org/prosite); Blastp to search for homologous protein sequences (www.ncbi.nlm.nih.gov/BLAST).

III- RESULTS

III.1- Preparation of total RNA from control and mineralized VSa16 cell cultures

III.1.1- *In vitro* mineralization of VSa16 cells

Confluent cultures grown in D-MEM (Fig. 3a and 3b) were supplemented for 3 weeks with ascorbic acid, calcium chloride and β-glycerophosphate to induce extracellular matrix mineralization. Mineralization of VSa16 cells was then evaluated using von Kossa staining method (Fig. 3c and 3d). Mineral nodules of various sizes were found in treated cultures at sites where cells condensed more (Fig. 3d), while no staining was observed in control cultures (Fig. 3c)

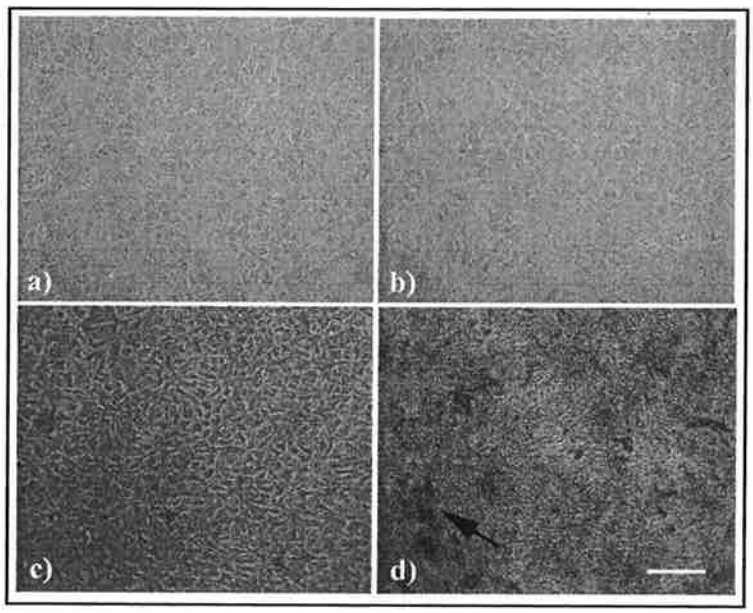


Figure 3: Phase contrast micrographs of *S. aurata* VSa16 cells grown until confluence in D-MEM (**a** and **b**) or silver-stained according to von Kossa technique after 3 weeks under control (**c**) or mineralizing conditions (**d**). Black arrow indicates silver stained mineral nodules. Bar represents 20 µm in **a** and **b**, and 100 µm in **c** and **d**.

III.1.2- Total RNA preparation

Total RNA from control and mineralized cells was extracted from 4×100-mm culture dishes and 10 µg of each preparation was size-separated in a 1% agarose formaldehyde gel (Fig. 4). Characteristic bands corresponding to 28S, 18S and 5S ribosomal RNAs were clearly visible demonstrating the good quality of the RNA preparations.

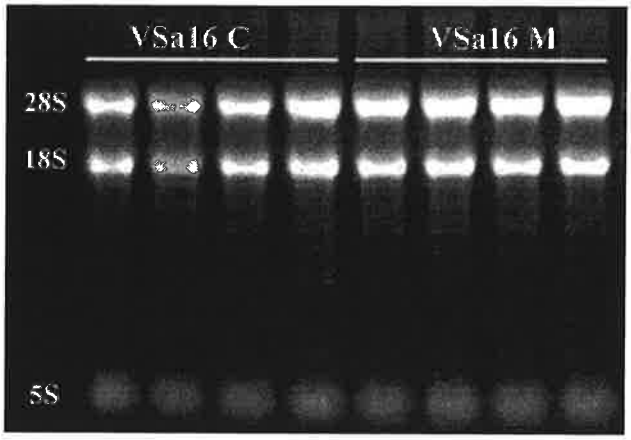


Figure 4: Ten micrograms of total RNA prepared from VSa16 cells grown under control (C) and mineralizing (M) conditions (4 plates each) and separated on a formaldehyde gel.

III.2- Suppression subtractive hybridization (SSH) and differential screening

III.2.1- SSH

PolyA+ RNA were purified from total RNA using a commercially available kit and 2 µg of either control or mineralized samples were reverse-transcribed to cDNA and subtracted according to the protocol provided by Clontech.

cDNAs up-regulated during mineralization (forward-subtraction) were then selectively amplified during the primary and secondary PCR reactions (Fig. 5). The result of the primary PCR was a smear of 0.2-2 kb, without distinct amplified bands whereas several bands were

observed after the secondary PCR. The pattern of amplification observed for the unsubtracted cDNA samples was clearly different from the subtracted one.

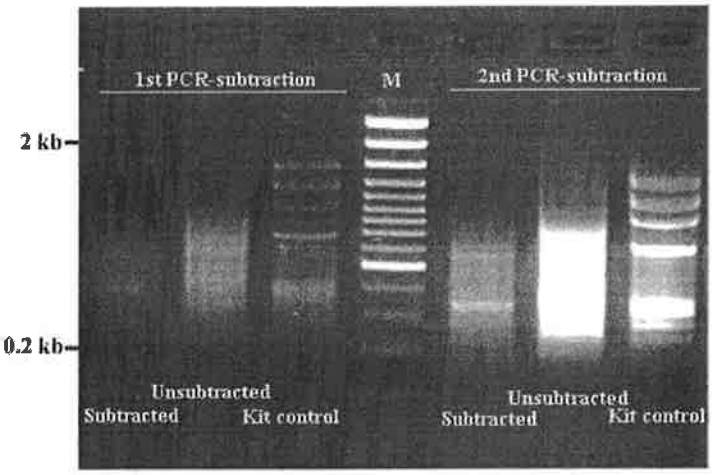


Figure 5: Primary and secondary PCR amplifications of VSa16 forward-subtracted cDNAs. Lane M, 100-bp DNA ladder (Fermentas, Appendix V).

A second subtraction needed for the differential screening of forward-subtracted cDNAs (see section III.2.2- Differential Screening) was prepared in the reverse direction (reverse-subtraction). Differentially expressed cDNAs from control sample were selectively amplified during the primary and secondary PCR reactions and results similar to those obtained for the forward-subtraction were observed (Fig. 6).

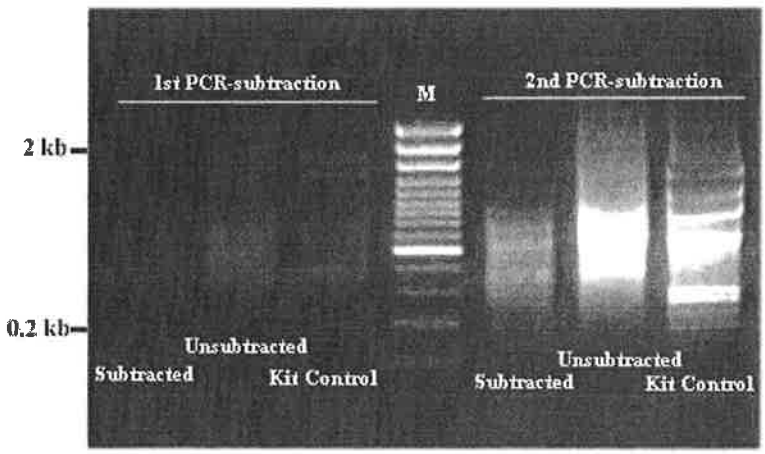


Figure 6: Primary and secondary PCR amplifications of VSa16 reverse-subtracted cDNAs. Lane M, 100-bp DNA ladder.

Forward-subtracted cDNAs were submitted to the Mirror Orientation Selection (MOS) technique, in order to reduce the background level as illustrated in Figure 7. Although, it was already possible to distinguish individual bands with SSH alone, the use of the MOS in addition to the SHH strongly increased their number and intensity.

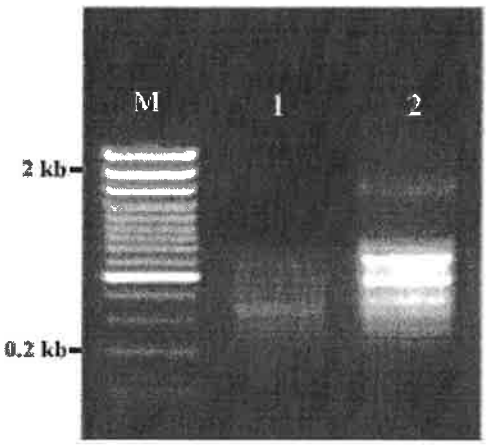


Figure 7: PCR amplification of forward-subtracted cDNAs after SSH (lane 1) or after SSH + MOS (lane 2). Lane M, 100-bp DNA ladder.

III.2.2- Differential screening

The search for up-regulated genes during mineralization was accomplished by hybridizing forward library (blotted on 2 identical membranes) with forward or reverse probes. Clones exhibiting a stronger signal when hybridized with forward probes (by comparison with membranes hybridized with reverse probes) were considered as positive clones and further analyzed. A total number of 672 clones from single SSH and 960 clones from SSH+MOS were selected (Fig. 8).

When comparing both approaches (SSH versus SSH+MOS), SSH+MOS clearly allowed the identification of more clones.

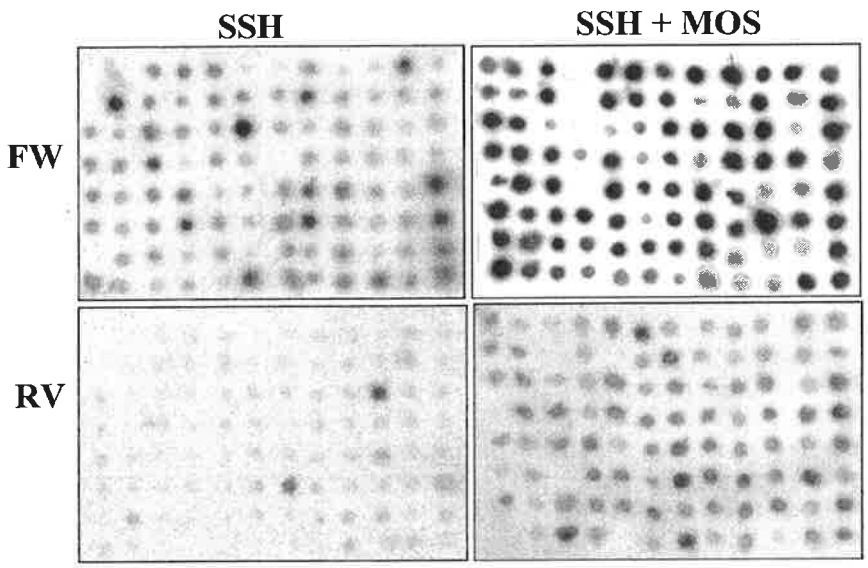


Figure 8: Screening of differentially expressed genes in a subtractive library prepared from VSa16 cells undergoing mineralization. DNA from 96 different bacterial clones obtained using SSH or SSH+MOS techniques were blotted onto nylon membranes and hybridized with the forward (FW) or reverse (RV) probes.

III.3- Identification of genes up-regulated during mineralization

More than 1600 positive bacterial clones were screened. From these, 194 were confirmed as differentially expressed (up-regulated) during mineralization. cDNA fragments corresponding to each clone were sequenced and identified by similarity search using Blast facilities at NCBI. A total number of 20 different genes representing different class of proteins were finally identified (Table II). These included proteins involved in cell metabolism and calcium binding, and proteins with no similarity with any sequences present in public databases. Interestingly, 2 genes accounted for more than 84% of the positive differential clones. These genes encode proteins identified as *S. aurata* osteopontin-like protein (the most abundant with 68% of the positive differential clones) and *S. aurata* sniffer-like protein (16% of the positive differential clones). This observation suggests that these 2 genes are likely to be strongly up-regulated during VSa16 mineralization.

Table II: Genes up-regulated during VSa16 cells mineralization

Name (abbreviation)	Occurrence (N= 194)	Size (bp)
Osteopontin-like protein (OP-like)*	133[1]	2138
Sniffer-like protein (SNIF)	31[2]	515
Unknown 1 (Unk1)	4	519
Cytochrome oxidase subunit I (COX I)	3	1437
Unknown 2 (Unk2)	3	199
Transgelin 2 (TRG2)	2	455
Mucin-like protein (MUC)	2	576
Ubiquitin-conjugating enzyme E2 (UBQE2)	2	168
β-actin (βACT)	2	444
Unknown 3 (Unk3)	2	235
Calcium-binding protein (S100-like)	1	648
Cytochrome c oxidase subunit VIb (COX VIb)	1	303
Putative fish transposase (fishTRP)	1	458
Ribosomal protein L23a (RPL23a)	1	245
Glucose 6 phosphate 1 dehydrogenase (G6PD)	1	399
Unknown 4 (Unk4)	1	389
Unknown 5 (Unk5)	1	606
Unknown 6 (Unk6)	1	263
Unknown 7 (Unk7)	1	515
Unknown 8 (Unk8)	1	266

[1] Three different fragments corresponding to different regions of OP-like cDNA were identified (OP-like1, 2 clones; OP-like2, 8 clones; OP-like3, 123 clones).

[2] Two different fragments corresponding to different regions of SNIF cDNA were identified (SNIF1, 20 clones; SNIF2, 11 clones).

Ten up-regulated genes were identified using the SSH technique alone (MUC, OP-like1, UBQE2, Unk4, G6PD, Unk3, S100-like, COX VIb, Unk6 and Unk7), 6 using the SSH/MOS technique (Transg2, βACT, Unk1, Unk2, fishTRP and RPL23a) and 6 were common to both techniques (SNIF, COX I, OP-like2, OP-like3, Unk5 and Unk8). It should be noted that OP-like3 was represented by 83 clones (43% of total clones).

From a total of 672 clones from SSH and 960 clones from MOS forward libraries, the percentage of confirmed genes was twice as much in the SSH than in the MOS technique (Table I).

Table I: Comparison of enriched subtractive cDNA libraries generated either by SSH or SHH+ MOS techniques.

Technique	Analysed clones	Putative differential clones	Confirmed genes
SSH	672	80 (12%)[a]	16 (20%)[b]
MOS	960	115 (12%)[a]	12 (10%)[b]

[a] Percentage of all analysed clones
[b] Percentage of putative genes

III.4- Cloning and characterization of *S. aurata* S100-like and OP-like full length cDNAs

III.4.1- Characterization of S100-like full length cDNA

Analysis of the 648-bp S100-like clone obtained previously using bioinformatic tools identified a complete coding sequence (cds) with an ATG initiation codon at position 43 in frame with a stop codon at position 302 (Fig. 9). This cds encodes a 86-aa protein that exhibited sequence identity (from 35% to 40%) to numerous members of the S100 calcium-binding protein family (including Ictacalcin, calbindin D9k, S100A1-10).

```
1   CAC TTT TTC AAG CCT CAC AGC TTT TCA TCC AAC TTT GCC ACC ATG
                                                        M  1
45  ACA GAT CTC CCT AAA GCA ATG GGA CTC CTC AGG ACA GTC TTC AAG
     T   D   L   P   K   A   M   G   L   L   R   T   V   F   K  16
91  AAT CAT GCT GGA AAA GAT GGA GAC CCA AAG TCT TTG AAC AAG AAG
     N   H   A   G   K   D   G   D   P   K   S   L   N   K   K  31
136 GAA CTC TCT GAA CTG CTC CGC GCT GAG TTT CCT GAG GCG GGA TCC
     E   L   S   E   L   L   R   A   E   F   P   E   A   G   S  46
181 ACA TCC AAA AAC GAA TTG GAC AAA TTC TTC AAG TCG CTG GAT AAC
     T   S   K   N   E   L   D   K   F   F   K   S   L   D   N  61
226 GAC GGG GAC GGT GTT GTT AGT TTT GAG GAG TTT GTG ACT TTT GCA
     D   G   D   G   V   V   S   F   E   E   F   V   T   F   A  76
271 GCA GCC CTG ACT GTG ATT TGC CAT GGG GAA TAA AAA CGC ACT GAC
     A   A   L   T   V   I   C   H   G   E  ***                86

316 TCA CAA CTT TCT TGT CTT ACT AAA TGT ATG CTA AAT GAT AAA AAA
361 ATC AAT AAA TAC ATT TAA AAC TCA GTG CTC CAA CTT GCA CCA GTA
406 TTA CAG TCA AAT ACA TAA AAA TTC ACT TTT CAT GAT CAA TAT GTT
451 CTG AGA AAT AAA ACT TTG TAA ACA CAC ACA GTT TTA TCA CAG ATG
496 TTT TGT TAC TGT TGG CTT GAG AGA TGT GAA GGT AAT GTG CTA AAT
541 CTC TGT GTG TAT ATC ACT GAT TTT GAA TAT CAG AAG TGA TGA ATG
586 AAG AAC CAA AAT TAA ATG TCA TAT TTG AAA ATT GAA AAA AAA AAA
631 AAA AAA AAA AAA GCT TGT
```

Figure 9: Nucleotide and deduced amino acid sequences of *S. aurata* S100-like cDNA. Nucleotides are numbered on the left and amino acid on the right. ATG start codon and TAA stop codon are indicated in bold. Sequence has been deposited in GenBank but an accession number has not been attributed yet.

III.4.1.1- Characterization of S100-like putative domains

In order to identify putative features of S100-like protein, sequence was analyzed using PROSITE, SignalP and NetPhos Internet tools. No signal peptide was found indicating that S100-like protein is not a secreted protein but an EF-hand domain responsible for calcium binding and putative serine and threonine phosphorylation sites were identified (Fig. 10). Analysis of protein sequence did not identified any proteolytic cleavage sites therefore the sequence presented in Figure 10 is likely to correspond to mature protein.

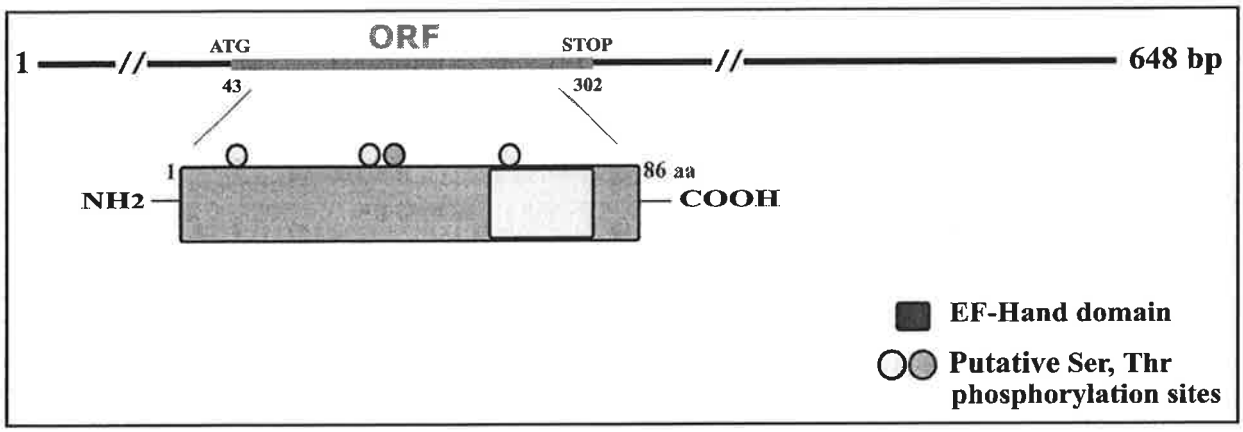


Figure 10: Structure of SaS100-like cDNA and protein.

III.4.2- Cloning and characterization of osteopontin-like full length cDNA

Non-overlapping clones OP-like1, OP-like2 and OP-like3, representing the same gene identified as *S. aurata* osteopontin-like protein, were used to design specific primers (Table III) in order to clone OP-like full length cDNA.

Table III- Primers used to clone OP-like full length cDNA.

Primers Name	Primers sequence
SaOP1-F01	5'- CGCTCCAGCCGCTGAACTCCTGAAGC –3'
SaOP2-F02	5'- CCACCCCTCAGCCCATCGACCCTACC –3'
SaOP2-R04	3'- GGTAGGGTCGATGGGCTGAGGGGTGG –5'
SaOP3-F03	5'- GGCGGGACCTGACACCACCACTGACA –3'

The complete OP-like cDNA (GenBank accession number AY651247) was amplified by PCR and RACE-PCR, cloned in pGEM-T Easy and subsequently sequenced. Full length cDNA is 2138-bp long with an ATG initiation codon at position 130 in frame with a stop codon at position 1253 (Fig. 11).

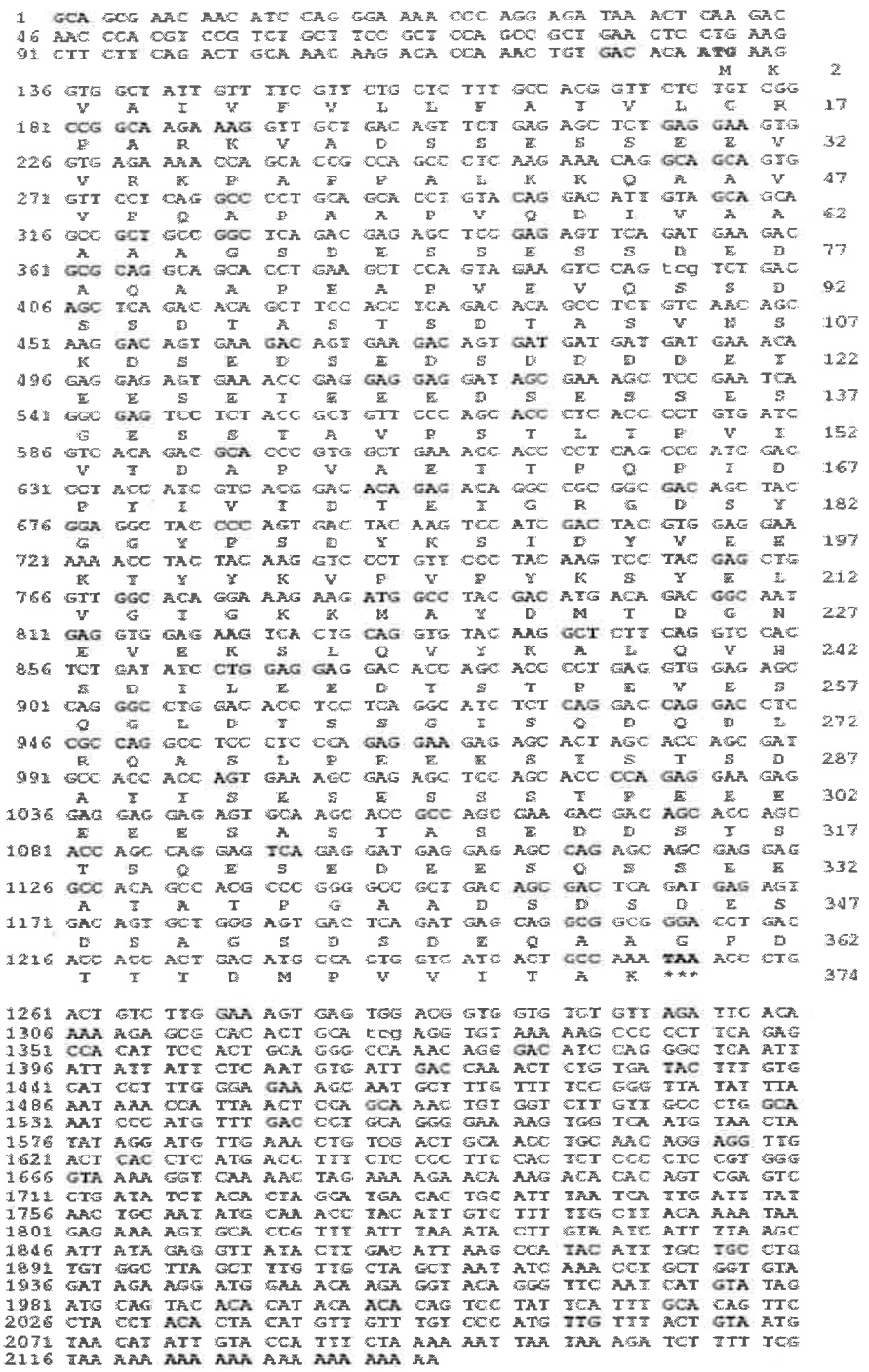


Figure 11: Nucleotide and deduced amino acid sequences of *Sparus aurata* osteopontin-like cDNA. Nucleotides are numbered on the left and amino acid on the right. ATG initiation codon and TAA stop codon are indicated in bold. Sequence has been deposited in GenBank with the accession number AY651247).

S. aurata OP-like cDNA encodes a 374-aa protein that exhibits ca. 40% identity with other OP-like obtained from *Oncorhynchus mykiss* (rainbow trout), *Salvelinus fontinalis* (brook trout), *Danio rerio* (zebrafish), and *Ictalurus punctantus* (channel catfish) (Table. IV).

Table IV- Percentage of sequence identity among OP-like proteins. *S. aurata* (SaOP-like), *O. mykiss* (OmOP-like1 and OmOP-like2), *S. fontinalis* (SfOP-like), *D. rerio* (DrOP-like) and *I. punctantus* (IpOP-like). Identity values were calculated aligned sequences using The Sequence Manipulation Suite program.

	SaOP-like	SfOP-like	DrOP-like	IpOP-like	OmOP-like1	OmOP-like2
SaOP-like						
SfOP-like	45.2					
DrOP-like	32.4	37				
IpOP-like	21.2	23.6	20.8			
OmOP-like1	44.1	75.1	36.2	25.8		
OmOP-like2	42.4	88.3	35.1	24.2	73.6	

III.4.2.2- Characterization of SaOP-like protein domains

Sequence of SaOP-like protein was analyzed using PROSITE, SignalP and NetPhos Internet tools. A 16-aa long signal peptide located in the N-terminus of the protein was identified demonstrating that OP-like is a secreted protein. A RGD domain demonstrating that maybe this protein is involved in cell-adhesion, a serine-rich domain and various putative serine, threonine and tyrosine phosphorylation sites where identified suggesting OP-like involvement in cell proliferation (Fig. 12).

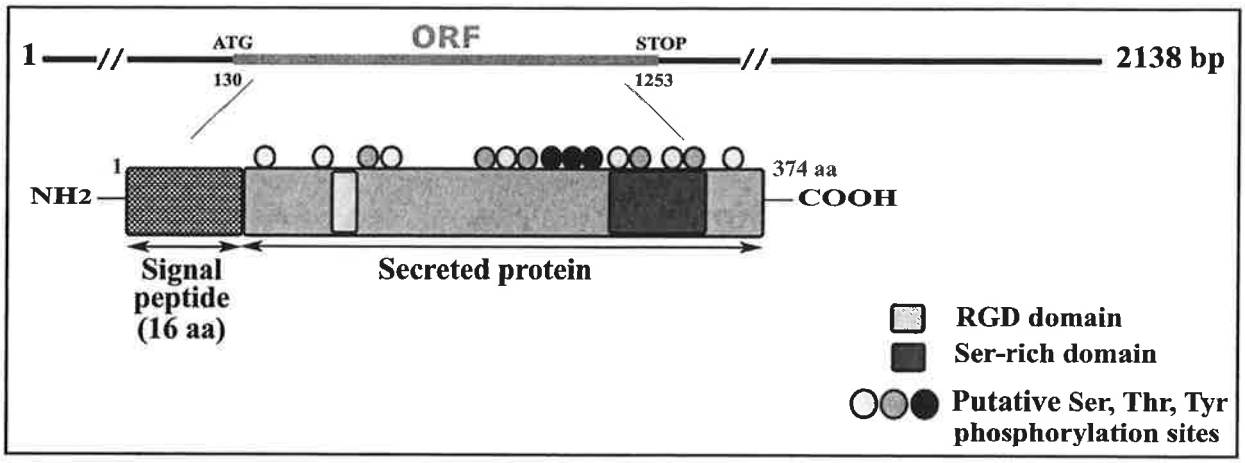


Figure 12: Structure of SaOP-like cDNA and protein.

III.5 Analysis of S100-like and OP-like gene expression

III.5.1- Expression patterns in *S. aurata* bone-derived cell lines

S100-like and OP-like gene expression patterns were determined by Northern blot in VSa13 and VSa16 bone-derived cell lines grown under control or mineralizing conditions for 3-4 weeks. Results indicated that OP-like gene expression was strongly up-regulated in mineralized cells (10 and 20 fold induction in VSa13 and VSa16, respectively) while only a weak up-regulation of S100-like gene expression was observed (1.1 and 2 fold induction in VSa13 and VSa16, respectively) as illustrated in Figure 13.

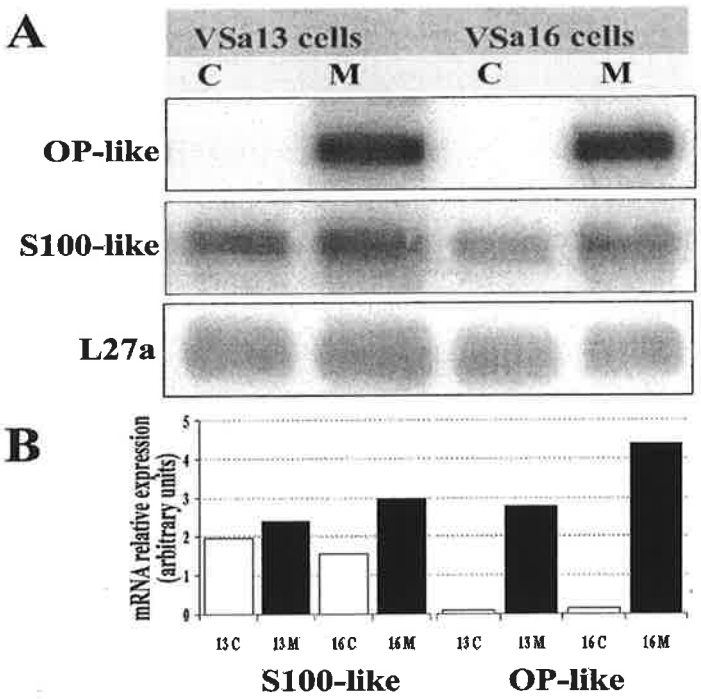


Figure 13: Gene expression of S100-like and OP-like in cells grown under control (C) or mineralizing (M) conditions. A, signals obtained from total RNA hybridized with S100-like, OP-like or L27a ^{32}P-radiolabeled probes. B, relative gene expression of S100-like and OP-like (after normalization with L27a).

III.5.2- Gene expression during larval development

S100-like and OP-like gene expression was investigated during *S. aurata* development using RNA prepared from larvae and juvenile fish. Using for that purpose samples from unfertilized eggs; 2/4, 8, 32, 128, 512 number of cells; 10, 12, 14, 16, 18, 20 and 24 hours after fertilization; 2, 3, 4, 6, 27, 37, 47, 50, 61, 82 and 91 days after hatching). Results indicated that both genes have a different expression pattern during development (Fig. 14). Expression of OP-like was only detected in 82 and 91 days after hatching (dah) samples, indicating a late expression. On the contrary, expression of S100-like was detected as early as gastrulation (10 hours after fertilization- haf) and was progressively up-regulated until 91 dah reaching 50-fold induction.

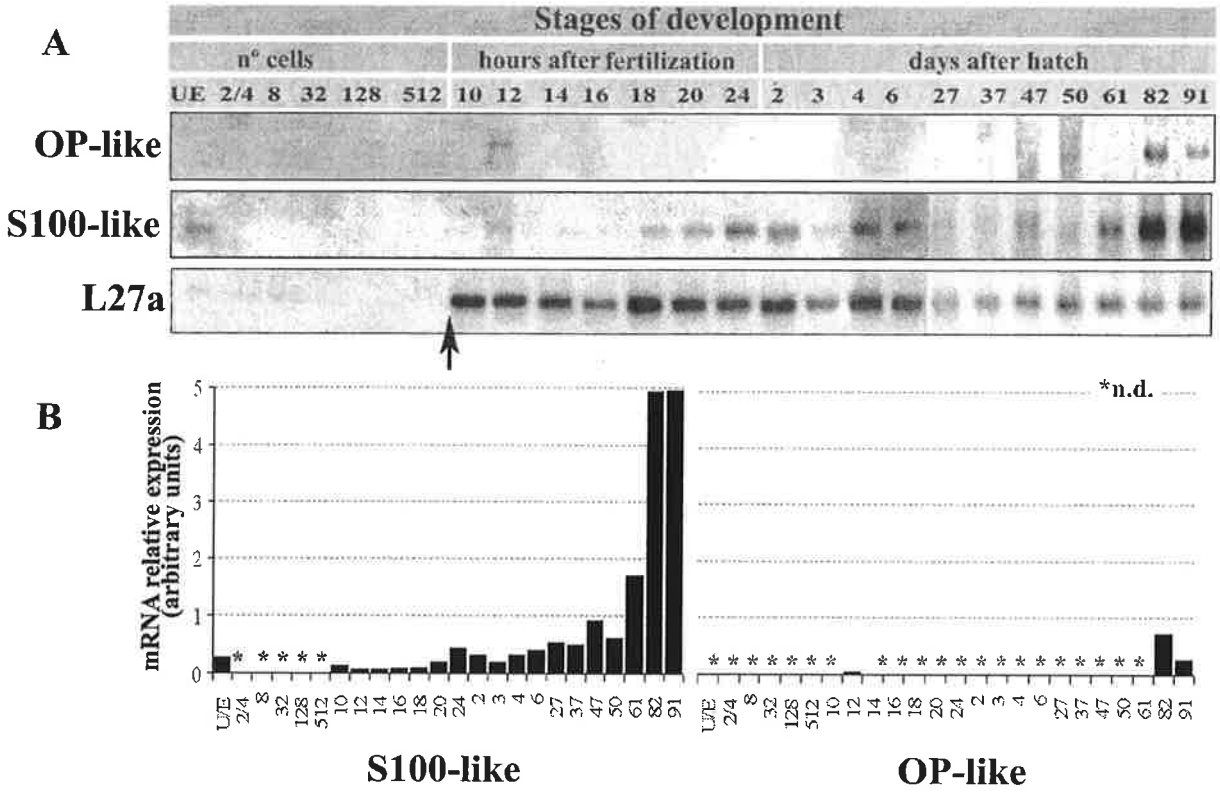


Figure 14: Expression of S100-like and OP-like proteins during *S. aurata* development: unfertilized eggs (UE); 2/4, 8, 32, 128, 512 number of cells; 10, 12, 14, 16, 18, 20 and 24 hours after fertilization; 2, 3, 4, 6, 27, 37, 47, 50, 61, 82 and 91 days after hatching. A- Signals obtained from total RNA hybridized with S100-like, OP-like and L27a ^{32}P-radiolabeled probes. B- Relative gene expression of S100-like and OP-like (after normalization with L27a). Black arrow indicates initiation of gastrulation. n.d. is for non-detected.

III.5.3- Gene expression in adult tissues

The tissue distribution of S100-like and OP-like transcript was investigated in a broad variety of adult tissues including brain, gall bladder, gonad, aorta, fin, spleen, gut, liver, kidney, branchial arches, heart and vertebra tissues (Fig. 15). OP-like was found to be highly expressed in branchial arches and vertebra samples (calcified cartilage and bone tissues, respectively) while not detected in other tissues.

On the contrary, S100-like gene expression is ubiquitous and RNA was found in most of the tissues studies (gall bladder, gonad, aorta, fin, branchial arches, heart and vertebra) with stronger signals in fin, branchial arches and vertebra. Altogether, these results suggest an important role for OP-like in tissue mineralization and a more general role for S100-like.

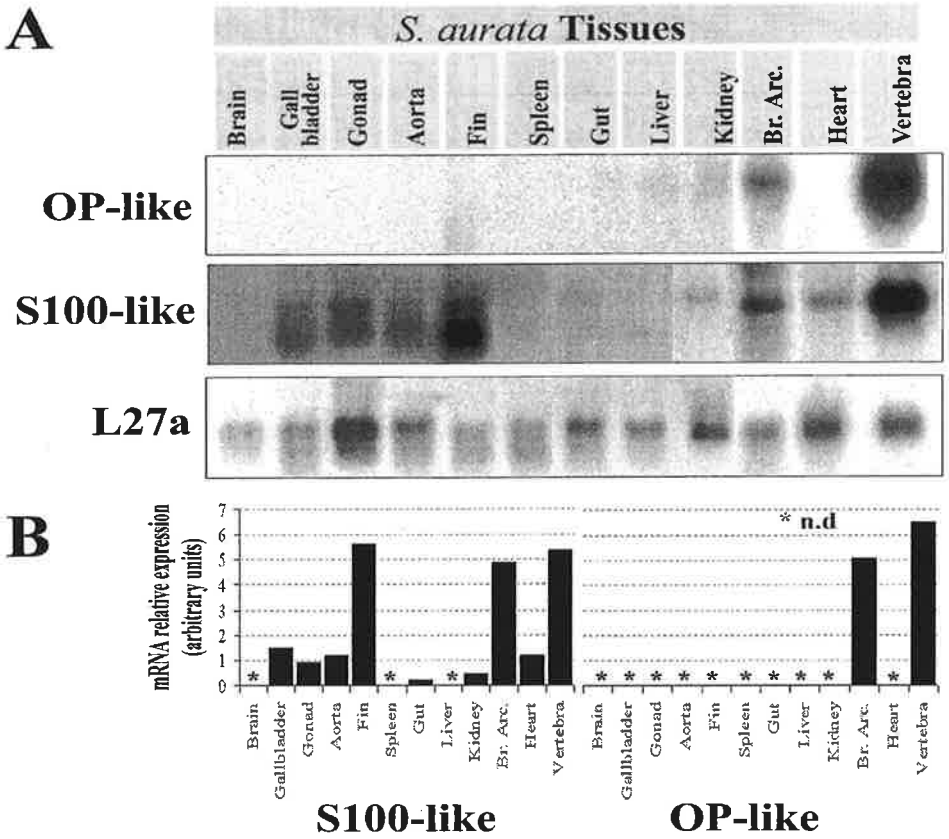


Figure 15: Tissue distribution of S100-like and OP-like gene expression in brain, gall bladder, gonad, aorta, fin, spleen, gut, liver, kidney, branchial arches, heart and vertebra. A- Signals obtained from total RNA hybridized with S100-like, OP-like and L27a ^{32}P-radiolabeled probes. B- Relative gene expression of S100-like and OP-like (after normalization with L27a). n.d. is for non-detected.

IV- DISCUSSION

The aim of this work was to identify differentially expressed genes during mineralization of *S. aurata* VSa16 cells. A robust subtractive hybridization approach was chosen, improved to give better results and has allowed the identification of 194 mineralized osteoblasts-enriched cDNA clones representing 20 different genes.

IV.1- Suitability/efficiency of the subtractive method used

Identifying a maximum of up-regulated genes during mineralization of VSa16 cells was our first interest. When using standard subtractive methods to identify up-regulated genes, you may face the following problem: abundant up-regulated transcripts will be over-represented in the subtracted library hiding rare up-regulated transcripts. The subtractive method developed by Clontech and used in this work has been chosen for its capability of enriching the subtracted library in low-abundance transcripts (normalization step), typically the most difficult to obtain (Diatchenko *et al.*, 1996; Cho & Park, 1998).

The efficiency of the normalization step will not be discussed here, since expression data concerning most of the 20 genes obtained are lacking (only 2 of them, OP-like and S100-like have such data). Other parameters that might prevent the good evaluation of normalization efficiency are: (i) PolyA+ RNA lost during the subtractive procedure, (ii) not all differentially expressed clones were identified and sent to sequence, (iii) screening one library is not sufficient to identify all up-regulated genes (this needs further confirmation with more libraries), and (iv) the use of an *in situ*-based instead of a classical PCR-based differential screening might prevent the identification of some differentially expressed clones.

Normalization efficiency of SSH couldn't be estimated but subtraction efficiency could, simply by analyzing results obtained after PCR subtraction and differential screening (Diatchenko *et al.*, 1996; Munir *et al.*, 2004). When analyzing PCR products, the fewer the products observed the better, since it will demonstrate specific amplification of target molecules (Diatchenko *et al.*, 1996), and this was clearly observed in our case, after both amplifications (SSH uses 2 rounds of PCR amplification). Control sample may contain few additional bands while subtracted sample should contain a smear together with clearly distinct bands, theoretically corresponding to the enriched and normalized low-abundance sequences (CLONTECH, 2002). Banding pattern of VSa16 unsubtracted cDNA was indeed found to be clearly different from banding pattern of those subtracted, in both forward and reverse subtractive reactions, thus demonstrating the successful subtraction of mineralization-specific cDNAs. In addition, few false positive clones were obtained after differential screening, further confirming the efficient subtraction.

Inserts from VSa16 normalized-subtracted cDNA library span from 0.2 to 0.9 kb long, a size very similar to inserts obtained from other libraries prepared using the same method (0.2-1 kb)(Carninci *et al.*, 2000; CLONTECH, 2002) suggesting that cDNA target molecules were preserved after subtraction (Carninci *et al.*, 2000).

Reducing the occurrence of false positive clones (not up-regulated) before the screening process was also of big interest. Some authors have hampered the efficiency of SSH arguing that background DNA molecules are present in the subtracted library, thus giving false positive results in the screening analysis (Rebrikov *et al.*, 2000; Boengler *et al.*, 2003). In order to prevent such problem, an extra step using the MOS method was added to the SSH. During this step, subtraction effect is enhanced by a PCR amplification using a short primer that will allow the amplification of mirror orientated molecules, *i.e.* only molecules cloned in one direction will be amplified while others are considered as background (Rebrikov *et al.*, 2000).

The analysis of MOS PCR products clearly showed a change in the cDNA fragment population, *i.e.* less smearing cDNAs and more recognizable distinct bands, demonstrating the successful reduction of background molecules by MOS method. In fact, using MOS coupled to SSH has allowed us to identify 6 additional genes, even though the screening process was more laborious due to a high proportion of repeated clones (Rebrikov *et al.*, 2000; Munir *et al.*, 2004).

Estimation of library enrichment is always misleading due to the absence of data on the initial abundance of target sequences. It is very hard to know what was subtracted in the first place (Diatchenko *et al.*, 1996; Boengler *et al.*, 2003) since target sequences are enriched by removal of common ones from control samples (Wang & Brown, 1991; Cho & Park, 1998). Usually, the best way to confirm cDNA enrichment for a given condition (in our case, enrichment in mineralization-specific cDNAs) is (i) by Northern blot analysis or other screening process like virtual Northern or slot blot analysis (Diatchenko *et al.*, 1996; Rebrikov *et al.*, 2000; Dey *et al.*, 2001; Boengler *et al.*, 2003; Du *et al.*, 2003; Tsoi *et al.*, 2004) and (ii) RT-PCR in cases needing high sensitivity (Tsoi *et al.*, 2004). Beyond that and as referred before, more libraries would be needed to confirm levels of enrichment.

The screening process was developed, in the course of the present work, allowing the detection of differentially expressed genes obtained from the subtractive library using a manual dot blot analysis and *in situ* screening of bacteria. When comparing differential screening of libraries submitted or not to MOS, it was clear that SSH+MOS library was enriched with more genes as demonstrated by the higher number of positive clones identified after MOS.

Altogether, results described here showed that (i) SSH is simple and efficient for generating libraries moderately enriched for differentially expressed genes of low and high abundance and (ii) SSH coupled to MOS clearly decreased the number of false positive clones allowing a faster and more efficient screening of the subtracted library.

IV.2- Differentially expressed genes

In this study, we have identified 20 different genes up-regulated during mineralization of VSa16 cells. From these, 8 were not homologous to any known gene, 4 were apparently related to calcium mechanisms, and the remaining 8 genes were involved in mechanisms of related to cell metabolism. Even though almost all genes obtained are new with respect to *S. aurata* gene pool (with exception of β-actin), some have already been given specific function in other vertebrates (essentially mammals). The identification of the 8 genes classified as unknown will clearly require additional analysis and their implication in mineralization mechanisms will not be discussed here. It is interesting to notice that genes Unk1, Unk2 and Unk3 were represented by more than 1 clone and are therefore good candidates as up-regulated genes.

Four candidates from the subtractive library were apparently related to calcium metabolism. They encode osteopontin-like (SaOP-like), S100-like (SaS100-like), mucin-like protein (SaMUC) and transgelin2 (SaTRG2).

SaOP-like gene was found to be strongly up-regulated during mineralization of VSa16 cells as demonstrated by Northern blot analysis, a result clearly supported by the fact that it was represented by 68% of the subtracted clones obtained after SSH+MOS. In mammals, osteopontin is a phosphorylated acidic glycoprotein implicated in a number of pathological and physiological events, including bone remodeling (Denhardt & Noda, 1998; Denhardt *et al.*, 2001; Huang *et al.*, 2004). The strong expression of OP-like gene observed in mineralized VSa16 cells could be related to the known role of osteopontin in cell adhesion and cell matrix attachment during osteoblast differentiation (Bellows *et al.*, 1992; Beck *et al.*, 2000; Denhardt *et al.*, 2001; Huang, *et al.*, 2004). In addition, the induction of OP-like gene expression during late larval development could be linked to the onset of mineralization, as seen in mammals (Beck *et al.*, 1998; Swaminathan, 2001; Doi *et al.*, 2002; Huang *et al.*, 2004; Maeda *et al.*, 2004).

The second differentially expressed gene that was studied in this work encodes a protein that belongs to the family of the S100 calcium-binding proteins. This family is the largest subgroup within the helix-loop-helix (EF-hands) calcium-binding protein family (Donato, 1999; Donato, 2001; Heizmann *et al.*, 2002; Heizmann *et al.*, 2003; Zimmer *et al.*, 2003; Fritz & Heizmann, 2004) and its members have been shown to be involved in calcium transduction, uptake and transport (Koltzscher *et al.*, 2003; Fritz & Heizmann, 2004) and in extracellular matrix mineralization (Balmain *et al.*, 2003; Berdal *et al.*, 1996; Duarte *et al.*, 2003; Somogyi *et al.*, 2003).

In general, few S100 proteins are expressed at high levels, making them rather difficult to isolate from tissues (Fritz & Heizmann, 2004). However, SaS100-like gene expression could clearly be detected in VSa16 cells by northern blot suggesting high expression levels.

It has not been possible to assign yet a definitive identity to *S. aurata* S100-like protein, mainly because its sequence is not clearly homologous to the sequence of one of the 20 members of the S100 protein family. In fact, only few fish sequences from S100 proteins are currently available (Bobe & Goetz, 2000; Donato, 2001; Heizmann *et al.*, 2002; Duarte *et al.*, 2003). A way to clearly assign an ID to this gene would be to collect or reconstruct sequences from protein of the S100 family, draw a phylogenetic tree and identify the group of genes within which SaS100-like clusters. This work is currently in progress.

The next gene identified through SSH+MOS encodes a protein similar to mammalian mucins, which are heavily glycosylated, and sometimes sulfated, proteins that bind calcium ions in the process of cell adhesion (Marin *et al.*, 2000).

Mucins have been shown to be associated with (i) skin secretions (Aguero *et al.*, 2002; Nagashima *et al.*, 2003; Long *et al.*, 2004), (ii) calcification in the buccal cavity, where salivary mucins strongly bind to teeth hydroxyapatite (Offner *et al.*, 1998; Raynal *et al.*, 2003), (iii) delay in calcium-phosphate precipitation (Afdhal *et al.*, 1995), (iv) mechanisms modulating the shape of calcium oxalates in urine (Akbarieh & Tawashi, 1991), (v) nucleation of calcium salts (Grases & Llobera, 1998), and more recently (vi) mollusks calcification (Marin *et al.*, 2000). The function of *S. aurata* mucin-like protein in mineralization of VSa16 cells could be associated with the control of ECM mineralization by binding and/or shaping hydroxyapatite crystals, or by delaying calcium-phosphate precipitation.

The last gene identified as being related to calcium metabolism encodes a protein similar to transgelin 2 (TRG2) that belongs to the family of calponins (Goodman *et al.*, 2003). It has been suggested that transgelin would be a target binding protein with calcium receptor properties that mediates interaction between other proteins such as calmodulin (Ikura, 1996; Ikura *et al.*, 2002; Zimmer *et al.*, 2003). In addition, transgelins are thought to play a role in cytokleston organization, since they also bind actin filaments (Yang *et al.*, 2003). Maybe in this case SaTRG2 may also have binding activity responsible for bone formation or may mediate interaction with other calcium-binding proteins. Further experiments would be needed to clearly define the role of SaTRG2 in osteoblast mineralization.

Represented by 16% of the clones identified, the gene encoding a sniffer-like protein (SaSNIF) is still under investigation (evaluation of gene expression levels and cloning of the full-length cDNA) and will be only briefly discussed here. Sniffer-like proteins are known to have an oxidoreductase activity involved in cell metabolism (Botella *et al.*, 2004) and their short chain dehydrogenase/reductase domain is required for efficient cholesterol and sterol metabolism (Qin *et al.*, 2000; Kasus-Jacobi *et al.*, 2003).

Cholesterol is a membrane lipid that not only influences membrane fluidity but it is also (i) a precursor of vitamin D which stimulates Ca^{2+} uptake from intestines and (ii) a precursor of steroids that control salt balance (Horton *et al.*, 1996). The *sniffer* gene was first discovered in *Drosophila melanogaster* where it was shown to protect cellular mechanisms against oxidative stress (Botella et al. 2004). To date, there is no data available about a possible link between sniffer-like proteins and mechanisms of mineralization. Further analyses are needed to clearly establish the role played by SaSNIF in the mineralization of VSa16 cells. The first step, the cloning of SaSNIF full length cDNA, has been recently initiated.

During the process of mineralization, the production of components required for extracellular matrix construction will strongly increase utilizing additional metabolic energy produced by cell machinery. It was therefore not a surprise to find genes encoding proteins involved in such mechanisms among the identified up-regulated genes. These include COX I and COX VIb known to be H^+ and Na^+ translocating transporters in the electron transport chain (Zubay, 1993), G6PD which has a transporter activity of sugar and inorganic phosphate in oxidative reactions (Horton *et al.*, 1996; Boren *et al.*, 2001) and for transposases, known to be responsible for DNA repair, replication and recombination (Horton *et al.*, 1996), maybe representing here membrane protein building blocks.

Proteins known to be involved in cell growth and proliferation were also uncovered, like ubiquitin-conjugating enzyme and also β-actin. Ubiquitin is synthesized by cells to regulate cell cycle progression and proliferation (Cai *et al.*, 2004) and is also involved in protein degradation (Martin *et al.*, 2002; Mochida *et al.*, 2003; Cai *et al.*, 2004). To date, there is not a clear link between ubiquitin and ubiquitin-related enzyme with mechanisms of mineralization and it is likely that this clone (found only once) might represent false positive up-regulated clone. Similarly, RPL23a and β-actin, which are both essential for the maintenance of a normal cell life (Wong *et al.*, 2000) are also likely to represent false positive up-regulated genes.

Genes identified by SSH were carefully analyzed and classified according to their role (putative for most of them) in the mineralization of VSa16 cells, and we decided to study some of them more in detail. *S. aurata* OP-like protein - because its transcript was represented by 68% of the clones identified by SSH - and SaS100-like protein - because it is a calcium-binding protein - were chosen to be studied at first place. Full-length cDNAs were obtained, protein sequences were analyzed using bioinformatic tools, and expression patterns were analyzed in various tissues and conditions.

IV.3- S100-like cloning and expression in S. aurata cells, stages of development and tissues

When fully aligned, the overall identity between SaS100-like and other vertebrate S100 proteins was moderately strong (30-35%). The structure of SaS100-like protein is very similar to those of other S100. It contains a characteristic calcium-binding motif (EF-hand) at the C-terminus, responsible for calcium regulation and binding (Ikura, 1996; Donato, 2001; Heizmann *et al.*, 2002; Heizmann *et al.*, 2003).

It has been reported that S100 proteins contribute to the characterization of the terminal differentiation of osteoblasts (Berdal *et al.*, 1996) but also of other cells (Balmain *et al.*, 2003) in parallel with other matrix proteins (Berdal *et al.*, 1996). Therefore it was not a surprise to observe that SaS100-like was expressed not only by osteoblast-like cells (VSa16), but also by chondrocyte-like cells (VSa13). Calcium binding proteins may play a direct role in calcium storage, when trapped within the bone matrix (Faucheux *et al.*, 1998), which explains SaS100-like in mineralized cells.

In addition, both calbindin (Christakos & Liu, 2004) as well as osteopontin (Denhardt & Noda, 1998; Denhardt *et al.*, 2001; Ishii *et al.*, 2004) are also known to protect against cellular degeneration by buffering calcium, preventing mitochondrial damage and consequent generation of oxygen free radicals (Christakos & Liu, 2004). S100-like could prevent *in S. aurata* damaging effect of high extracellular calcium concentrations observed during mineralization or in seawater.

It has been recently reported that requirements in calcium during *S. aurata* development increases as fish develops and that calcium accumulation is related to skeletal growth, due to higher skeleton densities (Guerreiro *et al.*, 2004). In addition, calcium-binding proteins are mainly involved in calcium storage (Faucheux *et al.*, 1998), and vertebrate embryos, including fish, require large amounts of calcium from extra-embryonic sources during early development (Tuan & Scott, 1977; Guerreiro *et al.*, 2004). This could explain the exponential expression of SaS100-like protein during *S. aurata* development. Furthermore, there is an enormous calcium uptake from water in marine fish that must be balanced through excretion mechanisms (Flick & Verbost, 1993). Accordingly SaS100-like slight expression on *S. aurata* unfertilized egg can also result from the deposition of calcium in the egg, where calcium is an important ionic component (Flanagan *et al.*, 2002).

Bone mineralization relies on calcium availability in the extracellular fluids and, unlike terrestrial vertebrates, which obtain this ion exclusively from the diet, marine fish obtain it from the calcium-rich sea water (Guerreiro *et al.*, 2004). The water-exposed gills, skin and intestine epithelia are the primary surfaces for calcium uptake, while the kidney (and possibly the intestine) is involved in excretion (Flick & Verbost, 1993).

Calcium-binding proteins in fish are related to skin (Manso *et al.*, 1997; Hsiao *et al.*, 2003) mostly due to mechanisms of absorption and excretion of calcium through different epithelia (Flick & Verbost, 1993; Miyazono, 2000; Guerreiro *et al.*, 2002; Hsiao *et al.*, 2003) but are also found in other tissues (Bobe & Goetz, 2000; Cao *et al.*, 2003; Kales *et al.*, 2004).

In fishes there has been an effort to isolate calcium-binding proteins like (i) trout calreticulin in liver (Kales *et al.*, 2004), (ii) trout calcium-binding 28K in gills (Manso *et al.*, 1997), (iii) Fugu calcitonin in brain (Clark *et al.*, 2002), (iv) channel catfish and zebrafish ictacalcin in skin (Porta *et al.*, 1996; Hsiao *et al.*, 2003) and (v) a S100 was also isolated by subtractive hybridization in zebrafish and trout (Cao *et al.*, 2003).

SaS100-like transcript was found in numerous tissues but expression was higher in bone and fin tissues. Fin epithelium is a region with high calcium requirements, when undergoing mineralization (Guerreiro *et al.*, 2004), which explains such high levels of expression in SaS100-like mRNA.

IV.4- OP-like cloning and expression in S. aurata cells, stages of development and tissues

The overall identity between (i) SaOP-like and other osteopontin-like proteins was found to be moderately strong (from 21 to 45%) and (ii) OP-like and OP proteins was found to be weak (from 10 to 17%). In fact, OP and OP-like proteins would nether be grouped in the same protein family on the basis of overall sequence percent identity. Only a deep analysis of protein domains and post-translational modifications, as well as a comparison of gene expression patterns in various tissues and under mineralizing conditions, has demonstrated common features and suggested a common origin for OP and OP-like proteins (Bobe & Goetz, 2001; this work). Interestingly, OP-like proteins are only found in fish and OP proteins only in mammals. We suggest that OP and OP-like could represent the same protein that has highly diverged since teleost branching. This hypothesis would need further experiments to be demonstrated.

Common features of OP and OP-like proteins are as followed: (i) numerous phosphorylation sites involved in signal transduction pathways (Blom *et al.*, 2004; Diella *et al.*, 2004) (ii) a serine rich motif at the C-terminus probably involved in protein calcium-binding activity (Denhardt *et al.*, 2001), (iii) a RGD domain involved in the binding of integrins and responsible for OP role in cell-matrix and cell-cell interactions (Denhardt & Noda, 1998; Denhardt *et al.*, 2001), (iv) up-regulated expression during tissue mineralization. It was also found at the N-terminus of OP-like a signal peptide that recognizes the receptors on the surface of the membrane and assures the secretion of the protein (Nielsen *et al.*, 1997; Nielsen *et al.*, 1999).

In agreement with other studies SaOP-like is up regulated during the mineralization of osteoblast-like cells. Although osteopontin is not exclusive of bone cells (Denhardt & Noda, 1998; Asou *et al.*, 2001; Denhardt *et al.*, 2001; Huang *et al.*, 2004), it is known to be strongly up-regulated in cells undergoing mineralization as a way to control the mineralization process by preventing mineral deposition (Denhardt & Noda, 1998; Brown *et al.*, 1992; Beck *et al.*, 2000; Gopalakrishnan *et al.*, 2001; Boskey, 2003). It was therefore not a surprise to observe increased OP-like expression in mineralized VSa13 cells , which exhibit a chondrocyte-like phenotype.

The onset of *S. aurata* skeleton development takes place few days after hatching, and ossification of the vertebral column (Faustino & Power, 1998) and cranial complex (Faustino & Power, 2001) continues during the first 70–90 days post hatching (Guerreiro *et al.*, 2004). Osteopontin has been reported to be specific for calcified tissues and to play a role in bone remodeling but not to be required for bone development (Denhardt & Noda, 1998; Denhardt *et al.*, 2001). It was therefore not a surprise to detect SaOP-like expression only at 82 and 91 days after hatching, probably at a time when bony structures are more abundant.

Osteopontin is known to be expressed in almost all mammalian tissues (Nomura *et al.*, 1988; Craig & Denhardt, 1991; Kerr *et al.*, 1991; Young *et al.*, 1990; Sakuma *et al.*, 2003) although differently regulated from one tissue to another (Sakuma *et al.*, 2003). Very few data are available on OP tissue distribution in fish. Some osteopontin-like genes were found in trout ovary, gills and skin (Bobe & Goetz, 2001) and very similar to OP in zebra fish enamel matrix (Kawasaki *et al.*, 2004). In this study, SaOP-like mRNA expression was exclusively detected in calcified tissues in complete agreement with results obtain in mammals.

Altogether, these results suggest that SaS100-like might be responsible for calcium homeostasis and storage in fishes and that SaOP-like could represent the *S. aurata* homologue of OP, given not its only to bone specificity but also to sequence similarities.

IV.5- Genes temporal pattern of expression

It has been extensively reported that, once the extracellular matrix synthesis begins, osteoblastic cells differentiate as genes encoding markers such as alkaline phosphatase (ALP), type-I collagen (ColI), osteonectin (ON), osteocalcin (OC), osteopontin (OP) and bone sialoproteins (BSPs) are activated (Aubin, 1998; Cooper *et al.*, 1998; Beck *et al.*, 2000; Karsenty, 2000; Maeda *et al.*, 2004). The work reported here was done on cells fully mineralized and the absence of bone-specific markers with an early expression pattern (Figure 17) was in fact not a surprise. Similarly, the presence of osteopontin-like gene was expected. If intermediate subtractive libraries (for example from RNA collected at 1, 2 and 3 weeks of treatment) would be constructed, it is likely that such bone markers would have been identified.

Since not many fish bone-specific genes are known and also that the 3'-UTR differs a lot between them, there is still the possibility that some of these late bone markers could be represented in the eight unknown genes uncovered by the subtractive library.

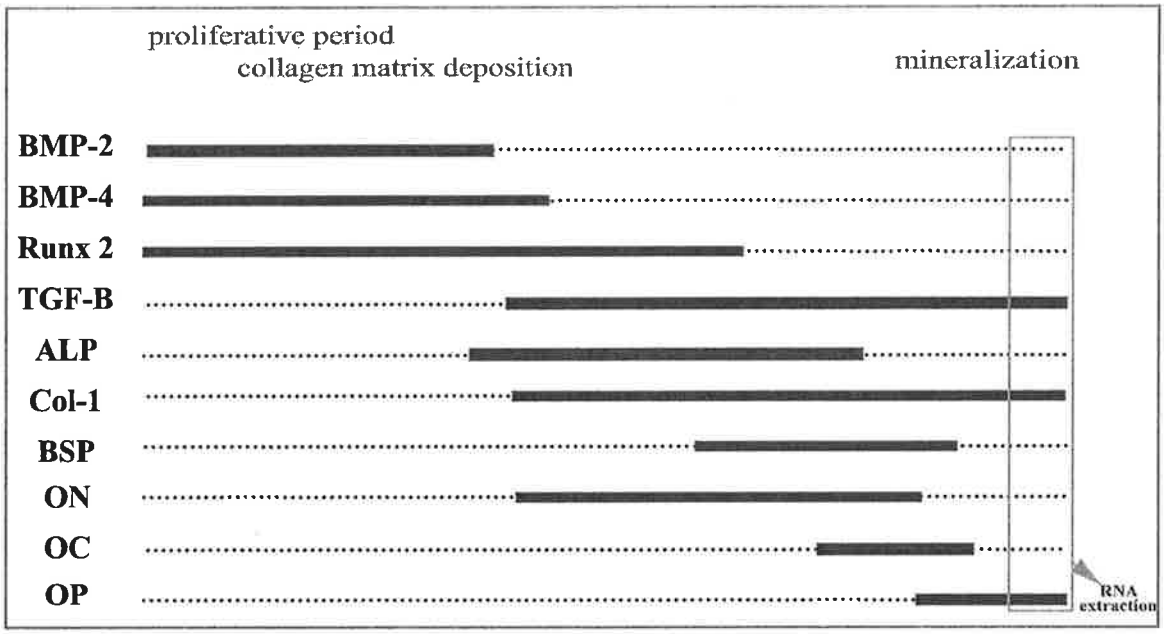


Figure 17: Schematic illustration of temporal expression of known osteoblastic markers. (BMP: bone morphogenetic protein, Runx: runt family transcription factor, TGF-β: transcription growth factor beta, ALP: alkaline phosphatase, Col-1: collagen type I, BSP: bone sialoprotein, ON: osteonectin, OC: osteocalcin and OP: osteopontin) (Adapted from (Beck *et al.*, 2000; Maeda *et al.*, 2004)).

Beyond the differences on temporal expression of osteoblasts markers a recent study can explain the reason why some late stage mineralizing genes, such as osteocalcin or collagen type I, were not detected in this experiment. Huang *et al.*, (2004) have reported that an increase in OP production led to significant decreases in the expression of specific-ECM genes, BMP-2-inducible alkaline phosphatase activity and also mineral deposition. Another possible explanation to the absence of OC and ColI clones in up-regulated gene list would be that OP-like high expression has strongly inhibited Oc and ColI gene expression in late stage of cell mineralization.

IV.6- Final considerations and perspectives

This study has identified 20 genes up-regulated during mineralization of fish osteoblast-like cells. Two of these genes have been further studied and were shown to be highly expressed in calcified tissues and in mineralized osteoblasts (OP-like) and to be important for larval development (S100-like). Both proteins are likely to be involved in calcium regulating mechanisms and mineral deposition during the process of fish tissue mineralization. Remaining genes are currently further analyzed by Northern blot to confirm up-regulation of gene expression during VSa16 mineralization. Full-length cDNA of positive clones will be obtained and protein sequences and expression patterns will be analyzed.

It would also be of great interest to identify genes down-regulated during the process of mineralization in VSa16 cells. This work is under progress: the subtracted library has been constructed and the cloning and screening are under progress.

Various additional subtractive libraries are about to be developed to identify more markers of osteoblast mineralization. As demonstrated previously, there is a temporal expression pattern of genes involved in the mineralization process and the identification of these genes will need subtractive libraries prepared from RNA prepared at early time points.

The exact role of osteopontin-like in mineralization should also be addressed more deeply. It would be interesting to over-express or knock-down SaOP-like gene expression and observe effects on cell mineralization and bone-specific gene expression.

Finally, the construction of subtractive libraries from VSa13 bone-derived cell line, with a chondrocyte-like phenotype, would bring additional data on bone- and cartilage-specific genes in *S. aurata*.

V- BIBLIOGRAPHIC REFERENCES

Afdhal, N., J. Ostrow, R. Koehler, N. Niu, A. Groen, A. Veis, D. Nunes, and G. Offner. (1995). Interaction of bovine gallbladder mucin and calcium-binding protein: effects on calcium phosphate precipitation. *Gastroenterology*. 109: 1661-72.

Akbarieh, M., and R. Tawashi. (1991). Calcium oxalate crystal growth in the presence of mucin. *Scanning Microsc.* 5: 1019-26.

Asou, Y., S.R. Rittling, H. Yoshitake, K. Tsuji, K. Shinomya, A. Nifuji, and D. Denhardt. (2001). Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption in ectopic bone. *Endocrinology*. 142: 1325–1332.

Aubin, J.E. (1998). Advances in the osteoblast lineage. *Biochem. Cell Biol.* 76: 899–910.

Aubin, J.E. (2001). Regulation of osteoblast formation and function. *Rev Endocr Metab Dis*. 2: 81-94.

Balmain, N., F. Moutahir, C. Heizman, and M. Lieberherr. (2003). Immunolocalization of S100A2 calcium-binding protein in cartilage and bone cells. *Cell Mol Biol*. 49: 485-486.

Beck, G., E.C. Sullivan, E. Moran, and B. Zerler. (1998). Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. *J Cell Biochem*. 68:269–280.

Beck, G., B. Zerler, and E. Moran. (2000). Phosphate is a specific signal for induction of osteopontin gene expression. *Proc. Natl. Acad. Sci. USA*. 97: 8352–8357.

Beck, G.R., E. Moran, and N. Knecht. (2003). Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. *Exp Cell Resh*. 288: 288-300.

Berdal, A., D. Hotton, J. Saffar, M. Thomasset, and A. Nanci. (1996). Calbindin-D9k and calbindin-D28k expression in rat mineralized tissues in vivo. *J Bone Miner Res*. 11: 768-79.

Bidwell, J., M. Alvarez, H. Feister, J. Onya, and J. Hock. (1998). Nuclear matrix proteins and osteoblast gene expression. *J Bone Miner Res*. 13: 155-167.

Blair, H.C., M. Zaidi, and P.H. Schlesinger. (2002). Mechanisms balancing skeletal matrix synthesis and degradation. *Biochem. J.* 364: 329-341.

Blom, N., T. Sicheritz-Pontén, RGupta, S. Gammeltoft, and S. Brunak. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics*. 4: 1633–1649.

Bobe, J., and F.W. Goetz. (2000). A S100 homologue mRNA isolated by differential display PCR is down-regulated in the brook trout (Salvelinus fontinalis) postovulatory ovary. *Gene*. 257: 187–194.

Bobe, J., and F.W. Goetz. (2001). A novel osteopontin-like protein is expressed in the trout ovary during ovulation. *FEBS Letters*. 489: 119-124.

Boengler, K., F. Pipp, W. Schaper, and E. Deindl. (2003). Rapid Identification of Differentially Expressed Genes by Combination of SSH and MOS. *Lab Invest*. 83: 759-761.

Boivin, G., and P.J. Meunier. (2003). The mineralization of bone tissue: a forgotten dimension in osteoporosis research. *Osteoporos Int*. 14: S19–S24.

Boren, J., M. Cascante, S. Marin, B. Comín-Anduix, J.J. Centelles, S. Lim, S. Bassilian, S. Ahmed, W. -N.P. Lee, and L.G. Boros. (2001). Gleevec (STI571) Influences Metabolic Enzyme Activities and Glucose Carbon Flow toward Nucleic Acid and Fatty Acid Synthesis in Myeloid Tumor Cells. *J Biol Chem*. 276: 37747–37753.

Boskey, A. (1999). Mineralization, structure, and function of bone. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 153-162.

Boskey, A. (2003). Bone mineral crystal size. *Osteoporos Int*. 14: S16–S21.

Botella, J., J. Ulschmid, C. Gruenewald, C. Moehle, D. Kretzschmar, K. Becker, and S. Schneuwly. (2004). The Drosophila carbonyl reductase sniffer prevents oxidative stress-induced neurodegeneration. *Curr Biol*. 14: 782-786.

Bowtell, D.D.L. (1999). Options available from start to finish for obtaining expression data by microarray. *Nat Gen suppl*. 21: 25-32.

Burgoyne, R., and J. Weiss. (2001). The neuronal calcium sensor family of Ca^{2+} binding proteins. *Biochem J*. 353: 1-12.

Cai, D., K.K.H. Lee, M. Li, M.K. Tang, and K.M. Chand. (2004). Ubiquitin expression is up-regulated in human and rat skeletal muscles during aging. *Arch Biochem Biophys*. 425: 42–50.

Cancela, M., M. Williamson, and P. Price. (1995). Amino acid sequence of bone Gla protein from the african clawed toad Xenopus laevis and the fish Sparus aurata. *Int J Pept Protein Res*. 46: 419-423.

Cao, Z., R.L. Tanguay, D. McKenzie, R.E. Peterson, and J.M. Aiken. (2003). Identification of a putative calcium-binding protein as a dioxin-responsive gene in zebrafish and rainbow trout. *Aquat Toxicol*. 63: 271-282.

Carninci, P., Y. Shibata, N. Hayatsu, Y. Sugahara, K. Shibata, M. Itoh, H. Konno, Y. Okazak, M. Muramatsu, and Y. Hayashizaki. (2000). Normalization and Subtractionof Cap-Trapper-Selected cDNAs to Prepare Full Length cDNA Libraries for Rapid Discovery of New Genes. *Genome Res*. 10: 1617–1630.

Chang, Y.-L., C. Standford, and J. Keller. (2000). Calcium and phosphate supplementation promotes bone cell mineralization: implications for hydroapatite (HA)-enhance bone formation. *J Biomed Mater Res*. 52: 270–278.

Cho, T.-J., and S.-S. Park. (1998). A simulation of subtractive hybridization. *Nucleic Acids Res.* 26: 1440–1448.

Christakos, S., and Y. Liu. (2004). Biological actions and mechanism of action of calbindin in the process of apoptosis. *J Steroid Biochem.* 89–90: 401–404.

Clark, M.S., L. Bendell, D.M. Power, S. Warner, G. Elgar, and P.M. Ingleton. (2002). Calcitonin: characterisation and expression in a teleost fish, Fugu rubripes. *J Mol Endocrinol.* 28: 111–123.

CLONTECH. (2002). Clontech PCR-Select TM cDA subtraction Kit user manual. BD Bisciences- CLONTECH.

Cooper, L., P. Yliheikkila, and S. Whitson. (1998). Spatiotemporal assesment of fetal bovine osteoblast culture indicates a role for BSP in promoting differentiation. *J Bone Miner Res.* 13: 620-632.

Croucher, P., and R. Russel. (1999). Growth factors. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 83-89.

Declercq, H., N.V.d. Vreken, E.D. Maeyer, R. Verbeeck, E. Schacht, L.D. Ridder, and M. Cornelissen. (2004). Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions:comparison of different isolation techniques and source. *Biomaterials.* 25: 757–768.

Denhardt, D., and M. Noda. (1998). Osteopontin expression and function: role in bone remodeling. *J Cell Biochem Suppls.* 30/31: 92-102.

Denhardt, D., M. Noda, A. O'Regan, D. Pavlin, and J. Berman. (2001). Osteopontin as a means to cope with environmental insults: regulation of inflamation, tissues remodeling, and cell survival. *J Clin Invest.* 107: 1055-1061.

Dey, R., H. -H. Son, and M. -I. Cho. (2001). Isolation and partial sequencing of potentially odontoblast-specific:enriched rat cDNA clones obtained by suppression subtractive hybridization. *Archives of Oral Biology.* 46: 249–260.

Diatchenko, L., Y. -F.C. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E.D. Sverdlov, and P.D. Siebert. (1996). Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA.* 93: 6025–6030.

Diella, F., S. Cameron, C. Gemünd, R. Linding, A. Via, B. Kuster, T. Sicheritz-Pontén, N. Blom, and T. Gibson. (2004). Phospho.ELM: A database of experimentally verified phosphorylation sites in eukaryotic proteins. *BMC Bioinformatics.* 5: 1-5.

Doi, M., A. Nagano, and Y. Nakamura. (2002). Genome-wide screening by cDNA microarray of genes associated with matrix mineralization by human mesenchymal stem cells in vitro. *Biochem Bioph Res Co.* 290: 381–390.

Donato, R. (1999). Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta.* 1450: 191-231.

Donato, R. (2001). S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol.* 33: 637–668.

Du, J. -J., K.-F. Dou, S.-Y. Peng, H.-S. Xiao, W.-Z. Wang, W.-X. Guan, Z.-H. Wang, Z.-Q. Gao, and Y.-B. Liu. (2003). cDNA suppression subtraction library for screening down-regulated genes in gastric carcinoma. *World J Gastroentero.* 9: 1439-1443.

Duarte, W., T. Shibata, K. Takenaga, E. Takahashi, K. Kaori, K. Ohya, I. Ishikawa, A. Yamaguchi, and S. Kasugai. (2003). S100A4: a novel negative regulator of mineralization and osteoblast diferentiation. *J Bone Miner Res.* 18: 493.

Ducy, P., and G. Karsenty. (2000). The family of bone morphogenetic proteins. *Kidney Intl.* 57: 2207-2214.

Ducy, P., T. Schinke, and G. Karsenty. (2000). The osteoblast: a sophisticated fibroblast under central surveillance. *Science.* 289: 1501-1504.

Faucheux, C., R. Bareille, and J. Amedee. (1998). Synthesis of calbindin-D28K during mineralization in human bone marrow stromal cells. *Biochem. J.* 333: 817±823.

Faustino, M., and D.M. Power. (1998). Development of osteological structures in the sea bream: vertebral column and caudal fin complex. *J. Fish Biol.* 52: 11-24.

Faustino, M., and D.M. Power. (2001). Osteologic development of theviscerocranial skeleton in sea bream: alternative ossification strategies in teleost fish. *J. Fish Biol.* 58: 537-572.

Ferland, G. (1998). The vitamin K-dependent proteins: an update. *Nutr Rev.* 56: 223-230.

Flanagan, J.A., L.A. Bendell, P.M. Guerreiro, M.S. Clark, D.M. Power, A.V.M. Canario, B.L. Brown, and P.M. Ingletona. (2002). Cloning of the cDNA for the putative calcium-sensing receptor and its tissue distribution in sea bream (*Sparus aurata*). *Gen Comp Endocr.* 127: 117–127.

Flick, G., and P. Verbost. (1993). Calcium transport in fish gills and intestine. *J exp. Biol.* 184: 17-29.

Fritz, G., and C. Heizmann. (2004). 3D structures of the calcium and zinc binding S100 proteins. *In* Handbook of metalloproteins. Vol. 3. W. Bode, A. Messerschmidt, and M. Cygler, editors. John Wiley & Sons, Ltd, Chichester. 529-540.

Furie, B., B. Bouchard, and C. Furie. (1999). Vitamin K-dependent bisynthesis of Y-carboxyglutamic acid. *Blood.* 93: 1798-1808.

Golub, E. (1996). Enzymes in mineralizing systems: state of the art. *Connect Tissue Res.* 35: 183-8.

Goodman, A., B. Goode, P. Matsudaira, and G. Fink. (2003). The *Saccharomyces cerevisiae* Calponin/Transgelin homolog Scp1 functions with fimbrin to regulate stability and organization of the actin cytoskeleton. *Mol Biol Cell.* 14: 2617–2629.

Gopalakrishnan, R., H. Ouyang, M. Somerman, L. McCauley, and R. Franceschi. (2001). Matrix Y-carboxyglutamic acid protein is a key regulator of PTH-mediated inhibition of mineralizationin MC3T3-E1 osteoblast-like cells. *Endocrinology.* 142: 4379-4388.

Grases, F., and A. Llobera. (1998). Experimental model to study sedimentary kidney stones. *Micron.* 29: 105-11.

Guerreiro, P., J. Fuentes, A. Canario, and D. Power. (2002). Calcium balance in seabream (*Sparua aurata*): the effect of oestradiol-17B. *J Endocrinol.* 173: 377-385.

Guerreiro, P.M., J. Fuentes, G. Flik, J. Rotllant, D.M. Power, and A.V.M. Canario. (2004). Water calcium concentration modifies whole-body calcium uptake in sea bream larvae during short-term adaptation to altered salinities. *J Expl Biol.* 207: 645-653.

Gundberg, C. (2003). Matrix proteins. *Osteoporos Int.* 14: S37–S42.

Hardingham, T. (1999). Proteoglycans and glycosaminoglycans. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 71-80.

Haudenschild, D.R., J.M. Mcpherson, R. Tubo, and F. Binette. (2001). Differential expression of multiple genes during articular chondrocyte redifferentiation. *Anat Rec.* 263: 91–98.

Heinegard, D., T. Saxne, and P. Lorenzo. (1999). Noncollagenous proteins: glycoproteins and related molecules. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 59-66.

Heizmann, C., G. Fritz, and B. Schafer. (2002). S100 proteins: stucture, functions and pathology. *Front Biosci.* 7: d1356-1368.

Heizmann, C., B. Schafer, and G. Fritz. (2003). The family of S100 Cell signaling proteins. *In* Handbook of cellular signalling. Vol. 2. R. Bradshaw and E. Dennis, editors. Copywright, Elsevier Science, USA. 87-93.

Helfrich, M., and M. Horton. (1999). Integrins and adhesion molecules. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 111-122.

Henthorn, P., J. Millán, and P. Leboy. (1999). Acid and alkaline phosphatases. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 127-146.

Horton, H., L. Moran, R. Ochs, J. Rawn, and K. Scrimgeour. 1996. Principles of biochemistry. Prentice Hall, New Jersey.

Hsiao, C.-D., M. Ekker, and H.-J. Tsai. (2003). Skin-specific expression of ictacalcin, a homolog of the S100 genes, during zebrafish embryogenesis. *Dev Dynam.* 228: 745-750.

Huang, W., B. Carlsen, G. Rudkin, M. Berry, K. Ishida, D.T. Yamaguchi, and T.A. Millera. (2004). Osteopontin is a negative regulator of proliferation and differentiation in MC3T3-E1 pre-osteoblastic cells. *Bone.* 34: 799–808.

Hunter, G., P. Hauschka, A. Poole, L. rosenberg, and H. Goldberg. (1996). Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. *Biochem J.* 317: 59-64.

Ishii, T., S. Ohshima, T. Ishida, T. Mima, Y. Tabunoki, H. Kobayashi, M. Maeda, T. Uede, L. Liaw, N. Kinoshita, I. Kawase, and Y. Saeki. (2004). Osteopontin as a positive regulator in the osteoclastogenesis of arthritis. *Biochem Bioph Res Co.* 316: 809–815.

Jong, D.S.d., B.L. Vaes, K.J. Dechering, A. Feijen, J.M. Hendriks, R. Wehrens, C.L. Mummery, E.J.v. Zoelen, W. Olijve, and W.T. Steegenga. (2004). Identification of novel regulators associated with early-phase osteoblast differentiation. *J Bone Miner Res* .19: 947–958.

Kales, S., K. Fujiki, and B. Dixon. (2004). Molecular cloning and characterization of calreticulin rainbow trout (*Onchorhynchus mykiss*). *Immunognetics.* 55: 717-723.

Karsenty, G. (1999). The genetic transformation of bone biology. *Genes & Development.* 13: 3037–3051.

Karsenty, G. (2000). Bone formation and factors affecting this process. *Matrix Biology.* 19: 85-89.

Karsenty, G. (2003). The complexities of skeletal biology. *Nature.* 423: 316-318.

Karsenty, G., and E. Wagner. (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev Cell.* 2: 389-406.

Kasus-Jacobi, A., J. Ou, Y. Bashmakov, J. Shelton, J. Richardson, J. Goldstein, and M. Brown. (2003). Characterization of mouse short-chain aldehyde reductase (SCALD), an enzyme regulated by sterol regulatory element-binding proteins. *J Biol Chem.* 278: 32380–32389.

Kato, Y., J. Windle, B. Koop, G. Mundy, and L.F. Bonewald. (1997). Establishment of an osteocyte cell line, MLO-Y4. *J Bone Miner Res.* 12: 2014-2023.

Kiss, C., J. Nishikawa, A. Dieckmann, K. Takada, G. Klein, and L. Szekely. (2003). Improved subtractive suppression hybridization combined with high density cDNA array screening identifies differentially expressed viral and cellular genes. *J Virol Methods.* 107: 195-203.

Kobori, M., Y. Ikeda, H. Nara, M. Kato, M. Kumegawa, H. Nojima, and H. Kawashima. (1998). Large scale isolation of osteoclast-specific genes by an improved method involving the preparation of a subtracted cDNA library. *Genes to Cells.* 3: 459-475.

Koltzscher, M., C. Neumann, S. Konig, and V. Gerke. (2003). Ca2+ -dependent binding and activation of dormant ezrin by dimeric S100P. *Mol Biol Cell.* 14: 2372-2384.

Lerner, U.H. (2000). Osteoclast formation and resorption. *Matrix Biology.* 19: 107-120.

Lian, J., and G. Stein. (1999). The cells of bone. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 165-177.

Loveridge, N. (1999). Bone: More Than a Stick. *J. Anim. Sci.* 77: 190-196.

Mackie, E. (2003). Osteoblasts: novel roles in orchestration of skeletal architecture. *Int J Biochem Cell B.* 35: 1301–1305.

Maeda, T., A. Matsunuma, I. Kurahashi, T. Yanagawa, and H. Yoshida. (2004). Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells. *J Cell Biochemy.* 92: 458-471.

Manso, M., M. Becerra, M. Becerra, and R. Anadón. (1997). Expression of a low-molecular weight (10kDa) calcium binding protein in glial cells of the brain of the trout (Teleostei). *Anat Embryol.* 196: 403416.

Marie, P. (2001). Différenciation, fonction et contrôle de l'ostéoblaste. *m/s Synthése.* 17: 1252-1259.

Marie, P. (2003). Fibroblast growth factor signaling controllong osteoblast differentiation. *Gene.* 316: 23-32.

Marin, F.r., P. Corstjens, B.d. Gaulejac, E.d.V.-D. Jong, and P. Westbroek. (2000). Mucins and Molluscan Calcification. *J Biol Chem.* 275: 20667–20675.

Marks, S.C., and P.R. Odgren. (2002). Structure and Development of the Skeleton. *In* Principles in Bone Biology. Vol. 1. J. Bilezekian, L. Raisz, and G. Rodan, editors. Academic Press, New York. 3-15.

Martin, S.A.M., S. Blaney, A.S. Bowman, and D.F. Houlihan. (2002). Ubiquitin-proteasome-dependent proteolysis in rainbow trout (Oncorhynchus mykiss): effect of food deprivation. *European J Physiol* 445(2): 257-66.

Miyazono, K. (2000). Positive and negative regulation of TGF-b signaling. *J Cell Science.* 113: 1101-1109.

Mochida, K., K. Fujii, A. Kakuno, T. Matsubara, N. Ohkubo, S. Adachi, and K. Yamauchi. (2003). Expression of ubiquitin C-terminal hydrolase is regulated by estradiol-17â in testis and brain of the Japanese common goby. *Fish Physiol Biochem.* 28: 435–436.

Mundy, G., M.D.D. Chen, M. Zhao, S. Dallas, C. Xu, and S. Harris. (2001). Growth regulatory factors and bone. *Rev Endocr Metab Dis.* 2: 105±115.

Munir, S., S. Singh, K. Kaur, and V. Kapur. (2004). Suppression subtractive hybridization coupled with microarray analysis to examine differential expression of genes in virus infected cells. *Biol. Proced. Online.* 6: 94-104.

Murshed, M., T. Schinke, M. McKee, and G. Karsenty. (2004). Extracellular matrix mineralization in regulated locally; different roles of two gla-containig proteins. *J Cell Biology*. 165: 625-630.

Nakajima, I., S. Muroya, R. -I. Tanabe, and K. Chikuni. (2002). Extracellular matrix development during differentiation into adipocytes with a unique increase in type V and VI collagen. *Biol Cell* 94: 197–203.

Nakashima, K., and B.d. Combrugghe. (2003). Transcriptional mechanisms in osteoblast differentiation and bone formation. *TRENDS in Genetics*. 19: 458-466.

Nielsen, H., S. Brunak, and G.v. Heijne. (1999). Machine learning approaches for the prediction of signal peptides and other protein sorting signals. *Protein Eng*. 12: 3–9.

Nielsen, H., J. Engelbrecht, S. Brunak, and G.v. Heijne. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng*. 10: 1–6

Offner, G.D., D.P. Nunes, A.C. Keates, N.H. Afdhal, and R.F. Troxler. (1998). The Amino-Terminal Sequence of MUC5B Contains Conserved Multifunctional D Domains: Implications for Tissue-Specific Mucin Functions. *Biochem Biophys Res Co*. 251: 350–355.

Pinto, J., M. Ohresser, and M. Cancela. (2001). Cloning of the bone Gla protein gene from the teleost fish Sparus aurata. evidence for overall conservation in gene organization and bone-specific expression from fish to man. *Bone*. 270: 77-91.

Pinto, J.P., N. Conceição, P.J. Gavaia, and M.L. Cancela. (2003). Matrix Gla protein gene expression and protein accumulation colocalize with cartilage distribution during development of the teleost fish Sparus aurata. *Bone*. 32: 201–210.

Pombinho, A.R., V. Laizé, D.M. Molha, S.M.P. Marques, and M.L. Cancela. (2004). Development of two bone-derived cell lines from the marine teleost Sparus aurata; evidence for extracellular matrix mineralization and cell-type-specific expression of matrix Gla protein and osteocalcin. *Cell Tissue Res*. 315: 393-406.

Porta, A., E. Bettini, O. Buiakova, and H. Bakker. (1996). Molecular cloning of ictacalcin: a novel calcium-binding protein from the cannel catfish, *Ictalurus punctatus*. *Mol Brain Res*. 41: 81-89.

Qin, Y. -M., M. Poutanen, and D. Novikov. (2000). Substrate specificities of peroxisomal members of short-chain alcohol dehydrogenase superfamily: expression and characterization of dehydrogenase part of *Candida tropicalis* multifunctional enzyme. *J. Lipid Res*. 41: 93–98.

Raouf, A., and A. Seth. (2002). Discovery of osteoblast-associated genes using cDNA microarray. *Bone*. 30: 463-471.

Raynal, B.D.E., T.E. Hardingham, J.K. Sheehan, and D.J. Thornton. (2003). Calcium-dependent protein interactions in MUC5B provide reversible cross-links in salivary mucus. *J Biol Chem*. 278: 28703–28710.

Rebrikov, D., O.V. Britanova, N.G. Gurskaya, K.A. Lukyanov, V.S. Tarabykin, and S.A. Lukyanov. (2000). Mirror Orientation Selection (MOS): a method for eliminating false positive clones from libraries generated by suppression subtractive libraries. *Nucleic Acids Res.* 28: 1-4.

Rochet, N., A. Loubat, J. -P. Laugier, P. Hofman, J. Bouler, G. Daculsi, G. Carle, and B. Rossi. (2003). Modification of gene expression induced in human osteogenic and osteosarcoma cells by culture on a biphaisc calcium phosphate bone substitute. *Bone.* 32: 602-610.

Safadi, F., J. Xu, S.L. Smock, R. Kanaann, A.-H. Selim, P. Odgreen, J. SC Marks, T.A. Owen, and S. Popoff. (2003). Expression of connective tissue growth factor in bone: Its role in osteoblast proliferation and differentiation in vitro and bone formation in vivo. *J. Cell. Physiol.* 196: 51–62.

Safadi, F.F., J. Xu, S.L. Smock, M.C. Rico, T.A. Owen, and S.N. Popoff. (2002). Cloning and characterization of osteoactivin, a novel cDNA expressed in osteoblasts. *J Cell Biochemy.* 84: 12±26.

Sakuma, T., Y. Higashibata, H. Kawahata, S. Yamada, M. Okabe, Y. Kitamura, and S. Nomura. (2003). Difference of osteopontin gene regulation between bone and kidney. *J Orthop Sci.* 8: 361–366.

Schinke, T., M. McKee, and G. Karsenty. (1999). Extracellular matrix calcification: where is the action? N*ature genetics supplement.* 21: 150-151.

Seibel, M., and S. Robins. (1999). Vitamin K-dependent proteins of bone and cartilage. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 43-53.

Simes, D., M. Williamson, J. Ortiz-Delgado, C. Viegas, P. Price, and M. Cancela. (2003). Purification of matrix Gla protein from a marine teleost fish, Argyrosomus regius: calcified cartilage and not bone as the primary site of MGP accumulation in fish. *J Bone Miner Res.* 18: 244-259.

Sommer, B., M. Bickel, W. Hofstetter, and A. Wetterwald. (1996). Expression of matrix proteins during the development of mineralized tissues. *Bone.* 19:371-380.

Sommerfeldt, D.W., and C.T. Rubin. (2001). Biology of bone and how it orchestrates the form and function of the skeleton. *Eur Spine J.* 10:S86–S95.

Somogyi, E., U. Petersson, K. Hultenby, and M. Wendel. (2003). Calreticulin-an endoplasmatic reticulum protein with calcium-binding activity is also found in the extracellular matrix. *Matrix Biology.* 22: 179-191.

Standford, C., P. Jacobson, E. Eanes, and L. Lembke. (1995). Rapidilly forming apatitic in an osteoblastic cell line (UMR 106-01 BSP). *J Biol Chem.* 270: 9420-9428.

Sugawara, Y., K. Suzuki, M. Koshikawa, and J. Iida. (2002). Necessity of enzimatic activity of alkaline phosphatase for mineralization of osteoblastic cells. *Jpn. J. Pharmacol.* 88: 262-269.

Swaminathan, R. (2001). Biochemical markers of bone turnover. *Clin Chim Acta.* 313: 95-105.

Tate, M.L.K., J.R. Adamson, A.E. Tami, and T.W. Bauer. (2004). The osteocyte. *Int J Biochem Cell B.* 36: 1–8.

Tsoi, S.C.M., K.V. Ewart, S. Penny, K. Melville, R.S. Liebscher, L.L. Brown, and S.E. Douglas. (2004). Identification of Immune-Relevant Genes from Atlantic Salmon Using Suppression Subtractive Hybridization. *Mar. Biotechnol.* 6: 199–214.

Tuan, R., and W.A. Scott. (1977). Calcium-binding protein of chorioallantoic membrane: Identification and developmental expression. *Proc. Natl. Acad. Sci. USA*. 74: 1946-1949.

von DER Mark, K. (1999). Structure and biosynthesis of collagens. *In* Dynamics of bone and cartilage metabolism. M. Seibel, S. Robins, and J. Bilezikian, editors. Academic Press, Florida. 3-18.

Wagner, T.U., J. Ret-m, T. Riemensperger, J.-N. Volff, R.W. Koster, R. Goerlich, M. Schartl, and C. Winkle. (2003). The Teleost Fish Medaka (*Oryzias latipes*) as a genetic model to study gravity dependent bone homeostasis *in vivo*. *Adv. Space.Res.* 32:1459-1465.

Wang, Z., and D. Brown. (1991). A gene expression screen. *Proc. Natl. Acad. Sci. USA*. 88:11505-11509.

Weiss, R., and N. Watabe. (1979). Studies on the biology of fish bone. III. Ultrastructure of osteogenesis and resorption in osteocytic (cellular) and anosteocytic (acellular) bones. *Calcif Tissue Int.* 28:43-56.

Witten, P., A. Hansen, and B. Hall. (2001). Features of Mono- and Multinucleated Bone Resorbing Cells of the Zebrafish Danio rerio and Their Contribution to Skeletal Development, Remodeling, and Growth. *J Morphol.* 250:197–207.

Wong, C.C., W.H. Poon, T.Y. Tsim, E.Y.K. Wong, and M.S. Leung. (2000). Gene expressions during the development and sexual differentiation of the olfactory bulb in rats. *Dev Brain Res* 119:187–194.

Yamaguchi, A., T. Komori, and T. Suda. (2000). Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocr Rev*. 21:393-411.

Yang, X.-Y., J.-H. Yao, L. Cheng, D.-W. Wei, J.-L. Xue, and D.-R. Lu. (2003). Molecular cloning and expression of a smooth muscle-specific gene SM22a in zebrafish. *Biochem Biophys Res Co*. 312:741–746.

Young, M.F. (2003). Bone matrix proteins: their function, regulation, and relationship to osteoporosis. *Osteoporos Int*. 14:S35–S42.

Zimmer, D., P. Sadosky, and D. Weber. (2003). Molecular mechanisms of S100-target protein interactions. *Microsc Res Tech*. 60:552-559.

Zubay, G. 1993. Biochemistry. Wm C Brown Communications, Inc., Dubuque.

APPENDIXS

APPENDIX I: solutions and protocols

I.1- Bacteria

- IPTG 200 µg/ml

IPTG	0.002 g
H_2O	up to 10 ml

Filter-sterilize and store at -20°C.

- X-Gal 20 mg/ml

X-Gal	0.100 g
DMF	up to 5 ml

Protect from light with aluminium foil.

Store at -20°C.

- LB medium (agar)

Agar	15 g
LB broth (Sigma)	20 g

Adjust volume with 1 l of ddH_20.

Autoclave 120°C, 20 min

Add 50 mg/l of ampicillin after cooling.

- LB agar plates

Supplement LB medium agar with 0.5 mM IPTG and 80 µg/ml X-Gal.

Pour 20 ml of LB medium agar into 100-mm Petri dishes.

Let the agar harden.

Store at 4°C.

- SOC medium

LB broth medium (Sigma)	1000 µl
Glucose (2 M)	10µl
$MgCl_2$ (2 M)	10 µl

$MgSO_4$ (2 M)	10 µl

I.2- Cell culture

- EDTA 0,5 M

EDTA	18.61 g
ddH_20	80 ml

Adjust pH to 8 with NaOH and volume to 100 ml of ddH_20.

- Solution T

NaCl (137 mM)	4 g
KCl (2.7 mM)	0.1 g
Na_2HPO_4 (15.8 mM)	1.4 g
KH_2PO_4 (1.23 mM)	0.1 g
EDTA (1.1 mM)	0.2 g
Trypsin (0.2%)	1 g
ddH_2O	450 ml

Adjust pH to 7.4 and volume to 500 ml with double distilled

Filter with a 0.2 µm filter. Store in aliquots at -20°C.

- PBS 10 x

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Adjust pH to 7.4 and add water to a final volume of one litter.

Filter with a 0.2 µm filter and autoclave 120°C, 20 min.

- Mineralizing medium (MM)

[Vit C final] = 50 µg/ml in water

[β-GP final] = 10 mM in water

[$CaCl_2$ final] = 4 mM in water

Add 5 µl of each agent per ml of medium.

- D-MEM (500 ml)

Penicillin (1 %)	5 ml
Fungizone (0.2 %)	1 ml
L-glutamine (1 %)	5 ml
FBS (10 %)	50 ml

I.3- RNA extraction

- Guanidine isothiocyanate

Guanidine isothiocyanate	250 g
DEPC treated water	293 ml
0.75 M sodium citrate, pH 7.0	17.6 ml
10% N-lauroyl sarcosine	26.4 ml

Stir at 65°C.

Filter with a 0.2 µm filter.

Aliquot in 50 ml tubes.

- Solution D

Guanidine isothiocyanate	50 ml
2-mercaptoethanol	360 µl

Store at 4°C.

I.4- Electrophoresis and Transfers

- DEPC treated water

DEPC	1000 µl
ddH_20	1000 ml

Leave stirring overnight at 37°C.

Autoclave 120°C, 20 min.

- 50x Tris-acetate (TAE)

Tris base (40 mM)	242 g
Glacial acetic acid (40 mM)	57.1 ml
EDTA (0.5 M)	100 ml
ddH2O	1 l

Adjust to pH 8.0

- 10X MOPS

MOPS	20.9 g
Anhydrous sodium acetate	2.05 g
EDTA (0.4 M)	12.5 ml

Adjust volume to 500 ml with double distilled water.

Adjust to pH 7.0 with 5 N NaOH.

- 20x SSC

NaCl (3 M)	175 g
Na-citrate (0.3M)	88.2 g
ddH_2O	1 l

Adjust to pH 7.

-10x SDS

SDS	100 g

Add 1 l of ddH_2O and stir until dissolved.

- RNA gel (1.2%)

Agarose	1.2 g
DEPC water	87 ml
10x MOPS	10 ml
Formaldehyde 37%	3 ml

Dissolve by boiling without the formaldehyde.

Add the formaldehyde after cooling.

- DNA gel (2%)

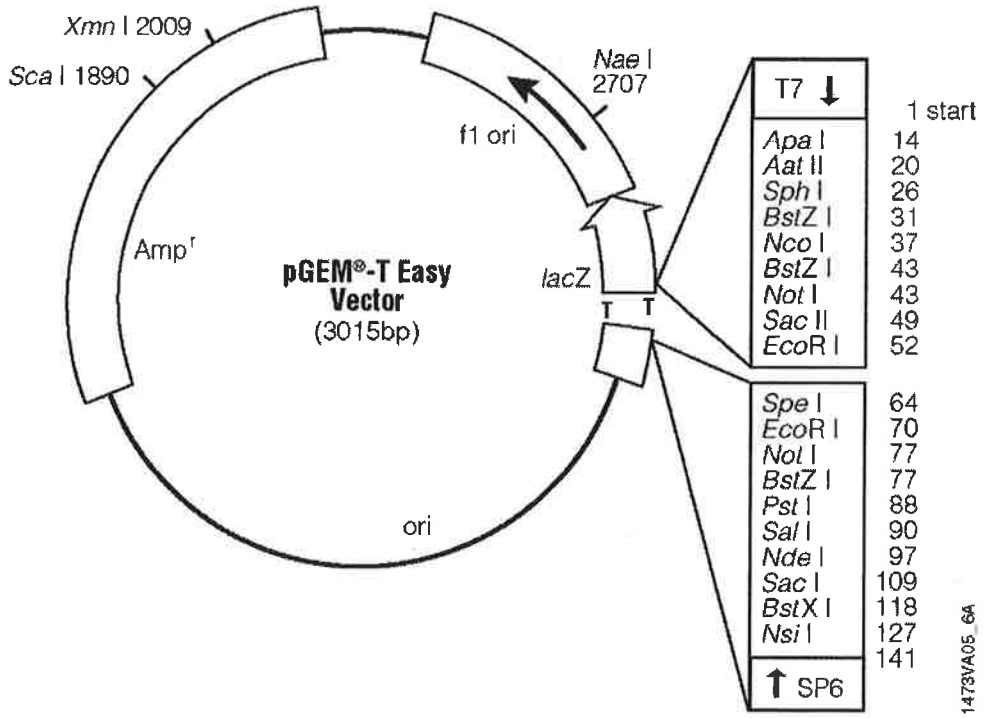
Agarose	1.4 g
1x TAE	70 ml
Ethidum bromide (1 mg/µl)	7 ml

Dissolve by boiling without the EtBr.

Add the EtBr after cooling.

APPENDIX II: pGEM-T Easy vector

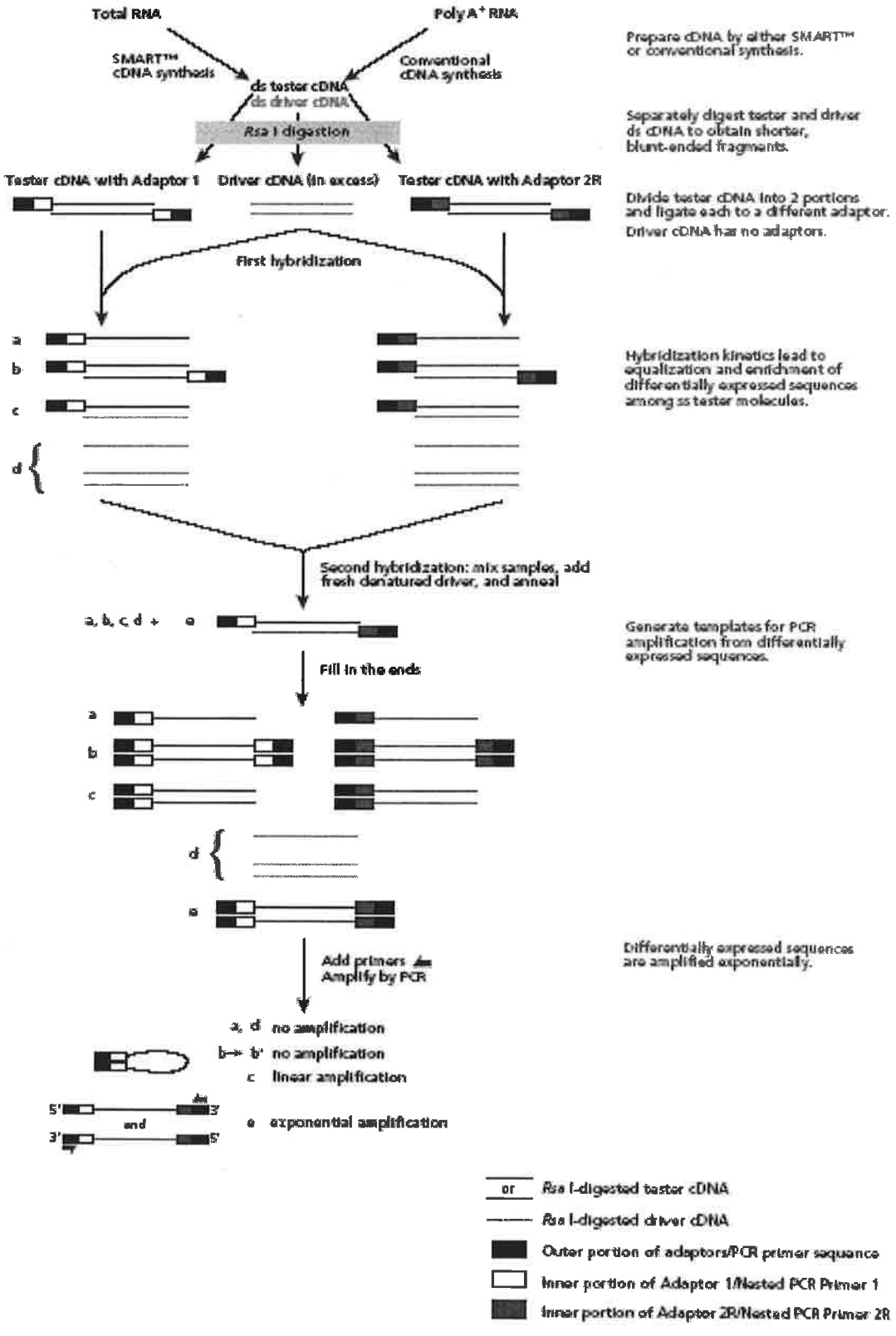
pGEM-T Easy vector sequence map (Promega)



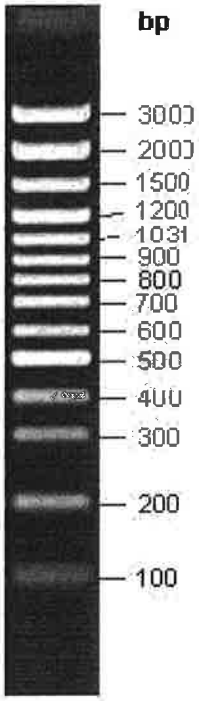
Appendix III: primers

P1	5'- CTA ATA CGA CTC ACT ATA GGG C-3'
NP1	5' - TCG AGC GGC CGC CCG GGC AGG T-3'
NP2R	5'- AGC GTG GTC GCG GCC GAG GT-3'
NP2Rs	5'- GGT CGC GGC CGA GGT-3'
AP1	5'- CCA TCC TAA TAC GAC TCA CTA TAG GGC-3'
AP2	5'- ACT CAC TAT AGG GCT CGA GCG GC-3'

Appendix IV: PCR-select cDNA subtraction technique

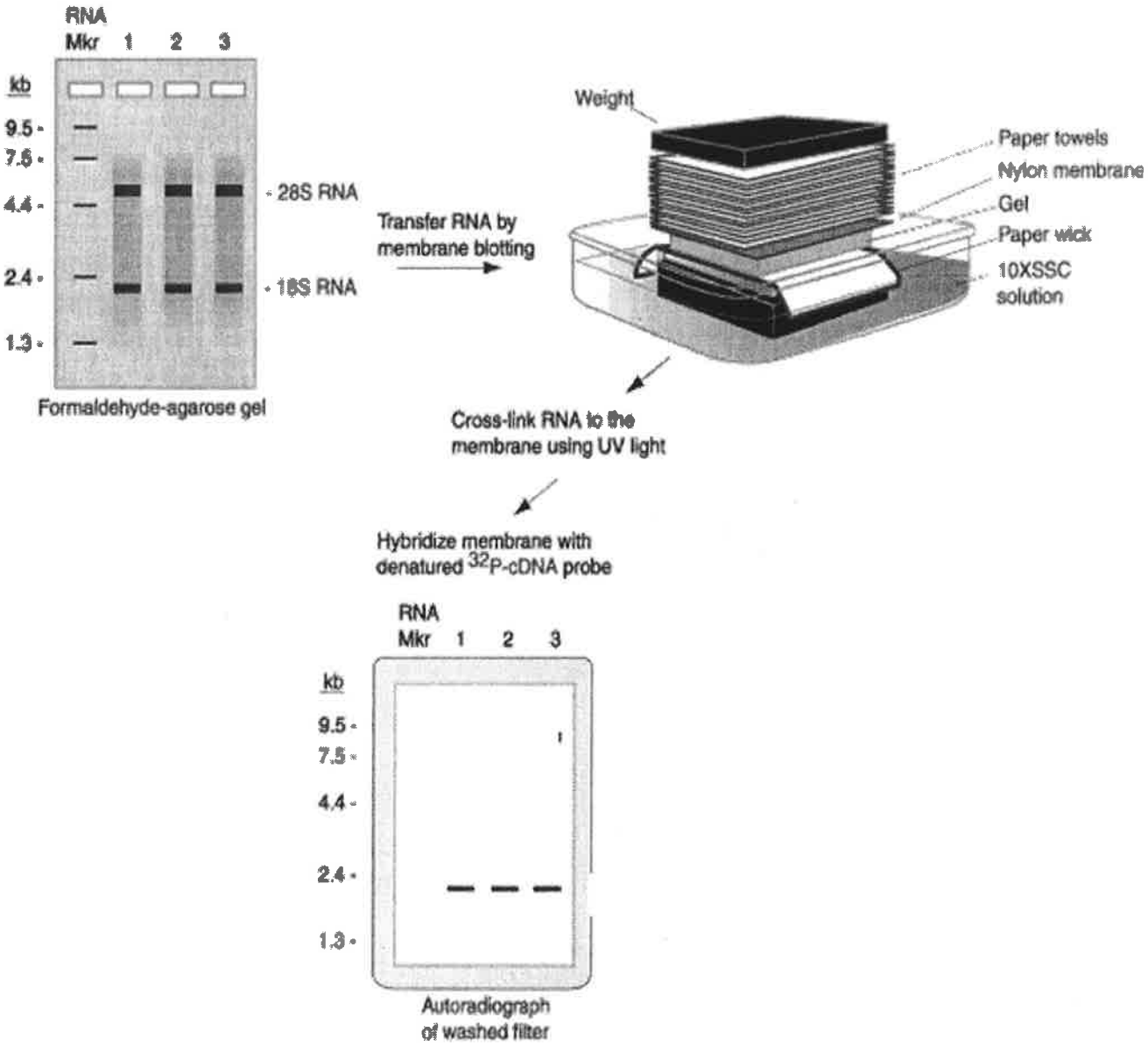


Appendix V: DNA ladder



GeneRuler™ 100bp DNA Ladder Plus- Fermentas

Appendix VI: Northern blot procedure



Appendix VII: articles and communications concerning this thesis

Published articles and communications:

Fonseca VG, Lago-Lestón A, Laizé V, Cancela ML. Rapid identification of differentially expressed genes by *in situ* screening of bacteria. Molecular Biotechnology (in press).

Fonseca VG, Laizé V, Cancela ML. Up-regulated genes during *in vitro* mineralization of fish osteoblast-like cells: Cloning and characterization of *Sparus aurata* osteopontin. XIVth Biochemistry National Congress. Vilamoura, 2-4 of December 2004.

Unpublished articles:

Fonseca VG, Laizé V, Cancela ML. Up-regulated genes during mineralization of *Sparus aurata* osteoblast-like cell line: Evidences for osteopontin as a key protein for tissue mineralization in fish (in prep).

Fonseca VG, Laizé V, Cancela ML. Molecular cloning of *Sparus aurata* S100-like calcium binding protein: Expression in mineralized fish bone-derived cell lines and tissues (in prep).