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ORIGINAL ARTICLE

Regulation of Extracellular Matrix Synthesis by Shell Extracts from the Marine Bivalve *Pecten maximus* in Human Articular Chondrocytes— Application for Cartilage Engineering

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Abstract

The shells of the bivalve mollusks are organo-mineral structures predominantly composed of calcium carbonate, but also of a minor organic matrix, a mixture of proteins, glycoproteins, and polysaccharides. These proteins are involved in mineral deposition and, more generally, in the spatial organization of the shell crystallites in well-defined microstructures. In this work, we extracted different organic shell extracts (acid-soluble matrix, acid-insoluble matrix, water-soluble matrix, guanidine HCl/EDTA-extracted matrix, referred as ASM, AIM, WSM, and EDTAM, respectively) from the shell of the scallop *Pecten maximus* and studied their biological activities on human articular chondrocytes (HACs). We found that these extracts differentially modulate the biological activities of HACs, depending on the type of extraction and the concentration used. Furthermore, we showed that, unlike ASM and AIM, WSM promotes maintenance of the chondrocyte phenotype in monolayer culture. WSM increased the expression of chondrocyte-specific markers (aggrecan and type II collagen), without enhancing that of the main chondrocyte dedifferentiation marker (type I collagen). We also demonstrated that WSM could favor redifferentiation of chondrocyte in collagen sponge scaffold in hypoxia. Thus, this study suggests that the organic matrix of *Pecten maximus*, particularly WSM, may contain interesting molecules with chondrogenic effects. Our research emphasizes the potential use of WSM of *Pecten maximus* for cell therapy of cartilage.

Keywords *Pecten maximus* · Shell extracts · Water-soluble matrix · Human articular chondrocytes · Cartilage engineering

Introduction

In vertebrates, the skeletal system is internal, whereas, in invertebrates, it is generally external and forms the outer shell that recovers and protects the tissues. Although both systems have evolved separately, convergent key factors are involved in the mineralization of these two skeletal systems (Matsushiro and Miyashita 2004). Shell structure of

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invertebrates especially that of marine mollusks is composed of the most abundant biogenic minerals. These shells are mainly composed (95–99%) of $CaCO_3$ crystals embedded in a thin organic cell-free extracellular matrix layer that controls the shell biomineral deposition (Mann 1988). This organic matrix is composed of several macromolecules, including polysaccharides (e.g., chitin), proteins, and glycoproteins that are present both in inter- and intracrystalline locations (Zhang and Zhang 2006; Marin et al. 2012).

Studies focused on the analysis of the shell organic matrix components of marine mollusks have identified a wide variety of shell proteins. Some of these molecules exhibit structural homologies with proteins encountered in vertebrates, especially in humans. For example, nacrein, the first shell protein identified to date, possesses two functional domains that exhibit high similarity to that of human carbonic anhydrase II (Miyamoto et al. 1996). Perlustrin, a nacre protein from the abalone *Haliotis laevigata*, is homologous to the insulin-like growth factor binding protein (IGF-BP) N-terminal module of vertebrates (Weiss et al. 2001). More recently, large screenings of shell matrix proteins via proteomics identified a large number of shell proteins with functional domains, such as TIMP, sushi, kunitz-like, collagen-like, and fibronectin-like, which are also identified in vertebrate systems, including human ones (Marie et al. 2012; Gao et al. 2015). This suggests that the homology in the gene sequence between mollusks and vertebrates may involve functional analogy. Several studies have shown biological effects of molluscan shell protein extracts on vertebrate models (Lopez et al. 1992; Mouriès et al. 2002; Liu et al. 2006; Latire et al. 2014). Wide effects, ranging from biomedicine (osteoinduction and bone repair), dermatology (effects on wound repair), and nutraceutical were observed. The exact composition of shell extracts is unknown and the biologically active molecules are not yet identified. Two groups of molecules can be isolated according to the extraction protocol: the acetic acid-soluble fraction, referred as ASM, contains hydrophilic proteins, whereas the acetic acid-insoluble fraction, also called AIM, rather contains hydrophobic ones, rich in glycine and alanine.

Many researchers have focused on the mechanical and biological properties of shell extracts, such as the osteoinductive activity of the nacre in vitro, and its biocompatibility with bone tissue in vivo (Lopez et al. 1992; Silve et al. 1992; Atlan et al. 1997). The same team has also shown that it was possible to compensate for bone loss in sheep, by implanting nacre in the bone defect (Atlan et al. 1999). These studies suggested that components from oyster shell nacre are able to induce bone formation in vitro and in vivo. Surprisingly, to our knowledge, no study was performed on cartilage. Based on the absence of data on cartilage, we hypothesized that components of organic matrix of molluscan shells could also modulate metabolic activity of articular chondrocytes.

The metabolic balance controls the synthesis or the degradation of cartilage extracellular matrix (ECM) produced by chondrocytes, leading to osteoarthritis (OA) if the degradation predominates. This degenerative disease is characterized by a slow progressive deterioration of cartilage that cannot be counterbalanced by the poor intrinsic healing capacity of this tissue. This process results in a destabilization of the cartilage matrix, which leads to the loss of its mechanical and biological properties (Demoor et al. 2014). During OA, the synthesis of type II collagen and aggrecan-the specific phenotypic markers of cartilage-significantly decreases in favor of type I collagen synthesis, the marker of chondrocyte dedifferentiation (Demoor et al. 2012). These events get worse by chondrocyte hypertrophy characterized by the synthesis of type X collagen and by an increase in the activity of metalloproteinases (MMPs). In this context, various drug treatments (analgesics, non-steroidal anti-inflammatory drugs, chondroprotective substances) decrease pain and inflammation without promoting the regeneration of cartilage. Moreover, reconstructive surgery (arthroscopic washes, debridement, and mosaicplasty) has also shown its limits. Considering the relative effectiveness of these treatments, cartilage engineering based on autologous chondrocyte implantation (ACI) seems more promising but needs improvement (Marlovits et al. 2006; Demoor et al. 2012, 2014).

We have recently shown that shell extracts from the edible king scallop *Pecten maximus* have biological effects on primary cultured human skin fibroblasts (Latire et al. 2014). Our study showed that shell extracts, especially ASM, regulate the ECM synthesis in human dermal fibroblasts. The objective of the present study was to investigate for the first time the effects of four shell extracts from *Pecten maximus* (ASM, AIM, EDTAM, and WSM) on human chondrocytes in vitro.

To this end, we performed four types of shell matrix extractions, either with acetic acid (ASM, AIM), with water (WSM), or with guanidine HCl/EDTA (EDTAM), and analyzed the biological activity of each of the resulting extract on human chondrocytes in primary culture. The metabolic activity and proliferation of chondrocytes exposed to the different shell extracts were respectively assayed using a WST-1 assay and a crystal violet assay. We also examined their possible toxicity using a cytotoxicity assay before studying chondrocyte phenotype with real-time RT-qPCR, Western blot, and enzyme-linked immunosorbent assays (ELISA) experiments. Our results indicated that the scallop shell organic matrix contains molecules involved in the modulation of the metabolic activity and of the ECM synthesis by chondrocytes. In particular, WSM showed very interesting chondrogenic effects. Finally, WSM was employed in redifferentiation experiments with dedifferentiated chondrocytes cultured in collagen sponges in hypoxia during 7 days, with a BMP-2 treatment as a positive control. Indeed, we previously showed that dedifferentiated chondrocytes seeded onto these collagen

sponges, and treated with 50 ng/ml of BMP-2 during 7 days in hypoxia, recovered a differentiated phenotype (Legendre et al. 2013). The effects of WSM on chondrocyte phenotype were quantified by RT-qPCR, Western blot, and ELISA. Altogether, our results showed that WSM of *Pecten maximus* could be effective in tissue engineering for articular cartilage repair.

Materials and Methods

Shell Matrices Extractions

The collection of shells of the scallop *Pecten maximus* and their reduction into fine powder was performed by Copalis (Boulogne-Sur-Mer, France). Briefly, the shells 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 shell-calcified layers (nacre and prisms) were then thoroughly rinsed with deionized 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 (Latire et al. 2014). Briefly, the shell powder was decalcified overnight in cold dilute acetic acid (10%, *v*/*v*). 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 Milli-Q water, freeze-dried and weighed. The supernatant, corresponding to the acid-soluble matrix (ASM), was extensively dialyzed (3.5 kDa cut-off, Spectra/Por dialysis membrane) against 10 l of Milli-Q water for 3 days (several water changes) before being freeze-dried and weighed.

The water-soluble matrix (WSM) was obtained by suspending shell powder in Milli-Q water (100 g/l) for 3 days at 4 °C with continuous stirring. The solution was centrifuged at 3250×*g* for 30 min at 4 °C. The supernatant was subsequently freeze-dried and the WSM pellet weighed. In parallel to the WSM extraction, an extraction from pure $CaCO_3$ was performed, as a negative control, using the same steps as the WSM extraction.

The guanidine HCl/EDTA-extracted matrix (EDTAM) was obtained according to the method described by Pfeilschifter et al. with some modification (Pfeilschifter et al. 1995). The shell powder was dissolved (1 g per 100 ml) in extraction solution composed of a 50 mM Tris–HCl buffer, 4 M guanidine hydrochloride, pH 7.4, 150 mM EDTA, and protease inhibitors (5 mM benzamidine–HCl, 1 mM phenylmethylsulfonyl fluoride, and 0.1 M ε-aminocaproic acid). After extraction with stirring at 4 °C for 3 days, the solution was centrifuged at 10,000×*g* for 30 min at 4 °C. The resulting supernatant was then dialyzed (3.5 kDa cut-off) against water for 3 days at 4 °C. Samples were then freeze-dried and weighed.

All the resulting lyophilized powders were resuspended (4 mg/ml) in phosphate-buffer saline, filtered (0.22 μm mesh) and diluted at the desired concentration (from 100 to 1000 μg/ml) before use.

Chondrocyte Culture

HACs were prepared from macroscopically healthy zones of femoral heads obtained from patients undergoing joint arthroplasty (age range 52–83 years) as previously described (Legendre et al. 2013). All patients signed an informed consent agreement form, which was approved by the local ethics committee. Chondrocytes were seeded at 4×10^4 cells/cm^2 in plastic dishes, in a 5% CO_2 atmosphere in a medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco®, Life Technologies) supplemented with 10% fetal calf serum (FCS; Gibco®, Life Technologies™), 100 IU/ml of penicillin, 100 μg/ml of erythromycin, and 0.25 μg/ml of fungizone. Chondrocytes, at 80% confluency, were incubated at passage 0 (P0) with different shell matrix extracts in DMEM, with 2% FCS for 24, 48, or 72 h.

Alternatively, after one passage, trypsinized chondrocytes were recovered and used for redifferentiation experiments in 3D collagen sponge scaffolds (Symatèse Biomatériaux, Chaponost, France) like previously described (Legendre et al. 2013; Ollitrault et al. 2015). These collagen sponge scaffolds (2 mm thick and 5 mm in diameter) were composed of native type I collagen (90–95%) and type III collagen (5–10%) from calf skin. The day after the seeding into the sponge, cells were incubated with the different shell matrix extracts or with 50 ng/ml of rhBMP-2 (inductOs, Wyeth Europa Ltd) in DMEM + 2% FCS in hypoxia (3% O_2) for 7 days. The medium was replaced at day 3. Hypoxic cultures were performed in a sealed chamber as previously described (Legendre et al. 2009). The medium was changed and collected on days 3 and 7, and sponges were harvested on day 7.

WST-1 Assay

Cells were seeded onto 96-well microplates at a density of 9200 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 WSM of $CaCO_3$ for 24, 48, and 72 h. The medium was then removed and 100 μl of WST-1 reagent (WST-1 cell proliferation kit; dilution 1:40 in DMEM) was added for 40 min. Absorbance was measured at 450 and 630 nm with a microplate reader.

Crystal Violet Assay

Cells were seeded and treated onto 96-well microplates like described above with shell matrix extracts 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 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 solubilized in 20% acetic acid solution for 15 min. Absorbance was measured at 600 nm with a microplate reader.

Cytotoxicity Assay

Cells were seeded and treated onto 96-well microplates as described above with shell matrix extracts for 24 h. The cytotoxicity of the different shells extracts was measured using the CytoTox 96® assay (Promega) as previously described (Legendre et al. 2009). The principle of this test is based on measurement of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Maximum LDH cell release was obtained by lysis of cells with 0.8% Triton X-100. Culture medium LDH background release was also measured. The percentage of toxicity was calculated as follows: (treatment absorbance − culture medium background absorbance) / maximum LDH absorbance × 100.

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted with TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions and reversed-transcribed into cDNA before real-time PCR amplification as previously described (Ollitrault et al. 2015). Real-time PCR was performed in an ABI Prism SDS 7000 thermocycler. All procedures were conducted in triplicate. The sequences of the forward and reverse primers were designed using Primer Express software (Table 1). Ribosomal protein L13a (RPL13a) was used as an endogenous reference gene. The mRNA amount was normalized to *RPL13A* mRNA, and analysis of relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Following treatments, cultures in monolayers or in collagen sponges were rinsed once with ice-cold PBS, crushed and lysed in the RIPA buffer to prepare cellular extracts as previously described (Legendre et al. 2003). Cellular extracts (20 μg) underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electrotransferred to a polyvinylidene difluoride transfer membrane (Millipore). Membranes were probed with type I collagen (Novotec), type II collagen (Novotec), type X collagen (Sigma-Aldrich), and GAPDH (Santa Cruz Biotechnology) antibodies overnight at 4 °C, and then washed and incubated for 1 h with secondary peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Inc.). The signals were revealed with Western Lightning Plus-ECL reagent (PerkinElmer, Inc.). As control extracts, cartilage small slices from macroscopically healthy zones of femoral heads of patients undergoing joint arthroplasty (called OA cartilage) or of healthy donors (called healthy cartilage) were ground crushed in liquid nitrogen, and protein extraction was carried with the RIPA buffer.

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

Genes	Sequences	Strand
COL1A1	5′-CACCAATCACCTGCGTACAGAA-3′	F
	5′-CAGATCACGTCATCGCACAAC-3′	R
COL2A1	5′-GGCAATAGCAGGTTCACGTACA-3′	F
	5′-CGATAACAGTCTTGCCCCACTT-3′	R
COL10A1	5′-AAACCAGGAGAGAGAGGACCATATG-3′	F
	5′-CAGCCGGTCCAGGGATTC-3′	R
ACAN	5′-TCGAGGACAGCGAGGCC-3′	F
	5′-TCGAGGGTGTAGCGTGTAGAGA-3′	R
MMP-1	5′-GAAGCTGCTTACGAATTTGCCG-3′	F
	5′-CAAAAGGAGCTGTAGATGTCCT-3′	R
MMP-13	5′-AAGGAGCATGGCGACTTCT-3′	F
	5′-TGGCCCAGGAGGAAAAGC-3′	R
RPL13A	5′-GAGGTATGCTGCCCCACAAA-3′	F
	5′-GTGGGATGCCGTCAAACAC-3′	R

Type I Collagen and MMP-1 ELISAs

ELISA tests were performed on the culture media of HAC cultures. Type I collagen measurements were evaluated in the culture media with the CICP MicroVue Bone Health kit (Quidel), according to the manufacturer's instructions. Active MMP-1 was assayed with the Fuorokine E human MMP-1 Fluorescent Assay (R&D) following the manufacturer's recommendations. Absorbances were measured at 405 nm with a microplate reader. The concentrations were calculated in ng/ml and normalized to the total protein content of each point.

Statistical Analysis

Data representative of a minimum of four experiments were presented as box plots. Box plots showed the minimum values; the 25th, 50th (median), and 75th percentiles; and the maximum values. Means were shown as crosses. Data representative of three experiments were presented as floating bars. Floating bars showed the minimum values, the 50th percentiles, and the maximum values. Means were also shown as crosses. The Kruskal-Wallis test was used for multiple comparisons. The Mann-Whitney U test was used to determine

significant differences between two groups of treatments. No adjustment was made for multiple comparisons. *P* values less than 0.05 were considered significant: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Results

Shell Extracts Differently Modulate the Metabolic Activity of Chondrocytes.

Primary articular chondrocytes were treated with 50, 100, 250, 500, and 1000 μg/ml of each shell extract (ASM, AIM, WSM, and EDTAM), for 24, 48, and 72 h. The metabolic activity of cells was then evaluated using a WST-1 assay (Fig. 1). In comparison to the untreated cells, a significant (i.e., statistically significant: $p < 0.05$ or less) increase, in the metabolic activity of HACs (from 25 to 35%) was observed in the presence of 100 μg/ml of ASM after 48 and 72 h of incubation and a non-significant (i.e., not statistically significant or NS) increase with 50, 250, and 500 μg/ml of ASM after 24, 48, and 72 h of incubation. Moreover, compared to control cells, the incubation with 1000 μg/ml of ASM did not appear to increase the metabolic activity after 24 and 72 h of incubation. Unlike ASM, AIM did not induce any change in the metabolic activity regardless of the incubation time, with only the exception of 500 and 1000 μg/ml of AIM after 24 h of incubation, where we observed a significant decrease (20%) in the metabolic activity. WSM had also no significant effect on the metabolic activity of HACs. WSM can contain dissolved $CaCO_3$, and we have shown that the control test with WSM of pure $CaCO_3$ from 50 to 1000 μg/ml for 24, 48, and 72 h has also no effect on the metabolic activity of HACs (supplementary Fig. S1). Finally, we observed a non-significant increase (up to 36%) in the metabolic activity of HACs in the presence of 50, 100, and 250 μg/ml of

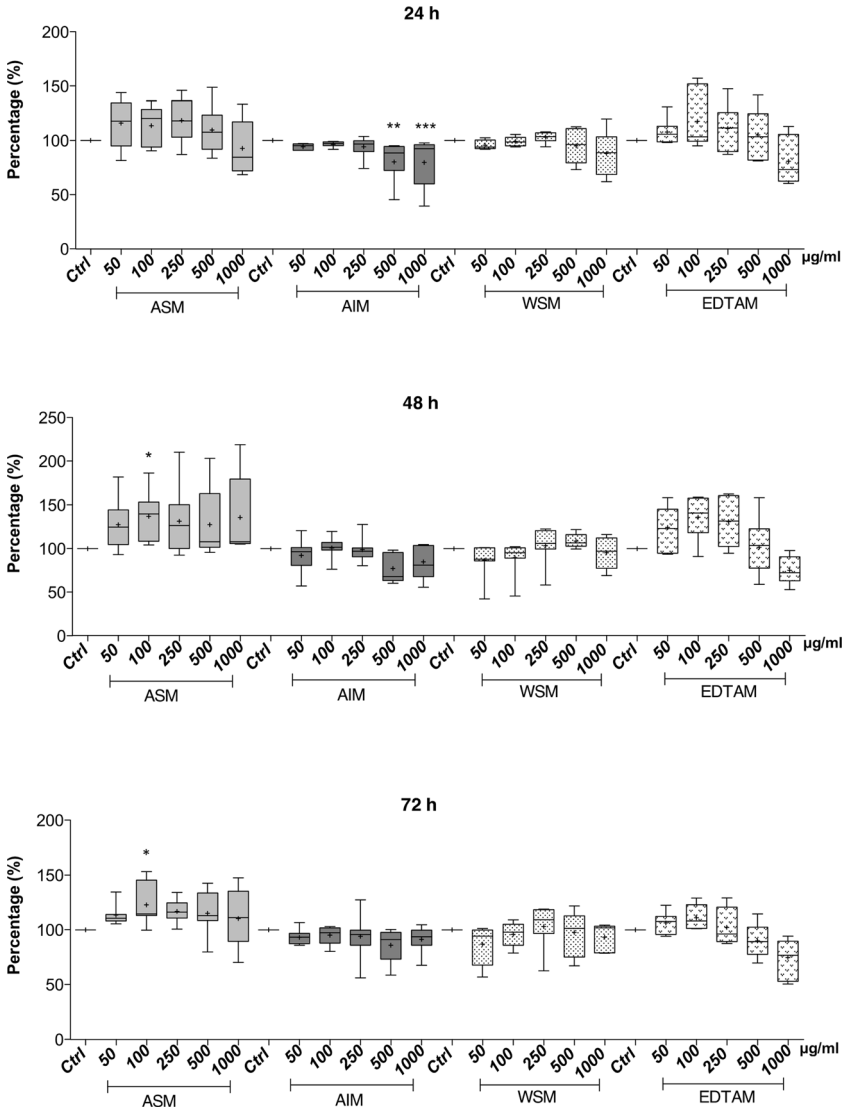

Fig. 1 Effect of shell matrix extracts on the metabolic activity of chondrocytes cultured in monolayers. HACs were cultured for 24, 48, and 72 h with ASM, AIM, WSM, and EDTAM (50–1000 μg/ml). The metabolic activity was measured and represented as box plots ($n = 7$). The significance of the results was assessed using the Kruskal-Wallis test. Statistical differences compared to the controls are indicated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

EDTAM for an incubation of 48 h. On the contrary, 1000 μg/ml of EDTAM seemed to decrease the metabolic activity of HACs (− 20%), an effect already observed after 24 h of incubation.

Shell Extracts Differentially Modulate Chondrocyte Proliferation.

The number of chondrocytes was quantified using a crystal violet assay after treatment with various shell extracts. We observed non-significant (i.e., not statistically significant or NS) increases (up to 45%) in the cell density from 50 to 250 μg/ml of ASM for 24, 48, and 72 h of incubation (Fig. 2). On the contrary, 1000 μg/ml of ASM appeared to decrease the cell density (NS). AIM had no significant effect on the cell density. WSM seemed to decrease the cell density at 1000 μg/ml after 48 h of incubation (37%) and to a lower extent at 500 and 1000 μg/ml after 72 h of incubation, but these effects were not statistically significant. EDTAM appeared to decrease the cell density at 500 and 1000 μg/ml since 48 h of incubation. Thus, 1000 μg/ml of EDTAM decreased the cell density of 42% at 48 h and of 49% at 72 h, but because of high standard deviations, it was not statistically significant.

Shell Extracts Differentially Affect the Viability of Chondrocytes.

We previously observed that high doses of shell extracts have a tendency to decrease the metabolic activity or the proliferation of chondrocytes. To determine whether these effects were associated with cell toxicity, the viability of shell extract-treated cells was assayed by measuring LDH release using the CytoTox 96® assay after 24 h of incubation (Fig. 3). We previously showed that 500 and 1000 μg/ml of ASM could decrease chondrocyte proliferation. The cytotoxicity assay showed that the decrease in the cell density was due to the cytotoxicity of ASM after 24 h of incubation, although not statistically significant (NS) because of high standard deviations. The toxicity increased 5 times with 1000 μg/ml of ASM. The concentration of 500 μg/ml of ASM also seemed cytotoxic because the toxicity increased 3.6 times (NS). AIM had little effect on the metabolic activity of HACs and chondrocyte proliferation, and we also showed that 500 and

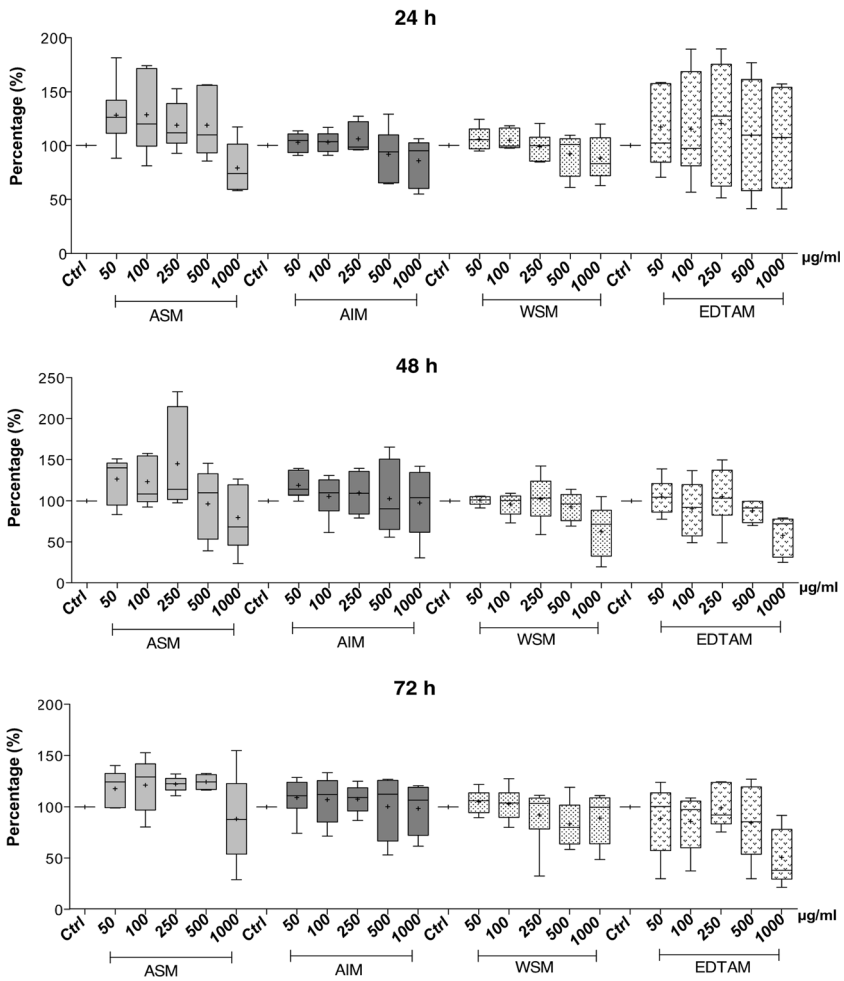

Fig. 2 Effect of shell matrix extracts on the proliferation of chondrocytes cultured in monolayers. HACs were cultured for 24, 48, and 72 h with ASM, AIM, WSM, and EDTAM (50–1000 μg/ml). The proliferation was measured and represented as box plots ($n = 6$). The significance of the results was assessed using the Kruskal-Wallis test (no significant difference)

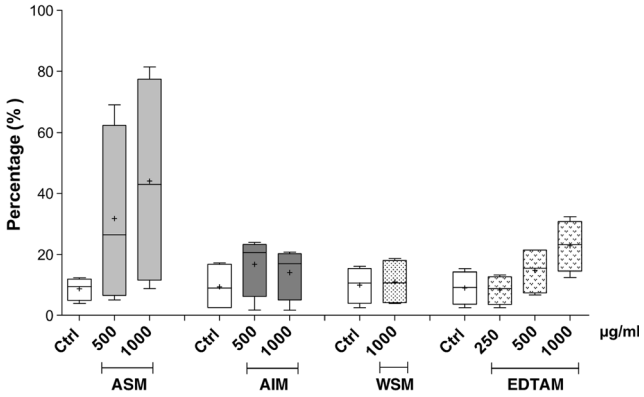

Fig. 3 Effect of selected shell matrix extracts on the cytotoxicity of chondrocytes cultured in monolayers. HACs were cultured for 24 h with varying concentrations (250–1000 µg/ml) of ASM, AIM, WSM, and EDTAM. The cytotoxicity was measured and represented as box plots ($n = 4$). The significance of the results was assessed using the Kruskal-Wallis test (no significant difference)

1000 µg/ml of AIM were not cytotoxic for chondrocytes. WSM had no effect on the metabolic activity of HACs but could decrease the cell density at 1000 µg/ml. This effect was not due to its cytotoxicity after 24 h of incubation. We found that 500 and 1000 µg/ml of EDTAM could decrease the metabolic activity and the cell density (NS). These effects were certainly linked to their cytotoxicity, because they increased the cytotoxicity of 1.7- and 2.5-fold respectively after 24 h of incubation (NS).

To summarize, the results showed that lower concentrations of ASM (until 250 µg/ml) could increase both metabolic activity and proliferation of HACs, whereas higher concentrations (500 and 1000 µg/ml) were themselves cytotoxic and then decreased the cell proliferation. EDTAM had similar effects, except an absence of positive effects on the proliferation at the lower concentrations. Higher concentrations of EDTAM also seemed to be cytotoxic for HACs. We found that only 500 and 1000 µg/ml of AIM significantly decrease the metabolic activity of HACs after 24 h of incubation, without changing the cell density. Moreover, this extract did not exhibit a cytotoxic activity at the higher concentrations after 24 h of incubation. Whatever its concentration, WSM has no effect on the metabolic activity of HACs, whereas it could decrease the cell proliferation at higher concentrations, without showing cytotoxic effects.

Shell Extracts Differentially Influence Gene Expression Profiles of Chondrocytes Cultured in Monolayers

Next, we tested the effects of shell extracts of *Pecten maximus* on chondrocyte gene expression. Taking into account the effects on the cytotoxicity, the extracts were used at 100 and 250 µg/ml for ASM, 500 and 1000 µg/ml for AIM, and finally 500 and 1000 µg/ml for WSM. We no longer used the EDTAM extract thereafter. Primary chondrocytes cultured in monolayers were treated for 48 h before analysis of the mRNA steady-state levels of chondrocyte phenotypic markers, type II collagen (*COL2A1*) and aggrecan (*ACAN*), chondrocyte dedifferentiation marker, type I collagen (*COL1A1*), chondrocyte hypertrophic marker, type X collagen (*COL10A1*), and finally metalloproteases (*MMP-1* and *MMP-13*) (Fig. 4). This phenotypic profile was completed by the calculation of two chondrocyte differentiation indexes corresponding to the ratio of type II collagen mRNA to type I collagen mRNA (*COL2A1*/*COL1A1* ratio) and of aggrecan mRNA to type I collagen mRNA (*ACAN*/*COL1A1* ratio). The increase of these ratios demonstrated the improvement of the chondrocyte phenotype, with the induction of markers specific to cartilage (type II collagen and aggrecan), and on the contrary the inhibition of dedifferentiation marker (type I collagen).

Regarding ASM, the treatment with 100 µg/ml significantly (i.e., statistically significant: $p < 0.05$ or less) decreased *COL2A1* and *ACAN* mRNA levels and non-significantly (i.e., not statistically significant or NS) increased *COL1A1* and *COL10A1* mRNA levels. As a result, 100 µg/ml of ASM decreased the *COL2A1*/*COL1A1* ratio (2-fold). In addition, 250 µg/ml of ASM significantly increased *COL1A1* and *COL10A1* mRNA levels and reduced *ACAN*/*COL1A1* ratio (2-fold). The two concentrations of ASM did not significantly influence the expression of *MMP-1* and *MMP-13*.

AIM, whatever its concentration, significantly decreased *ACAN* mRNA levels (0.4-fold; $p < 0.05$) and tended to decrease *COL2A1* mRNA levels. At the same time, 500 µg/ml of AIM tended to increase *COL1A1* mRNA levels. Hence, 500 and 1000 µg/ml of AIM significantly decreased *COL2A1*/*COL1A1* mRNA ratio (1.3-fold). AIM at 1000 µg/ml increased the steady-state mRNA levels of *COL10A1* (2-fold), but it was not statistically significant. AIM had the same effect on *MMP-1* and *MMP-13* mRNA levels. Thus, 500 µg/ml of AIM significantly decreased the expression of both genes (2- and 2.5-fold, respectively), whereas 1000 µg/ml of AIM significantly increased their expression (1.5- and 1.7-fold).

WSM at 500 and 1000 µg/ml increased *COL2A1* and *ACAN* mRNA levels (1.8- and 1.6-fold respectively for *COL2A1* and 1.5-fold with 500 µg/ml for *ACAN*). Moreover, we observed no statistically significant effect of WSM on the steady-state mRNA levels of *COL1A1*, *MMP-1*, and *MMP-13*. By contrast, 500 and 1000 µg/ml of WSM increased the expression of *COL10A1* (5- and 2-fold, respectively), but this effect was only statistically significant for 1000 µg/ml. However, as described above, WSM did not increase the expression of *MMP-13*, which is also considered as a marker of hypertrophy. As a result, WSM increased the two differentiation indexes *COL2A1*/*COL1A1* and *ACAN*/*COL1A1* (maximum 1.5 times).

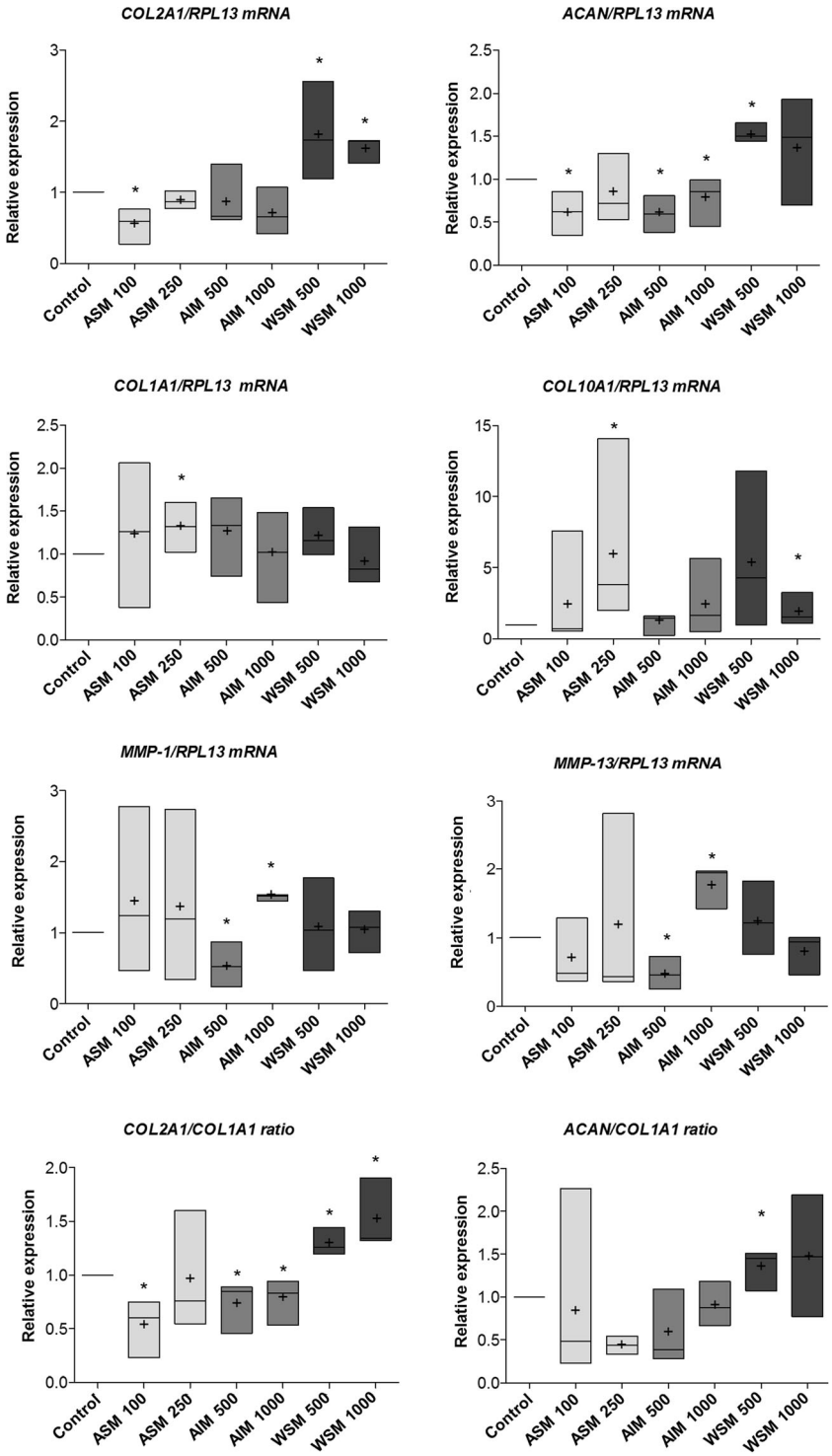

Fig. 4 Effect of shell matrix extracts on *COL2A1*, *COL1A1*, *COL10A1*, *MMP-1*, and *MMP-13* mRNA expression in chondrocytes cultured in monolayers. HACs were cultured for 48 h with varying concentrations (100–1000 μg/ml) of ASM, AIM, and WSM. The relative expression of each gene was determined and represented as floating bars of three independent experiments performed in triplicate. Statistically significant differences between the untreated and treated cells were determined using the Mann-Whitney U test (*$p < 0.05$)

Shell Extracts Differentially Modulate ECM Synthesis in Chondrocytes Cultured in Monolayers

The effect of shell extracts on the synthesis of type I and type II collagens was evaluated by Western blot in cellular extracts using a treatment with WSM of $CaCO_3$ as a control (Fig. 5a). HACs were treated for 48 h in monolayers. Cartilage extract from healthy donor was also used as a positive control. We showed that 100 μg/ml of ASM decreases the synthesis of both type I and type II collagens, whereas 250 μg/ml of ASM can increase type I collagen synthesis. Only 1000 μg/ml of AIM slightly increased the synthesis of both type I and type II procollagens. Five hundred and 1000 μg/ml of WSM increased type II collagen synthesis (immature forms)

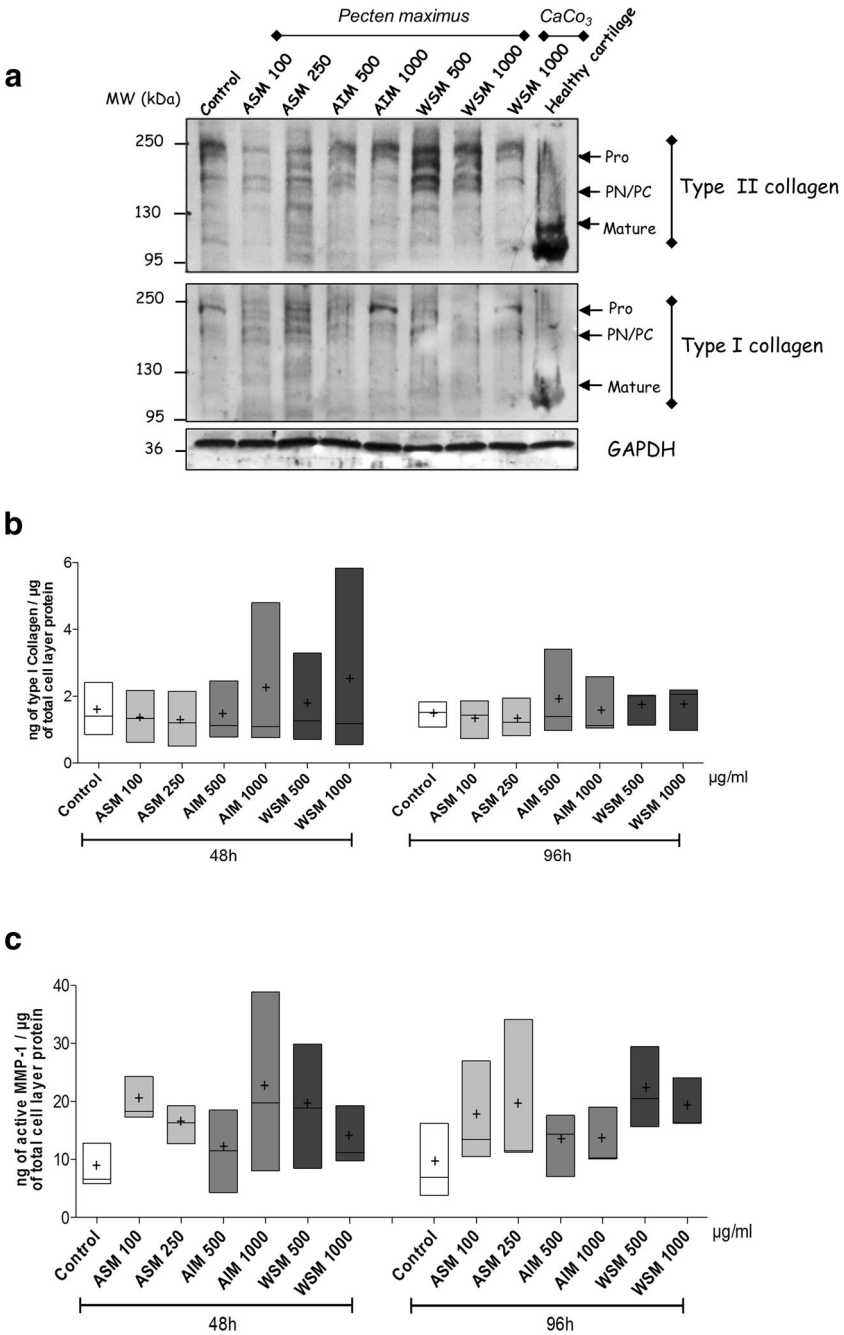

Fig. 5 Effect of shell matrix extracts on collagen synthesis and on active MMP-1 production in chondrocytes cultured in monolayers. **a** HACs were cultured for 48 h with varying concentrations (100–1000 µg/ml) of ASM, AIM, and WSM. HACs were also treated with 1000 µg/ml of WSM of $CaCO_3$. Healthy human cartilage extract was used as a control. Representative blots of three independent Western blots are shown. **b**, **c** HACs were cultured for 48 or 96 h as described above. The synthesis of type I collagen and active MMP-1 was evaluated by ELISA. The results are represented as floating bar ($n = 3$). Statistically significant differences between the untreated and treated cells were determined using the Mann-Whitney U test (no significant difference)

compared to the control after 48 h of incubation. Concomitantly, type I collagen synthesis was inhibited in the presence of the same doses. WSM of $CaCO_3$ did not modulate type I and type II collagen syntheses.

The effects of shell extracts on the synthesis of type I collagen and active MMP-1 were then measured using ELISA assays in the culture media with HACs cultured in monolayers for 48 and 96 h (Fig. 5b, c). The data showed no statistically significant variation of type I collagen release after 48 and 96 h of incubation, whatever the type of extract of *Pecten maximus* used (Fig. 5b). Nevertheless, 1000 µg/ml of both AIM and WSM tended to increase its synthesis after 48 h of incubation (42 and 61%, respectively, NS). This effect disappeared after 96 h of incubation.

Furthermore, 100 and 250 µg/ml of ASM tended to increase the active MMP-1 synthesis after 48 and 96 h of incubation in the culture media (2.3-fold for example at 48 h with 100 µg/ml of ASM; NS, Fig. 5c). Five hundred and 1000 µg/ml of AIM induced no change in active MMP-1 release, except with 1000 µg/ml at 48 h, which induced a non-statistically significant increase in active MMP-1 levels (2.6-fold). Finally, a non-statistically significant increase of active MMP-1 synthesis was observed in the presence of 500 µg/ml of WSM after 48 and 96 h of

incubation (2.3- and 2.4-fold) and with 1000 μg/ml after 96 h (2-fold).

To summarize, ASM (250 and 500 μg/ml) could increase both metabolic activity and proliferation of HACs but could also deteriorate the chondrocyte phenotype (decrease of differentiation indexes, tendency to stimulate the expression and activity of metalloproteases and the expression of the hypertrophic marker, type X collagen). AIM (500 and 1000 μg/ml) had little effect on the proliferation and the metabolic activity of chondrocytes, and such as ASM, it could be damaging to the chondrocyte phenotype. On the contrary, WSM (500 and 1000 μg/ml), which did not affect the metabolic activity of HACs and could decrease the cell proliferation at higher concentration, could favor the chondrocyte phenotype. WSM increased the differentiation indexes by inducing the expression of *COL2A1* and *ACAN*, without significantly affecting the catabolic or hypertrophic pathway. It appeared that only WSM had convincing chondrogenic effects in primary chondrocytes cultured in monolayers.

WSM of *Pecten maximus* Has Chondrogenic Effect in 3D Culture

Next, we used WSM extracts with dedifferentiated chondrocytes cultured in collagen sponges in hypoxia during 7 days, with a BMP-2 treatment as a positive control, as previously described (Legendre et al. 2013). We first evaluated the effects of WSM on gene expression profiles of chondrocytes cultured in collagen sponges by RT-qPCR, and next on ECM synthesis by Western blot and ELISA.

As expected, BMP-2 used at 50 ng/ml stimulated the steady-state mRNA levels of *COL2A1* (7-fold, $p < 0.05$), and also those of *COL1A1* (2.6-fold, NS) and *COL10A1* (2.8-fold, NS), but not of *ACAN* (Fig. 6). In addition, BMP-2 did not increase the mRNA expression of *MMP-1* and *MMP-13*.

The results obtained with the WSM treatments (Fig. 6) are in accordance with those observed in monolayer. Indeed, we observed a slight increase in *COL2A1* and *ACAN* mRNA levels in the presence of 1000 μg/ml of WSM (1.4- and 2.2-fold respectively, but NS), with no change in *COL1A1* mRNA levels. As a result, *COL2A1*/*COL1A1* and to a lesser extent *ACAN*/*COL1A1* ratios were upregulated in these conditions. The *COL2A1*/*COL1A1* ratio under WSM treatment is equivalent to the one observed with BMP-2, known to be one of the best chondrogenic inducers. Regarding the marker of hypertrophy, type X collagen, WSM (500 and 1000 μg/ml) did not significantly influence its gene expression. By contrast, WSM increased the expression of *MMP-1* and *MMP-13* mRNAs. Thus, WSM at 500 and 1000 μg/ml increased in a dose-dependent manner *MMP-1* mRNA levels (6- and 11-fold respectively), but with data only statistically significant for 1000 μg/ml. *MMP-13* mRNA levels were also increased (2.4- and 3-fold respectively), with data only statistically significant with 500 μg/ml of WSM.

The effects of WSM on collagen biosynthesis were then evaluated by Western blot, with 1000 μg/ml of WSM of $CaCO_3$ as a control treatment (Fig. 7). An extract of OA cartilage from OA donor was also used as a control. Five hundred and 1000 μg/ml of WSM of *Pecten maximus* did not increase type II collagen synthesis compared to the BMP-2 treatment (Fig. 7a). WSM of *Pecten maximus* strongly decreased all isoforms of type I collagen, whereas BMP-2 had no effect (Fig. 7b). WSM of $CaCO_3$ only slightly decreased PN/PC isoforms of type I collagen. BMP-2 and the two types of WSM did not modulate the synthesis of the 59 kDa form of type X collagen expected in denatured-reduced conditions (as attested in OA cartilage extract; Fig. 7b).

Type I collagen release in the culture media was also measured by ELISA in the same conditions of culture (Fig. 7c). Unlike BMP-2, and as expected from the Western blot analysis, the WSM of *Pecten maximus* (500 and 1000 μg/ml) decreased type I collagen release (2- and 3-fold respectively), but these effects were not statistically significant. WSM of $CaCO_3$ also decreased type I collagen release (1.7-fold, NS), but to a lesser extent than WSM of *Pecten maximus*.

Discussion

Several studies showed conservation of biological activities of molluscan shell proteins on mammalian cells (Westbroek and Marin 1998; Marin et al. 2008). For example, two studies highlighted the bioactivity of the nacre in the process of bone repair in vertebrates (Silve et al. 1992; Atlan et al. 1997). Others studies suggest that scallop shell extracts may be used for wound and skin repair (Torita et al. 2004; Liu et al. 2006). Furthermore, our group had demonstrated positive effects of shell extracts from *Pecten maximus* on the regulation of ECM synthesis in human dermal fibroblasts (Latire et al. 2014). These results suggest that the shell components may have biological activities in various mammalian tissues. Thus, the objective of our work was to evaluate the potential biological activities of the shell matrix compounds extracted from the organic fraction of the scallop *Pecten maximus* on the metabolism of human chondrocytes. We analyzed the expression of ECM components, i.e., the typical phenotypic markers (type II collagen and aggrecan) and non-specific markers (type I and type X collagen) of hyaline cartilage. We also focused on the catabolic pathway, via the analysis of the expression of MMPs. This study was performed both in monolayers with primary chondrocytes and in collagen sponge scaffolds with dedifferentiated chondrocytes. In the latter case, the three-dimensional scaffold constructs were incubated in hypoxia. Indeed, in a previous study using dedifferentiated

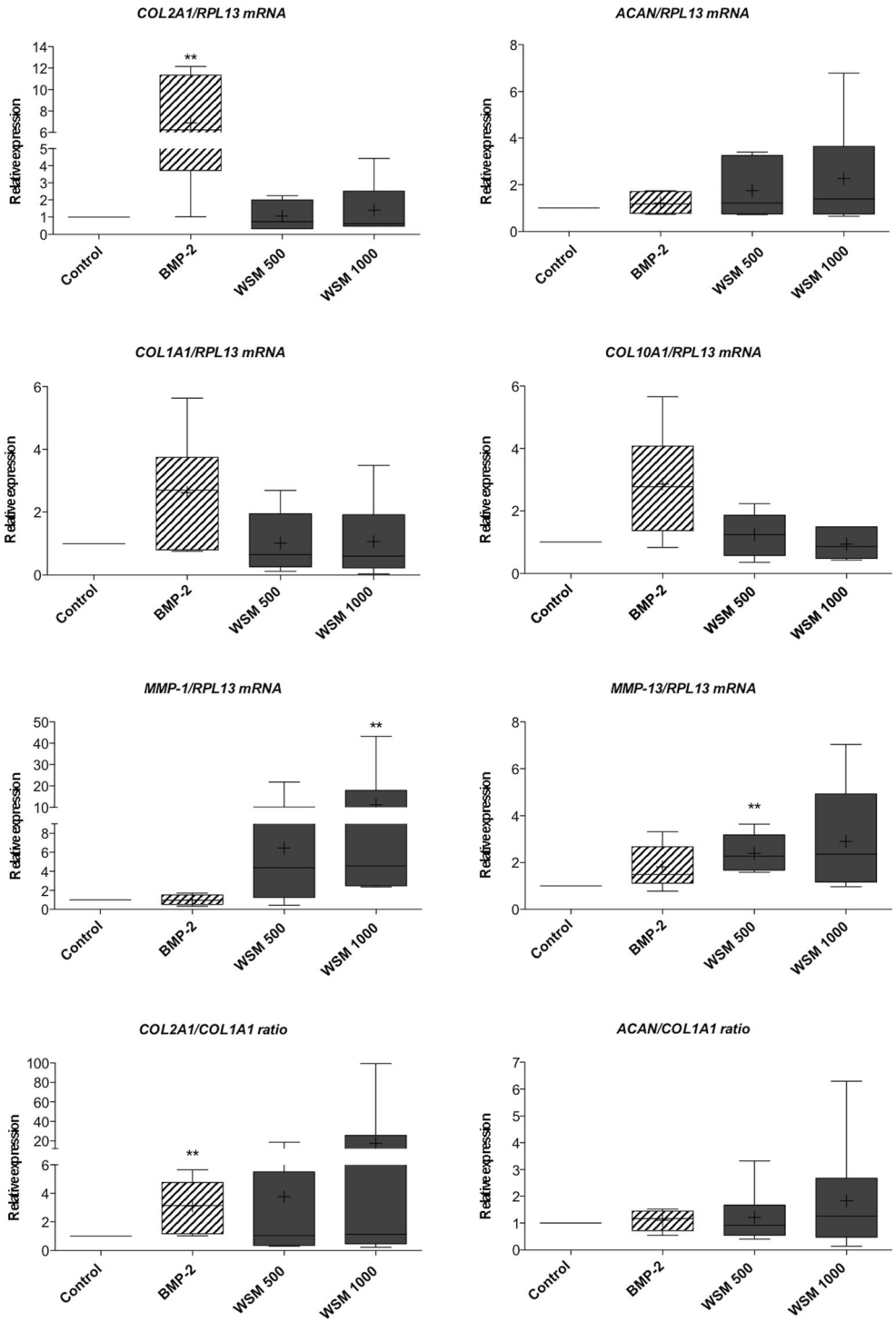

Fig. 6 Effect of WSM on *COL2A1*, *COL1A1*, *COL10A1*, *MMP-1*, and *MMP-13* mRNA expression in chondrocytes cultured in 3D in hypoxia. HACs were cultured for 7 days with 500 or 1000 μg/ml of WSM and with BMP-2 (50 ng/ml). The relative expression of each gene was determined and represented as box plots of four experiments performed in triplicate. Statistically significant differences between the untreated and treated cells were determined using the Mann-Whitney U test (**$p < 0.01$)

chondrocytes from OA patients, we demonstrated that the recovery of differentiated phenotype was better in hypoxia ($3\%O_2$) than in normoxia, after 7 days of culture with BMP-2 in type I/III collagen sponges (Legendre et al. 2013).

In the first part of the study, we found that various extracts of *Pecten maximus* had different biological effects on HACs, depending both on their concentration and on the time of incubation. The two extracts, ASM and EDTAM, showed cytotoxic effects at higher concentrations (starting at 500 μg/ml). If we excluded fractions with adverse effects, ASM (until 250 μg/ml) increased both cell metabolism and proliferation (about 25%), whereas AIM had no significant effect from 48 h of incubation. WSM had no effect on the metabolic activity but could decrease cell proliferation at high concentrations (500 and 1000 μg/ml). By contrast, EDTAM (until 250 μg/ml) could stimulate the metabolic activity, with no effect on

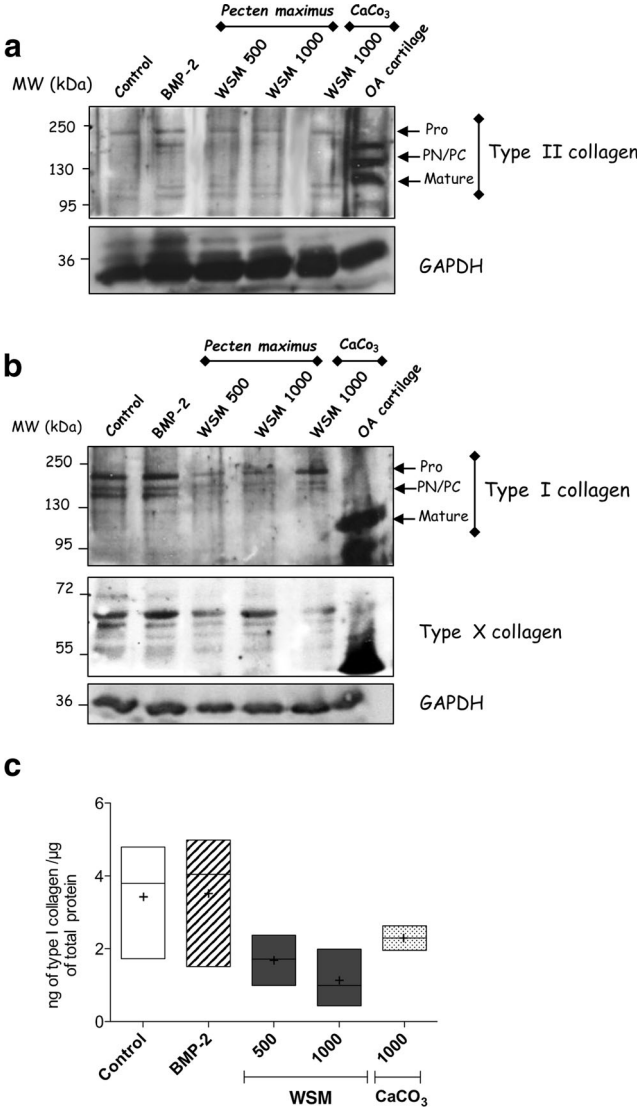

Fig. 7 Effect of WSM on collagen synthesis in chondrocytes cultured in 3D in hypoxia. HACs were cultured for 7 days, with 500 or 1000 µg/ml of WSM of *Pecten maximus* and 1000 µg/ml of WSM of $CaCO_3$ and BMP-2 (50 ng/ml). Osteoarthritis human cartilage extract was used as a control. **a**, **b** Representative blots of three independent Western blots are shown. **c** Type I collagen synthesis was also evaluated by ELISA. The results are represented as floating bar ($n = 3$). Statistically significant differences between the untreated and treated cells were determined using the Mann-Whitney U test (no significant difference)

cell proliferation. Several studies have already demonstrated that such extracts from molluscan shells affect the metabolism or the proliferation of vertebrate cells in vitro (Mouriès et al. 2002; Rousseau et al. 2003; Shen et al. 2006; Torita et al. 2007; Lee et al. 2011), but to our knowledge, no study was interested in the effects of shell extracts on chondrocyte metabolism. On the other hand, these studies suggest that molluscan shell extracts contain one or more signal molecules capable of activating proliferation of mammalian cells, but the signaling mechanisms involved in this regulation are not yet understood.

We previously demonstrated that ASM of *Pecten maximus* (1000 µg/ml) stimulates cell metabolism, but not proliferation of dermal fibroblasts (Latire et al. 2014). Another study demonstrated that ASM from the shell of the Japanese scallop *Patinopecten yessoensis* increased the metabolism of fibroblasts (Torita et al. 2007). In our study, ASM of *Pecten maximus* stimulated both chondrocyte metabolism and proliferation.

Regarding WSM of *Pecten maximus*, we previously found that it had no effect on the metabolic activity or the proliferation of fibroblasts (Latire et al. 2014), whereas in the present study, it had also no effect on the metabolic activity of chondrocytes, but it can decrease their proliferation at higher concentrations. In the literature, biological activities of WSM from molluscan shells were studied on various vertebrate cells (Lamghari et al. 1999; Almeida et al. 2000; Mouriès et al. 2002; Rousseau et al. 2003; Shen et al. 2006) and WSM could affect cell proliferation. For example, WSM from the shell of the pearl oyster *Pinctada maxima* decreased the cell density and increased the ALP activity of bone marrow stromal cells (Lamghari et al. 1999). Similar results were obtained on the cell density of fibroblasts isolated from human fetal lung (Almeida et al. 2000).

In the case of AIM of *Pecten maximus*, the results presented here with chondrocytes revealed no effect on the metabolic activity and on the cell density, as we previously observed with human dermal fibroblasts.

EDTAM could stimulate the metabolic activity of chondrocytes but had cytotoxic effects at the highest concentrations. The effects of EDTAM extracts on vertebrate cells were not extensively studied in the literature (Pereira-Mouriès et al. 2002), and we have not pursued our experiments because the process of extraction using EDTA cannot be applied for cell therapy applications.

In the second part of our study, we analyzed the effects of AIM (500–1000 µg/ml), ASM (100–250 µg/ml), and WSM (500–1000 µg/ml) on ECM biosynthesis in primary HACs cultured in monolayers. We found that ASM and AIM tended to decrease the expression of chondrogenic markers of differentiated chondrocytes, type II collagen, and aggrecan, in favor of the markers of the dedifferentiated and hypertrophic chondrocytes, type I collagen, and type X collagen, respectively. Accordingly, ASM and AIM decreased the differentiation indexes *COL2A1*/*COL1A1* and *ACAN*/*COL1A1*. Moreover, depending on the concentration used, they could stimulate catabolic pathway by stimulating the expression of *MMP-1* and *MMP-13*, and also active MMP-1 synthesis. Thus, ASM and AIM from the shell of *Pecten maximus* clearly favored the dedifferentiation of chondrocytes. These results are consistent with our previous study on dermal fibroblasts, where we found that ASM of *Pecten maximus* stimulates the synthesis of components of the skin ECM (Latire et al. 2014). In particular, we showed that ASM favors the synthesis of

type I collagen, the major marker of fibroblastic ECM, by the recruitment of *trans*activating factors (Sp1, Sp3, and c-krox) in the − 112/− 61 bp *COL1A1* promoter region. These results are also in accordance with several studies on wound healing performed in the presence of shellfish extracts (Torita et al. 2007; Lee et al. 2011).

On the contrary, WSM of *Pecten maximus* helped to maintain the chondrocyte phenotype, by increasing the expression of *COL2A1* and *ACAN* (from 1.5- to 2-fold), but not of *COL1A1* in chondrocytes cultured in monolayers. As a result, WSM favored the chondrocyte differentiation indexes *COL2A1*/*COL1A1* and *ACAN*/*COL1A1*. However, WSM could also increase *COL10A1* mRNA levels (from 2- to 5-fold), even if type X collagen was not detectable at the protein level in chondrocytes treated during 48 h in monolayers. The effects of WSM on catabolic pathway seemed limited in 2D culture, with no significant induction of *MMP-1* and *MMP-13* mRNA levels, and a weak induction of active MMP-1 synthesis (about 2-fold, not statistically significant).

In this context, we finally tested the effects of WSM on chondrocyte redifferentiation using dedifferentiated chondrocytes cultured in type I/III collagen sponges in hypoxia, during 7 days with BMP-2 treatment, as a positive control for redifferentiation (Legendre et al. 2013; Ollitrault et al. 2015). Although WSM had also chondrogenic effects in collagen sponge scaffold in hypoxia, we observed differences compared to monolayer culture system in normoxia. The effect of WSM on type II collagen expression was less evident at mRNA and protein levels. It was also the case for the induction of *ACAN* mRNA levels because of high standard deviations. As in monolayers, WSM induced no change in *COL1A1* mRNA levels, but in addition, it decreased type I collagen protein synthesis. By contrast, in type I collagen sponges and low oxygen tension, we observed no induction of *COL10A1* expression, and conversely, an induction of *MMP-1* and *MMP-13* mRNA levels. We cannot exclude an effect of hypoxia on type I and type X collagen downregulation, because it is generally admitted that low oxygen tension inhibits type I collagen expression and hypertrophy in redifferentiated chondrocytes (Markway et al. 2013; Legendre et al. 2013; Demoor et al. 2014). However, in our culture conditions, WSM increased the expression of *MMP-13*, also considered as a late chondrocyte hypertrophic marker. It seemed that WSM has less chondrogenic effect than BMP-2, but also less hypertrophic effect, and unlike BMP-2, WSM decreased type I collagen synthesis. As a consequence, it would be interesting to test the combined action of WSM and BMP-2 on chondrocyte redifferentiation experiments.

We showed for the first time that WSM of *Pecten maximus* could help to maintain the chondrocyte phenotype in 2D culture, and also favors redifferentiation of the chondrocytes in collagen sponge scaffold in hypoxia. Moreover, it seemed that WSM could also induce ECM remodeling. These effects seem to be specific of chondrocytes. Indeed, WSW of *Pecten maximus* tended to increase fibrotic markers (type I and type III collagens) and did not affect active MMP-1 synthesis in human dermal fibroblast cultures (Latire et al. 2014). Many in vivo and in vitro studies provide strong evidence of the osteogenic activity of WSM from the nacre (Lopez et al. 1992; Lamghari et al. 1999; Mouriès et al. 2002; Rousseau et al. 2003; Chaturvedi et al. 2013). In particular, WSM from *Pinctada maxima* was extensively studied. It stimulated the alkaline phosphatase activity of rat bone marrow cells (Lamghari et al. 1999), the osteoblast differentiation and matrix mineralization of MC3T3-E1 pre-osteoblast cell line from mouse calvaria (Rousseau et al. 2003), and had the same osteoinductive effects as BMP-2 on MRC5 human fetal lung fibroblasts (Mouriès et al. 2002). These studies suggest that nacre may contain signaling molecules, like BMPs, capable of activating osteogenic differentiation. Since BMPs are mainly related to bone and cartilage formation (Bessa et al. 2008) and in view of BMP-like effects in our study on chondrocytes, we can suspect the presence, in the WSM from the shell of *Pecten maximus*, of proteins that induce similar effects. A recently published, proteomic analysis of the acid-soluble and acid-insoluble shell matrix of *P. maximus* identified 46 proteins, 20 of which exhibit sequence homologies with proteins from other mollusk models (Arivalagan et al. 2017). Interestingly, although the analysis did not identify BMP-related proteins, one of the detected proteins possesses an EGF-like domain while 22 others are totally unknown, leaving the possibility that one or more of them exhibit signaling functions of BMP-type. Bédouet et al. used dialysis-based size-fractionation through both 1 kDa and 6–8 kDa cut-off membranes to investigate the composition of WSM from *Pinctada margaritifera* at the molecular level (Bédouet et al. 2006). Reversed-phase chromatography and mass spectrometry allowed them to suspect the presence of "cytokine-like" peptides among 100 molecules of low molecular weight. Orthologs of BMP-2/-4 have been already identified in mollusks such as *Crassostrea gigas*, *Patella vulgata*, and *Pinctada fucata* (Lelong et al. 2001; Nederbragt et al. 2002). Others families of growth factors (EGF, IGF-BP) and matrix proteins could be involved in the chondrogenic effects of WSM, as identified in the shells from *Pinctada margaritifera* and from *Haliotis laevigata* (Weiss et al. 2001; Marie et al. 2012). For example, Marie et al. identified EGF-like proteins in the prismatic shell layer of the pearl oyster by mass spectrometry and by in silico analysis of amino acid sequence alignment (Marie et al. 2012). Moreover, we cannot exclude the combined effect of minerals such as calcium with others soluble factors, which are as yet uncharacterized, to WSM-induced chondrogenic effects, as described for nacre's ability to heal wounds (Lee et al. 2011).

In this study, we found that each shell extract from *Pecten maximus* differentially modulates the metabolic activity of

HACs, depending on the type of extract and on its concentration. Moreover, whereas ASM and AIM favored dedifferentiation of articular chondrocytes, WSM promoted maintenance of the chondrocyte phenotype in monolayer culture and redifferentiation of chondrocytes cultured in collagen sponge scaffold in hypoxia. Our research has highlighted that the organic matrix of WSM from *Pecten maximus* may contain molecules with chondrogenic effects which can be useful in cell therapy of articular cartilage. More investigations with dialysis fractionation and proteomic analysis will be necessary to identify what molecules are biologically active on chondrocytes.

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Compliance with Ethical Standards

All patients signed an informed consent agreement form, which was approved by the local ethics committee.

Conflict of Interest The authors declare that they have no conflicts of interest.

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