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MARINE INVERTEBRATE CELL CULTURE

Expression of biomineralisation genes in tissues and cultured cells of the abalone *Haliotis tuberculata*

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Abstract Mollusc shell biomineralisation involves a variety of organic macromolecules (matrix proteins and enzymes) that control calcium carbonate ($CaCO_3$) deposition, growth of crystals, the selection of polymorph, and the microstructure of the shell. Since the mantle and the hemocytes play an important role in the control of shell formation, primary cell cultures have been developed to study the expression of three biomineralisation genes recently identified in the abalone *Haliotis tuberculata*: a matrix protein, Lustrin A, and two carbonic anhydrase enzymes. Mantle cells and hemocytes were successfully maintained in primary cultures and were evaluated for their viability and proliferation over time using a semi-automated assay (XTT). PCR and densitometric analysis were used to semi-quantify the gene expression and compare the level of expression in native tissues and cultured cells. The results demonstrated that the three genes of interest were being expressed in abalone tissues, with expression highest in the mantle and much lower in the hemocytes and the gills. Biomineralisation genes were also expressed significantly in mantle cells, confirming that primary cultures of target tissues are suitable models for in vitro investigation of matrix protein secretion.

Keywords Biomineralisation · Gene expression · Primary culture · *Haliotis tuberculata*

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Introduction

The shell of marine mollusc is a composite biomaterial made of calcium carbonate ($CaCO_3$) intimately associated with organic matrix components that are secreted by mantle epithelial cells. Shell biomineralisation involves a variety of proteins, glycoproteins, chitin and lipids (the organic matrix) that control $CaCO_3$ deposition, growth of crystals, the selection of polymorph, and the microstructure of the shell.

The European abalone *Haliotis tuberculata* is a relevant model organism to study the basic mechanisms of shell formation, owing to its highly ordered columnar nacre and its potential applications in

jewellery and biomimetics (Lin and Meyers 2005). There is already detailed knowledge on abalone shell microstructure and mineralogy (Dauphin et al. 1989; Lin and Meyers 2005; Nakahara et al. 1982) and more recent studies focused on the onset of shell mineralization in the European abalone *H. tuberculata* (Jardillier et al. 2008; Auzoux-Bordenave et al. 2010). To further understand the biological control of shell formation, Gaume et al. (2011) evidenced a correlation between the main steps of shell formation and the enzymatic activities of both alkaline phosphatase and carbonic anhydrase. Shell matrix proteins are quite well referenced within the *Haliotids*, although to a lesser extent than in Bivalves, including to date Lustrin A (Shen et al. 1997), AP7–AP24 (Michenfelder et al. 2003), Perlucin and Perlustrin (Weiss et al. 2000), Perlwapin (Treccani et al. 2006) and Perlinhibin (Mann et al. 2007). More recently, the comparison of peptide sequences obtained by proteomics with translated transcripts from EST library allowed the finding of twelve novel shell proteins in the abalone *Haliotis asinina* (Marie et al. 2010; Jackson et al. 2006). In the European abalone *H. tuberculata*, two carbonic anhydrase enzymes were characterized from the shell-forming mantle tissue (Le Roy et al. 2012). From the shell matrix, only few peptides sequences were found to match with the EST library from *H. asinina* mantle (Bédouet et al. 2012). Furthermore, a partial sequence of the *Lustrin A* gene has been recently identified (GenBank accession number: HM852427) and its expression is being studied over the development of the European abalone (Gaume et al. 2013).

Although some putative functions have been proposed based on the origin and the structure of these matrix proteins, we are still far from understanding their role in the biomineralisation process. It is now well established that mantle epithelial cells are responsible for the secretion of matrix proteins and enzymes whereas circulating hemocytes are involved in ion transportation during shell formation and regeneration (Wilbur 1964; Awaji and Suzuki 1998). A number of publications have reported the use of primary cell cultures from marine molluscs for studies on biomineralisation (Suja and Dharmaraj 2005; Awaji and Machii 2011; Auzoux-Bordenave and Domart-Coulon 2010; Gong et al. 2008a). The formation of calcium carbonate crystals was reported in mantle explant cultures from the pearl oyster *Pinctada fucata* (Suja and Dharmaraj 2005; Machii and Wada 1989) and from the abalone *Haliotis varia* (Suja and Dharmaraj 2005; Machii and Wada 1989). More recently, the expression of three genes encoding matrix proteins was reported in mantle explant cultures from the pearl oyster *P. fucata*, demonstrating that primary cultures were suitable for in vitro investigations of matrix protein secretion (Gong et al. 2008b). On the other hand, hemocyte primary cultures were used to investigate the regulation of collagen synthesis in vitro (Serpentini et al. 2000) and the expression of heat-shock proteins under thermal stress (Farcy et al. 2007). Furthermore, it has been suggested that hemocytes might be directly involved in shell mineralization by storing $CaCO_3$ as intracellular deposits and delivering crystals to the mineralization front (Mount et al. 2004). However, the role of circulating hemocytes and their cooperation with mantle epithelial cells during shell mineralization still needs to be clarified.

To further understand the cellular and molecular mechanisms involved in the shell formation process, the present work aimed at studying the expression of three biomineralisation genes recently identified in the abalone *H. tuberculata,* a matrix protein, Lustrin A, and two carbonic anhydrase enzymes. Since the mantle and the hemocytes play an important role in the control of shell formation, the level of gene expression was investigated in both native tissues and derived primary cultures. The gills, which are not directly involved in shell mineralization, were used as a reference tissue. The explant culture method was used for the establishment of mantle primary cultures, since it provides a reliable in vitro model containing all the cell types present in the tissue of origin.

Materials and methods

Source and maintenance of animals

European adult abalones (*H. tuberculata*), 70 to 90 mm in shell length, were collected from the North coast of Brittany (Roscoff, France). Animals were maintained at the laboratory in an 80L tank supplied with aerated natural seawater. Seasonal conditions of water temperature, salinity and photoperiod were used. Abalones were fed once a week with the red algae *Palmaria palmata*. 2 days before experiments, the abalones were starved and sea water was UV treated to prevent contamination.

Mantle cell cultures

Parts of the mantle, in contact with the shell edge, were removed from the animals and stored at 4 °C for 2 days in an antiseptic solution containing 200 U/mL penicillin, 200 µg/mL streptomycin, 250 µg/mL gentamycin and 2 µg/mL amphotericin B (Sigma, St. Louis, MO, USA) with the solution changed once. Tissues were then minced into 2 mm^3 explants which were placed, at a density of 12–16 explants per well, to adhere onto the bottom of 6 well plates (Dutscher, Brumath, France). Explants were left to adhere for 1 h before 1 mL of a modified Leibovitz L-15 medium (L-15, Sigma, adjusted to 1,100 mosmol/L by addition of mineral salts (20.2 g/L NaCl, 0.54 g/L KCl, 0.6 g/L $CaCl_2$, 1 g/L $MgSO_4.7H_2O$, 3.9 g/L $MgCl_2.6H_2O$) and supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 200 µg/mL gentamycin and 1 µg/mL amphotericin B (Sigma) was added in each well. Culture plates were kept in an incubator at 19 °C and observed daily with an inverted light microscope (Telaval 3, Zeiss, Oberkochen, Germany).

After 5–6 days of primary culture, a large quantity of mantle cells were generated from the explants and the cells were transferred to 96-well plates for cell viability assay (XTT), as previously described (Auzoux-Bordenave et al. 2007). RNA extractions were performed from cultured cells, 3 to 14 day-old, and from fresh tissue (t0).

Hemocyte cell culture

Hemolymph was withdrawn from the abalone with a sterile syringe inserted into the sinus located below the mouth. Extracted hemolymph was filtered on a 40 µm-sieve and then diluted in a 1:1:1 ratio with a sterile anticoagulant solution (383 mM NaCl, 115 mM glucose, 37 mM sodium dihydrogen citrate, 11 mM EDTA, 100 U/mL penicillin, 100 µg/mL streptomycin, diluted in ultra-pure water, pH 7.5) and an antiseptic solution (200 U/mL penicillin, 200 µg/mL streptomycin, 250 µg/mL gentamicin, 2 µg/mL amphotericin B, diluted in filtered sea water, pH 7.4). Hemocytes were plated at 2.10^7 cells per 75 cm^2 culture flask (Dutscher) for mRNA extraction, or at a cell density from 20,000 to 80,000 cells/well in 96-well microplates (Dutscher) for XTT assay. Cells were allowed to adhere for 1 h before the replacement of the salt-solution with modified L-15 medium (1,100 mosmol/L, pH 7.4). Plates were incubated at 19 °C. RNA extractions were performed from cultured hemocytes, 3 to 14 day-old, and from freshly sampled hemolymph (t0).

XTT reduction assay

Cell viability was evaluated using the XTT assay (Roche Laboratory, Meylan, France), based on the reduction of a tetrazolium salt into yellow formazan salt by active mitochondria (Mosmann 1983). As with the MTT assay, previously adapted to marine mollusc cells, the XTT assay provided a global evaluation of the number of viable cells (Domart-Coulon et al. 2000). For each experiment, 50 µL of XTT/PMS mixture (0.02:5) was added to each well containing 100 µL of cell suspension. Plates were incubated for 6 h at 19 °C in the dark. The optical density (OD) was measured by spectrophotometry at 490 nm with a 655 nm reference wave-length (Bio-Tek plate reader, Colmar, France).

Data were the mean ± SE of six values expressed as mOD 490 nm/655. Statistical analysis was performed using a two-sample t test, with a significant difference at $P < 0.05$.

Total RNA extraction

Mantle cells and hemocytes were collected from the culture flasks at 3, 5, 7, 10, 12 and 14-day culture with a cell scraper, passed through a 70 µm cell strainer (VWR, Fontenay sous Bois, France) and centrifuged at 3,000g. Around 25.10^6 cells were used per extraction and homogeneized using TRIzol® (Invitrogen, Carlsbad, CA, USA), 1 mL Trizol/10^7 cells, according to the method from Chomczynski and Sacchi (1987).

Total RNA was also extracted from fresh tissues of adult abalones. Hemolymph was first collected using sterile syringe inserted into the sinus below the mouth. 5 mL of hemolymph were filtered on a 40 µm-sieve (VWR) and immediately centrifuge at 3,000g for 15 min. After removal of supernatant, 1 mL of Trizol was added to the pellet of hemocytes. Epithelium of the right and left lobes of the mantle (around 200 mg) was collected with a sterile scalpel and immediately mixed with 1 mL of Trizol. Gills (around 200 mg) were extracted with scissors from the pallial cavity and immediately homogeneized with 1 mL of Trizol. Tissues were then grounded in a 1.5 mL microtube using a micro pestle. After a centrifugation at 12,000g for 10 min at 4 °C, supernatant was collected in a clean micro-centrifuge tube.

RNA from homogenized cell and tissue samples was then isolated by adding chloroform (0.2 mL), then centrifuged (15 min 12,000*g* at 4 °C). The RNA, contained in the aqueous phase, was precipitated with isopropanol (0.5 mL). After rinsing with 75 % ethanol (1 mL), total RNA was dried and resuspended in RNase-free water (50 mL). To avoid genomic DNA amplification during PCR analyses, total RNA was treated with Desoxyribonuclease I (Sigma) following the manufacturer's instructions. The quality and quantity of isolated RNA were determined with a UV/visible spectrophotometer (NanoView Plus GE Healthcare, Velizy-Villacoublay, France).

Reverse transcriptase PCR

Reverse transcription was performed from 1 µg of total RNA using the M-MLV Reverse Transcriptase (Promega, Charbonnières les Bains, France) according to the manufacturer's recommendations. Oligo (dT) primers (Promega) and total RNA were incubated 10 min at 70 °C and chilled on ice. Then in vitro transcription was carried out at 25 °C for 5 min, 50 °C for 1 h and 70 °C for 15 min in a 20 µl reaction mixture containing 0.5 mM dNTPs, 0.5 units of RNasine® (Promega) and 200 units of M-MLV reverse transcriptase. Specific primers were used to perform the PCR amplification of *Lustrin A* (*LusA*), *Carbonic anhydrases 1* (*HtCA1*) and *2* (*HtCA2*). mRNA encoding the cytoskeleton protein actin was used as a reference to normalize the expression levels of Lustrin A and carbonic anhydrases. *Actin* was previously shown to be a suitable reference gene for gene expression studies in abalone tissues (Le Roy et al. 2012) and cell cultures (Farcy et al. 2007). The stability of *Actin* expression was also checked in mantle cells and hemocytes at different times of primary culture. Sequences of the primers used are presented in Table 1.

For *Lustrin A*, specific primers were designed based on the amino acid sequence of Lustrin A identified in *H. tuberculata* (Genbank accession number: HM852427). The primers are located between the basic and the WAP domains of the protein, PCR generating a 362 bp amplification product. For *Carbonic anhydrase* genes (*HtCA1* and *HtCA2*) specific primers designed from the amino acid sequences of the Carbonic anhydrases recently identified in *H. tuberculata* were used (Genbank accession numbers: AEL22200 and AEL22201), generating 246 and 160 bp amplification products respectively (Le Roy et al. 2012).

PCR amplification was performed using Platinum® Taq DNA Polymerase (Invitrogen). To semi-quantify the levels of *Lustrin A*, *HtCA1*, *HtCA2* and *Actin* expression in various tissues and cell cultures over time, the linear phase of PCR amplification was determined experimentally for each gene. Each PCR was performed in a final volume of 100 µl, according the following program: 3 min at 94 °C, 30 sec at 94 °C, 30 sec at annealing temperature ranging from 50 to 60 °C, 1 min at 72 °C and final extension 10 min at 72 °C. PCR was run for 50 cycles. 10 µl of each PCR reaction was collected every five cycles from 20 to 50 cycles and samples were then separated on a 1.5 % agarose gel electrophoresis and photographed. After analysis with Image J software, an annealing temperature of 55 °C and 35 cycles were determined as optimal PCR conditions, allowing comparison of gene expression.

Semi quantitative gel analysis

Densitometric analysis was undertaken using the method found on the image J software (NIH) information website (Image J information and documentation portal: http://imagejdocu.tudor.lu). Gel backgrounds were removed and images clarified, lanes were then

Table 1 Specific primer sequences used for the PCR amplification of *Lustrin A*, *Carbonic anhydrases 1* (*HtCA1*) and *2 (HtCA2)*, and *Actin* genes in *H. tuberculata*

Primer name	Expected size (bp)	Sequence
Lustrin A 31F	362	5′-CGACCTGCCGCAAGACCCTT-3′
Lustrin A 31R	362	5′-GCCTTACGGCGGGGCAACTT-3′
HtCA1-1F	246	5′-TGGACATTGCCTGTGTTGTT-3′
HtCA1-1R	246	5′-TCACTTCCTCGGGGTCTATG-3′
HtCA2-2F	160	5′-CGCCGACTTTATCTGAGAGC-3′
HtCA2-2R	160	5′-GTCTCCCACGAAGTGGTTGT-3′
Actin-F	534	5′-CCATCTACGAGGGATATGCC-3′
Actin-R	534	5′-CAATCCAGACGGAGTATTTCC-3′

drawn onto the gels and measurements of the intensity of the bands was taken. The area under of the peaks were measured and the intensity of *Lustrin A* and *Carbonic anhydrases* were converted into a ratio with *Actin* for comparison between gels.

Results

Characterization of mantle cell cultures

Mantle cells were successfully maintained in primary cultures as previously described (Auzoux-Bordenave et al. 2007). Explant mantle cultures allowed for the production of a large quantity of cells, with a majority of epithelial cells (i.e. round, columnar and glandular cells) and circulating hemocytes as previously reported for abalone mantle cells (Auzoux-Bordenave et al. 2007). After 5–6 days of primary culture, the migrating cells were transferred to 96-well plates and checked for their morphology and cell viability over time (Fig. 1).

The morphology of mantle sub-cultures at 1, 7 and 14 days respectively is illustrated in Fig. 1a–c. Epithelial cells represented the main cell population and included round cells, 6–10 µm in diameter, and columnar cells, 15–25 µm in length, that are typical of the mantle epithelium.

The XTT assay was used to measure the metabolic activity of mantle cells in vitro. Figure 1d shows the XTT response of sub-cultured mantle cells as a function of cell density, over a 12 day period. The evolution of XTT response of mantle cells showed a slight decrease during the first 4 days in vitro and an increase in global cell metabolism with cell density.

Characterization of hemocyte primary cultures

Hemocytes were successfully maintained in primary cultures as previously described (Auzoux-Bordenave et al. 2007). The morphology of hemocytes at 1, 8 and 14 days of culture is illustrated in Fig. 2a–c. The hemocytes quickly adhered to the flasks and two cell populations were easily recognizable: small and large round hyalinocytes, 5–12 µm in diameter, and fibroblast-like cells, 50–100 µm in length, displaying long pseudopodia and forming cellular networks. The proportion of these two cell populations changed over culture time and hemocyte culture exhibited a high plasticity. The evolution of XTT response of hemocytes over culture time is shown in Fig. 2d. Hemocyte viability is maintained for 14 days and increased for the highest densities. A high variability was found at the highest cell density due to cells clustering within the wells.

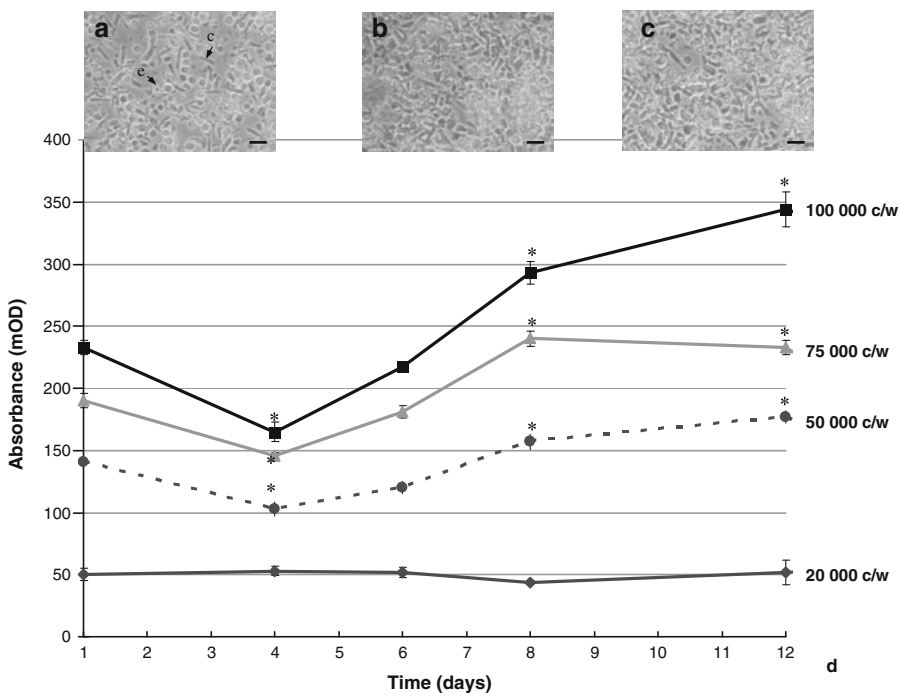

Fig. 1 Characterization of mantle cell sub-cultures. Morphology of mantle cell sub-cultures at 1 day (*a*), 7 days (*b*) and 14 days (*c*). *c* columnar cells; *e* round epithelial cells. *Scale-bar* 50 µm. *d* Evolution of XTT response of mantle cells over a 12 days period showing a global increase of cell metabolism. Cells were plated at four densities ranging from 20,000 to 100,000 cells/well in 96-well microplates. Data are the mean ± SE of six values expressed as mOD 490 nm/655. *Indicate a significant difference ($P < 0.05$) from absorbance measured at day 1

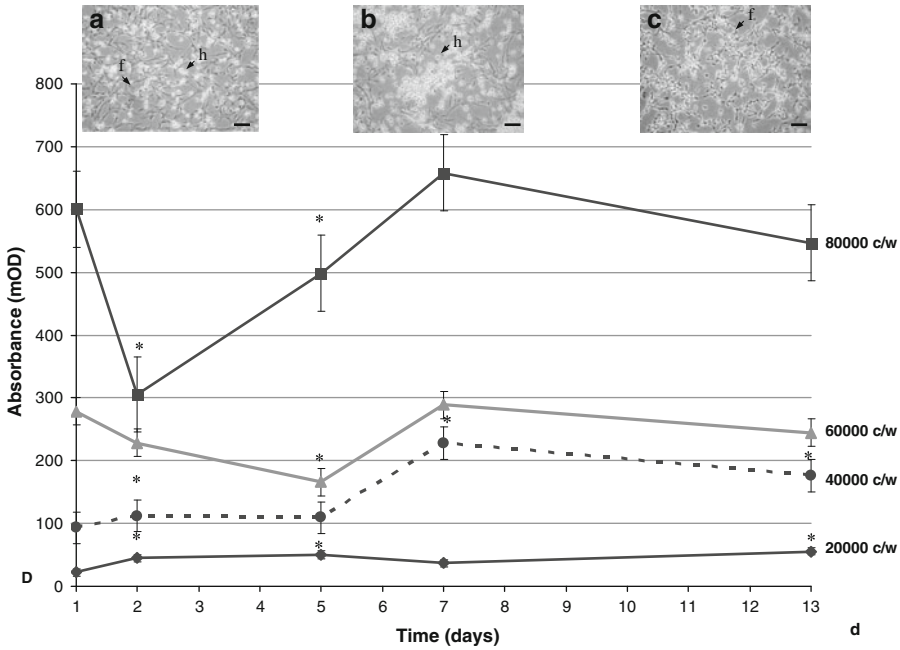

Fig. 2 Characterization of hemocyte primary cultures. Morphology of cultured hemocytes at 1 day (*a*), 8 days (*b*) and 14 days (*c*). *f* fibroblast-like cells; *h* hyalinocytes. *Scale-bar* 50 µm. *d* Evolution of XTT of response of hemocytes over a 13 days period. Cells were plated at four densities ranging from 20,000 to 80,000 cells/well in 96-well microplates. Data are the mean ± SE of six values expressed as mOD 490 nm/655. *Indicate a significant difference ($P < 0.05$) from absorbance measured at day one

In the following, native tissues and derived primary cultures were used in parallel assays to study the expression of biomineralising genes.

Gene expression in tissues

The levels of expression of three genes involved in the biomineralisation process—*Lustrin A* and two *Carbonic anhydrases*—were analysed by PCR in native tissues of the European abalone (Fig. 3). The three genes are expressed in abalone tissues as shown by the electrophoresis gel with the bands corresponding to the amplification products of *Carbonic anhydrase 2* (150 bp), *Carbonic anhydrase 1* (250 bp), and *Lustrin A* (350 bp) genes, respectively (Fig. 3a). The band at 500 bp corresponds to the housekeeping gene *Actin* used as a reference.

Semi quantification of gene expression shows that *Lustrin A* is highly expressed in the mantle compared to the levels measured in the gills and hemocytes (Fig. 3b). *Carbonic anhydrase 1* is also expressed in the mantle, to a lesser extent than *Lustrin A*, and is weakly expressed in the gills and hemocytes. *Carbonic anhydrase 2* is expressed in all three tissues to an equal extent.

Gene expression in cultured cells

Total RNA was extracted at increasing times of primary cultures to investigate the expression of biomineralisation genes in mantle cells (Fig. 4) and hemocytes (not shown). The relative expression of *Lustrin A*, *Carbonic anhydrase 1* and *Carbonic anhydrase 2* genes was analysed in mantle cells over 14 days in primary culture (Fig. 4a). *Lustrin A* and both *Carbonic anhydrase* genes are significantly expressed in cultured mantle cells. The levels of expression of *Lustrin A* and *Carbonic anhydrase 2* were higher compared to that of *Carbonic anhydrase 1* at any time of cell culture (Fig. 4b). After 10 days of primary culture the levels of expression of the three genes was significantly decreased in mantle cells. In cultured hemocytes, none of the three genes were expressed over time in cell culture (data not shown).

Discussion

To further understand the cellular process of matrix protein secretion, the present work aimed at studying the expression of three biomineralisation genes in both native tissues and derived primary cultures from the European abalone *H. tuberculata*.

Previous studies have shown that primary explants cultures from the mantle were suitable in vitro models to study the process of biomineralisation, maintaining cell viability and cell-to-cell interactions for periods ranging from 10 to 30 days in vitro (Kleinschuster et al.

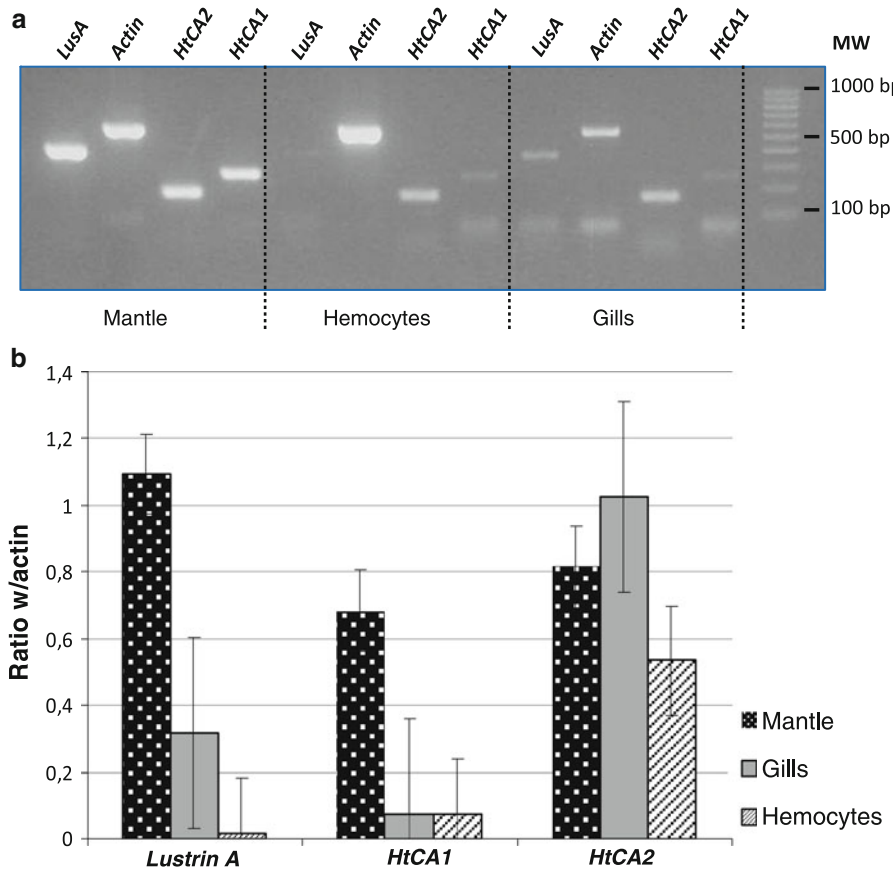

Fig. 3 RT-PCR analysis of the relative expression levels of *Lustrin A*, *Carbonic anhydrase 1* and *Carbonic anhydrase 2* genes in tissues of *H. tuberculata.* **a** Electrophoresis gel showing the three bands corresponding to the amplification products of *Carbonic anhydrase 2* (150 bp), *Carbonic anhydrase 1* (250 bp), and *Lustrin A* (350 bp) genes in the mantle, the hemocytes and the gills of the abalone. The band at 500 bp corresponds to the housekeeping gene *Actin* used as a reference. *LusA: Lustrin A; HtCA1: Carbonic anhydrase 1; HtCA2: Carbonic anhydrase 2*; *bp* base pairs; *MW* molecular weight. **b** Relative expression of the three genes in the mantle, in the gills, and in freshly collected hemocytes. Semi quantitative gel analysis was performed using the ImageJ software package (NIH). The intensity of the bands was converted into a ratio with *Actin* gene as a reference. *Indicate a significant difference with the level of expression in the mantle ($P < 0.05$)

1996; Auzoux et al. 1993; Machii and Wada 1989; Awaji and Suzuki 1998). In this study, mantle cells were sub-cultured after 5–6 days of explant primary culture, and were checked for morphology and cell metabolism along cell culture, according to the methods previously described (Auzoux-Bordenave et al. 2007). Mantle cells were successfully maintained in sub-cultures for up to 12 days with a significant metabolic activity as shown by the XTT cell response. The initial decline over the first 4 days is probably due to cell adaptation to their in vitro environment. Since we did not observe any proliferation features, the increase of the global activity would rather correspond to an increase of individual cell metabolism. This is consistent with previous studies showing only few dividing cells in primary cultures of adult tissues (Rinkevich 1999).

Since hemocytes are supposed to participate in the biomineralisation process, we also developed primary cultures from circulating cells. Two main cell categories were recognized in vitro according to previous characterization of abalone *H. tuberculata* hemocytes (Travers et al. 2008; Gaume et al. 2012). Hemocytes displayed a significant metabolic activity for up to

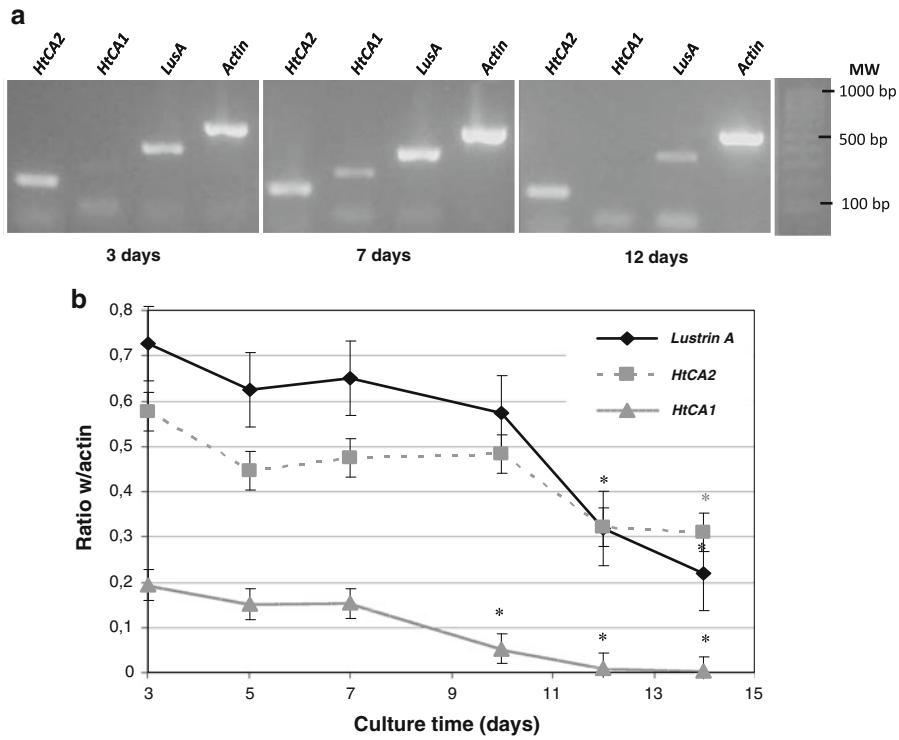

Fig. 4 RT-PCR analysis of the relative expression levels of *Lustrin A*, *Carbonic anhydrase 1* and *Carbonic anhydrase 2* genes in mantle cell cultures from *H. tuberculata.* **a** Electrophoresis gel showing the PCR amplification of *Carbonic anhydrase 2* (150 bp), *Carbonic anhydrase 1* (250 bp), and *Lustrin A* (350 bp) genes at day 3, 7 and 12 of cell culture. The band at 500 bp corresponds to the housekeeping gene *Actin* used as a reference. *LusA: Lustrin A; HtCA1: Carbonic anhydrase 1; HtCA2 : Carbonic anhydrase 2*; *bp* base pairs; *MW* molecular weight. **b** Relative expression of *Lustrin A*, *HtCA1* and *HtCA2* genes in cultured mantle cells over 14 days in vitro. Semi quantitative gel analysis was performed using the Image J software package (NIH). The intensity of the bands was converted into a ratio with *Actin* as a reference gene. *Indicate a significant difference from expression at day 3 ($P < 0.05$)

13 days in vitro. As observed previously, hemocytes exhibited an increased metabolic response compared to mantle cells and a higher heterogeneity due to cell clustering in the wells (Boulo et al. 1991; Auzoux-Bordenave et al. 2007).

The patterns of expression of *Lustrin A* and *Carbonic anhydrase* genes were analysed in the mantle, the hemocytes and the gills of the European abalone *H. tuberculata*. *Lustrin A* was expressed in all three tissues with significantly higher expression levels in the mantle than in the gills and hemocytes. These results are in accordance with previous studies showing the specific expression of *Lustrin A* in the mantle of *Haliotis rufescens* (Shen et al. 1997). Furthermore, the expression and localisation of *Lustrin A* transcripts were evidenced in mantle cells of juvenile abalones *H. asinina* and *H. tuberculata* (Gaume et al. 2013; Jackson et al. 2007). The tissue expression patterns of *Lustrin A* found in this study support the role of this protein in the biomineralisation process.

Carbonic anhydrase 1 (HtCA1) was also significantly expressed in the mantle, that is consistent with previous data on the specific expression of *HtCA1* in abalone mantle (Le Roy et al. 2012). Together, the high levels of expression of *Lustrin A* and *HtCA1* support the role of the mantle tissue in the secretion of matrix proteins. On the other hand, we found that the *Carbonic anhydrase 2* gene was expressed equally in the mantle, the gills and the hemocytes, suggesting a more general role for this enzyme. By contrast, Le Roy et al. (2012) reported a specific expression of *HtCA1* and *HtCA2* in the abalone mantle. The different patterns of expression found in abalone tissues in the

present study suggest different roles for the two carbonic anhydrases. HtCA1 has been characterized as a secreted protein (Le Roy et al. 2012) which may participate to calcium carbonate formation at the mineralizing site. The mantle-specific expression found in our study is consistent with a specific role of HtCA1 in biomineralising process. However, the incorporation of HtCA1 into the shell matrix network has not yet been demonstrated. HtCA2 has been previously characterized as a membrane-associated protein which could play a different role, for example in ionic transportation and pH regulation (Le Roy et al. 2012). The expression of *HtCA2* in the mantle, the gills and the hemocytes is consistent with a non specific role of this enzyme in basal cell metabolism such as intracellular CO_2 exchanges and proton transfer. Furthermore, the expression of *HtCA2* in the gills is in accordance with previous carbonic anhydrase activities reported in abalone gills, showing a higher carbonic anhydrase activity (1.7 times) than in the mantle (Duvail and Fouchereau-Peron 2001; Gaume et al. 2011).

Thus, two forms of carbonic anhydrase (non specific and mantle-specific) might be expressed differentially in abalone tissues according to the seasonality and the growth activity:

- During active biomineralising period (spring), one form of carbonic anhydrase would be specifically expressed in the mantle, allowing an acceleration of $CaCO_3$ precipitation at the mineralization site,
- During slow growth period (winter), the other carbonic anhydrase form might be generally expressed in all tissues, acting as a key-cell regulator in pH regulation and ionic transmembrane transports.

Only few studies reported seasonal variations in the expression of biomineralising genes. In the mantle of the pearl oyster *P. fucata*, the levels of expression of *MSI60* and *N16* genes were higher in winter season, while no seasonal variation was found in the *Nacrein* expression level (Miyazaki et al. 2008). Nacrein contains two anhydrase carbonic anhydrase domains and GXN repeat domains, thus it is supposed to have a dual function in both CO_2 hydration and $CaCO_3$ interactions (Le Roy et al. 2012). Since the present experiments were performed on adult abalones collected between February and April (slow growth period), the *HtCA2* expressed in the various abalone tissues would rather correspond to a generalist Carbonic anhydrase, regulating basal cell metabolism during slow biomineralising activity.

The expression of shell matrix protein was previously reported in mantle cell cultures from the pearl oyster *P. fucata* (Gong et al. 2008b). In the present study, primary cultures from the abalone were used to investigate the expression of *Lustrin A*, *HtCA1* and *HtCA2* in both hemocytes and mantle cells. The patterns of expression of the three biomineralising genes in mantle cells are in accordance with relative expression measured in abalone mantle tissue. *Lustrin A* was significantly expressed for up to 10 days in vitro, confirming the maintenance of cell functionality and the role of mantle cells in the synthesis of this matrix protein. In the pearl oyster *P. fucata*, Gong et al. (2008b) evidenced the expression of three shell matrix proteins, *MSI7*, *MSI60* and *Nacrein*, in mantle cell cultures for up to 12 days in vitro. Nacrein was also detected in the culture medium confirming the protein secretion from the mantle explants.

The levels of expression for *HtCA2* gene were also maintained at a significant level in vitro, that is consistent with a more general role of this enzyme in the maintenance of basal cell metabolism. *HtCA1*, however, exhibited a significantly lower expression in vitro than the other two genes. Since *HtCA1* was expressed at a higher level in the mantle tissue in vivo, the low expression found in primary culture might be related to a down-regulation of this gene following the adaptation to in vitro conditions. After 12 days of cell culture, the expression levels dropped significantly implying that mantle cells stop producing these proteins. Since cell-to-cell interactions are involved in the regulation of matrix gene expression, the decrease in gene expression may be likely due to the lack of cell signalling from the rest of organism. These results indicate that the optimal time for studying gene expression is between 0 and 10 days in vitro, as previously observed in primary cultures from marine molluscs (Gong et al. 2008b; Awaji and Machii 2011). Biomineralising genes were also expressed in freshly collected hemocytes to a lower extent than in the mantle. However, the expression levels were not maintained over time in vitro although the metabolic activity was still significant. From these results, we can assume that hemocytes are not directly involved in the basal biomineralization processes but may only be utilized for shell repair, as shown by Fleury et al. (2008).

The expression of *Lustrin A* and *Carbonic anhydrases* genes in both shell-forming tissue and cultured cells of the abalone support the role of these proteins in the biomineralisation process. Furthermore, the in vitro expression of the three genes demonstrated that cultured mantle cells maintain the functional capacities of shell formation, providing further evidence that primary cultures are reliable models for in vitro investigation of matrix protein secretion. *Lustrin A*, as expected, was confirmed to be very specific to the mantle cells and was expressed to a larger extent than carbonic anhydrases. Since the regulation of biomineralisation genes appeared complex, with various sets of genes being activated spatially and temporally in different tissues, at different stages of the life cycle (Jackson et al. 2007), more in depth studies will be necessary to clarify the respective roles and the interactions between multifunctional matrix proteins such as Lustrin A and biomineralising enzymes. *In situ* hybridisation and immunohistochemical experiments would help to specify the localisation of biomineralising proteins within the tissues and the cells. The development of cell typing and the improvement of cell requirements in vitro would be also essential to determine the functions of epithelial and circulating cells in ion transport, shell matrix synthesis and secretion as well as calcium carbonate deposition.

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References

Auzoux S, Domart-Coulon I, Doumenc D (1993) Gill cell cultures of the butterfish clam *Ruditapes decussatus*. J Mar Biotechnol 1:79–81

Auzoux-Bordenave S, Domart-Coulon I (2010) Marine invertebrate cell cultures as tools for biomineralization studies. Journal des Sciences Halieutique et Aquatique 2:42–47

Auzoux-Bordenave S, Fouchereau-Peron M, Helléouet M-N, Doumenc D (2007) CGRP regulates the activity of mantle cells and hemocytes in abalone primary cell cultures (*Haliotis tuberculata*). J Shellfish Res 26:887–894

Auzoux-Bordenave S, Badou A, Gaume B, Berland S, Helléouet M-N, Milet C, Huchette S (2010) Ultrastructure, chemistry and mineralogy of the growing shell of the European abalone *Haliotis tuberculata*. J Struct Biol 171:277–290. doi:10.1016/j.jsb.2010.05.012

Awaji M, Machii A (2011) Fundamental studies on in vivo and in vitro pearl formation—contribution of outer epithelial cells of pearl oyster mantle and pearl sacs. Aqua-BioSci Monogr 4:1–39. doi:10.5047/absm.2011.00401.0001

Awaji M, Suzuki T (1998) Monolayer formation and DNA synthesis of the outer epithelial cells from pearl oyster mantle in coculture with amebocytes. In Vitro Cell Dev Biol Anim 34:486–491

Bédouet L, Marie A, Berland S, Marie B, Auzoux-Bordenave S, Marin F, Milet C (2012) Proteomic strategy for identifying mollusc shell proteins using mild chemical degradation and trypsin digestion of insoluble organic shell matrix: a pilot study on *Haliotis tuberculata*. Mar Biotechnol 14: 446–458. doi:10.1007/s10126-011-9425-0

Boulo V, Hervio D, Morvan A, Bachere E, Mialhe E (1991) In vitro culture of mollusc hemocytes. Functional study of burst respiratory activity and analysis of interactions with protozoan and procaryotic pathogens. In Vitro 27:42A

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

Dauphin Y, Cuif J-P, Mutvei H, Denis A (1989) Mineralogy, chemistry and ultrastructure of the external shell-layer in ten species of *Haliotis* with reference to *Haliotis tuberculata* (Mollusca: Archaeogastropoda). Bull Geol Inst University Uppsala 15:7–38

Domart-Coulon I, Auzoux-Bordenave S, Doumenc D, Khalanski M (2000) Cytotoxicity assessment of antibiofouling compounds and by-products in marine bivalve cell cultures. Toxicol In Vitro 14:245–251. doi:10.1016/s0887-2333(00)00011-4

Duvail L, Fouchereau-Peron M (2001) Calcium metabolism related markers during the growth of *Haliotis tuberculata*. Invertebr Reprod Dev 40:209–216

Farcy E, Serpentini A, Fiévet B, Lebel J-M (2007) Identification of cDNAs encoding HSP70 and HSP90 in the abalone *Haliotis tuberculata*: transcriptional induction in response to thermal stress in hemocyte primary culture. Comp Biochem Physiol B: Biochem Mol Biol 146:540–550. doi:10.1016/j.cbpb.2006.12.006

Fleury C, Marin F, Marie B, Luquet G, Thomas J, Josse C, Serpentini A, Lebel JM (2008) Shell repair process in the green ormer *Haliotis tuberculata*: a histological and microstructural study. Tissue Cell 40:207–218

Gaume B, Fouchereau-Peron M, Badou A, Helléouet M-N, Huchette S, Auzoux-Bordenave S (2011) Biomineralization markers during early shell formation in the European abalone *Haliotis tuberculata*, Linnaeus. Mar Biol 158:341–353. doi:10.1007/s00227-010-1562-x

Gaume B, Auzoux-Bordenave S, Bourgougnon N, Roig B, Le Bot B, Bedoux G (2012) In vitro effects of triclosan and methyl-triclosan on the marine gastropod *Haliotis tuberculata*. Comp Biochem Physiol C: Toxicol Pharmacol 156:87–94

Gaume B, Denis F, Van Wormhoudt A, Huchette S, Jackson DJ, Auzoux-Bordenave S (2013) Characterization and expression of *Lustrin A* biomineralizing gene during shell formation of the European abalone *Haliotis tuberculata*, in preparation

Gong N, Li Q, Huang J, Fang Z, Zhang G, Xie L, Zhang R (2008a) Culture of outer epithelial cells from mantle tissue to study shell matrix protein secretion for biomineralization. Cell Tissue Res 333:493–501

Gong N, Ma Z, Li Q, Li Q, Yan Z, Xie L, Zhang R (2008b) Characterization of calcium deposition and shell matrix protein secretion in primary mantle tissue culture from the marine pearl oyster *Pinctada fucata*. Mar Biotechnol 10:457–465

Jackson D, McDougall C, Green K, Simpson F, Worheide G, Degnan B (2006) A rapidly evolving secretome builds and patterns a sea shell. BMC Biol 4:40

Jackson D, Worheide G, Degnan B (2007) Dynamic expression of ancient and novel molluscan shell genes during ecological transitions. BMC Evol Biol 7:160

Jardillier E, Rousseau M, Gendron-Badou A, Fröhlich F, Smith D, Martin M, Helléouet M-N, Huchette S, Doumenc D, Auzoux-Bordenave S (2008) A morphological and structural study of the larval shell from the abalone *Haliotis tuberculata*. Mar Biol 154:735–744

Kleinschuster SJ, Parent J, Walker CW, Farley CA (1996) A cardiac cell line from *Mya arenaria* (Linnaeus, 1759). J Shellfish Res 15:695–707

Le Roy N, Marie B, Gaume B, Guichard N, Delgado S, Zanella-Cléon I, Becchi M, Auzoux-Bordenave S, Sire J-Y, Marin F (2012) Identification of two carbonic anhydrases in the mantle of the European abalone *Haliotis tuberculata* (Gastropoda, Haliotidae): phylogenetic implications. J Exp Zool B Mol Dev Evol 318B:353–367

Lin A, Meyers MA (2005) Growth and structure in abalone shell. Mater Sci Eng A 390:27–41

Machii A, Wada K (1989) Some marine invertebrates tissue culture. In: Mitsuhashi J (ed) Invertebrate cell system applications, vol 2. CRC Press, Boca Raton, pp 225–233

Mann K, Siedler F, Treccani L, Heinemann F, Fritz M (2007) Perlinhibin, a cysteine-, histidine-, and arginine-rich miniprotein from abalone (*Haliotis laevigata*) nacre, inhibits in vitro calcium carbonate crystallization. Biophys J 93:1246–1254

Marie B, Marie A, Jackson D, Dubost L, Degnan B, Milet C, Marin F (2010) Proteomic analysis of the organic matrix of the abalone *Haliotis asinina* calcified shell. Proteome Sci 8:54

Michenfelder M, Fu G, Lawrence C, Weaver JC, Wustman BA, Taranto L, Evans JS, Morse DE (2003) Characterization of two molluscan crystal-modulating biomineralization proteins and identification of putative mineral binding domains. Biopolymers 70:522–533. doi:10.1002/bip.10536

Miyazaki Y, Usui T, Kajikawa A, Hishiyama H, Matsuzawa N, Nishida T, Machii A, Samata T (2008) Daily oscillation of gene expression associated with nacreous layer formation. Front Mater Sci Chin 2:162–166

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63. doi:10.1016/0022-1759(83)90303-4

Mount AS, Wheeler AP, Paradkar RP, Snider D (2004) Hemocyte-mediated shell mineralization in the eastern oyster. Science 304:297–300

Nakahara H, Bevelander G, Kakei M (1982) Electron microscopic and amino acid studies on the outer and inner shell layers of *Haliotis rufescens*. Venus (Japan. J Malacol) 41:33–46

Rinkevich B (1999) Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. J Biotechnol 70:133–153

Serpentini A, Ghayor C, Poncet JM, Hebert V, Galéra P, Pujol JP, Boucaud-Camou E, Lebel J-M (2000) Collagen study and regulation of the *de novo* synthesis by IgF-I in hemocytes from the gastropod mollusc, *Haliotis tuberculata*. J Exp Zool 287:275–284

Shen X, Belcher AM, Hansma PK, Stucky GD, Morse DE (1997) Molecular cloning and characterization of Lustrin A, a matrix protein from shell and pearl nacre of *Haliotis rufescens*. J Biol Chem 272:32472–32481. doi:10.1074/jbc.272.51.32472

Suja CP, Dharmaraj S (2005) In vitro culture of mantle tissue of the abalone *Haliotis varia* Linnaeus. Tissue Cell 37:1–10

Travers M-A, Mirella da Silva P, Le Goic N, Marie D, Donval A, Huchette S, Koken M, Paillard C (2008) Morphologic, cytometric and functional characterization of abalone (*Haliotis tuberculata*) hemocytes. Fish Shellfish Immunol 24:400–411

Treccani L, Mann K, Heinemann F, Fritz M (2006) Perlwapin, an abalone nacre protein with three four-disulfide core (whey acidic protein) domains, inhibits the growth of calcium carbonate crystals. Biophys J 91:2601–2608

Weiss IM, Kaufmann S, Mann K, Fritz M (2000) Purification and characterization of perlucin and perlustrin, two new proteins from the shell of the mollusc *Haliotis laevigata*. Biochem Biophys Res Commun 267:17–21

Wilbur KM (1964) Shell formation and regeneration. In: Wilbur KM, Yonge CM (eds) Physiology of Mollusca. Academic Press, New York, pp 243–282