Cells Isolated from Regenerating Caudal Fin of *Sparus aurata* Can Differentiate into Distinct Bone Cell Lineages

Parameswaran Vijayakumar[1,2*] vijayparamesh@gmail.com,
João Cardeira[1,3],
Vincent Laizé[1],
Paulo J. Gavaia[1],
M. Leonor Cancela[1,4*] lcancela@ualg.pt

[1]Centre of Marine Sciences (CCMAR)
University of Algarve
Campus de Gambelas, 8005-139 Faro, Portugal
[2]Centre for Ocean Research
Sathyabama Institute of Science and Technology
Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai, 600 119, Tamil ~~n~~Nadu, India
[3]Department of Developmental Genetics
Max Planck Institute for Heart and Lung Research
61231, Bad Nauheim, Germany
[4]Department of Biomedical Sciences and Medicine (DCBM) and Algarve Biomedical Center (ABC)
University of Algarve
Campus de Gambelas, 8005-139, Faro, Portugal

Abstract

Teleosts have the ability to regenerate their caudal fin upon amputation. A highly proliferative mass of undifferentiated cells called blastema forms beneath wound epidermis and differentiates to regenerate all missing parts of the fin. To date, the origin and fate of the blastema cells remain unclear. However current hypothesis suggests that the blastema comprises of lineage restricted cells those arise by de-differentiation. To investigate the mechanisms of cell differentiation of caudal fin regeneration, primary cultures were initiated from the explants of 2 -days post-

amputated (dpa) regenerating caudal fin (which consists of predominately blastema and wound epidermis cells) of juvenile gilthead seabream (*Sparus aurata*). These cells were subcultured for over 30 passages and were named as BSa2. After 10th passage, they were characterized by their ability to differentiate towards bone cell lineages, by performing extracellular matrix mineralization through immunocytochemistry, histology, and RT-PCR. Exogenous DNA was efficiently delivered into these cells by nucleofection. Assessment of lineage-specific markers revealed that BSa2 cells were capable of osteo/chondroblastic differentiation. BSa2 cells were also found to be capable of osteoclastic differentiation, as demonstrated through TRAP-specific staining and pit resorption assay. The process of fin regeneration was found to be complete at 14 dpa and de novo bone formation started at 6 dpa. Thus we developed the first successful cell line viz., BSa2, from *S. aurata* 2 dpa regenerating caudal fin with the ability of multilineage differentiation and capable of in vitro mineralization. The availability of such in vitro cell systems will stimulate research on the mechanisms of cell differentiation during fin regeneration and provide new insights into the mechanisms of bone formation.

Keywords

Blastema

Caudal fin

Regeneration

Cell line

Seabream

Differentiation

Introduction

Epimorphic regeneration is a dynamic developmental process involved in reconstruction of organ or tissues upon amputation or injury. In mammals, the ability to regenerate is limited but can be seen in some structures, including the liver, skin, skeletal muscle, and digit tips (Illingworth 1974; Muller et al. 1999; Han et al. 2005; Carlson 2005; Hata et al. 2007; Yoshizato 2007). In contrast, tremendous regenerative capabilities can be seen in urodele amphibians and in teleost fish. These organisms regenerate their limbs, fins, heart, spinal cord, scales, eye lens, and

many internal organs (reviewed by Akimenko et al. 2003; Nye et al. 2003; Poss et al. 2003; Slack 2003; Nakatani et al. 2007; Gemberling et al. 2013; Tornini and Poss 2014; Pfefferli and Jaźwińska 2015; Watson and Kwon 2015; Wehner and Weidinger 2015; Sehring and Weidinger 2019). In particular, zebrafish and medaka models have been used intensely to study the process of fin regeneration (Katogi et al. 2004; Whitehead et al. 2005; Nishidate et al. 2007; Lee et al. 2009; Yoshinari et al. 2009; Grotek et al. 2013; Petrie et al. 2014; Blum and Begemann 2015; Owlarn et al. 2017; König et al. 2018; Dasyani et al. 2019). The process of fin regeneration starts by forming a thickened wound epidermis followed by formation of the blastema, a mass of highly proliferative, undifferentiated tissue responsible for the reconstitution of the lost fin (Brockes and Kumar 2005; Stoick-Cooper et al. 2007). Many studies have suggested that differentiated cells de-differentiate and contribute to blastema formation~~,~~ but remain lineage committed (Knopf et al. 2011; Sousa et al. 2011; Tu and Johnson 2011; Stewart and Stankunas 2012). Many in vivo studies were performed on blastema to better understand the molecular and genetic processes involved in fin regeneration in teleost (Akimenko et al. 2003; Poss et al. 2003; Blum and Begemann 2012; Tornini et al. 2016) and on the origin and differentiation of the osteoblastic cells responsible for regenerating skeletal elements (Singh et al. 2012). But, on the other hand, in vitro studies to investigate these processes have been hampered due to the absence of a suitable cell culture system from teleost. Thus, appropriate in vitro cell systems are required for better understanding the processes leading to cell differentiation during fin regeneration.

In the present study, we used gilthead seabream (*Sparus aurata*) as a model teleost, a well-established model for marine fish which is now increasingly used due to the growing availability of a number of genomic and transcriptomic sequencing data, as well as microarrays and other molecular tools. Furthermore, *S. aurata* is an important species for fisheries and aquaculture in the Mediterranean basin and in southern Portugal, and thus, it is not only of economic relevance to understand the mechanisms of regeneration in this fish, but also no specific studies are available on this subject for this species. Here, we report for the first time the process of fin regeneration in *S. aurata*. Further we describe the establishment and initial characterization of a new cell line derived from 2 dpa regenerating caudal fin of *S. aurata*, ~~and~~ designated as BSa2. Cells from this culture can be triggered to differentiate towards osteoblastic, chondrocytic, and osteoclastic cell lineages, capable of in vitro mineralization and represent a valuable tool for fin regeneration studies in teleost.

Materials and Methods

Maintenance, Fin Amputation, and Regeneration Analysis

Nine healthy juvenile gilthead seabream (*S. aurata*) (8–10 g in weight) were collected from Ramalhete rearing facilities at CCMAR-University of Algarve and maintained in 100-L water tanks with strong aeration (100 mL min^{-1}), at a temperature of 22 °C and fed twice a day with artificial food (Ibersoja 3 mm). On day 0, fish were rapidly anesthetized with 100 ppm of 2-phenoxyethanol in seawater. After transferring anesthetized fish onto an inverted Petri dish placed on the stage of a stereomicroscope and carefully flattening the caudal fin, the bottom half of the caudal fin was amputated two segments before the cleft using a sterile scalpel (Fig. 1), and then the fish quickly recovered in sea water and transferred back to their tanks. The regenerating area of the caudal fin was photographed at different time points (0 to 14 days) using a stereomicroscope (Leica MZ 6) equipped with a digital camera. Re-amputation of regenerating fins was carried at different time points (0 to 14 days post-amputation) using the same procedure as above. Re-amputated fins were used for further analysis.

Detection of Mineral in *S. aurata* Regenerating Caudal Fin

Samples (bottom half of each caudal fin) were fixed in 4% buffered paraformaldehyde, washed in PBS, and stained with alizarin red S (C.I. 58005, Sigma), as previously described by Gavaia et al. (2000). The tissue was incubated in 1% KOH, 1.5% H_2O_2 for 30 min to remove pigmentation and gradually transferred to glycerol for preservation. Photographs were taken under a fluorescence stereomicroscope Leica MZ 7.5 (Excitation 530–560–emission 580) equipped with an F-View camera.

Histological Sections

Samples were embedded in paraffin (Panreac) and sectioned longitudinally in a Microm 340 M microtome (Microm, Germany). Sections of 5 µm were stained with Azan combination solution (Chroma, Muenster—Germany) according to the manufacturer's instructions. Slides were mounted in DPX (Sigma) medium, analyzed and photographed in an Axioimager Z2 microscope equipped with an AxioCam ICc3 digital camera (Zeiss, Germany).

Development of 2 Ddpa Regenerating Caudal Fin Cells from *S. aurata*

At 2 and 4 dpa, 3 fishes were ~~anaesthetized~~anesthetized, and their caudal fins were rinsed with 70% ethanol for surface contaminants. Subsequently the regenerated fins of the 3 fishes were re-amputated to collect the cells (please refer the detailed procedure for fin amputation above). Cells were washed 2× with Leibovitz's L-15 medium supplemented with 1% antibiotics (500 IU/ml of penicillin and 500 µg/ml of streptomycin), then minced into small fragments (approximately 1–2 mm in size) and placed into 35-mm cell culture dishes containing 500 µl of L-15 medium supplemented with 15% FBS, 1% antibiotic, and 1% fungizone. Cell culture dishes were incubated at 28 °C with culture medium replaced once in 3 days. Confluent cultures were sub-cultured using trypsin-EDTA solution and seeded into a 6-well plate containing 2 ml of L-15 medium. Cell cultures were routinely sub-cultured (1:2) at confluence by trypsinization.

Cryopreservation

BSa2 cells from subconfluent cultures were trypsinized, harvested by centrifugation (2000 × ~~×~~ *g* for 5 min) and resuspended at a density of 10^6 cells/ml in ice-cold medium supplemented with 10% cell culture grade dimethyl sulphoxide (Sigma-Aldrich, St. Louis, MO). Cell suspensions were dispensed into 2-ml cryotubes (Sarstedt), placed at – 80 °C in a cell freezing device (Mr. frosty, Nalgene, Rochester, NY) overnight and then transferred into liquid nitrogen. Frozen cells were recovered in 9 ml of culture medium and placed in a flask at 28 °C. Medium was renewed after 8 h and viability was tested using the trypan blue dye exclusion method (Strober 2001).

Cell Proliferation

Proliferation of BSa2 cells was determined at passage 14 using different FBS concentrations by the CellTiter 96 non-radioactive proliferation assay kit (Promega, Madison, WI) according to manufacturer's instructions. Briefly, the cells were seeded at a density of 1.5 × 10^3 cells/well in 96-well plates. At appropriate times, cells were incubated for 2 h with 20 µl of reagent mixture and then cell proliferation was determined from absorbance measurements at 490 nm using spectrophotometer.

Transfection

Transfection efficiency of BSa2 cells was determined by using pEGFP-N1 (Clontech, Mountain View, CA) and pmaxGFP (Lonza, Cologne, Germany) vectors expressing the green fluorescent protein (GFP) under the control of CMV promoter. BSa2 cells were seeded at a density of 5 × 10^5 cells/well in 6-well plates and cultured until sub-confluence. pEGFP-N1 vector (1 µg)

was delivered into cells using FuGENE6 or FuGENE HD (both from Roche, Mannheim, Germany) according to manufacturer's instructions, or through calcium-phosphate coprecipitation (Pfitzner et al. 1995); pmaxGFP vector was delivered into BSa2 cells through nucleofection. In this case, 6 × 10^5 cells were resuspended in 100 µl of buffer L or V (Nucleofection optimization kit, Lonza) and nucleofected with 2 µg of pmaxGFP vector using Amaxa Nucleofector (Lonza). Cells were seeded into 6-well plates and incubated for 6 h at 28 °C. Culture medium was changed 48 h after transfection and 6 h after nucleofection, and the percentage of GFP positive cells was determined by flow cytometry (BD Biosciences, San Jose, CA).

Alkaline Phosphatase Activity

BSa2 cells were seeded at the density of 3 × 10^5 cells/well in 6-well plates and cultured in medium supplemented with 10 mM β-glycerophosphate, 4 mM calcium chloride, and 50 µg/ml of ascorbic acid or left untreated (control), and supplemented medium was renewed twice a week. After 3 weeks of treatment, cells were washed with PBS, fixed in 1% (v/v) glutaraldehyde solution (prepared in PBS) for 10 min, washed twice with PBS, and then incubated in the dark in a buffer (100 mM Tris-HCl [pH 9.5], 50 mM MgCl2, 100 mM NaCl, 0.1% Tween20) containing bromo-chloro-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT, Sigma-Aldrich). The development of a blue signal indicative of alkaline phosphatase (AP) activity was observed by microscopy. After 1 week of treatment, the ALP activity was also measured in cell extracts by spectrophotometry according to the method described by Tiago et al. (2008).

ECM Mineralization

Confluent BSa2 cell cultures were placed in medium supplemented with 10 mM β-glycerophosphate, 4 mM calcium chloride, and 50 µg/ml of ascorbic acid or left untreated (control). Supplemented medium was renewed twice a week. After 3 weeks of treatment, levels of mineralized ECM were assessed through Alizarin Red S staining (40 mM at pH 4.2; Sigma-Aldrich) and von Kossa's silver nitrate staining (Bills et al. 1974). For alizarin red S (AR-S) staining, the cells were washed three times with PBS at room temperature, fixed with 4% formaldehyde (in PBS) for 1 h at 4 °C, washed three times with distilled water, and then incubated with 40 mM AR-S for 15 min at RT with gentle agitation. After discarding the stain,

the cells were washed four times with MilliQ water. For the quantification of dye, the stained ECM was distained with 10% cetylpyridinium chloride for 15 min at RT and absorbance measured at 550 nm by spectrophotometry. For von Kossa's silver nitrate staining, the cells were washed three times with PBS at room temperature, fixed with 4% formaldehyde (in PBS) for 1 h at 4 °C, washed three times with distilled water, and then incubated with 5% silver nitrate for 30 min under ultraviolet light. Formation of mineralized nodules in the ECM was observed under an inverted light microscope. Relative levels of ECM mineralization were determined by densitometric methods with Quantity 1 software (Bio-Rad, Amadora, Portugal).

Proteoglycan Production

BSa2 cells were seeded normally or cultured in high density micromass-culture (Shakibaei et al. 1993), briefly 10 µL drops of a concentrated BSa2 cell suspension (5×10^6 cells/mL) were placed in around 1 cm intervals on each well and allowed to attach at 28 °C for overnight. The medium was then replaced with chondrogenic medium supplemented with 6.25 µg/ml of human insulin, 50 nM ascorbate 2-phosphate, and 10 ng/ml of human transforming growth factor-β1 (TGF-β1) (all from Sigma-Aldrich), or left untreated (control). Supplemented medium was renewed twice a week. After 3 weeks of treatment, the presence of proteoglycan deposition in the matrix was assessed through Alcian Blue 8GS staining (Scott and Dorling 1965). For this, the cells were washed twice with PBS, fixed for 1 h with 4% formaldehyde (in PBS), washed twice with 0.1 N HCl, and then incubated for 5 h at room temperature with 0.5% (w/v) Alcian blue 8GX (in 0.1 N HCl at pH 1.0). Unbound dye was removed by extensive washes with distilled water. ECM staining was observed under an inverted light microscope and quantified, after solubilization in 1% SDS, at 595 nm.

Osteoclast Differentiation

BSa2 cells were plated at a density of 1×10^5 cells/13-mm cover slips (Sarstedt) in a 6-well plate and then cultured in medium supplemented with 25 ng/ml of human macrophage-colony-stimulating factor (M-CSF) and 40 ng/ml of human receptor activator of nuclear factor κB ligand (RANKL) (Peprotech, Rocky Hill, NJ) or left untreated (control). Supplemented medium was renewed twice a week. Osteoclast differentiation was assessed through tartrate-resistant acid phosphatase (TRAP) staining (modified protocol after Burstone 1959). At 2 and 3 weeks, cultures were fixed with 4% (v/v) formalin (in PBS) for 15 min, washed in PBS, pre-incubated

for 10 min at 20 °C in 0.1 M veronal-acetate buffer containing 50 mM sodium tartrate at pH 5.5 and then incubated for up to 6 h at 20 °C in a working solution containing 0.066 mg/ml of naphthol-ASBI-phosphate, 0.04% (w/v) magnesium chloride, 0.0013 mg/ml of hexazotized pararosanilin, and 50 mM sodium tartrate in veronal-acetate buffer at pH 5.5 (all from Sigma-Aldrich). Development of the characteristic red staining was followed and when appropriate, stopped with demineralized water. Coverslips were mounted with antifade mounting medium Mowiol (Sigma-Aldrich) (Valnes and Brandtzaeq 1985). The number of positive cells per coverslip was counted under an inverted light microscope.

Bone Resorption Assay (Osteologic Discs)

To evaluate bone resorption by pit formation, BSa2 cells were cultured for 3 weeks on BD BioCoat Osteologic hydroxyapatite discs (BD Biosciences, Bedford, MA) at the density of 1 × ~~x~~ 10^6 cells and maintained in the same culture condition as indicated above in osteoclastic differentiation. After 3 weeks, cells were removed with a solution of 20% sodium hypochlorite for 5 min. To better analyze the resorption lacunae, the discs were stained with von Kossa's, and bone resorption activity was observed under bright field microscopy.

Immunofluorescence Staining

For immunophenotyping, BSa2 cells grown on 13-mm glass coverslips supplemented with osteogenic, chondrogenic, and osteoclastic cocktails were briefly fixed with 100% ice cold-methanol for 10 min, washed with PBS, permeabilized with 0.1% Triton X-100 for 5 min, and blocked in PBS containing 1% bovine serum albumin (BSA). Mouse monoclonal antibodies against type 2 collagen/Col2 (II-II6B3), chondroitin sulfate proteoglycans/Cspgs (9BA12) (Developmental Studies Hybridoma Bank, Iowa City, IA) for chondrogenic differentiation were diluted 1:200, and rabbit polyclonal antibody against teleost osteocalcin1 (Oc1) (Simes et al. 2004) for osteogenic differentiation were diluted 1:200. All the above dilutions were made in PBS with 1% BSA and directly added to the fixed cells at room temperature for 2 h. Cells were washed with 1:10 dilution of blocking buffer in PBS then incubated with Alexa fluor 594-linked secondary antibody (either anti-mouse IgG or anti-rabbit IgG, Invitrogen) for 45 min at room temperature in the dark. Cells were washed several times with PBS, mounted in Mowiol (Sigma) and observed using an Axio observer Z2 fluorescence microscope (Zeiss) with Apotome, equipped with an Axiocam camera (Zeiss).

RNA Preparation and RT-PCR

Total RNA was extracted from BSa2 cells treated/non treated with mineralization medium using the method described by Chomczynski and Sacchi (1987). RNA integrity was confirmed following 1% (w/v) agarose/MOPS-formaldehyde gel electrophoresis, and RNA quantity was determined through spectrophotometry (NanoDrop 1000; Thermo Scientific). Total RNA (1 µg) was treated with RNase-free DNase I (Promega) during 30 min at 37 °C and reverse-transcribed at 37 °C for 1 h using the Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen), RNase Out (Invitrogen) and an oligo-d(T) primer [5′-ACGCGTCGACCTCGAGATCGATG(T)13–3′].

Analysis of Gene Expression by Quantitative Real-Time PCR

Levels of gene expression were determined through real-time PCR amplification carried out using gene-specific primers (Supplementary Table 1) and normalized using the reference gene *rpl27a*. A reaction mixture containing 1× Sso Fast EvaGreen supermix (Bio-Rad), 10 µM of forward and reverse primers, and 1:10 dilution of reverse-transcribed RNA was submitted to the following PCR conditions: 4 min at 95 °C, 40–50 cycles (each cycle is 30 s at 95 °C, 45 s at 68 °C). *rpl27a* relative gene expression was used to normalize gene expression levels. Amplifications were performed by using the StepOnePlus Real-Time PCR system (Applied Biosystems). The relative expression ratio of target genes was calculated by the $^{\Delta\Delta}$Ct method.

Results

Process of Fin Regeneration

Similar to zebrafish and medaka, *S. aurata* fins can also fully regenerate their missing structures in 14 days following amputation (Fig. 2a~~A~~). Within 12 hpa, the wound was healed by the rapid migration of the epithelial cells to cover the surface of the stump. Formation of wound epidermis, followed by a blastema was detectable at 48 hpa. The complete formation of blastema can be seen at 72 hpa (Fig. 2a~~A~~). The alizarin staining revealed that the onset of mineralization took place between 5 and 6 dpa (Fig. 2b~~B~~). At 9 dpa, the mineralized lepidotrichia were already starting to bifurcate (Fig. 2b~~B~~). Azan staining revealed the formation of a wound epidermis at 48 hpa (Fig. 2c~~C~~) followed by migration of mesenchymal cells located under the wound epidermis which migrated distally, leading to the completion of blastema formation at 72 hpa (Fig. 2d~~D~~).

No bone was yet regenerating at 4 dpa (Fig. 2eE). The formation of new bone could only be seen at 6 dpa (Fig. 2fF–2, Gg), confirming the results achieved through alizarin red staining. Further, the outgrowth of caudal fins at 7, 9, and 14 dpa showed the progression of the lepidotrichia regenerative process (Fig. 2hH–2, Jj).

Establishment of the BSa2 Cell Line

Primary cultures were obtained from 2 and 4 dpa caudal fin. The cells quickly migrated from the explants and formed a monolayer in 3 days, and these were subcultured at intervals of 3–5 days. The cells were split in a ratio of 1:2. After two passages, the cells derived from 4 dpa died gradually. Only the cells from the 2 dpa explants developed into a cell line and were designated as BSa2 (refer to Fig. 1 for the detailed procedure for obtaining the regenerating caudal fin explants for cell culture). BSa2 cell line was subcultured for more than 30 passages. The morphology of BSa2 cell line showed polygonal phenotype (Fig. 3Aa) and the cells were found to be contact-inhibited. Phenotype was maintained after more than 30 passages. Cell monolayer was routinely dissociated every 3–5 days using trypsin-EDTA and divided 1:2. The growth of BSa2 cells was supported well in L-15 medium, without supplementing with fish serum. Cryopreservation of BSa2 cells at different passages was achieved successfully as evaluated by percentage of recovery and survival from cryopreservation.

Characterization of BSa2 Cell Line

To determine the optimal requirement of serum in BSa2 cells, growth rate was determined using 4 different FBS concentrations, and 15% FBS was found to be optimal (Fig. 3bB). The average viability of the cells after cryopreservation was estimated at about 90%, and surviving cells actively divided subsequently (results not shown). DNA delivery into BSa2 cells at passage 20 was assayed through lipofection, nucleofection or using polymers, and evaluated through GFP fluorescence. While lipofection and polymer-assisted transfection resulted in very few GFP-positive cells (efficiency was less than 2%; Fig. 3cC), nucleofection was found to be more efficient (highest efficiency was 12% in buffer V using electrical program 4; Fig. 3Cc).

Osteoblastic and Chondroblastic Differentiation

To determine the differentiation potential of BSa2 cells into an osteoblastic lineage, confluent cell cultures were treated for 3 weeks with osteoblastic cocktail and mineral deposition within the extracellular matrix (ECM) was evaluated. The presence of numerous calcium-phosphate

nodules of various sizes was evidenced in BSa2 ECM through von Kossa's (VK) and alizarin red S (AR-S) stainings (Fig. 4a, A and 4C, respectively). No nodules were observed in untreated cells (insert in Fig. 4a A and, 4C c). ECM mineralization was further quantified through densitometry (VK; Fig. 4b B) and spectrophotometry (AR-S; Fig. 4D d) analysis. Further, BSa2 cell cultures exposed for 3 -weeks to the osteogenic cocktail exhibited a strong alkaline phosphatase (ALP) activity (Fig. 4e E), although some ALP activity was observed in untreated cells (insert in Fig. 4E e); this was further confirmed through enzymatic assays of ALP activity (Fig. 4F f).

To determine the differentiation potential of BSa2 cells into the chondrocytic lineage, the high density micromass-culture cells were treated for 3 -weeks with chondroblastic cocktail and evaluated through the measurement of sulfated proteoglycans production. Microscopic observation revealed the presence of a Alcian blue staining in the differentiated BSa2 cells (Fig. 4g G) corresponding to cartilage associated proteoglycans. No staining was observed in untreated cells (insert in Fig. 4g G). Proteoglycan content was further confirmed through spectrophotometry analysis (Fig. 4H h).

Osteogenic and chondrogenic differentiation of BSa2 cells were further confirmed by immunocytochemistry using antibodies specific for osteoblast-specific Osteocalcin1 (Fig. 5A a- 5 D d) and cartilage-associated proteins including chondroitin sulphate proteoglycans (Fig. 5e E, - 5H h) and type 2 collagen (Fig. 5I- 5L i, l) respectively. No signal was detected in negative controls (Fig. 5M- 5P m, p), cells left untreated or at week 0 (data not shown).

Osteoclast Differentiation

Differentiation into osteoclastic lineage was triggered by treating confluent BSa2 cells for 3 -weeks with RANKL and M-CSF and evaluated through the measurement of TRAP activity. While no TRAP positive cells were detected at time 0 (T0) (Fig. 6a A), several TRAP positive cells were observed at 2 -week (T2) (Fig. 6B b), and even more TRAP positive cells were observed at 3 -week (T3) (Fig. 6C c). The number of TRAP positive cells was 5 times higher in cultures stimulated for 3 -weeks (Fig. 6D d) when compared to with 2 -week. Osteoclasts obtained from BSa2 were tested further for their function in terms of bone resorption (pit formation), as shown in Fig. 7A- 7C a–c. The BSa2 cells were able to produce resorption lacunae on osteologic discs after 3 -weeks of stimulation (Fig. 7b B).

Expression of osteoclast related genes, namely, osteoclast-stimulating factor-1 (OSTF-1) and cathepsin K (CTSK) were evaluated by qPCR in total RNA isolated from BSa2 cell cultures treated for 3 -weeks with RANKL and M-CSF and compared ~~to~~ with control cells (Fig. 7~~D~~ 7~~E~~d, e). The qPCR analysis of osteoclast marker gene expression revealed the up-regulation OSTF-1 and CTSK during the 1st and 3rd week of treatment.

Gene Expression ~~d~~During Mineralization of BSa2 Cells

Expressions of several bone and cartilage-related genes (*spp1*, *sparc*, *col1a1*, *oc1*, *bmp2*, and *s100*) were evaluated by qPCR in total RNA isolated from BSa2 cell cultures undergoing osteoblast differentiation (1 -week) and ECM mineralization (3 -weeks) and compared ~~to~~ with control cells (Fig. 8). The qPCR analysis of osteoblast marker gene expression revealed the up-regulation of non-collagenous ECM proteins (*spp1* and *sparc*) and matrix proteins (*col1a1*) during 3rd week of treatment.

Discussion

Development of a cell culture derived from 2 dpa regenerating caudal fin of *S. aurata* opens the possibility to investigate in vitro the cellular and molecular mechanisms associated to epimorphic regeneration of caudal fin. Here we present, for the first time, the establishment of a cell line derived from 2 dpa regenerating caudal fin of *S. aurata*, with the ability of differentiating into osteoblastic, chondrocytic, or osteoclastic lineages. We have further identified that the process of fin regeneration in *S. aurata* is similar to that of other teleosts like zebrafish and medaka (Poss et al. 2003; Katogi et al. 2004). The primary cultures were initiated from 2 and 4 dpa regenerating caudal fin using explant culture, but only the cells from 2 dpa regenerating caudal fin survived well and derived as a cell line, viz., BSa2, suggesting that 4 dpa cells were already in a more committed stage and less prone to proliferate actively under the in vitro conditions used. The cells were cultured in a standard medium without any further supplements like conditioned medium or fish serum and sub-cultured for more than 30 passages keeping a stable morphology (showed polygonal phenotype). Growth rate of BSa2 cells was shown to be optimal using 10% and 15% FBS. Poorer results were obtained for lower concentration of FBS 5% or higher concentration of 20%, possibly due to the absence of optimal growth factors found in 5% and increased levels in 20% which resulted in a cytotoxic effect on BSa2 cell proliferation. This data is in contrast with the primary cultures from zebrafish blastema cells where 5% is optimal, and

higher concentrations lead to lower proliferation rates (Parameswaran et al.~~,~~ unpublished data), highlighting the fact that cells derived from similar tissues but from different organisms have indeed different requirements, a fact already observed for other cell types like bone derived cells (Pombinho et al. 2004; Vijayakumar et al. 2013).

BSa2 cells were successfully cryopreserved using the methods already developed for mammalian cells and also successfully applied to marine fish cell lines previously (Marques et al. 2007) which allowed a permanent maintenance in the laboratory without the need to keep them continuously in culture. The efficient delivery of DNA vectors into the cells is important for successful gene targeting approach (Koller et al. 1989). BSa2 cells were successfully transfected using nucleofection, and therefore, they represent a suitable tool for the evaluation of promoter efficiency using various gene constructs and expression studies.

Osteoblasts are cells specialized in the production of an extracellular matrix susceptible to mineralization (Blair et al. 2002). Mechanisms of osteoblast differentiation have been studied in vitro using essentially primary cultures or lines of cells isolated from mammalian bone (Declercq et al. 2004) or from bone marrow (Aubin and Triffit 2002) or cartilage (Alsalameh et al. 2004), and also from fish bone and stem cells (Pombinho et al. 2004; Parameswaran et al. 2012). Generally mineralization of mammalian osteoblast requires medium supplementation with ascorbic acid (for the correct formation of collagen fibers) and β-glycerophosphate (a phosphate donor for the formation of hydroxyapatite crystals) (Costa and Fernandes 2000; Fournier and Price 1991; Kellermann et al. 1990; Otsuka et al. 1999). However in fish pre-osteoblasts, ECM mineralization was achieved in a consistent way only when in addition to the two supplements used for mammalian cells, calcium was also added in order to achieve a sustainable formation of hydroxyapatite crystals (Pombinho et al. 2004; Parameswaran et al. 2012). Accordingly, BSa2 cells treated with the same cocktail, as for other fish cell lines, were able to mineralize their ECM and showed an increased expression of osteoblastic markers at sites of cell aggregation and ECM mineralization (Fig. 4~~A, 4C and 4E~~a, c, e). Furthermore, these cells expressed high alkaline phosphatase activity, a marker of bone formation in vivo, which showed that alkaline phosphatase activity positively affected the predisposition of these cells to differentiate towards an osteoblastic-like phenotype, which is similar to the report of Vijayakumar et al. 2013. Expression of osteoblastic specific osteocalcin was also detected confirming the presence of

mature osteoblastic cells. These data provide evidence that BSa2 cells can differentiate into an osteoblastic phenotype.

Chondrocytes are cells specialized in the production and maintenance of a cartilaginous matrix. Differentiation of BSa2 cells towards chondrocytic lineage was achieved by high density mass-culture, as described for primary cultures of mouse chondrocytes isolated from limb buds (Shakibaei et al. 1993), and using a chondroblastic cocktail developed for mammalian cells (Zuk et al. 2002). This cocktail was supplemented with transforming growth factor-β1 (TGF-β1), a key factor in the regulation of cellular differentiation in cartilage formation. Using the above cocktail, in particular TGF-β1, triggered the BSa2 cells to form three-dimensional aggregates producing highly sulfated proteoglycans present in cartilage matrix (Zuk et al. 2002). Similar results have been reported by Ogawa et al. 2004 in a study where adipose-derived stem cells treated with TGF-β1 resulted in three-dimensional aggregates that positively stained with ~~a~~Alcian blue (Ogawa et al. 2004). Microscopic observations revealed the presence of ~~a~~Alcian blue staining in the matrix secreted by differentiated BSa2 cells corresponding to cartilage associated proteoglycans. Similar results have previously been reported by Parameswaran et al. (2012) in a study where fish blastula-derived stem-like cells treated with transforming growth factor-β1 resulted in three-dimensional aggregates that positively stained with ~~a~~Alcian blue. Expression of chondrocyte specific proteins, including chondroitin sulfate proteoglycans and type 2 collagen, were also detected mostly at the sites where cells formed condensations. These data strongly suggest the ability of BSa2 cells to differentiate towards a chondroblastic phenotype and produce a cartilaginous matrix.

Based on the above results, we propose that BSa2 cells can differentiate into osteoblastic and chondrocytic cell lineages upon appropriate stimulation and thus represent a promising model for investigating skeletal-lineage cell differentiation upon regeneration.

Osteoclasts differentiated from hematopoietic monocyte/macrophage precursor cells under the control of two main cytokines, M-CSF, which is required for proliferation, survival, and expression of RANK in osteoclast precursors, and RANKL, which is critical for activation and survival of osteoclasts and for their fusion into large multinucleated cells. Upon treatment with M-CSF and RANKL, BSa2 cells developed an osteoclastic phenotype with an increased production of TRAP. Furthermore, these cells had the capability to produce resorption lacunae upon seeding on osteologic discs, similar to what was previously reported for mammalian cells

(García-Pérez et al. 2006). The mRNA levels of osteoclast markers, such as osteoclast-stimulating factor-1 (OSTF-1) and Cathepsin K (CTSK), were found to be increased in BSa2 cells under RANKL/M-CSF treatment. OSTF-1 is an intracellular protein produced by osteoclasts that indirectly induces osteoclast formation and bone resorption (Reddy et al. 1998). The cDNA of *S. aurata* OSTF-1 was reconstructed using ESTs from our laboratory (V. Laizé Pers. Comm.). We found that the expression of this gene was up-regulated (1.2 fold) at 1 week of treatment and 1.4 fold at 3 weeks of treatment, compared to with control cells. Cathepsin K, a lysosomal cysteine proteinase, is a key enzyme in the degradation of demineralized bone ECM, and it is highly expressed by osteoclasts (Drake et al. 1996). Its expression has been shown to be up-regulated (1.4 fold) at 1 week of treatment and 1.4 fold at 3 weeks of treatment, compared to with control cells. To our surprise, the mRNAs of OSTF-1 and CTSK were also expressed in control cells. This was the reason for us to believe that these cells are behaving like progenitors, and even in normal culture conditions, these cells possess spontaneous differentiation capacity. According to these results, we assumed that blastema cells differentiated into osteoclasts. But further studies are needed to explore this hypothesis. Based on the above results, we concluded that RANKL/M-CSF might induce BSa2 cells to differentiate and function as osteoclasts. Expression of ECM proteins by BSa2 cells showed a pattern consistent with the expected type of differentiation. Osteopontin (Spp1) and osteonectin (Sparc) are two non-collagenous proteins present in osteoblast-produced ECM (Bellows et al. 1999; Gorski 1992). Spp1 is a highly phosphorylated sialoprotein and a prominent component of bone and teeth-mineralized ECM (Sodek et al. 2000), and Sparc is a matricellular glycoprotein with calcium-binding domains that has been associated with regulation of ECM mineralization in osteoblasts (Yan and Sage 1999). These two proteins have been reported to play a role in the regulation of mineralization (Bellows et al. 1999), attachment of osteoblasts and osteoclasts to the bone matrix, and/or attraction of cells to the bone matrix (Cowles et al. 1998). Expression of both these genes in BSa2 cells was stable in control cells and significantly up-regulated in cells undergoing differentiation (3.8 and 2.2 fold increase for *spp1* and *sparc*, respectively). During ECM mineralization, upon exposure to the osteogenic cocktail, *spp1* was highly upregulated with a 39.6 fold increase while *sparc* expression increased to 3.8 fold relatively to the control cells. An increase in *spp1* expression during mineralization in BSa2 cells is in agreement with previous reports in mammalian (Kärner et al. 2009; Zur Nieden et al. 2003) and fish (Tiago et al. 2011) cell systems. In VSa13 and

VSa16 cells, *spp1* has been shown to be highly up-regulated (~ 100×~~x~~) during mineralization of the ECM (Tiago et al. 2011) similar to the results obtained in this study. Up-regulation of *sparc* expression during ECM mineralization in BSa2 cells is in agreement with previous reports in mouse embryonic stem cells and human mesenchymal stem cells (MSC) (Zur Nieden et al. 2003; Kulterer et al. 2007), but surprisingly, this results are contradictory to previous expression data in gilthead seabream bone-derived VSa13 and VSa16 cells (Laizé et al. 2005), where the authors reported a decrease in expression during ECM mineralization by 73% and 62% respectively. This might possibly be due to the fact that VSa13 and VSa16 are derived from vertebral tissues while BSa2 cells are derived from regenerating caudal fin, where they act like multipotent cells and indeed regulation of gene expression may differ in each cell type.

Type I collagen (*col1a1*) is known to be an early marker of osteoprogenitor cells and major structural component of bone ECM (Bou-Gharios and De Crombrugghe 2008; Jikko et al. 1999). Its expression has been found to be increased in cells undergoing differentiation (1.3 fold increase). Maximum expression was reached during ECM mineralization (3.2 fold increase) compared ~~to~~ with control cells, similar to the reports of pre-osteoblastic VSa16 cells (Viegas et al. 2012), and also in agreement with previous reports using human mesenchymal stem cells (MSC) (Kulterer et al. 2007), where the authors found that maximum expression was reached at 21 -days of osteogenic differentiation treatment.

Bone morphogenetic protein 2 (Bmp2) is a glycosylated protein involved in the induction of osteoblast differentiation and ECM mineralization through Bmp receptors and smad signaling pathway (Wang et al. 1988; Takuwa et al. 1991); our laboratory has identified only one *bmp2* isoform in *S. aurata*, which was involved in tissue mineralization (Rafael et al. 2006).

We found that this *bmp2* expression in BSa2 cells to be down-regulated during both the differentiation and ECM mineralization processes, contrary to what was reported for VSa13 cells (Rafael et al. 2006). Like previously discussed for *sparc* gene expression, this might be due to different origins of cell lines and specific gene expression patterns.

The osteocalcin (Oc) gene encodes a ~ 6-kDa noncollagenous bone-specific protein. The protein is secreted by osteoblasts and is thought to play a role in osteoblast differentiation and in controlling hydroxyapatite crystal formation during ECM mineralization at late stages of bone formation (Hauschka et al. 1989). In several teleosts, including *S. aurata*, two osteocalcin genes have been identified. The *oc1* (the ortholog of mammalian *OC*) was down-regulated during both

the differentiation and ECM mineralization. These data are contradictory to the previous expression data in mammalian (Kärner et al. 2009; Zur Nieden et al. 2003) cell systems. S100 is a calcium-binding protein involved in various cellular activities such as signal transduction, cell differentiation, gene transcription, calcium homeostasis, and cell cycle progression (Heizmann et al. 2002; Heizmann and Fritz 2009). We have previously reported the discovery of a novel S100-like calcium-binding protein in *S. aurata* and emphasized its potential role as a marker of mineralizing cartilage in developing fish (Fonseca et al. 2011). We found that the expression of this gene was up-regulated during the differentiation (12.5 fold increase) and ECM mineralization (4 fold increase), whereas, control cells showed no expression, similar to the reports of Fonseca et al. (2011) and Tiago et al. (2011) where they found up-regulation of *s100* gene expression in chondrocytic and osteoblastic cells undergoing ECM mineralization. qPCR analysis of osteoblast marker gene expression revealed the up-regulation of non-collagenous ECM proteins (*spp1* and *sparc*), matrix proteins (*col1a1*) and thus further demonstrated the osteoblastic potential of BSa2 cell line.

In conclusion, we have shown the establishment of the first successful cell line from the 2 dpa regenerating caudal fin of *S. aurata* with the ability of multilineage differentiation and capability of in vitro mineralization upon appropriate treatment. The availability of such in vitro tool will stimulate the research on the mechanisms of cell differentiation during fin regeneration and will open the possibility to acquire new insights into the mechanisms of bone formation. Although BSa2 is the first cell line of its kind, we expect the technique described in this article can also be used successfully to develop regenerating caudal fin cell cultures or cell lines from other fish species.

Acknowledgments

~~This work was co-funded by the Portuguese Science and Technology Foundation (FCT) through FISHCELL (PTDC/MAR/105313/2008) research project and by the European Community (EC) through ASSEMBLE (FP7/227799) research project. PV is supported by a post-doctoral grant (SFRH/BPD/39189/2007) from the FCT.~~ The monoclonal antibodies used in this study were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Flow cytometry and fluorescence imaging were performed in the facilities of the

Department of Biomedical Sciences and Medicine, University of Algarve. We thank Dr. Jorge Dias for providing animals for experiment.

Funding Information

This work was co-funded by the Portuguese Science and Technology Foundation (FCT) through FISHCELL (PTDC/MAR/105313/2008) research project and by the European Community (EC) through ASSEMBLE (FP7/227799) research project. PV is supported by a post-doctoral grant (SFRH/BPD/39189/2007) from the FCT.

Compliance with Ethical Standards

Animal handling and experimentation was performed by qualified operators legally accredited by the Portuguese Direção Geral de Alimentação e Veterinária (DGAV) and following the EU and Portuguese legislation for animal experimentation and welfare (Directive 86/609/CEE and 2010/63/EU, Portaria 1005/92, 466/95 and 1131/97). Experimental procedures were performed under authorization (0421/000/000) from the DGAV, complying with the decreto de lei 113/2013 de 7 de Agosto, from the Portuguese legislation.

Conflict of Interest

The authors declare that they have no competing interests.

A. Electronic supplementary material

ESM 1 (DOCX 18 kb)

References

Akimenko MA, Marí-Beffa M, Becerra J, Géraudie J (2003) Old questions, new tools, and some answers to the mystery of fin regeneration. Dev Dyn 226:190–201

Alsalameh S, Amin R, Gemba T, Lotz M (2004) Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 50:1522–1532

Aubin JE, Triffit JT (2002) Mesenchymal stem cells and osteoblast differentiation. In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of bBone bBiology. Vol. 1. Academic Press, San Diego, pp. 59–81

Bellows CG, Reimers SM, Heersche JN (1999) Expression of mRNAs for type-I collagen, bone sialoprotein, osteocalcin, and osteopontin at different stages of osteoblastic differentiation and their regulation by 1,25 dihydroxyvitamin D_3. Cell Tissue Res 297:249–259

Bills CE, Eisenberg H, Pallante SL (1974) Complexes of organic acids with calcium phosphate: tThe von Kossa stain as a clue to the composition of bone mineral. Johns Hopkins Med J 128:194–207

Blair HC, Zaidi M. Schlesinger, PH (2002) Mechanisms balancing skeletal matrix synthesis and degradation. Biochem J 364:329–341

Blum N, Begemann G (2012) Retinoic acid signaling controls the formation, proliferation and survival of the blastema during adult zebrafish fin regeneration. Development. 139: 107–116.

Blum N, Begemann G (2015) Osteoblast de- and redifferentiation are controlled by a dynamic response to retinoic acid during zebrafish fin regeneration. Development 142, 2894–2903.

Bou-Gharios G, De Crombrugghe B (2008) Type I collagen structure, synthesis and regulation. In: Principles of bone biology. Bilezikian JP, Raisz LG, Martin TJ, editors. Elsevier, Amsterdam. pp. 285–318

Brockes JP, Kumar A (2005) Appendage regeneration in adult vertebrates and implications for regenerative medicine. Science 310:1919–1923

Burstone MS (1959) Histochemical demonstration of acid phosphatase activity in osteoclasts. J Histochem Cytochem 7:39–41

Carlson BM (2005) Some principles of regeneration in mammalian systems. Anat. Rec. B New Anat. 287:4–13

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal BiochemAnal BioChem 162:156–159

Costa MA, Fernandes MH (2000) Long-term effects of parathyroid hormone, 1,25-dihydroxyvitamin d_3, and dexamethasone on the cell growth and functional activity of human osteogenic alveolar bone cell cultures. Pharmacol Res 42:345–353

Cowles EA, DeRome ME, Pastizzo G, Brailey LL, Gronowicz GA (1998) Mineralization and the expression of matrix proteins during in vivo bone development. Calcif Tissue Int 62:74–82

Dasyani M, Tan WH, Sundaram S, Imangali N, Centanin L, Wittbrodt J, Winkler C (2019) Lineage tracing of col10a1 cells identifies distinct progenitor populations for osteoblasts and joint cells in the regenerating fin of medaka (Oryzias latipes). Dev BiolDev Biol. 455:85–99.

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

Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, Lee-Rykaczewshi E, Coleman L, Rieman D, Barthlow R, Hastings G, Gowen M (1996) Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem 271:12511–12516

Fonseca VG, Rosa J, Laizé V, Gavaia PJ, Cancela ML (2011) Identification of a new cartilage-specific S100-like protein up-regulated during endo/perichondral mineralization in gilthead seabream. Gene Expr Patterns 11:448–455

Fournier B, Price PA (1991) Characterization of a new human osteosarcoma cell line OHS-4. J Cell Biol 114:577–583

García-Pérez MA, Noguera I, Hermenegildo C, Martínez-Romero A, Tarín JJ, Cano A (2006) Alterations in the phenotype and function of immune cells in ovariectomy-induced osteopenic mice. Hum Reprod 21:880–887

Gavaia PJ, Sarasquete C, Cancela ML (2000) Detection of mineralized structures in early stages of development of marine *Teleostei* using a modified alcian blue-alizarin red double staining technique for bone and cartilage. Biotech Histochem 75:79–84

Gemberling M, Bailey TJ, Hyde DR, Poss KD (2013) The zebrafish as a model for complex tissue regeneration. Trends GenetTrends Genet. 29: 611–620.

Gorski JP (1992) Acidic phosphoproteins from bone matrix: a structural rationalization of their role in biomineralization. Calcif Tissue Int 50:391–396

Grotek B, Wehner D,Weidinger G (2013) Notch signaling coordinates cellular proliferation with differentiation during zebrafish fin regeneration. Development. 140:1412–1423.

Han M, Yang X, Taylor G, Burdsal CA, Anderson RA, Muneoka K (2005) Limb regeneration in higher vertebrates: developing a roadmap. Anat Rec B New Anat 287:14–24

Hata S, Namae M, Nishina H (2007) Liver development and regeneration: from laboratory study to clinical therapy. Develop Growth DifferDev Growth Differ 49:163–170

Hauschka PV, Lian JB, Cole DE, Gundberg CM (1989) Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. Physiol Rev 69:990–1047

Heizmann CW, Fritz G (2009) The family of S100 cell signaling proteins. In: Bradshaw RA, Dennis EA, editors. Handbook of cCell Ssignalling. 2nd. Oxford: Academic Press. pp. 983–994

Heizmann CW, Fritz G, Schäfer BW (2002) S100 proteins: structure, functions and pathology. Front Biosci 7:d1356–1368

Illingworth CM (1974) Trapped fingers and amputated finger tips in children. J Pediatr Surg 9:853–858

Jikko A, Harris SE, Chen D, Mendrick DL, Damsky CH (1999) Collagen integrin receptors regulate early osteoblast differentiation induced by BMP-2. J Bone Miner Res 14:1075–1083

Kärner E, Bäckesjö CM, Cedervall J, Sugars RV, Ahrlund-Richter L, Wendel M (2009) Dynamics of gene expression during bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. Biochim Biophys Acta 1790:110–118

Katogi R, Nakatani Y, Shin-I T, Kohara Y, Inohaya K, Kudo A (2004) Large-scale analysis of the genes involved in fin regeneration and blastema formation in the medaka, *Oryzias latipes*. Mech Dev 121:861–872

Kellermann O, Buc-Caron MH, Marie PJ, Lamblin D, Jacob F (1990) An immortalized osteogenic cell line derived from mouse teratocarcinoma is able to mineralize in vivo and in vitro. J Cell Biol 110:123–132

Knopf F, Hammond C, Chekuru A, Kurth T, Hans S, Weber CW, Mahatma G, Fisher S, Brand M, Schulte-Merker S, Weidinger G (2011) Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. Dev Cell 20:713–724

Koller BH, Hagemann LJ, Doetschman T, Hagaman JR, Huang S, Williams PJ, First NL, Maeda N, Smithies O (1989) Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. Proc Natl Acad Sci U S AProc Natl Acad Sci USA 86:8927–8931

König D, Page L, Chassot B, Jazwinska A (2018) Dynamics of actinotrichia regeneration in the adult zebrafish fin. Dev BiolDev. Biol. 433: 416–432.

Kulterer B, Friedl G, Jandrositz A, Sanchez-Cabo F, Prokesch A, Paar C, Scheideler M, Windhager R, Preisegger KH, Trajanoski Z (2007) Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. BMC Genomics 8:70

Laizé V, Pombinho AR, Cancela ML (2005) Characterization of *Sparus aurata* osteonectin cDNA and in silico analysis of protein conserved features: evidence for more than one osteonectin in Salmonidae. Biochimie 87:411–420

Lee Y, Hami D, De Val S, Kagermeier-Schenk B, Wills AA, Black BL, Weidinger G, Poss KD (2009) Maintenance of blastemal proliferation by functionally diverse epidermis in regenerating zebrafish fins. Dev Biol 331:270–280

Marques CL, Rafael MS, Cancela ML, Laizé V (2007) Establishment of primary cell cultures from fish calcified tissues. Cytotechnology 55:9–13

Muller TL, Ngo-Muller V, Reginelli A, Taylor G, Anderson R, Muneoka K (1999) Regeneration in higher vertebrates: limb buds and digit tips. Semin Cell Dev Biol 10:405–413

Nakatani Y, Kawakami A, Kudo A (2007) Cellular and molecular processes of regeneration, with special emphasis on fish fins. Develop Growth DifferDev Growth Differ 49:145–154

Nishidate M, Nakatani Y, Kudo A, Kawakami A (2007) Identification of novel markers expressed during fin regeneration by microarray analysis in medaka fish. Dev Dyn 236:2685–2693

Nye HL, Cameron JA, Chernoff EA, Stocum DL (2003) Regeneration of the urodele limb: a review. Dev Dyn 226:280–294

Ogawa R, Mizuno H, Watanabe A, Migita M, Shimada T, Hyakusoku H (2004) Osteogenic and chondrogenic differentiation by adipose-derived stem cells harvested from GFP transgenic mice. Biochem Biophys Res Commun 313:871–877

Otsuka E, Yamaguchi A, Hirose S, Hagiwara H (1999) Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. Am J PhysAm J Physiol 277:C132–138

Owlarn S, Klenner F, Schmidt D, Rabert F, Tomasso A, Reuter H, Mulaw MA, Moritz S, Gentile L, Weidinger G, Bartscherer K (2017) Generic wound signals initiate regeneration in missing-tissue contexts. Nat CommunNat Commun. 8:2282.

Parameswaran V, Laizé V, Gavaia PJ, Cancela ML (2012) ESSA1 embryonic stem like cells from gilthead seabream: aA new tool to study mesenchymal cell lineage differentiation in fish. Differentiation 84:240–251

Petrie TA, Strand NS, Yang CT, Rabinowitz JS. Moon RT (2014) Macrophages modulate adult zebrafish tail fin regeneration. Development 141: 2581–2591.

Pfefferli C, Jaźwińska A (2015) The art of fin regeneration in zebrafish. Regeneration (Oxf). 19:72–83

Pfitzner E, Becker P, Rolke A, Schüle R (1995) Functional antagonism between the retinoic acid receptor and the viral transactivator BZLF1 is mediated by protein-protein interactions. Proc Natl Acad Sci U S AProc Natl Acad Sci USA 92:12265–12269

Pombinho AR, Laizé V, Molha DM, Marques SM, Cancela ML (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

Poss KD, Keating MT, Nechiporuk A (2003) Tales of regeneration in zebrafish. Dev Dyn 226: 202–210

Rafael MS, Laizé V, Cancela ML (2006) Identification of *Sparus aurata* bone morphogenetic protein 2: molecular cloning, gene expression and in silico analysis of protein conserved features in vertebrates. Bone 39:1373–1381

Reddy S, Devlin R, Menaa C, Nishimura R, Choi SJ, Dallas M, Yoneda T, Roodman GD (1998) Isolation and characterization of a cDNA clone encoding a novel peptide (OSF)

that enhances osteoclast formation and bone resorption. J Cell Physiol 177:636–645

Scott JE, Dorling J (1965) Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. Histochemie 5:221–233

Sehring IM, Weidinger G (2019) Recent advancements in understanding fin regeneration in zebrafish. Wiley Interdiscip Rev Dev BiolWiley Interdiscip Rev Dev Biol. 14:e367.

Shakibaei M, Schröter-Kermani C, Merker HJ (1993) Matrix changes during long-term cultivation of cartilage (organoid or high-density cultures). Histol Histopathol 8:463–470

Simes DC, Williamson MK, Schaff BJ, Gavaia PJ, Ingleton PM, Price PA, Cancela ML (2004) Characterization of osteocalcin (BGP) and matrix gla protein (MGP) fish specific antibodies: vValidation for immunodetection studies in lower vertebrates. Calcif Tissue Int 74:170–180

Singh SP, Holdway JE, Poss KD (2012) Regeneration of amputated zebrafish fin rays from de novo osteoblasts. Dev Cell 22: 879–886

Slack JM (2003) Regeneration research today. Dev Dyn 226:162–166

Sodek J, Ganss B, McKee MD (2000) Osteopontin. Crit Rev Oral Biol Med 11:279–303

Sousa S, Afonso N, Bensimon-Brito A, Fonseca M, Simões M, Leon J, Roehl H, Cancela ML, Jacinto A (2011) Differentiated skeletal cells contribute to blastema formation during zebrafish fin regeneration. Development 138:3897–3905

Stewart S, Stankunas K (2012) Limited dedifferentiation provides replacement tissue during zebrafish fin regeneration. Dev BiolDev Biol. 365: 339–349.

Stoick-Cooper CL, Moon RT, Weidinger G (2007) Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. Genes Dev 21:1292–1315

Strober W (2001) Trypan blue exclusion test of cell viability. Curr Protoc Immunol aA.3B.1-aA.3B.2. Curr Protoc Immunol a.3B.1-a.3B.2

Takuwa Y, Ohse C, Wang EA, Wozney JM, Yamashita K (1991) Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells, MC3T3-E1. Biochem Biophys Res Commun 174:96–101

Tiago DM, Cancela ML, Aureliano M, Laizé V (2008) Vanadate proliferative and anti-mineralogenic effects are mediated by MAPK and PI-3K/Ras/Erk pathways in a fish chondrocyte cell line. FEBS Lett 582:1381–1385

Tiago DM, Laizé V, Bargelloni L, Ferraresso S, Romualdi C, Cancela ML (2011) Global analysis of gene expression in mineralizing fish vertebra-derived cell lines: new insights into anti-mineralogenic effect of vanadate. BMC Genomics 12:310

Tornini VA, Poss KD (2014) Keeping at arm's length during regeneration.Dev Cell.29:139-45.

Tornini VA, Puliafito A, Slota LA, Thompson JD, Nachtrab G, Kaushik AL, Kapsimali M, Primo L, Di Talia S, Poss KD (2016) Live monitoring of blastemal cell contributions during appendage regeneration. Curr BiolCurr Biol. 26: 2981–2991.

Tu S, Johnson SL (2011) Fate restriction in the growing and regenerating zebrafish fin. Dev Cell 20:725–732

Valnes K, Brandtzaeq P (1985) Retardation of immunofluorescence during microscopy. J Histochem Cytochem 33:755–761

Viegas MN, Dias J, Cancela ML, Laizé V (2012) Polyunsaturated fatty acids regulate cell proliferation, extracellular matrix mineralization and gene expression in a gilthead seabream skeletal cell line. J Appl Ichthyol 28:427–432

Vijayakumar P, Laizé V, Cardeira J, Trindade M, Cancela ML (2013) Development of an in vitro cell system from zebrafish suitable to study bone cell differentiation and extracellular matrix mineralization. Zebrafish 10:500–509

Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, Luxenberg DP, Sibley BS, Wozney JM (1988) Purification and characterization of other distinct bone-inducing factors. Proc Natl Acad Sci U S AProc Natl Acad Sci USA 85:9484–9488

Watson CJ, Kwon RY (2015) Osteogenic programs during zebrafish fin regeneration. Bonekey Rep. 4:745.

Wehner D, Weidinger G (2015) Signaling networks organizing regenerative growth of the zebrafish fin.Trends GenetTrends Genet. 31: 336–343.

Whitehead GG, Makino S, Lien CL, Keating MT (2005) fgf20 is essential for initiating zebrafish fin regeneration. Science 310:1957–1960

Yan Q, Sage EH (1999) SPARC, a matricellular glycoprotein with important biological functions. J Histochem Cytochem 47:1495–1506

Yoshinari N, Ishida T, Kudo A, Kawakami A (2009) Gene expression and functional analysis of zebrafish larval fin fold regeneration. Dev Biol 325:71–81

Yoshizato K (2007) Growth potential of adult hepatocytes in mammals: highly replicative small hepatocytes with liver progenitor-like traits. Develop Growth Differ ~~Dev Growth Differ~~ 49:171–184

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295

Zur Nieden NI, Kempka G, Ahr HJ (2003) In vitro differentiation of embryonic stem cells into mineralized osteoblasts. Differentiation 71:18–27

Fig. 1 Schematic diagram showing the amputation of *S. aurata* 2 dpa regenerating caudal fin for cell culture. Amputation of the caudal fin (**a**~~**A**~~) and re-amputation (~~**B**~~**b**) procedures towards blastema collection (~~**C**~~**c**). Minced explants seeded in cell culture dish (~~**D**~~**d**). Dotted lines indicate amputation and re-amputation planes. Blastema is indicated in red.

Fig. 2 Process of caudal fin regeneration in *S. aurata*. Full regeneration of caudal fin structures was achieved at approximately 14 dpa (**a**~~**A**~~). Formation of wound epidermis can be seen at 48 hpa. At 48–72 hpa, a mass of highly proliferative cells called blastema can be detected above each of the amputated fin rays. Later the blastema grew distally and differentiated to replace all the missing fin structures (**a**~~**A**~~). Progression of the mineralization process detected using alizarin red staining (**b**~~**B**~~). The onset of mineralization took place between 5 and 6 dpa (*arrow*). At 9 dpa, the mineralized lepidotrichia were already starting to bifurcate (asterisk). The dashed lines roughly represent the sites of amputation (~~**B**~~**b**). Azan stained sections (5 mm) of regenerating caudal fin of *S. aurata* at different regeneration stages (~~**C**~~**c**-**j**~~**J**~~). Aniline blue staining show mineralized fin rays. Migration and accumulation of epidermal cell layers at 48 hpa, forming the wound epidermis (asterisk) (~~**C**~~**c**); non-epidermal cells migrating to the amputation site (arrow), to complete the formation of blastema (highly proliferative regenerating tissue) at 72 hpa (~~**D**~~**d**); no bone was regenerating yet at 4 dpa (~~**E**~~**e**); new bone was already regenerating (arrowhead) at 6 dpa (~~**F**~~**f**); higher magnification of the newly mineralized bone in ~~**G**~~**g**, clearly

showing bone forming cells (orange cells in the periphery of the mineralized rays stained as blue); outgrowth of the caudal fins at 7, 9 and 14 dpa, respectively (hH jJ). Bar is 0.3 mm in Bb, 50 mm in Cc–gG, j,J and 100 mm in Hh–iI.

Fig. 3 Characterization of BSa2 cells. Phase contrast micrographs of confluent cultures of *S. aurata* cells at passage (P) 15 and 26 (Aa). Proliferation of BSa2 cells at passage 14 cultured for 15 days in medium supplemented with different FBS concentrations, and values calculated from atleastat least 6 biological replicates (n = 6) (Bb). Efficiency of DNA delivery into BSa2 cells assessed through GFP fluorescence. Insert shows GFP signal in BSa2 cells nucleofected using buffer v and program 4 (v-4). Results are the mean of the flow cytometry analysis from three separate experiments (n = 3) (Cc). Bar is 200 µm and 100 µm in insert **C**.

Fig. 4 Extracellular matrix mineralization (Aa–dD), alkaline phosphatase activity (Ee, Ff) and sulfated proteoglycan production (Gg, Hh) in BSa2 cells. Phase contrast micrographs of von Kossa-stained BSa2 cells at passage 18 treated for 3 weeks with osteogenic cocktail. Mineral nodules appear in black. Insert, untreated cells (Aa). Levels of ECM mineralization by densitometry analysis of von Kossa staining after 3 weeks of treatment, and values calculated from 3 biological replicates (Bb). Phase contrast micrographs of alizarin red-stained BSa2 cells at passage 18 treated for 3 weeks with osteogenic cocktail. Mineral nodules appear in red. Insert, untreated cells (cC). Levels of ECM mineralization by spectrophotometry analysis of AR-S staining after 3 weeks of treatment, and values calculated from 3 biological replicates (Dd). Phase contrast micrographs of mineralizing cell cultures stained for alkaline phosphatase activity (purple color) at passage 18 treated for 3 weeks with osteogenic cocktail. Insert, untreated cells (Ee). Levels of Alp activity in 1-week mineralizing cell cultures, and values calculated from 3 biological replicates (Ff). Phase contrast micrographs of aAlcian blue-stained BSa2 cells at passage 23 treated for 3 weeks with chondrogenic cocktail. Sulfated proteoglycans appear in blue (white arrows). Insert, untreated cells (Gg). Levels of sulfated proteoglycans by spectrophotometry analysis of AB staining after 3 weeks of treatment, and values calculated from 3 biological replicates (hH). Bar is 200 µm in A, C, E and 50 µm in G. ND means: not detected.

Fig. 5 Osteoblastic and chondroblastic differentiation of BSa2 cells confirmed by immunofluorescence labeling. A–D, cCells immunolabeled with anti-osteocalcin1 antibody (a–d); E–H, cells immunolabeled with anti-chondroitin sulphate proteoglycans antibody (e–h); I–L,

cells immunolabeled with anti-collagen 2 antibody (i, l); M–P, Negative control (m–p). A,E,I,M cCells labeled with DAPI (blue) (a, e, i, m); B,F,J,N cells labeled with Alexa (red) (b, f, j, n); C,G,K,O shows DIC image (greygray) (c, g, k, o); D,H,L,P merged images of DAPI (blue) (d, h, l, p), Alexa 594 (red) and DIC (greygray) for T3 (3 weeks treatment). Bar is 10 μm.

Fig. 6 Osteoclastic differentiation of BSa2 cells at passage 14 assessed through tartrate-resistant acid phosphatase (TRAP) staining. Micrographs of BSa2 cells seeded on plastic cover slips at time 0 (Aa) and after exposure to RANKL and M-CSF after 2 weeks (Bb) and 3 weeks (Cc). Inserts in C show other TRAP-positive cells in different areas of the cover slips. Number of TRAP-positive cells per coverslip after 2 weeks (T2) and 3 weeks (T3) of treatment (dD). TRAP-positive cells are indicated by arrows. All the images were taken with DIC contrast. Bar is 10 μm. ND means: not detected.

Fig. 7 Osteoclastic differentiation of BSa2 cells at passage 23 assessed through pit resorption assay using osteologic discs and real-time PCR analysis using osteoclast specific genes. Micrographs of BSa2 cells seeded on osteologic discs exposed to RANKL and M-CSF after 3 weeks (Bb) or left untreated (Aa). Quantification of resorbed area (Ppercent of resorption/ total disc area) presented as mean ± standard deviation calculated from 3 different osteologic discs (Cc). Images Aa and Bb are at × 40X magnification. Inserts in aA shows other resorption pits in different areas of the discs. Real-time PCR analysis of osteoclast specific genes in BSa2 cells treated for 3 weeks with RANKL/M-CSF (d,D E,e). ***ctsk***, Cathepsin K; ***ostf-1,*** Osteoclast-stimulating factor-1. Values are presented as mean ± standard deviation calculated from 3 biological replicates. Asterisks indicate values statistically significant from control and cardinals indicate values statistically significant from 1st and 3rd week of RANKL/M-CSF treated culture (one-way ANOVA $P < 0.05$).

Fig. 8 Real-time PCR analysis of bone-related genes in BSa2 cells treated for 3 weeks with osteoblastic cocktail. ***spp1***, osteopontin; ***oc1***, osteocalcin1;, ***sparc***, osteonectin;; ***bmp2***, bone morphogenetic protein 2;; ***col1a1***, collagen type 1;, ***s100***, s100-like calcium-binding protein. **C** means: control cultures; **M**: means mineralizing cultures. Values are presented as mean ± standard deviation calculated from 3 biological replicates. Asterisks indicate values statistically significant from T0 and cardinals indicate values statistically significant from mineralization and control (one-way ANOVA $P < 0.05$).