The role of matrix proteins in the control of nacreous layer deposition during pearl formation

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To study the function of pearl oyster matrix proteins in nacreous layer biomineralization in vivo, we examined the deposition on pearl nuclei and the expression of matrix protein genes in the pearl sac during the early stage of pearl formation. We found that the process of pearl formation involves two consecutive stages: (i) irregular calcium carbonate (CaCO₃) deposition on the bare nucleus and (ii) CaCO₃ deposition that becomes more and more regular until the mature nacreous layer has formed on the nucleus. The low-expression level of matrix proteins in the pearl sac during periods of irregular CaCO₃ deposition suggests that deposition may not be controlled by the organic matrix during this stage of the process. However, significant expression of matrix proteins in the pearl sac was detected by day 30–35 after implantation. On day 30, a thin layer of CaCO₃, which we believe was amorphous CaCO₃, covered large aragonites. By day 35, the nacreous layer had formed. The whole process is similar to that observed in shells, and the temporal expression of matrix protein genes indicated that their bioactivities were crucial for pearl development. Matrix proteins controlled the crystal phase, shape, size, nucleation and aggregation of CaCO₃ crystals.

Keywords: pearl formation; Pinctada fucata; nacre matrix proteins; real-time PCR; gene expression

1. INTRODUCTION
The beauty and lustre of pearls result from the surface nacreous layer. The nacreous layer is composed of calcium carbonate (CaCO₃) aragonite platelets and biological macromolecules, such as chitin and matrix proteins. Aragonite platelets of the nacreous layer are arranged in continuous parallel layers that are separated by interlamellar organic sheets and intercrystalline organic membranes [1–3]. The accurate and orderly assembly of the crystals and organic matrix not only adds lustre by reflecting light uniformly from the layered compartment [4], but it also strengthens the nacreous layer to provide fracture resistance [5]. The process of biomineralization of the nacreous layer is of great economic interest to the pearl aquaculture industry and to those who study biomimetic materials.

Many studies have focused on oyster shell formation because the nacreous layer of shells is structurally similar to the nacreous layer of pearls. Many matrix proteins have been separated from the mineralized shell and purified and the genes encoding these proteins have been identified by searching mollusc cDNA libraries [6]. Six matrix proteins have been isolated from the nacre organic matrix of the pearl oyster Pinctada fucata: nacrein, a nacre protein that has carbonic anhydrase activity [7]; N16, N40 and P10, which induce aragonite formation [8–10]; ACCBP [11], an extrapallial fluid protein that induces the formation of amorphous CaCO₃ (ACC); and N19, a nacre protein that plays an important role in CaCO₃ precipitation [12]. In addition, eight nacre matrix genes have been identified that encode nacrein, N16, MSI60 [13], pearlin [14], ACCBP, N19, MS17 [15] and Pif [16]. Pearlin, MSI60 and MS17 are involved in the formation of the organic framework of the nacreous layer, and Pif can induce the formation of flat CaCO₃ crystals in vivo on chitin-coated glass plates. The role of these matrix proteins in crystal formation has been examined [17–21], but few studies have examined the regulation of nacreous layer biomineralization in vivo because few methods exist to experimentally control nacreous layer formation. In addition, studies of nacre development are hampered by the rapid transmutation of the larval bivalve.

Pearls are secreted by the pearl sac. In nature, the pearl sac is induced by parasites or gravel. Natural pearl formation is rare, so most studies have focused on pearl aquaculture. The pearl sac consists of mucous cells, cells containing large acidophilic granules and epidermal cells; this structure is homologous to the cellular composition of the outer epithelium of the mollusc mantle pallial zone [22,23] that secretes nacre [13]. The role of the pearl sac in nacreous layer biomineralization is thought to mirror that of the oyster mantle. The gene-expression patterns of shell matrix proteins in pearl sacs were

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detected in recent studies, but these studies did not evaluate the expression of genes that encode shell matrix proteins in relation to the biomineralization process of the nacreous layer [24,25]. Thus, the regulation of these shell matrix proteins in biomineralization in vivo remains unknown.

In this study, we examined both the gene expression of five nacre matrix proteins in the pearl sac and the CaCO$_3$ deposition that occurred during the early stages of pearl formation. In this special stage, the surface of the implanted nucleus is enveloped by the nacreous layer [26]. Results of this study may aid in the development of new methods to identify the role of nacre matrix proteins in the formation of the nacreous layer in vivo.

2. MATERIAL AND METHODS

(a) Biological materials

Pearl oysters (P. fucata) were cultured at the Guofa Pearl Farm, Beihai, Guangxi Province, China. Aragonitic nuclei and mantle grafts were implanted in the gonads of a total of 800 individuals on 10 April 2009.

(b) Typical pearl and pearl sac selection

On the 5th, 10th, 15th, 20th, 25th, 30th and 35th day following implantation (i.e. day 0), 60 oysters with aragonitic nuclei were harvested and the pearl sacs were carefully isolated from the gonad tissues. The nuclei were then separated from the pearl sacs for study.

(c) Evaluation of CaCO$_3$ deposition

The elemental composition of the CaCO$_3$ material deposited on the aragonitic nucleus was assessed by energy-dispersive X-ray (EDX) spectra on a scanning electron microscope (SEM). In every sample, a surface area of approximately 16 mm$^2$ was selected for examination, and the per cent element composition was assessed for times for each pearl. The morphological observations and identification of the material deposited on the nuclei were then performed using Raman spectra and SEM.

(d) Total RNA extraction and cDNA synthesis

Total RNA was extracted from the pearl sac of P. fucata using an RNazol isolation kit (Biotex Laboratories, Inc.) according to the manufacturer’s instructions. We determined the integrity of the RNA by fractionation on formaldehyde-denaturing 1.2 per cent agarose gels and staining with ethidium bromide. The quantity of RNA was determined by measuring OD$_{260}$ nm with an Uatospec 3000 UV–Visible Spectrophotometer (Amersham). Approximately two microgram of pearl sac RNA was used as a template for cDNA synthesis using BD PowerScript Reverse Transcriptase (BD Biosciences Clontech). Six samples of each stage were prepared.

(e) Real-time polymerase cycle reaction (PCR) and data analysis

Five genes encoding shell matrix proteins (nacrein, N19, N16, MS17 and ACCBP) were targeted, and a housekeeping gene (actin) was used as the internal standard for each sample. Table 1 lists the nucleotide sequences for each gene primer pair. Real-time quantitative PCR was performed using the Mx3000P RT-PCR System (Stratagene) on triplicate samples in a reaction mix of SYBR Premix Ex Taq (Takara, China). For amplification, the cycle programme had 40 cycles, with each cycle consisting of 95°C for 5 s followed by 60°C for 20 s. The threshold cycle ($C_q$) of each sample was measured using Mx3000P software. The cDNA present after seven cycles was detected for each sample, and repeated three times per sample. In the data analysis, we standardized the $-\Delta\Delta C_q$ of each gene by taking the 5th day as the zero point. Data analysis was performed using ANOVA.

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence</th>
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<tbody>
<tr>
<td>N19-F</td>
<td>5'-CCAGATTCTCAACTGATCTCTAGGA-3'</td>
</tr>
<tr>
<td>N19-R</td>
<td>5'-GCCCTATCCCTCAAAAAGTG-3'</td>
</tr>
<tr>
<td>nacrein-F</td>
<td>5'-GAGCCCAAGGAGTGGGAAA-3'</td>
</tr>
<tr>
<td>nacrein-R</td>
<td>5'-GCTCCATAGGTGGAAAGCA-3'</td>
</tr>
<tr>
<td>ACCBP-F</td>
<td>5'-GACATGGAACCAAGATTGGG-3'</td>
</tr>
<tr>
<td>ACCBP-R</td>
<td>5'-CTGTGGCTTGAATGGTGGA-3'</td>
</tr>
<tr>
<td>N16-F</td>
<td>5'-TGCGGAGCTTACTCATACTGG-3'</td>
</tr>
<tr>
<td>N16-R</td>
<td>5'-CCACTCTGAAAGTCATTGCTA-3'</td>
</tr>
<tr>
<td>MS17-F</td>
<td>5'-GATCGTTTATATGCTCTG-3'</td>
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<td>TCT-3</td>
<td></td>
</tr>
<tr>
<td>MS17-R</td>
<td>5'-CTCTCGCGCCGATGACAT-3'</td>
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<td>5'-CTCCTACTGAAAGGCCCCTG-3'</td>
</tr>
<tr>
<td>actin-R</td>
<td>5'-ATGCCTGGAATAGGGATTCTGG-3'</td>
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3. RESULTS

(a) CaCO$_3$ deposition on the nucleus

On the 5th day after implantation, the mantle fragments and the gonad formed the completed pearl sac. Over the course of pearl maturation, SEM images revealed that the early nucleus had a bare surface that was gradually enveloped by the mature nacreous layer (figure 1). In the early stages, the CaCO$_3$ deposition was irregular, but it became smooth by the 20th day (figure 1). At this point, the deposition pattern started to change. On the 25th day, large crystals with shapes similar to the polycrystalline aragonite formed on the mature–prismatic transition in bivalve shells were observed [27]. On the 30th day, divided nucleation occurred on these large crystals as they grew larger and formed aggregates. A new layer covered these large crystals. On the 35th day, the nacreous layer formed around the nucleus (figure 1).

The surface of the nucleus on the 5th day was smoother than that at implantation (figure 1), but it became rougher by day 10 owing to the deposition of aragonite crystals. By day 20, the surface was smooth once again. From day 25 to 30, the surface was covered with yellow deposits (figure 1), and by day 35 these deposits were covered by the nacreous layer. On day 35, photographs revealed a smoother surface and a mature pearl lustre.

(b) Analysis of the deposited CaCO$_3$ on the nucleus

In every stage of pearl formation, the surface showed calcium, carbon and oxygen peaks in the EDX spectra, but their relative carbon content decreased. From day 20 to day 35, the carbon content increased again, only to decrease by the 35th day.

Raman analysis confirmed the aragonitic nature of the crystals over the seven time periods (figure 3), with the exception of the deposition on day 30 (figure 3b). On
day 30, Raman spectra showed no aragonitic or calcitic peaks. We speculate that deposition onto the nuclear surface at day 30 was in the form of ACC. The baseline Raman spectrum on day 5 was higher than that on day 0. From day 5 to 15, the baseline of the Raman spectra decreased (figure 3a). However, from day 15 to 35, the baseline Raman spectra continually increased (figure 3b).

(c) **Gene expression of matrix proteins in the pearl sac during the early stages of pearl formation**

Significant expression of matrix proteins was detected by the 30th day after implantation (figures 4 and 5). In the earlier stages, the expression levels of nacrein, N19 and N16 were very low, but they increased to reach a maximum by the 30th day, and the value remained high until the end.
of the experiment (i.e. the 35th day; figure 4a–c). The expression of MS17 also increased during development and was highest on day 30, but it declined by day 35 (figure 5a). Similarly, expression of ACCBP was highest at day 30 and decreased dramatically by day 35 (figure 5b).

4. DISCUSSION

(a) The pearl formation process

Over the course of pearl formation, the deposition of aragonites changed from large irregular crystals to smaller ordered crystals by day 35, with a concomitant increase in surface lustre. The whole process involves two consecutive stages.

The first stage involves irregular CaCO₃ deposition. The carbon content suddenly increased on day 5 compared with day 0 (figure 2), indicating an increase in the surface organic matrix. In a CaCO₃ system, the absolute content of Ca does not change when considerable quantities of organic matrix are added, but the relative content of Ca is affected as the absolute contents of C and O increase. Therefore, in this study we used the C : Ca ratio to evaluate the change of organic matrix in the deposition which is mainly composed of CaCO₃. The high baseline Raman spectra may have reflected this change. Cochenne-Lauréau et al. reported that pearl sacs secreted an organic layer after implantation [28], so the increase in carbon content on day 5 could have resulted from this secretion. This was not visible in the SEM images, but regular camera photographs revealed that the surface was smoother at day 5 than at day 0 (figure 1). After day 5, the number of CaCO₃ aragonites around the nucleus increased until the aragonites formed a smooth surface. During the middle stages (days 5–20), the carbon content decreased, as did the organic matrix and the baseline Raman spectrum. In previous studies, the CaCO₃ first deposited on the nucleus was the aragonitic [29] or calcitic [30] prismatic layer found in the cross section of the pearl, and the nacreous layer then formed on the prismatic layer. In the early stages in our study, the CaCO₃ depositions were all aragonites. Until the 15th day, the deposition did not appear to be accurately controlled by the organic matrix.

However, at day 20, the surface became smooth, and the prismatic layer may have formed between the 15th and 20th day. Observing the cross section of the nucleus at different stages of development may be a feasible method of studying this process further.

The second stage of the pearl formation process involved regular CaCO₃ deposition. On the 25th day, large aragonite crystals appeared on the surface of the nucleus, and smaller crystals were inhibited from forming. During this period, the organic matrix increased, as indicated by the increase in carbon content. The baseline Raman spectrum also increased and was significantly higher than on day 20, suggesting that the deposition of CaCO₃ crystals was now controlled by the organic matrix. These larger aragonite crystals were similar to polycrystalline aragonites formed on the nacre–prism transition in bivalve shells [27]. Polycrystalline aragonites occurred at the growth front of the nacreous layer and formed in the organic matrix of the nacre–prism transition in bivalve shells. Because we collected samples every 5 days in this study, we did not confirm if there was an organic layer on the nuclei before day 5. In bivalve shells, the nacreous layer then forms on the polycrystalline aragonites [31]. In the present study, the process mirrored that observed in bivalve shells. By the 30th day, a thin layer had formed on the large aragonites, and nucleation occurred. The deposits aggregated and formed a continuous layer covering the large aragonites. The organic matrix continued to increase (as indicated by the carbon content and Raman spectra), and the outer crystals began to decrease in size. These results indicated that the deposition of CaCO₃ was controlled by the organic matrix. By day 35, the nacreous layer had formed on the nucleus. In this period, aggregation was inhibited, and the sizes of crystals became even smaller than those present on day 30.

(b) The role of the matrix proteins in the early formation of pearls

Relatively high expression of nacrein was detected between the 30th and 35th day, indicating that nacrein was involved...
in ACC and nacreous layer formation. The highest levels of nacrein expression were observed on day 35. At day 35, the organic carbon content was lower than at day 30, which is consistent with an increased CaCO₃ content. In the earlier periods, the expression level of nacrein was relatively low. This finding suggests that nacrein, which catalyses HCO₃⁻ formation [7], is a carbonic anhydrase specific for biomineralization. The mineralization of aragonites during the early stages must have been controlled by other carbonic anhydrases in the pearl sac.

Matrix protein N19 can inhibit the crystallization of CaCO₃ [12]. It was highly expressed by the 30th day, suggesting that it was involved in ACC formation by inhibiting crystallization. On the 35th day, N19 expression...
remained relatively high, but was lower than that on day 30, indicating a reduced activity that may have allowed aragonite platelets to form more regular shapes. The developmental changes in N16 expression mirrored those of N19. This matrix protein induced the aragonite formation when fixed on the substrate, but it inhibited crystal formation without the substrate [8]. Thus, on day 30 it performed a function similar to that of N19, whereas on day 35 it induced aragonite formation during the creation of the nacreous layer. MSI7 participates in the formation of the nacre framework, and it can accelerate the nucleation and precipitation of CaCO$_3$ [15]. The highest levels of MSI7 expression occurred on day 35, which suggests that MSI7 was involved in nacreous layer formation. However, expression was also significant at day 30, suggesting that MSI7 also might play a role in the nucleation of CaCO$_3$ deposition during this period.

The highest expression of ACCBP also occurred on day 30 as the thin layer formed on large aragonites. ACCBP is an ACC-binding protein and can inhibit calcite formation and induce ACC formation [11]. The significant expression of ACCBP at this stage supports our speculation that the thin layer consists of ACC and the divided nucleation is under the control of other matrix proteins. After the nacreous layer had formed, the expression level of ACCBP decreased, which suggests that ACCBP is very important for the formation of the first nacreous layer.

The gene-expression patterns of these matrix proteins also varied among sampling periods, but the overall tendency of these variations mirrored those of the
proteins. The data obtained can help in establishing a correspondence between gene-expression patterns and the CaCO₃ deposition process. The highest levels of matrix protein expression occurred between days 30 and 35. During this period, the CaCO₃ deposition was ordered, which indicates that it was under the control of the organic matrix. In the earlier periods, the relative expression levels were lower, and the results from SEM, element content analysis and Raman spectra indicated that CaCO₃ deposition was independent of the organic matrix. These results suggest that matrix proteins were critical for the formation of the nacreous layer. Nacrein provided the HCO₃ for the nacreous layer formation.

N16, N19, MS17 and ACCBP controlled the size and morphological characteristics of CaCO₃ crystals and inhibited calcite formation. On day 30, a special morphogenetic change occurred in the initial organization of the crystallites. The initial organization was over to some extent as the nacreous layer was formed on day 35. At the same time, the expression level of ACCBP decreased to a very low level after the formation of the nacreous layer. Thus, ACCBP is critical for this organization. In recent studies, a polypeptide representing the N-terminal domain of ACCBP was shown to form supramolecular assemblies in solution that contained amorphous-appearing deposits [32]. Supramolecular assemblies constitute the base for nacreous layer biomineralization, and the position of supramolecular assemblies results in the initial organization of crystals.

It should be noted that the matrix proteins studied herein are all from the nacreous layer, and only five were studied. Moreover, the mechanism for the transition in the manner of crystal deposition that occurred between days 20 and 25 after implantation remains unknown. Further research is required to identify additional matrix proteins and to determine their stage-specific expression patterns.

This work was supported by the National Basic Research Programme of China (2010CB124045), the National High Technology Research and Development Programme of China (2010AA09Z405) and the National Natural Science Foundation of China (U0831001, 40876068). Mr Teter Jacob is thanked specially for his help with the manuscript preparation.

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