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ORIGINAL ARTICLE

Shell extracts of the edible mussel and oyster induce an enhancement of the catabolic pathway of human skin fibroblasts, in vitro

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Abstract Mollusc shells are composed of more than 95% calcium carbonate and less than 5% organic matrix consisting mostly of proteins, glycoproteins and polysaccharides. In this study, we investigated the effects of matrix macromolecular components extracted from the shells of two edible molluscs of economic interest, i.e., the blue mussel *Mytilus edulis* and the Pacific oyster *Crassostrea gigas*. The potential biological activities of these organic molecules were analysed on human dermal fibroblasts in primary culture. Our results demonstrate that shell extracts of the two studied molluscs modulate the metabolic activities of the cells. In addition, the extracts caused a decrease of type I collagen and a concomitant increase of active MMP-1, both at the mRNA and the protein levels. Therefore, our results suggest that shell extracts from *M. edulis* and *C. gigas* contain molecules that promote the catabolic pathway of human dermal fibroblasts. This work emphasises the potential use of these shell matrices in the context of anti-fibrotic strategies, particularly against scleroderma. More generally, it stresses the usefulness to valorise bivalve shells that are coproducts of shellfish farming activity.

Keywords Biological activity · Extracellular matrix · Fibroblast · Mollusc · Shell matrix

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Introduction

The mollusc shell is a natural composite material; it consists of inorganic components including calcium carbonate and of a mineral-occluded organic matrix network that constitutes between 0.1 and 5% (w/w) of the shell weight. Numerous studies have shown that this matrix is essential for controlling biomineral formation in the shell, and that it contains several macromolecules including polysaccharides, lipids, proteins and glycoproteins in addition to smaller molecules such as pigments, free amino acids and short peptides (for review, see Marin et al. 2012).

This mineralised structure has attracted great interests in the field of biomaterials due to its exceptional mechanical properties (Kaplan 1998). More recently, novel potential applications have emerged in the biomedical, cosmetic and nutraceutical industries: nacre also called mother-of-pearl—by far the most studied biomineralization together with bone—is a well-known example in this respect; it retains in its structure signalling molecules recognised by bone cells from vertebrate organisms. These molecules do not only stimulate certain cellular mechanisms but can also ensure their regulation. For example, it has been demonstrated that the activity of nacre can stimulate bone-forming cells, including stem cells, for repairing or maintaining tissues and can also lead to the final formation of physiologically active and healthy bones (Atlan et al. 1997, 1999; Liao et al. 2002; Milet et al. 2004). In vitro and in vivo studies have shown that nacre has excellent biocompatibility and osteogenic properties (Lopez et al. 1992; Rousseau et al. 2003, 2008). The compatibility of nacre signalling molecules with mammalian cells in osteogenesis provides strong evidence for the conservation—across Evolution—of molecular signals that trigger biomineralisation (Lopez et al. 1992; Westbroek and Marin 1998; Atlan et al. 1999; Lamghari et al. 2001). These results suggest that the complex machinery of biomineralisation in mammals and in molluscs share common structures that allow them to cross the barrier of the immune system and activate cells from another organism (Perreira-Mouriès et al. 2002b).

Beside their remarkable effects on bone-forming cells, nacre extracts were found to act on vertebrate skin cells, in particular on dermal fibroblasts. They are indeed effective in maintaining a normal healthy skin and in facilitating the regeneration of a wounded skin (Lee et al. 2011). The first studies on the implantation of nacre powder into the skin of rats have shown to improve the synthesis of the extracellular matrix, such as type I and type III collagens, and the production of components involved in adhesion and cell communication (Lopez et al. 2000).

Shell matrices from non-nacreous molluscs were also found to induce effects on skin cells. For example, extracts from the giant Ezo scallop *Patinopecten yessoensis* shell enhance the turnover rate of the epidermal layer and increase the recovery efficiency of the dorsal skin in UV-injured rats (Liu et al. 2006). Recently, Latire et al. (2014) have demonstrated that shell matrix components from the great scallop *Pecten maximus* regulate the synthesis of the extracellular matrix in primary-cultured human skin fibroblasts. More precisely, shell matrix components stimulate the synthesis of type I and III collagens, as well as that of sulphated GAGs. The increased expression of type I collagen is likely mediated by the recruitment of transactivating factors (Sp1, Sp3 and human c-Krox). These works emphasise the potential use of the shell matrix of scallops for dermo-cosmetic applications.

Although nacre is present in numerous marine and freshwater mollusc shells, it is striking to observe that most—if not all—studies on the effects of nacre focused on a single model, the pearl oyster (*Pinctada sp.*) (e.g., Berland et al. 2005; Perreira-Mouriès et al. 2002a; Rousseau et al. 2003), while the few additional works based on non-nacreous bivalves took the scallop as a model (e.g. Liu et al. 2002, 2006; Torita et al. 2007; Latire et al. 2014). In the present study, we have expanded the scope of investigation by using another nacreous bivalve, the blue mussel *Mytilus edulis*, but also one non-nacreous species, the Pacific oyster *Crassostrea gigas*. Both are of commercial interest. The shell of *Mytilus edulis* is of the nacroprismatic type (Taylor et al. 1969; Mutvei 1972; Carter 1990). It consists of a bi-layered organo-mineral structure, with an outer prismatic layer and an internal nacreous one (Dalbeck et al. 2006). The prisms appear as calcite rectilinear microneedles, oblique to the outer surface of the shell, and they are individually enveloped by an organic sheath (Taylor et al. 1969; Marie et al. 2011a). The mussel nacre, in turn, consists of a typical "brick wall" type structure containing aragonite tablets approximately 0.5 μm thick, surrounded by an organic matrix (Mutvei 1972; Feng et al. 1999; Carter 1990; Marie et al. 2011a). The shell of *Crassostrea gigas* is

made of calcite. It contains neither nacreous microstructures nor aragonite (Lee and Choi 2007) except the ligament and the myostracal layer (Taylor et al. 1969; Carter 1990). This shell is a multi-layered organo-mineral structure composed of four different microstructures (Carter 1990; Checa et al. 2007). The outer calcified part is composed of calcite prisms of 20–200 μm in height and 5–40 μm in width, while the middle and inner shell layers include usually a combination of three types of microstructures: regularly foliated, crossed foliated and chalky deposits (Carter 1990). The foliated structure consists of arrow point ending tabular laths measuring 2–5 μm wide and 200–250 μm thick (Checa et al. 2007; Marie et al. 2011b). The chalky structures are composed of platy calcite petals of variable sizes. The two predominant microstructures are the chalky and the foliated ones. Interestingly, recent proteomic analyses performed on the shell matrices of *Crassostrea gigas* on the one hand, and on *Mytilus sp.* (including *M. edulis*) on the other hand suggest that the shell protein content of these two genera are different but that they may partially overlap (Marie et al. 2011a, b). Partial overlapping of protein compositions may be a general finding encountered among the skeletal matrices of several calcifying metazoans (Jackson et al. 2015).

In this context, we compared the biochemical effects of different shell extracts from *M. edulis* or *C. gigas* on the metabolism and synthesis of the extracellular matrix (ECM) components of primary human skin fibroblasts by using an in vitro approach. ECM is a dynamic structure in which a large number of macromolecules interact. The composition of the ECM is subjected to a balance between an anabolic phase (synthesis of the ECM) and a catabolic phase (ECM degradation). Both processes, in balance, are essential in maintaining the integrity of the tissue. Thus, the expression of collagens, such as type I collagen, has been investigated after incubations in the presence of shell extracts. Collagens are major components of the ECM, and type I collagen is the most abundant protein found in the dermis. We have also analysed the effects of these extracts on the activity of MMPs (i.e., MMP-1), which are enzymes implicated in the ECM catabolic pathway (collagen degradation), and the expression of TIMPs, i.e., their inhibitors. Finally, we studied the effect of the shell components on the expression of p65, a NF-*k*B (Nuclear Factor *Kappa* B) subunit, which is a transcription factor known to inhibit the transcription of the *COL1A1* gene (Beauchef et al. 2012; Bigot et al. 2012).

Materials and methods

Sample collection

Human dermal samples were obtained from skin biopsies of healthy donors undergoing mammary hypertrophy surgery. Surgeries were made by the Surgery Service of St Martin Clinic (Caen, France). All patients signed informed consent agreement forms. This study with human sample was approved by the local Ethics Committee for research with human samples (Comité de Protection des Personnes Nord Ouest III) of the ''Centre Hospitalier Universitaire'' of Caen (France).

Extractions of shell matrices

The shells (mussel *Mytilus edulis* and oyster *Crassostrea gigas*) were collected from various fisheries located along the channel coast of France. Shells were brushed and incubated in NaOCl (10%, v/v) overnight to remove superficial organic contaminants. The calcified layers of the shell (nacre and prisms) were then thoroughly rinsed with deionised water, dried and then crushed into fine powder (<200 μm).

All subsequent extractions were performed at 4 °C. The acid extraction was prepared as previously described (Marin et al. 2005; Latire et al. 2014). Shell powder was decalcified overnight in cold diluted acetic acid (10%, v/v) that was progressively added (250 μL every 10 s). The solution was centrifuged at 3250 g for 30 min at 4 °C. The resulting pellet, corresponding to the acid-insoluble matrix (AIM), was rinsed several times with MilliQ water, freeze-dried and weighed. The supernatant, corresponding to the acid-soluble matrix (ASM), was extensively dialysed (3.5 kDa cut-off, Spectra/Por dialysis membrane, Biovalley SA, Marne la Vallée, France) against 10 L of MilliQ water for three days (several water changes) before being freeze-dried and weighed.

Water-soluble matrix (WSM) was obtained by suspending shell powder in MilliQ water (100 g/L) for three days at 4 °C with continuous stirring. The solution was centrifuged at 3200 g for 30 min at 4 °C. The supernatant was subsequently freeze-dried, and

the WSM pellet was weighed. To make sure that calcium did not interfere with the WSM extract, a control using $CaCO_3$ salt was included in the experiments. In parallel, a $CaCO_3$ extract was performed using the same steps as the WSM extraction.

All extracts were resuspended in phosphate-buffer saline (Invitrogen, Saint Aubin, France) (4 mg/mL) and filtered (0.22 µm mesh) before use.

Cell culture

Skin samples minced in small squares (1 cm^2) were enzymatically digested for 15 h with thermolysin (40 U, Sigma-Aldrich, Saint Quentin Fallavier, France) supplemented with gentamycin (4 µg/ml) and Fungizone (2.5 µg/mL) to facilitate separation of the epidermis from the dermis. Fibroblasts were extracted from the dermis by an additional treatment with *Clostridium hystolyticum* type 1 collagenase (2 mg/mL, Fisher Scientific, Illkirch, France) for 2 h at 37 °C. The cell suspension was filtered through a 70-µm cell strainer and then centrifuged for 10 min at 300 g to obtain a cell pellet. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, Fisher Scientific, Illkirch, France) supplemented with 10% foetal calf serum (FCS, Fisher Scientific, Illkirch, France), antibiotics and antifungals (gentamycin, 4 µg/mL, Fungizone, 2.5 µg/mL, ciprofloxacin, 10 µg/mL) and 2% Mycokill. Cells were seeded at 2.5×10^6 cells in 75 cm^2 culture flasks in DMEM with 10% FCS and antibiotics in a 5% CO_2 environment. They were passaged with a trypsin (0.05%) and EDTA (0.25 mM) solution (Fisher Scientific, Illkirch, France) after reaching confluence. All experiments were performed on cells between passages 3 and 8. For each analysed parameter, the number of replicates were indicated in the figure caption.

WST-1 assay

The WST-1 assay principle is based on the conversion of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) into a colored dye by mitochondrial dehydrogenase enzymes. The reaction produces a color change which is directly proportional to the amount of mitochondrial dehydrogenase enzymes in a given culture. As a result, the assay actually measures the net metabolic activity of cells (Peuster et al. 2003; Velten et al. 2004).

Cells were seeded onto 96-well microplates at a density of 2000 cells/well. After reaching 80% confluency, the cells were incubated in DMEM with 2% FCS in the absence or presence of shell matrix extracts or $CaCO_3$ extract for 24, 48 and 72 h. The medium was then removed, and 100 µL of WST-1 reagent (WST-1 cell proliferation kit, Roche Diagnostics, Meylan, France; 1:40 dilution in DMEM) was added for 40 min. Absorbance was measured at 450 and 630 nm with a microplate reader.

Crystal violet assay

Crystal violet (N-exametylpararosaniline) is a basic dye which stains cell nuclei and was used to determine cell number. The color is directly proportional to the amount cells in a given culture (Gillies et al. 1986; Kueng et al. 1989). Cells were seeded onto 96-well microplates at a density of 2000 cells/well. After reaching 80% confluency, the cells were incubated in DMEM with 2% FCS in the absence or presence of shell matrix extracts or $CaCO_3$ extract for 24, 48 and 72 h. The medium was then removed, and the wells were washed twice with PBS. The cells were stained with 0.1% crystal violet (Sigma-Aldrich, Saint Quentin Fallavier, France) dissolved in a PBS/Ca^{2+} solution for 30 min. The stained product was subsequently washed three times with PBS. Finally, the stained cells were solubilised in a 20% acetic acid solution for 15 min. Absorbance was measured at 600 nm with a microplate reader.

Type I collagen and MMP-1 ELISAs

At the end of the incubations, the cells were washed twice with PBS. Then, fibroblasts were lysed in RIPA buffer supplemented with leupeptin (1 µg/mL), PMSF (10 µg/mL), aprotinin (1 µg/mL) and pepstatin (1 µg/mL) as described previously (Legendre et al. 2003). Samples were centrifuged (12,000 g for 30 min at 4 °C), and supernatants containing cellular proteins were stored at −20 °C until analysis. Protein concentration was measured using a Protein Assay kit (Biorad) using bovine serum albumin as standard. Type I collagen measurements were evaluated in the culture medium with a CICP MicroVue Bone Health kit (TECOmedical, Versaille, France), according to

the manufacturer's instructions. Absorbance was determined at 405 nm with a microplate reader. Active MMP-1 was assayed with the Fluorokine E Human active MMP-1 Fluorescent Assay (R&D System, Lille, France) following the manufacturer's recommendations. Absorbance was measured at 405 nm with a microplate reader. The results were normalised to the cell layer protein amounts.

RNA extraction and real-time (RT) PCR analysis

Cells were seeded onto 12-well microplates and incubated in the absence or presence of shell matrix extracts for 48 h and 96 h without removal of the culture medium. Total RNA was extracted with TRIzol (Fisher Scientific, Illkirch, France) according to the manufacturer's instructions. 1.5 µg of the total RNA was treated with 1.5 U DNase I (Fisher Scientific, Illkirch, France) at room temperature for 15 min to remove any DNA contaminants.

Reverse transcription was conducted using 1 µg of total RNA treated with DNase I, 20 µM oligodT, 200 U Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV-RT, Fisher Scientific, Illkirch, France), 40 U RNaseOUT (Fisher Scientific, Illkirch, France) and 20 µM of each dNTP. Real-time PCR was performed in an ABI Prism SDS 7000 thermocycler. All procedures were conducted in triplicate. Controls of non-template cDNA were included in the PCR experiments. The sequences of the forward and reverse primers were designed using the Primer Express software (Table 1). Amplifications were performed as previously described (Latire et al. 2014). The amplification conditions were 40 cycles of 10 s at 95 °C and 60 s at 60 °C, followed by a protocol for the melting curve: 80 cycles of 10 s with an increase of 0.5 °C between each cycle from 55 to 95 °C. The mRNA amount was normalised to *RPL13A* mRNA, and analysis of relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Data analysis

The results are expressed as the mean ± S.D. Each experiment was repeated at least three times, and the means were calculated from triplicates for each experiment. The significance of the differences between the mean values was estimated by using Student's *t* test.

Results

Modulation of the metabolic activity of fibroblasts by shell extracts

The metabolic activity of fibroblasts exposed to different shell extracts from the mussel *M. edulis* and

Table 1 Primers used in real-time (RT) PCR experiments

Gene of interest	Primer sequence	Expected amplicon size (pb)
RPL13A	5′ GAGGTATGCTGCCCCACAAA 3′	75
	5′ GTGGGATGCCGTCAAACAC 3′	
COL1A1	5′ CACCAATCACCTGCGTACAGAA 3′	118
	5′ CAGATCACGTCATCGCACAAC 3′	
COL1A2	5′ AAAACATCCCAGCCAAGAACTG 3′	91
	5′ TCAAACTGGCTGCCAGCAT 3′	
COL3A1	5′ TCTTGGTCAGTCCTATGCGGATA 3′	89
	5′ CATCGCAGAGAACGGATCCT 3′	
MMP-1	5′ GAAGCTGCTTACGAATTTGCCG 3′	122
	5′ CCAAAGGAGCTGTAGATGTCCT 3′	
TIMP-1	5′ GTGTCTGCGGATACTTCCACAG 3′	131
	5′ AGCTAAGCTCAGGCTGTTCCAG 3′	
p65	5′ TAGGAAAGGACTGCCGGGAT 3′	101
	5′ CCGCTTCTTCACACACTGGA 3′	

the oyster *C. gigas* was analysed using the WST-1 assay. In these experiments, the cells were exposed for 24, 48 or 72 h to various concentrations of extracts ranging from 50 to 1000 µg/mL (Fig. 1). No significant variation in the metabolic activity of fibroblasts was measured up to 100 µg/mL of ASM from *M. edulis* (Fig. 1a). A very significant increase ($p < 0.001$) was detected when the cells were incubated for 48 and 72 h with 250 µg/ml of ASM. Then, the metabolic activity increased very significantly ($p < 0.001$) in a dose-dependent manner regardless of the incubation time. The highest concentration (1000 µg/ml) increased the metabolic activity by 233% in comparison to the control (0.488 ± 0.02 vs 0.209 ± 0.01) after 72 h of incubation. The ASM of *C. gigas* had no effect on fibroblast metabolic activity up to a concentration of 50 µg/ml (Fig. 1b). A slight but significant increase ($p < 0.05$) was detected when the cells were incubated for 48 and 72 h with 100 µg/ml of ASM. Then, higher concentrations (from 250 to 1000 µg/ml) of ASM drastically ($p < 0.001$) decreased the cell metabolic activity. The highest concentration (1000 µg/ml) decreased the metabolic activity by 76% in comparison to the control (0.095 ± 0.01 vs 0.277 ± 0.01) after 72 h of incubation.

Concerning the effect of WSM, no significant variation in the metabolic activity of fibroblasts was measured for up to 500 µl/ml of WSM from *M. edulis* (Fig. 1c). At 1000 µg/ml, the WSM increased the cell metabolic activity significantly regardless of the incubation time. This increase reached 45% in comparison to the control (0.386 ± 0.01 vs 0.265 ± 0.01) after 72 h of incubation. On the contrary, WSM of *C. gigas* did not modulate the metabolic activity of fibroblasts regardless of the concentration and the incubation time (Fig. 1d). $CaCO_3$ extract was used as a negative control, and we did not observe any significant difference with the control, regardless of the concentration and the incubation time (data not shown).

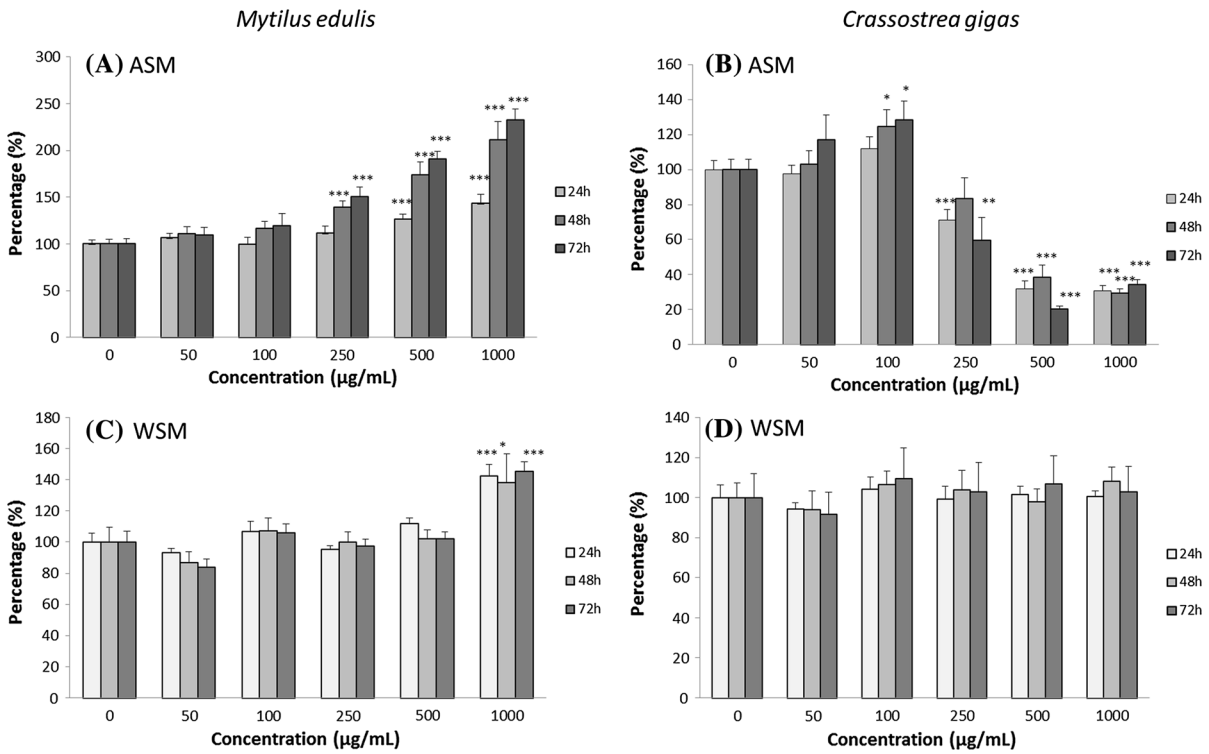

Fig. 1 Effect of shell matrix extracts on fibroblast metabolic activity evaluated by the WST-1 assay after culturing the cells in the presence of varying concentrations of extracts (50–1000 µg/ml) for 24, 48 and 72 h. Effects of acid-soluble matrix (ASM) (**a**) and water- soluble matrix (WSM) (**c**) from *Mytilus edulis* and of ASM (**b**) and WSM (**d**) from *Crassostrea gigas* were evaluated. Significant differences compared with controls are indicated by *asterisks* (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **a**: n = 3, **b**: n = 6, **c**: n = 3, **d**: n = 3

Modulation of cell proliferation by shell extracts from *M. edulis*

The number of fibroblasts exposed to different shell extracts was quantified using the crystal violet assay. In these experiments, cells were exposed for 24, 48 and 72 h to various concentrations of extracts (from 50 µg/mL to 1000 µg/mL) (Fig. 2). No significant variation of the cell density was measured after 24 h of incubation regardless of the concentrations of ASM and WSM from *M. edulis* (Fig. 2a, c). Cell density significantly increased when cells were incubated for 48 h with 500 µg/mL ($p < 0.01$) or for 72 h with 250 µg/mL ($p < 0.01$) of ASM (Fig. 2a). The highest concentration (1000 µg/mL) increased the cell density by 161% in comparison to the control (0.530 ± 0.03 vs 0.328 ± 0.04) after 72 h of incubation. Concerning the WSM *of M. edulis*, cell density significantly increased when cells were incubated for 48 or 72 h with 500 µg/mL ($p < 0.05$) of extract (Fig. 2c). A maximal significant ($p < 0.05$) increase of cell density of 24% compared with the control (0.295 ± 0.02 vs 0.237 ± 0.02) was observed for the highest concentration after 72 h of incubation. The cell density was not significantly modulated with *C. gigas* extracts compared with the control shell extract, regardless of the type of extraction used (ASM or WSM) and the incubation time (from 24 to 72 h) (Fig. 2b, d).

These results show that higher concentrations of ASM (from 250 µg/mL) and WSM (1000 µg/mL) from *M. edulis* stimulated both the metabolic activity and the cell proliferation of fibroblasts. Only the 100 µg/mL concentration of ASM from *C. gigas* increased the metabolic activity of fibroblasts without having an effect on cell density, whereas higher doses decreased the metabolic activity without having an effect on cell density. The WSM of *C. gigas* did not modulate the metabolic activity or fibroblast proliferation.

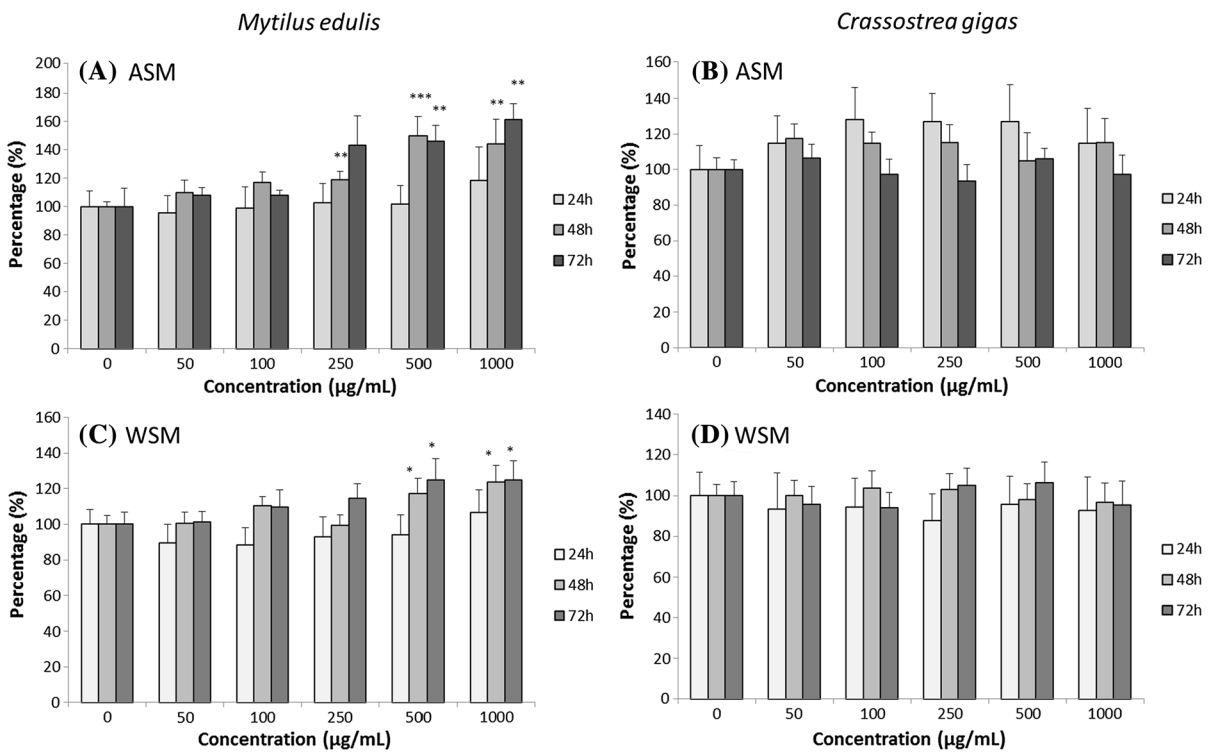

Fig. 2 Effect of shell matrix extracts on fibroblast proliferation evaluated by the crystal violet staining assay after culturing the cells in the presence of varying concentrations of extracts (50–1000 µg/ml) for 24, 48 and 72 h. The effects of acid-soluble matrix (ASM) (**a**) and water-soluble matrix (WSM) (**c**) from *Mytilus edulis* and of ASM (**b**) and WSM (**d**) from *Crassostrea gigas* were analysed. Significant differences compared with controls are indicated by *asterisks* (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **a**: n = 3, **b**: n = 6, **c**: n = 3, **d**: n = 3

Reduction of collagen gene expression and synthesis by shell extracts

We then studied the effects of shell extracts on type I collagen expression at the mRNA and the protein levels by using real-time PCR (Fig. 3) and ELISA (Fig. 4), respectively. *M. edulis* extracts (ASM and WSM) significantly decreased the *COL1A1* steady-state mRNA levels regardless of the incubation time used. A maximal significant ($p < 0.001$) decrease of 82% compared with the control was measured after 48 h of incubation with 250 µg/mL of ASM (Fig. 3a). Concerning the *C. gigas* extracts, ASM decreased *COL1A1* steady-state mRNA amounts after 48 h of incubation in a dose dependent manner, but due to high standard deviations, this decrease was not significant (Fig. 3a). On the contrary, after 96 h of incubation, ASM significantly ($p < 0.05$) decreased *COL1A1* mRNA level. WSM induced a significant decrease of mRNA levels by 75% compared with the control after 48 h of incubation and decreased the COL1A1 mRNA levels by 60% after 96 h of incubation, but this was not significant due to a large variability (Fig. 3b).

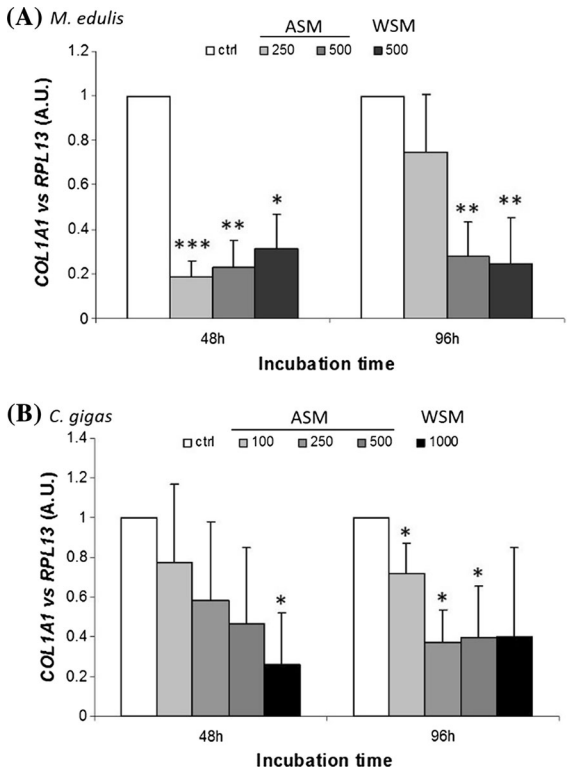

Fig. 3 Effect of shell matrix extracts on *COL1A1* mRNA expression in fibroblasts. Total RNAs were reverse-transcribed into cDNA, and real-time PCR assays were performed to evaluate *COL1A1* mRNA expression levels. The effects of shell extracts from *Mytilus edulis* (**a**) and *Crassostrea gigas* (**b**) were evaluated. Values are the mean of triplicate samples ± SD. Statistical analyses were performed with the Student's *t* test, and differences compared with controls are indicated by *asterisks* (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **a**: n = 4, **b**: n = 3

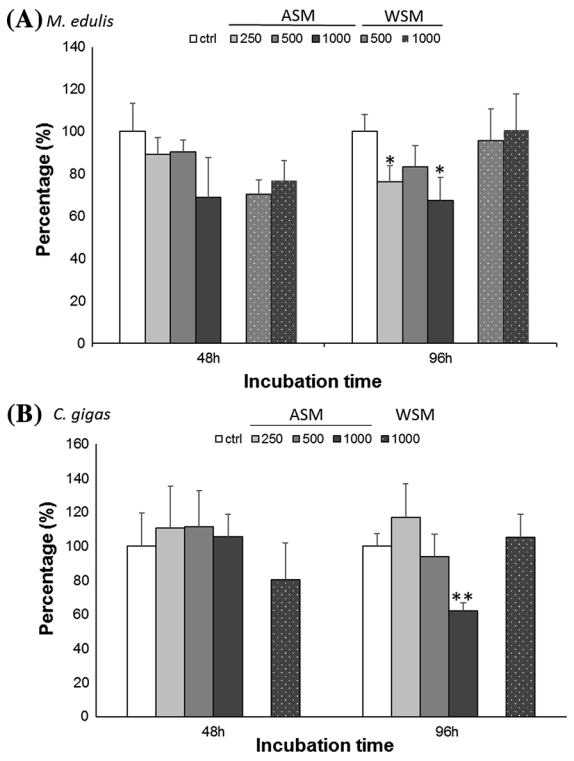

Fig. 4 Effect of shell matrix extracts on type I collagen synthesis in fibroblasts. Collagen synthesis was determined by ELISA after culturing the cells in the presence or absence of varying concentrations of extracts for 48 h and 96 h. Shell extracts were obtained from *Mytilus edulis* (**a**) and *Crassostrea gigas* (**b**). Values are the mean of triplicate samples ± SD. Statistical analyses were performed with the Student's *t* test and differences compared with controls are indicated by *asterisks* (*$p < 0.05$, **$p < 0.01$). **a**: n = 3, **b**: n = 2

When directly measured by ELISA, the synthesis of type I collagen was not significantly modulated by extracts after 48 h of incubation regardless of the types of extracts and shells (Fig. 4a, b, left histograms). However, a significant decrease of collagen synthesis was measured using 1000 µg/mL of both ASM extracts at 96 h. The decreases were 32 and 38% for 1000 µg/mL of ASM from *M. edulis* and *C. gigas*, respectively (Fig. 4a, b, black histograms on the right graphs).

The ASM and WSM of *M. edulis* decreased *COL1A1* mRNA expression but only a corresponding decrease of protein synthesis was observed for 250 and 1000 µg/ml of ASM. In a similar manner, the ASM and WSM from *C. gigas* decreased the mRNA levels but only 1000 µg/ml of ASM from *C. gigas* significantly decreased the corresponding type I collagen protein synthesis.

Role of MMP-1 in the decrease observed on type I collagen production

MMP-1 is known to be the main enzyme responsible for the degradation of collagen whereas TIMP-1 is its inhibitor. We evaluated the effects of shell extracts on the mRNA levels of these two components (Fig. 5) and on the MMP-1 activity (Fig. 6). *MMP-1* mRNA levels were significantly increased by the ASM extracts from *M. edulis* and *C. gigas* regardless of the incubation time. The maximal increases observed were 12-fold (Fig. 5a) and 31-fold (Fig. 5b) at 48 h for the mussel and oyster ASM extracts, respectively. The WSM enhanced *MMP-1* mRNA levels by 2.6-fold after 48 h of incubation. In addition, the mussel extract had no significant effect on *TIMP-1* mRNA levels (Fig. 5c) whatever the incubation time. On the contrary, the oyster extract induced a slight but significant decrease of the *TIMP-1* mRNA level. This decrease was measured after 48 or 96 h of incubation time for various concentrations of ASM and only after 48 h of incubation for 1000 µg/mL of WSM (Fig. 5d).

In addition to inducing changes in *MMP-1* mRNA levels, the synthesis of active MMP-1 was significantly modified by the extracts. The effects of *M. edulis* extracts were only significant after 96 h of incubation with changes of 250% and above for all concentrations of ASM (in particular at 250 µg/mL), whereas WSM had less effect (130% for 1000 µg/mL at 96 h) (Fig. 6a, right). Only 1000 µg/mL of ASM of *C. gigas* significantly ($p < 0.001$) increased the synthesis of active MMP-1 after 48 and 96 h of incubation (ninefold and 23-fold, respectively) (Fig. 6b).

To summarise, the ASM of *M. edulis* induced the expression of *MMP-1* at the mRNA and the protein levels but did not significantly influence the *TIMP-1* mRNA amounts; the WSM of *M. edulis* had similar but lesser effects. The WSM of *C. gigas* had no effects on the expression of *MMP-1* and very little effects on *TIMP-1*. The ASM of *C. gigas* stimulated the expression of *MMP-1* (mRNA and protein) but at the same time tended to decrease *TIMP-1* mRNA levels.

Stimulation of the expression of the p65 subunit of NF-κB by mussel shell extracts

We then analysed the effects of shell extracts on the mRNA levels of p65, a NF-kB subunit known to negatively regulate the transcription of the *COL1A1* gene. Our results, shown in Fig. 7, indicated that 500 µg/ml of ASM from mussel enhanced significantly ($p < 0.05$) (by 2.5-fold) the mRNA levels of p65 after 48 h of incubation (Fig. 7a, left histogram). This effect was not recorded after 96 h of incubation (Fig. 7a, right histogram). Other concentrations of mussel shell extracts did not show any significant effects on the mRNA amounts of p65 (Fig. 7a). None of the shell extracts of the oyster induced significant effects on the expression of mRNA of p65 (Fig. 7b).

Discussion

The shell biomineralisation process among molluscs leads to a wide variety of structures and architectures, emerging from the subtle interplay between extracellular organic matrices of complex protein content and the calcifying epithelial tissues that produce them. Numerous studies have shown the biocompatibility and the biological activities of these molluscan mineral-occluded macromolecules on vertebrates by in vitro and in vivo approaches (Lopez et al. 1992, 2000; Atlan et al. 1997; Torita et al. 2007; Latire et al. 2014). The shell macromolecules possess the ability to modulate the synthesis of matrix components of the osteogenic tissue and of the dermis in vertebrates (Lopez et al. 1992, 2000; Atlan et al. 1997; Liao et al. 2002; Rousseau et al. 2003, 2008; Torita et al. 2007).

In this study, we examined the potential biological activities of the shell matrix compounds extracted from the organic fraction of two marine molluscs of economic interest (*M. edulis* and *C. gigas*) on the metabolism of human dermal fibroblasts in primary culture. In particular, we focused on the expression of extracellular matrix elements involved in the anabolic pathway (for example, type I collagen) and on the expression of factors involved in the catabolic pathway (MMP-1).

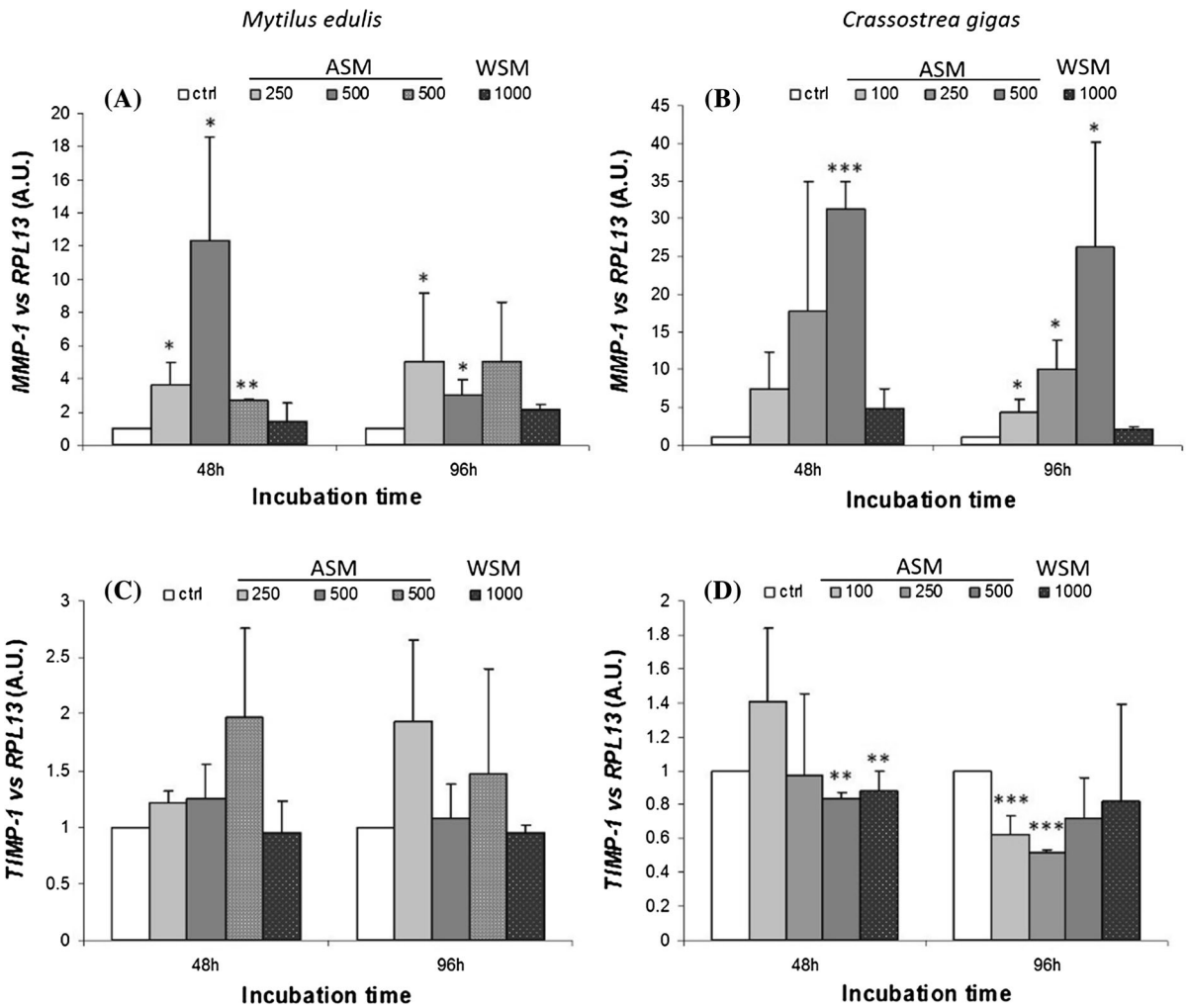

Fig. 5 Effect of shell matrix extracts on *MMP-1* and *TIMP-1* mRNA expression in fibroblasts. Cells were cultured in the presence of varying concentrations of extracts (100–1000 µg/ml) for 24, 48 and 72 h. Total RNAs were reverse-transcribed into cDNA, and real-time PCR assays were performed to determine *MMP-1* and *TIMP-1* mRNA expression levels. The effects of shell extracts from *Mytilus edulis* (**a**, **c**) and from *Crassostrea gigas* (**b**, **d**) were analysed. Significant differences compared with controls are indicated by *asterisks* (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **a**, **c**: n = 4, **b**, **d**: n = 3

Our results revealed that the ASM (at a concentration 250 µg/ml) and WSM (at a concentration of 1000 µg/ml) of *Mytilus edulis* induced an increase in the metabolic activity of cells. This effect was accompanied by an increase in cell density, which suggests that these fractions stimulated cell metabolism and proliferation. Only a concentration equal to 100 µg/ml of ASM of *C. gigas* increased the metabolic activity of cells, whereas higher concentrations inhibited the metabolic activity of fibroblasts. This effect was not accompanied by an increase in cell density, indicating that the ASM of *C. gigas* stimulated cell metabolism but not proliferation. Moreover, all concentrations of the WSM from *C. gigas* produced no effects on the metabolic activity or on the cell density.

In the literature, studies have already demonstrated that such components affect the metabolism or proliferation of vertebrate cells maintained in vitro. For example, pre-osteoblastic cells from mice or from the bone marrow of rat femurs showed an increase in their proliferation activities when these cells were incubated in the presence of WSM from *Pinctada maxima (*Perreira-Mouriès et al. 2002b; Rousseau et al. 2003). Similarly, a study on the effect of the pearl mussel

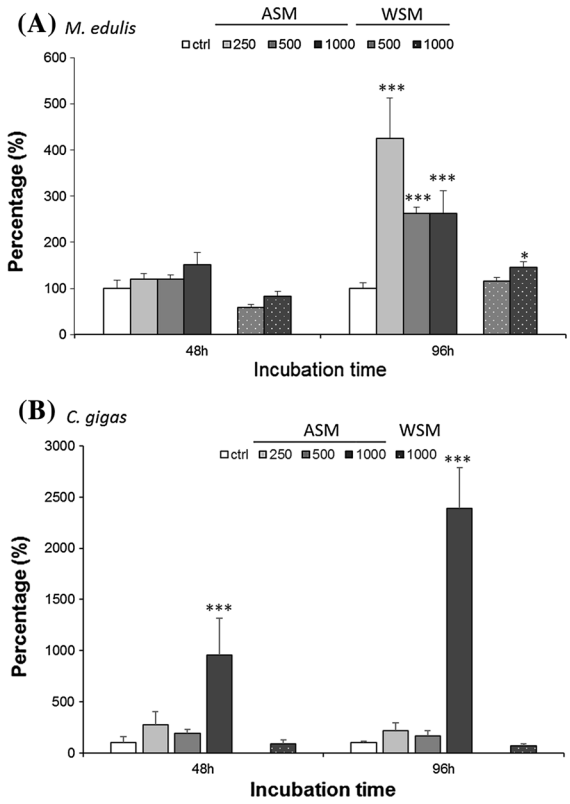

Fig. 6 Effect of shell matrix extracts on fibroblast MMP-1 activity. The levels of active MMP-1 were determined by ELISA after culturing the cells in the presence or absence of varying concentrations of extracts for 48 and 96 h. Shell extracts were obtained from *Mytilus edulis* (**a**) and *Crassostrea gigas* (**b**).Values are the mean of triplicate samples ± SD. Statistical analyses were performed with the Student's *t* test, and differences compared with controls are indicated by *asterisks* (*$p < 0.05$, ***$p < 0.001$). **a**: n = 3, **b**: n = 2

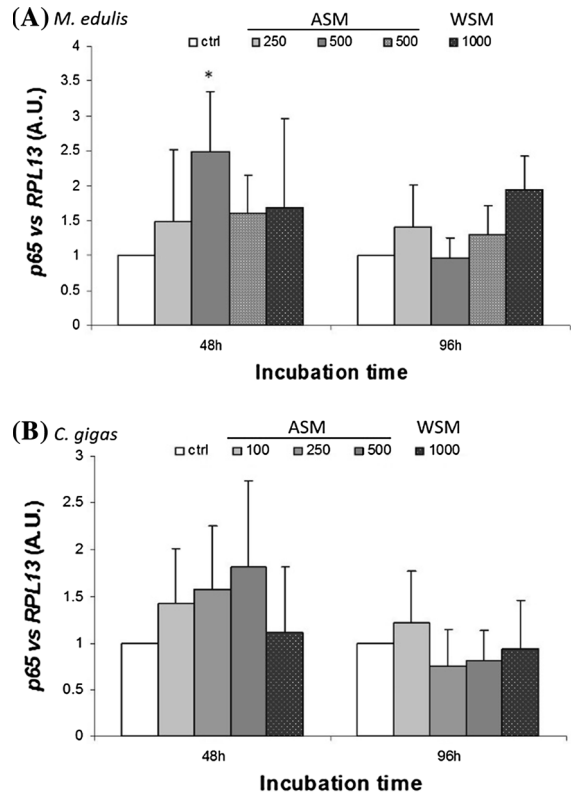

Fig. 7 Effect of shell matrix extracts on p65 mRNA expression in fibroblasts. Total RNAs were reverse-transcribed into cDNA, and real-time PCR assays were performed to determine p65 mRNA expression levels. Shell extracts were obtained from *Mytilus edulis* (**a**) and *Crassostrea gigas* (**b**). Values are the mean of triplicate samples ± SD. Statistical analyses were performed with the Student's *t* test, and differences compared with controls are indicated by *asterisks* (*$p < 0.05$). **a**: n = 4, **b**: n = 3

Hyriopsis cumingii Lea on the proliferation of osteoblastic lineage cells revealed an increase in the proliferation of these cells (Shen et al. 2006). In addition, the WSM of nacre from a pearl oyster *Pteria martensii* stimulated the proliferation of the NIH-3T3 cell line of mouse fibroblasts, in the presence of 25 mg/ml of the extract. Experiments performed on human dermal fibroblasts demonstrated that the ASM from the shell of the Japanese scallop *Patinopecten yessoensis* induced an increase in the metabolic activity of these cells after incubation in the presence of the extract (Torita et al. 2007). Recently, Latire et al. (2014) showed that the ASM from *Pecten maximus*, when used at concentration of 1000 µg/mL, induced a significant increase of the metabolic activity of fibroblasts in primary culture. However, this extract had no effect on cell density. Moreover, various concentrations of WSM of the same species produced no effects on the metabolic activity or on the cell density. Therefore, the results obtained in our work with extracts from the shell of *M. edulis or C. gigas* are in agreement with the literature data and clearly indicate that these matrix components have biological activities, at least in regard to the general cellular activities of human dermal fibroblasts.

We investigated the extracellular matrix (ECM) synthesised by fibroblasts. ECM is a dynamic structure in which a large number of macromolecules interact. The composition of the ECM is subjected to a balance between an anabolic phase (synthesis of the ECM) and a catabolic phase (ECM degradation). Both processes, in balance, are essential in maintaining the integrity of

the tissue. Our results showed that the shell extracts of *M. edulis* and of *C. gigas* have similar effects on the synthesis of type I collagen. That is, a decrease in the *COL1A1* mRNA levels in the presence of ASM and WSM was detected. At the protein level, our results revealed an inhibition of the synthesis of type I collagen by fibroblasts when cells are exposed to shell extracts, especially in the presence of ASM of *M. edulis*.

This reduction in type I collagen synthesis was associated with a significant increase of MMP-1, either at the mRNA or at the protein activity levels. MMP-1 belongs to the family of collagenases (Birkedal-Hansen et al. 1993). Its stimulation leads to a decrease of the amount of ECM components such as collagens. In addition, *C. gigas* extracts decreased the mRNA levels of TIMP-1 whereas *M. edulis* extracts had no effect on TIMP-1 mRNA levels. Although we did not evaluate TIMP-1 at the protein level, this finding should be in favour of an increase in the ECM catabolic pathway because TIMP-1 is the natural inhibitor of MMP-1, which reduces collagen degradation (Baker et al. 2002). Thus, an increase in the synthesis and activity of MMP-1 may have a beneficial role, as this enzyme is necessary for tissue repair. That is, in a skin wound, following the inflammation steps, tissue repair through the formation of a granulation tissue and tissue remodelling occurs via the action of MMPs to maintain a balance in ECM synthesis (Ravanti and Kähäri 2000).

Finally, from a mechanistic point of view, our results showed that ASM from *M. edulis* induces an important decrease of COL1A1 transcripts levels because it enhances more importantly p65 mRNA. For ASM from *C. gigas*, the COL1A1 inhibition is less pronounced because a weaker increase in p65 mRNA is detected resulting in a COL1A1 mRNA decrease of less magnitude. Probably the same mechanism of action is involved for both ASM fractions coming from *M. edulis* and *C. gigas*, and the difference in the magnitude of the effects on COL1A1 and p65 is certainly due to the bioactive molecule(s) concentration, which is lower in ASM from *C. gigas* compared to *M. edulis*. This NF *kappa* B sub-unit inhibits type I collagen genes and is largely responsible for the decrease in the expression of collagen synthesis of dermal fibroblasts (Beauchef et al. 2012; Bigot et al. 2012). In the literature, it has been shown that shell extracts from *Patinopecten yessoensis*, *Hyriopsis cumingii* or *Pecten maximus* increased the mRNA levels or the protein synthesis of type I collagen and TIMP-1 of vertebrate fibroblasts in culture (Torita et al. 2007; Jian-Ping et al. 2010; Latire et al. 2014). To our knowledge, our work represents the first study indicating a stimulation of the catabolic pathway by shell extracts.

What could be the shell molecules responsible for such effect? Could they be identical in the nacroprismatic mussel *Mytilus edulis* and in the foliated oyster *Crassostrea gigas?* At the present, we do not know yet which molecules in the shell extracts may stimulate the catabolic pathway of human skin fibroblasts. Mollusc shells contain indeed numerous macromolecules, including soluble proteins, glycoproteins, hydrophobic proteins, chitin, soluble polysaccharides and lipids (Lowenstam and Weiner 1989; Bédouet et al. 2001; Marie et al. 2011a; Marin et al. 2012), the individual functions of which in biomineralization are far from being elucidated. It is however remarkable to observe that the shell extracts of *Mytilus edulis* and of *Crassostrea gigas*, beyond dissimilar effects on cell proliferation, induce very similar effects, that is, the activation of the fibroblast catabolic pathway. Such a finding might suggest that the inducing factors could be similar, or at least, that functional domains responsible for the effect could be similar, whatever the shell proteins that exhibit these functional domains. Recently, the identification of some shell matrix proteins from the shell of *Crassostrea gigas* has been performed (Marie et al. 2011b; Oliveira et al. 2012). Among the putative protein candidates to investigate further, gigasin-2 contains two conserved epidermal growth factor (EGF) domains. EGF triggers many biological responses in vertebrates especially during wound healing processes (Grazul-Bilska et al. 2003; Barrientos et al. 2008; Berlanga-Acosta et al. 2009). Mimura et al. (2006) demonstrated that EGF down-regulates *de novo* synthesis and promotes degradation of type I collagen by increasing MMP-1 expression in cultured human dermal fibroblasts. More precisely, EGF regulates type I collagen expression at the post-transcriptional level, while it up-regulates MMP-1 expression mostly at the transcriptional level. In both cases, the MEK/ERK signalling pathway is involved. Therefore, our results are in accordance with the literature but complementary studies are needed to confirm the implication of gigasin-2 in the regulation of type I collagen and MMP-1 synthesis by fibroblasts.

In addition, although many macromolecules are present in the shells, recent studies have also revealed the presence of many low molecular weight molecules (Bédouet et al. 2006; Rousseau et al. 2008). For example, more than one hundred low molecular weight molecules in the nacre of *Pinctada margaritifera* have been identified (Bédouet et al. 2006). The authors speculate that a number of these molecules correspond to signalling molecules that can be recognised by cell membrane receptors and subsequently trigger an increase of their metabolic activities. Among these molecules, ''cytokine-like'' peptides are suspected, but their presence has not been demonstrated. Such molecules may be present in the shell organic matrices of bivalves, which would explain the responses (e.g., fibroblast proliferation, activation of catabolic pathway and NF *kappa* B induction) of human dermal fibroblasts exposed with shell extracts.

Recently, lipids of the micronized nacre powder of the oyster *Pinctada margaritifera* were extracted and the biological activity was tested on human skin explant (Rousseau et al. 2006). These authors showed that lipids extract had brought to the stratum corneum the elements necessary to a rapid reconstruction of the intercellular cement, by acting on the expression levels of transglutaminase and filaggrin which are essential for the regulation of epidermal homeostasis. However, molecule among the nacre lipid extract and molecular mechanisms responsible for the activation of these ECM molecules was not yet identified. Even though extraction procedure was different, we could not exclude the fact that some lipids could be partly responsible of the effects observed in our study.

Finally, above all, we cannot exclude that the effects that we record on human skin fibroblasts may result from a synergistic action of different molecules of the shell matrix, including high and low molecular weight components.

Conclusions

Our results indicate that shell extracts from *M. edulis* and *C. gigas* contain molecules that promote the catabolic pathway of human dermal fibroblasts in primary culture because they cause a decrease in the synthesis of type I collagen and a concomitant increase in the level of active MMP-1. These results are original and pioneering in the sense that, to the best of our knowledge, there are no data in the literature demonstrating the effects of shell extracts on the ECM catabolic pathway.

The use of shell extracts from *M. edulis* and *C. gigas* may be interesting in the context of anti-fibrotic strategies, particularly against scleroderma. Scleroderma is a complex disease characterised mainly by the perturbation of dermal homeostasis, leading to an excessive synthesis of ECM components, particularly collagen (Denton and Abraham 2001; Denton and Black 2005). Our results show that these shell extracts decrease the synthesis of collagen and increase the level of endogenous active MMP-1 released by fibroblasts. However, a more complete study is required by analysing other ECM components such as glycosaminoglycans (GAGs) or adhesive glycoproteins (fibronectin). These compounds are also increased by fibroblasts during fibrotic processes (Moinzadeh et al. 2012).

Finally, our work stresses once again the usefulness to valorise the shells of bivalves of economic interest that are coproducts of shellfish farming activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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