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Calcium carbonate mineralization mediated by *in vitro* cultured mantle cells from *Pinctada fucata*

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ABSTRACT

Formation of the molluscan shell is believed to be an extracellular event mediated by matrix proteins. We report calcium carbonate mineralization mediated by *Pinctada fucata* mantle cells. Crystals only appeared when mantle cells were present in the crystallization solution. These crystals were piled up in highly ordered units and showed the typical characteristics of biomineralization products. A thin organic framework was observed after dissolving the crystals in EDTA. Some crystals had etched surfaces with a much smoother appearance than other parts. Mantle cells were observed to be attached to some of these smooth surfaces. These results suggest that mantle cells may be directly involved in the nucleation and remodeling process of calcium carbonate mineralization. Our result demonstrate the practicability of studying the mantle cell mechanism of biomineralization and contribute to the overall understanding of the shell formation process.

1. Introduction

Pinctada fucata is a marine bivalve mollusk that is widely used to culture pearls. This species is also used frequently in biomineralization research [1]. The oyster shell consists of the prismatic layer composed of calcite and the nacreous layer composed of aragonite [2]. Previous studies have reported that the molluscan shell forms during an extracellular event mediated by the organic matrix that provides the framework and induces heterogeneous crystal nucleation [3–6]. *In vitro* crystallization experiments have demonstrated that matrix proteins control crystal growth and morphology [7,8]. However, the supersaturated solutions used in these experiments had much higher Ca^{2+} concentrations than what has been reported in the natural environment, which questions the effect of matrix proteins on shell mineralization.

Scientists are now beginning to understand the cellular mechanisms of shell formation, which will contribute to the overall understanding of the biomineralization process. Intracellular crystal growth has been observed in eastern oyster hemocytes, which lends evolutionary significance to the cell-mediated mineralization hypothesis [9]. However, this crystal growth was observed during the shell repair process, and there is no direct proof that the natural shell formation process adopts the same mechanism. It is commonly believed that the mantle, which secretes various matrix proteins, is the most important organ in shell formation [10,11] and whether mantle cells are directly involved in normal calcium carbonate deposition in the shell is now a research focus.

Primary cell culture is an innovative approach to investigate the cellular mechanism of biomineralization [12,13] and a primary *P. fucata* mantle cell culture has been established by Gong et al. [14]. Gong et al. and Ren et al. observed calcium carbonate crystals precipitating in the mantle cell culture [15,16]. Furthermore, it was demonstrated that mantle cells still have the ability to secrete matrix proteins in primary cell culture. Xiang et al. was also the first to report the existence of amorphous calcium carbonate (ACC) inside mantle cells [17]. Considering the remarkable contribution of mantle cells to shell formation, it is essential to establish *in vitro* mineralization models to research the related cellular mechanisms.

In this study, growth of calcium carbonate crystals was mediated by primary *P. fucata* mantle cells in an *in vitro* culture environment similar to natural living conditions. The crystals were characterized by light microscopy, scanning electron microscopy (SEM), and Raman spectroscopy. An *in vitro* calcium carbonate crystallization experiment was conducted to investigate the effect of substances secreted by the mantle cells on the crystal growth pattern. The results will help reveal mantle cell function during

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calcium carbonate deposition and contribute to further understanding of the shell formation process.

2. Material and methods

2.1. Cell cultures

A primary mantle cell culture was established according to a method described previously with a slight modification [15]. *P. fucata* mantle edge tissue was excised and soaked in disinfecting solution for 30 min. The disinfecting solution was prepared by dissolving 100 IU/mL gentamicin, 500 IU/mL penicillin, 2 μg/mL nystatin, and 1 mg/mL streptomycin in molluscan balanced salt solution (MBSS) [15].

After rinsing several times with D-MBSS (MBSS without Ca^{2+} and Mg^{2+}), the tissue fragments were placed in Petri dishes (Greiner Bio-one, Solingen, Germany) with 500 μL culture medium (Pf-CM2.5 with 10% fetal bovine serum). One L Pf-CM2.5 contained: Medium 199 and Leibovitz-15 medium (1 L, Gibco/Invitrogen, Carlsbad, CA, USA), 3.57 g HEPES, 10 mg ATP, 10.22 g NaCl, 128.9 mg taurine, 400 mg lactalbumin hydrolysate, 40 mg ascorbic acid, 50 mg kanamycin, 1 mg nystatin, 100 mg streptomycin, and 100,000 IU penicillin. pH was set to 7.2.The cells were cultured in a 25 °C incubator without CO_2, and an additional 200-μL aliquot of culture medium was added every 3 days. Cells migrating from the mantle tissue fragments were collected on day 7.

Gonadal cells were extracted from gonads for the control group by using a sterile syringe and incubated for 30 min in disinfecting solution. After rinsing the cells several times in D-MBSS, they were cultured in the same culture medium as that used for the mantle tissues.

2.2. Cell-mediated calcium carbonate crystallization

Sterilized and siliconized cover glasses were placed in 6-cell culture well plates. Primary mantle cells or gonadal cells incubated in culture medium containing 25 μM Ca^{2+} were added to each cover glass ($n = 6$/group). The total volume of medium in each well was 500 μL, at a cell concentration of 1×10^5/mL. The cells were cultured in an incubator (25 °C without CO_2), and an additional 200-μL aliquot of mixed culture medium was added to each well every 3 days.

2.3. Characterization of $CaCO_3$

2.3.1. Light microscopy

The culture plates were observed under a microscope (Nikon Eclipse Ti–U, Kanagawa, Japan) every day to evaluate the crystal deposition process. Photographs were taken to record the growth and morphological changes of the crystals.

2.3.2. SEM and energy-dispersive X-ray spectrometry (EDS)

Two cover glass-bearing crystals were removed from the wells and washed twice with D-MBSS to observe the structural details and analyze the elemental composition of the calcium carbonate deposits. The cells and crystals were fixed in 4% paraformaldehyde for 15 min. The samples were washed twice with Milli-Q water and dehydrated in an ascending ethanol series (50–100%). After freeze drying for 1 h, all samples were gold coated and observed under SEM (JSM-7001F field emission SEM, Tokyo, Japan) and subjected to EDS at an acceleration voltage of 15 kV.

2.3.3. Raman spectroscopy

Two samples were washed with Milli-Q water and 75% ethanol solution for 10 min each. Glass slides were dried at room temperature, and the crystal polymorph was analyzed by Raman spectroscopy (RM2000; Renishaw, Bedford, UK) at an excitation wavelength of 514 nm. The samples were scanned for 30 s in the 100–1200 cm^{-1} spectral range.

2.4. In vitro calcium carbonate crystallization assay

A saturated calcium bicarbonate solution was prepared according to the method of *Xiang* et al. [17]. Twelve-day-old medium that had been used in cultures was collected and filtered through a 0.22-μm membrane filter to remove cells and insoluble substances. The crystallization experiment was conducted by adding uncultured/cultured medium (4 μL) to the freshly prepared saturated solution (76 μL). The mixed solution was dropped on a siliconized cover glass (20 μL/drop). The cover glass was washed with Milli-Q water after 48 h to remove the crystallization solution, and the crystals were characterized by light microscopy and Raman spectroscopy.

3. Results

3.1. Calcium carbonate crystal growth mediated by primary mantle cells

No calcium carbonate crystals grew in either the uncultured cell medium or the gonadal cell culture (Fig. 1), indicating that crystals do not nucleate spontaneously in the culture medium used and that normal cell metabolic activities do not induce calcium carbonate crystallization.

However, a large number of crystals appeared in the primary mantle cell culture and were deposited over the entire cover glass. More crystals were observed near the mantle cell aggregates (Fig. 1F). We also noticed that larger crystals occurred nearest the cell aggregates. Some of the adjacent crystals merged with each other.

The growth of calcium carbonate crystals mediated by the primary mantle cells is illustrated in Fig. 2. Crystal size and morphology changed over time. No crystals were detected by light microscopy until day 7, and the size of the crystals increased with time. The calcium carbonate crystals were initially polyhedron shaped with one sharp end (Fig. 2A). A bright ridge was observed on the surface of these crystals, which were only a few microns in length when they appeared, but ultimately grew to >50 μm (Fig. 2B). The EDS analysis showed that the crystals were composed of Ca, C, and O (Fig. 2J). Raman spectroscopy demonstrated that the crystals were calcite (Fig. 2M). However, the polyhedron-shaped crystals were gradually replaced by crystals with a flatter appearance after 13 days (Fig. 2C). This crystal type was 50–100 μm in length. Adjacent crystals aggregated together as time passed (Fig. 2D), and the edges of some crystals became round (Fig. 2E). The rounded rectangular-shaped crystals were calcite and composed of Ca, C, and O (Fig. 2K and N). Round crystals were observed by light microscopy after 22 days (Fig. 2F). The EDS analysis and Raman spectroscopy demonstrated that the round crystals were composed of calcium carbonate (Fig. 2L and O).

The SEM images show more details of the calcium carbonate crystals (Fig. 2G–I). Each crystal seemed to be piled up in numerous highly ordered tiny pieces with the characteristics of a typical biomineralization product. The crystals dissolved in 10% EDTA (pH 8.0) solution but a semi-transparent film remained. This film was fragile and contained cracks and holes (Fig. 3A and B). The EDS results indicated that the film was composed of 73% C (Fig. 3C). Therefore, the fragile film was likely some crystal organic framework.

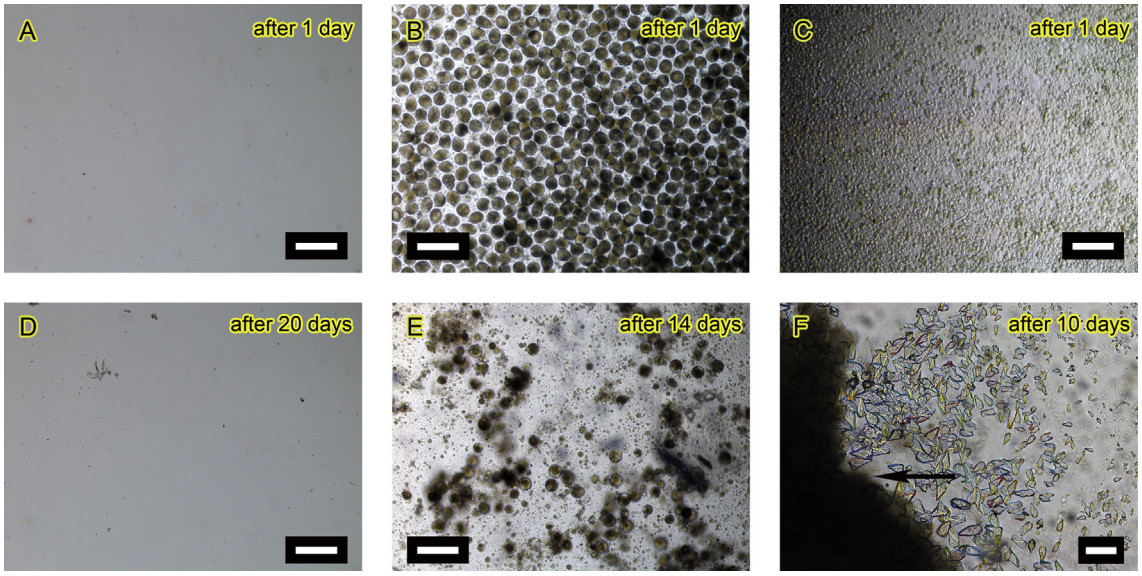

Fig. 1. Calcium carbonate crystallization in uncultured medium (A, D). Gonadal cell culture. (B, E). Mantle cell culture (C, F). No crystals appeared in the uncultured medium after 20 days. Most gonadal cells died after 14 days, and no crystals grew. However, large crystals formed in the area close to the mantle cell aggregates (arrow). Scale bar = 100 μm.

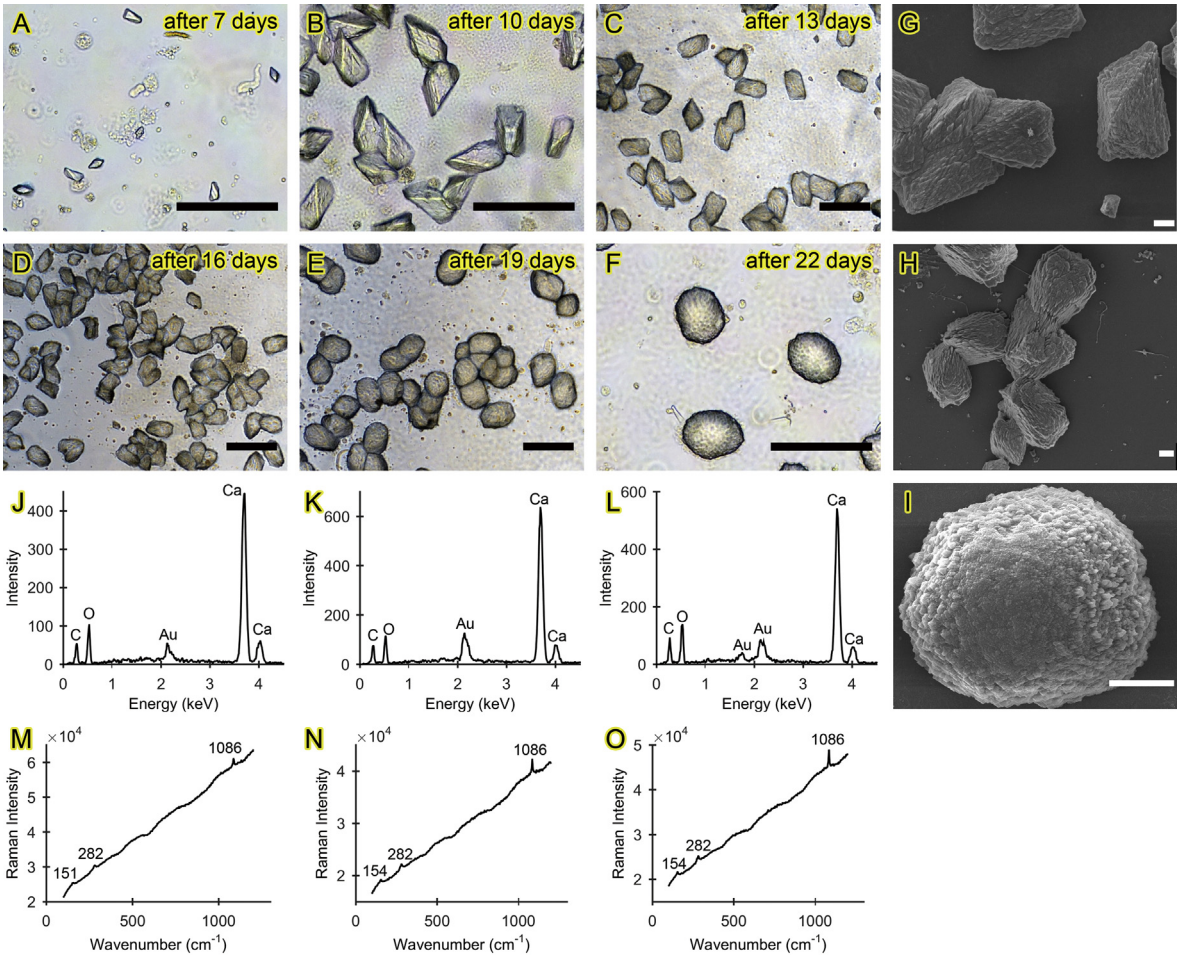

Fig. 2. Crystal growth process in the mantle cell culture. (A) Crystals began to appear after 7 days. (B) The number of polyhedron-shaped crystals increased after 10 days. (C) The polyhedron-shaped crystals became flat after 13 days. (D) Adjacent crystals aggregated after 16 days. (E) Round rectangular-shaped crystals appeared after 19 days. (F) Round crystals appeared after 22 days. Scale bar (A–F) 100 μm. (G–I) Scanning electron microscopic images of polyhedron-shaped crystals, round rectangular-shaped crystals, and round crystals, respectively. Scale bar = 20 μm. (J–L) Energy dispersive X-ray spectra of the crystals in (G–I); these crystals were composed of Ca, C, and O. (M–O) Raman spectrum of the crystals in (G–I); these crystals were all composed of calcite.

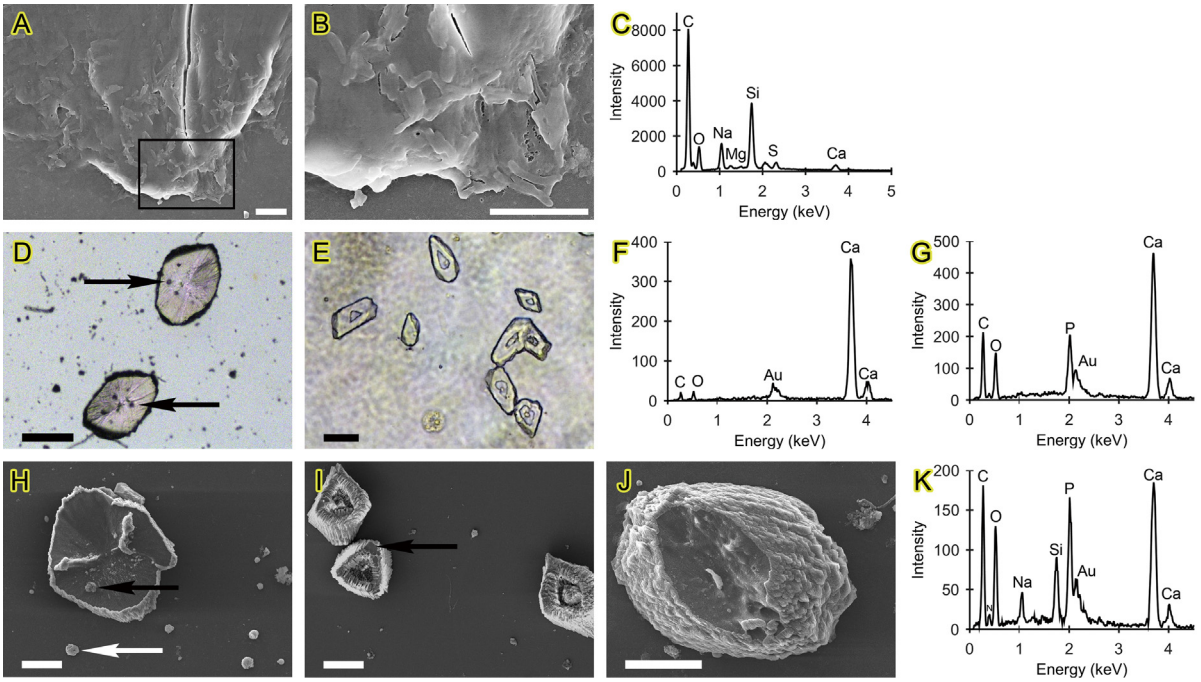

Fig. 3. Mantle cell remodeled crystals. (A) The organic frame of the crystals was fragile and was cracked. (B) Magnification of the box in (A); little holes were observed on the organic frame. (C) The energy dispersive X-ray spectral (EDS) analysis of the organic frame showed that it contained C, O, S, Na, Si, Mg, and Ca. Scale bar (A, B) 4 μm. (D) Crystals with flat and smooth surfaces. Cell-like particles attached on to the top of the crystals (arrows). (E) Crystals with a hole inside. (H), (I) Scanning electron microscopic (SEM) images of the crystals in (D) and (E). (J) SEM image of crystal with a partly etched surface. Scale bar (D–J) 40 μm. (F) EDS analysis of crystals in (H); the crystals were composed of Ca, C, and O. (G) EDS analysis of the cell-like particles in (H) (black arrow); the particles were composed of Ca, C, O, and P. (K) EDS analysis of the cell-like particles in (H) (white arrow); the cell-like particles were composed of Ca, C, O, P, N, Na, and Si.

3.2. Crystals with mantle cell remodeled surfaces

As culture time passed, some of the crystals developed surfaces that appeared to have been remodeled by the mantle cells. Fig. 3D shows one type of such a crystal. These crystals had flat and smooth surfaces and were very similar to transparent glass discs. The size of these crystals was above average, which was 50–100 μm in diameter. Round particles were observed on the surfaces of some of these crystals (Fig. 3D, arrows). We assumed that the particles were mantle cells. SEM revealed that the glass disc-shaped crystals were also composed of tiny pieces, and their sides were as rough as those of the other crystals (Fig. 3H). Some particles adhered on the smooth surfaces of the crystals. The round particles with the black arrow had a 10-μm diameter, which was similar to the size of mantle cells reported previously [17]. The results of the EDS analysis of the crystal and the two round particles suspected to be mantle cells are shown in Fig. 3F, G, and K. According to the results, the crystals were composed of Ca, C, and O. The particles adhering to the crystal surface (black arrow) consisted of Ca, C, O, and P. In addition to these four chemical elements, the particle near the crystal (black arrow) consisted of Na, N, and Si. Phosphorus is a very important component of cell membranes. We were quite sure that the round cell-like particles were mantle cells based on the relatively high phosphorus ratio.

The crystals shown in Fig. 3E were similar with the polyhedron-shaped crystals, but they had a small hole inside, as shown in more detail by SEM (Fig. 3I). These crystals had a 20-μm diameter hole on the inside upper surface and were piled up in thin sheets. Some parts of the crystal surfaces were smooth (Fig. 3I, arrow). We also noticed another interesting type of crystal (Fig. 3J), which was similar to the one shown in Fig. 2I, but it was largely concave. The inner surface of this crystal was smoother than the outer surface, suggesting that it was being remodeled. We deduced that this crystal might become the glass disc-shaped crystal.

3.3. Influence of medium used in culture on calcium carbonate crystal growth

To investigate the effect of substances secreted by mantle cells on crystal growth, 12-day-old cultured medium was collected and filtered in preparation for the crystallization experiment. No crystals appeared in a solution of 12-day-old cultured medium containing 25 μM Ca^{2+} after 15 days (Fig. 4A). We deduced that the substances secreted by mantle cells, such as matrix proteins and carbonic anhydrase, do not mediate crystal nucleation and growth independently.

However, the substances secreted by the cells performed differently in the *in vitro* calcium carbonate crystallization assay. The calcium carbonate deposits in the control group were the flower-shaped crystals shown in Fig. 4B (arrow). Raman spectroscopy confirmed that the crystals were calcite (Fig. 4C). The difference between the morphology of the crystals that appeared in the control group and natural calcite may have been due to the nutrition contained in the uncultured medium. Fig. 4D shows the calcium carbonate deposits in the experimental group. Most of these crystals were dumbbell shaped (arrow), and Raman spectroscopy confirmed that they were calcite (Fig. 4F). The crystals in the control and experimental groups were both calcite, but there was a big morphological difference. In addition, the crystals in the experimental group were larger (Fig. 4D), and the adjacent crystals had aggregated (Fig. 4E). These results show that the substances secreted into the cultured medium by the mantle cells may have strongly influenced the crystal growth pattern and rate.

4. Discussion

Formation of the molluscan shell is generally believed to be an extracellular event mediated by matrix proteins secreted by mantle tissues. However, whether mantle cells are directly involved in the

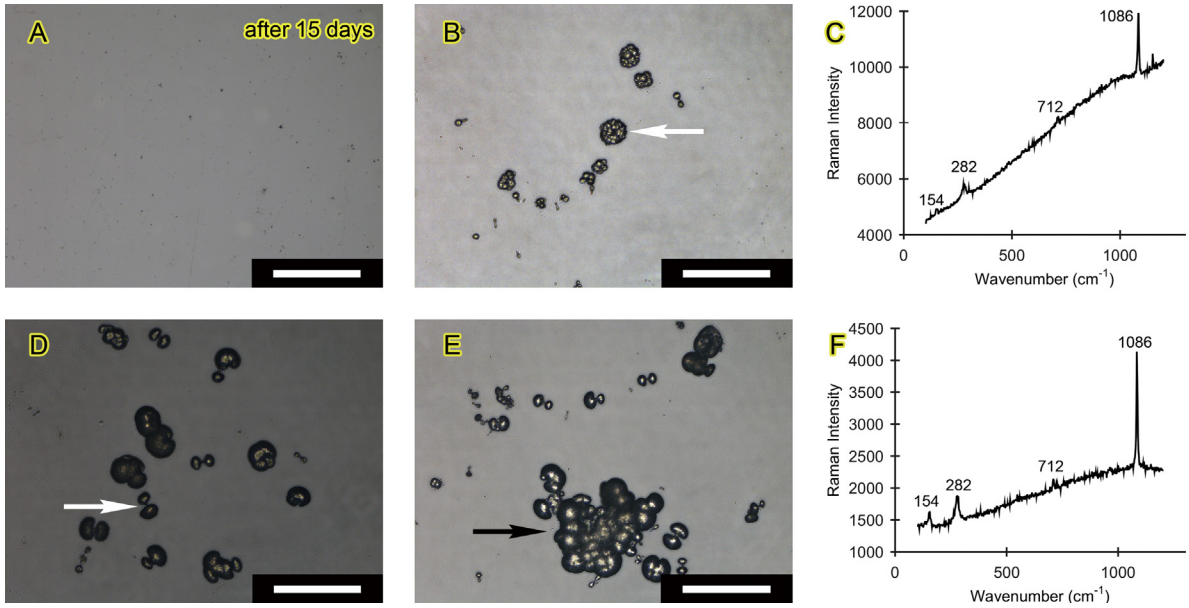

Fig. 4. *In vitro* calcium carbonate crystallization. (A) No crystals appeared in the 12-day-old cultured medium containing 25 μM Ca^{2+}. (B) The effect of uncultured medium on *in vitro* calcium carbonate crystallization; most of the calcium carbonate deposits were flower shaped (arrow). (C) Raman spectrum of the flower-shaped crystals in (A); the characteristic calcite peaks are at 154, 282, 712, and 1086 cm^{-1}. (D) and (E) The effects of 12-day-old cultured medium on *in vitro* calcium carbonate crystallization. The crystals were dumbbell shaped (white arrow) and aggregated (black arrow). (F) The Raman spectrum of the dumbbell-shaped crystals in (D); the crystals were calcite. Scale bar = 100 μm.

mineralization process remains unknown. It has been demonstrated previously that *in vitro* cultured mantle cells retain their ability to secrete mineralization-related enzymes and matrix proteins that can be detected in the cultured medium [17]. However, in our study, no calcium carbonate crystals grew in cultured medium containing 25 μM Ca^{2+} (Fig. 4A), suggesting that the organic cell products, particularly the soluble matrix proteins, did not induce crystal nucleation independently when the Ca^{2+} concentration was relatively lower.

Crystal nucleation can be intracellular during biologically controlled mineralization, such as spicule formation in sea urchins [18]. Mount et al. reported the involvement of oyster hemocytes in intracellular crystal formation [9,19]. In our study, calcium carbonate crystals only appeared when mantle cells were present, strongly suggesting that mantle cells are responsible for the crystal nucleation process. This suggestion can be confirmed by the existence of ACC crystals in mantle cells [17]. ACC is a highly disordered phase and a precursor of calcite and aragonite [20,21]. We deduced that mantle cells may mediate crystal nucleation by secreting ACC into the mineralization front and that ACC was transformed into calcite or aragonite later with the help of matrix proteins.

EDTA (10%) is widely used as mineral-dissolving solution in shell research [22]. The semi-transparent film that remained after the crystals were treated with EDTA was probably an organic matrix. The film outlines resembled the shapes of corresponding crystals. Thus, the film may provide frames for the crystals to grow on. The mantle is responsible for synthesizing matrix proteins that participate in forming the shell framework [11], and the prismatic extracellular matrix originates from the surface of the outer mantle epithelial cells [19]. Thus, we assumed that the mantle cells were likely instructing synthesis of the organic frames by secreting the proper proteins in the correct locations. This suggestion could help explain why more crystals formed in areas closer to the cell aggregates (Fig. 1F).

The crystals were larger and had a different morphology when cultured medium was added to the saturated $NaHCO_3$ solution, in which the calcium carbonate deposits precipitated spontaneously (Fig. 4). This result suggests that the soluble substances secreted by mantle cells increase crystal growth rate and contribute to regulate crystal morphology. This phenomenon was also observed in the mantle cell culture. The crystals near the cell aggregates were always larger and aggregated gradually as culture time passed (Fig. 1F). This observation may have been due to the relatively high matrix protein concentration in these areas.

All of the calcium carbonate crystals growing in the mantle cell culture were calcite, which may have been caused by the region of mantle tissue we chose for the primary cell culture. The mantle is divided into the edge, pallial, and center tissues [23]. The mantle edge secretes matrix proteins related to the prismatic layer, whereas the mantle pallial and center mainly secrete matrix proteins involved in forming the nacreous layer. The prismatic layer consists of calcite, and the nacreous layer is composed of aragonite [2]. We found that tissues from the mantle edge provided more cells; thus, we cultured the mantle tissue from this region to obtain the primary cells. These cells mainly secrete matrix proteins that mediate the formation of calcite, indicating why all of the crystals that grew in culture were calcite.

The crystals growing in the mantle cell culture had some characteristics typical of biomineralization products [24]. They all consisted of superimposed units, which is quite different from calcium carbonate formed under natural conditions. Crystals with such morphology may be the result of cooperation between cells and organic proteins. Zhang et al. proposed that mantle cells could possibly be involved in the complex assembly and modification processes of diverse proteins that lead to formation of the oyster shell [25]. The organic matrix helps control the growth pattern of biological minerals [26]. The crystal's appearance changed with time in our culture. The polyhedron-shaped crystals appeared first and were gradually replaced by round rectangular-shaped crystals. In the end, round crystals were observed after 20 days of culture. The changes in crystal morphology may have been caused by the accumulation of different matrix proteins or by some unknown crystal remodeling mechanism. This finding is worthy of further research.

In the early stage of our culture, most crystals had rough surfaces but some crystals with smooth facets began to appear later. The crystals shown in Fig. 3H had a unique flat and smooth appearance. Mantle cells were observed adhering to the smooth surfaces of these crystals, suggesting that mantle cells participate in the crystal remodeling process. The existence of two other types of crystals confirmed this suggestion. The crystals in Fig. 3I and J had partially smooth surfaces, and they appeared to have been etched. If the etching process continued, they likely became the glass-disc-shaped crystals (Fig. 3H). Therefore, these crystals are considered "semi-finished products". Unfortunately, we did not observe any mantle cells on the surfaces of the "semi-finished" crystals, possibly because the surfaces of these crystals were not smooth enough for the mantle cells to adhere firmly and they were washed off during the procedures after crystallization. Crystals being remodeled by cells has also been observed by Mount et al., who found that hemocytes dissolve the crystals to form plate-like structures.

Mount et al. and Xiang et al. both proposed that mantle cells deliver crystals to the mineralization front and remain attached to the front [9,17]. We conclude that mantle cells are likely to be directly involved in the entire shell formation process, including nucleation, growth, and remodeling. Our results demonstrate the practicability of studying the shell mineralization process at the cellular level using a mantle cell culture and provide evidence that mantle cells participate in the calcium carbonate mineralization process. Applying our results will be crucial for a further understanding of biologically controlled shell formation.

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References

[1] N. Inoue, R. Ishibashi, T. Ishikawa, et al., Can the quality of pearls from the Japanese pearl oyster (*Pinctada fucata*) be explained by the gene expression patterns of the major shell matrix proteins in the pearl sac? Mar. Biotechnol. 13 (2011) 48–55.
[2] S. Weiner, L. Addadi, Design strategies in mineralized biological materials, J. Mater. Chem. 7 (1997) 689–702.
[3] A.M. Belcher, X.H. Wu, R.J. Christensen, et al., Control of crystal phase switching and orientation by soluble mollusc-shell proteins, Nature 381 (1996) 56–58.
[4] H.A. Lowenstam, S. Weiner, On Biomineralization, Oxford Unversity Press, New York, 1989.
[5] L. Addadi, D. Joester, F. Nudelman, et al., Mollusk shell formation: a source of new concepts for understanding biomineralization processes, Chemistry 12 (2006) 980–987.
[6] C. Zhang, R. Zhang, Matrix proteins in the outer shells of molluscs, Mar. Biotechnol. 8 (2006) 572–586.
[7] Q. Feng, Z. Fang, Z. Yan, et al., The structure-function relationship of MSI7, a matrix protein from pearl oyster *Pinctada fucata*, Acta Biochim. Biophys. Sin. 41 (2009) 955–962.
[8] Z. Ma, J. Huang, J. Sun, et al., A novel extrapallial fluid protein controls the morphology of nacre lamellae in the pearl oyster, *Pinctada fucata*, J. Biol. Chem. 282 (2007) 23253–23263.
[9] A.S. Mount, A.P. Wheeler, R.P. Paradkar, et al., Hemocyte-mediated shell mineralization in the eastern oyster, Science 304 (2004) 297–300.
[10] C. Joubert, D. Piquemal, B. Marie, et al., Transcriptome and proteome analysis of *Pinctada margaritifera* calcifying mantle and shell: focus on biomineralization, BMC Genomics 11 (2010) 613.
[11] C. Zhang, L. Xie, J. Huang, et al., A novel matrix protein family participating in the prismatic layer framework formation of pearl oyster, *Pinctada fucata*, Biochem. Biophys. Res. Commun. 344 (2006) 735–740.
[12] I.J. Domart-Coulon, D.C. Elbert, E.P. Scully, et al., Aragonite crystallization in primary cell cultures of multicellular isolates from a hard coral, *Pocillopora damicornis*, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 11885–11890.
[13] J.M. Poncet, A. Serpentini, B. Thiebot, et al., In vitro synthesis of proteoglycans and collagen in primary cultures of mantle cells from the nacreous mollusk, *Haliotis tuberculata*: a new model for study of molluscan extracellular matrix, Mar. Biotechnol. 2 (2000) 387–398.
[14] N. Gong, Q. Li, J. Huang, et al., Culture of outer epithelial cells from mantle tissue to study shell matrix protein secretion for biomineralization, Cell. Tissue Res. 333 (2008) 493–501.
[15] N. Gong, Z. Ma, Q. Li, et al., Characterization of calcium deposition and shell matrix protein secretion in primary mantle tissue culture from the marine pearl oyster *Pinctada fucata*, Mar. Biotechnol. 10 (2008) 457–465.
[16] D. Ren, O. Albert, M. Sun, et al., Primary cell culture of fresh water *Hyriopsis cumingii* mantle/pearl sac tissues and its effect on calcium carbonate mineralization, Cryst. Growth. Des. 14 (2014) 1149–1157.
[17] L. Xiang, W. Kong, J. Su, et al., Amorphous calcium carbonate precipitation by cellular biomineralization in mantle cell cultures of *Pinctada fucata*, PLoS One 9 (2014) e113150.
[18] E. Beniash, L. Addadi, S. Weiner, Cellular control over spicule formation in sea urchin embryos: a structural approach, J. Struct. Biol. 125 (1999) 50–62.
[19] M.B. Johnstone, N.V. Gohad, E.P. Falwell, et al., Cellular orchestrated biomineralization of crystalline composites on implant surfaces by the eastern oyster, *Crassostrea virginica* (Gmelin, 1791), J. Exp. Mar. Biol. Ecol. 463 (2015) 8–16.
[20] L. Addadi, S. Raz, S. Weiner, Taking advantage of disorder: amorphous calcium carbonate and its roles in biomineralization, Adv. Mater. 15 (2003) 959–970.
[21] I.M. Weiss, N. Tuross, L. Addadi, et al., Mollusc larval shell formation: amorphous calcium carbonate is a precursor phase for aragonite, J. Exp. Zool. 293 (2002) 478–491.
[22] A.P. Wheeler, K.W. Rusenko, D.M. Swift, et al., Regulation of in vitro and in vivo $CaCO_3$ crystallization by fractions of oyster shell organic matrix, Mar. Biol. 98 (1988) 71–80.
[23] T. Takeuchi, K. Endo, Biphasic and dually coordinated expression of the genes encoding major shell matrix proteins in the pearl oyster *Pinctada fucata*, Mar. Biotechnol. 8 (2006) 52–61.
[24] J. Stolarski, M. Mazur, Nanostructure of biogenic versus abiogenic calcium carbonate crystals, Acta Palaeontol. Pol. 50 (2005) 847–865.
[25] G. Zhang, X. Fang, X. Guo, et al., The oyster genome reveals stress adaptation and complexity of shell formation, Nature 490 (2012) 49–54.
[26] A.G. Fincham, J. Moradian-Oldak, J.P. Simmer, The structural biology of the developing dental enamel matrix, Struct. Biol. 126 (1999) 270–299.