論文内容の要旨

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論文題目　**Global gene expression analysis on the formation of pearl sac and pearl by allografting in *Pinctada fucata***

（*Pinctada fucata* 同種間移植による真珠袋および真珠形成過程の網羅的遺伝子発現解析）

The bivalve mollusk, Pinctada fucata is renowned worldwide for its ability of producing high quality spherical pearl and accounts for more than 90% of seawater pearl production. Pearls are the result of mollusk's capability to produce calcified shell materials in response to an injury to the mantle tissue. Mollusk shell is mainly composed of calcium carbonate (CaCO3) crystals (>90% w/w) surrounded by an organic matrix (0.01% to 5% w/w) of proteins, lipids and polysaccharides. As in shell biomineralization, pearl formation is also regulated by the extracellular organic matrix secreted by the mantle tissue of mollusk.

Mantle grafting is the commonly practiced method for producing spherical pearls. The process involves a surgical implantation where a small piece (3 × 3 mm) of mantle tissue is excised from a suitable donor oyster and then implanted into the gonad of a host oyster along with an inorganic nucleus. Once transplanted, the outer epithelial cells of the graft start to proliferate and differentiate to give rise a monolayer of the secretory epithelium around the nucleus termed as 'pearl sac'. The newly formed pearl sac gradually secretes various matrix proteins onto the nucleus in order to produce a lustrous pearl. Therefore, it is very reasonable to claim that pearl sac formation is the critical step of pearl culture which eventually determines the success of culture.

Under normal condition, outer epithelium of the mantle is a stable and mitotically inactive tissue, whereas the inner epithelial cells, contrarily, proliferate intermittently for the renewal of the tissue. Upon a mantle injury, the outer epithelial cells multiply actively to regenerate the injured site. The pearl sac formation simply resembles the wound healing process that occurs after a mantle injury. During mantle grafting, the external epithelial cells of the graft become active soon after surgical implantation and start to proliferate into a pearl sac. Other components of the graft, such as inner epithelial cells, muscle fibres and connective tissues eventually disappear. It is assumed that the outer epithelium contains proliferating stem cells, but the feature of those cells is unclear. So, identification of genes involved in epithelial cell proliferation and differentiation is of utmost important to understand the mechanisms of pearl formation.

Shell or pearl biomineralization is a highly controlled biological process regulated by the cascades of a substantial number of genes. Though the mechanism of pearl formation has been studied largely, but the complex physiological process by which pearl sac and pearl is formed has not been properly understood yet. Using an RNAseq approach, here, we aimed to reveal the genes involved in the development of pearl sac and pearl, and the sequential expression patterns of different shell matrix proteins (SMPs) secreted from the pearl

sac during different stages of pearl formation. We also examined the pearl layers to scrutinize the microstructural characterization of the surface depositions on pearls. In the last part of the study, we tried to establish a suitable method of gene editing in P. fucata using CRISPR/Cas9 system.

1. Genes expressed during the proliferation of mantle epithelial cells into pearl sac

To describe the genes engaged in pearl sac formation, we performed RNA sequencing of mantle graft and the later pearl sac at different stages of pearl formation. During grafting experiments for three months, we collected nine samples: donor mantle epithelial cells, donor mantle pallium, donor mantle pallium on grafting, and mantle pallium each from the host at 24 hours, 48 hours, 1 week, 2 weeks, 1 months and 3 months post grafting. In the wound healing process, pearl sac was developed by two weeks of culture as indicated by the up-regulated Gene Ontology (GO) terms relevant to epithelial cell proliferation and differentiation. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that immune genes were highly expressed ($P < 0.05$) between 0 h – 24 h in a donor dependent-manner and 48 h – 1 w in a host dependent-manner.

We screened out a number of genes including JAG1, RFX3, STRC, FGFR2, SAV1, RAC1, DMD, RGMA, PTK7, MAF, MEF2A, SFRP5, TGM1, FZD1, GRHL2, TEAD1, PRKDC, LAMC1, EGFR, CASP8, CDC42, RSPO2, MTSS1, MATN1, SULF1, SPG20 and LRP6 that may be involved in the proliferation and differentiation of mantle epithelial cells into pearl sac. Furthermore, it is the first time that we identified some stem cell marker genes including ABCG2, SOX2, MEF2A, HES1, MET, NRP1, ESR1, STAT6, PAX2, FZD1 and PROM1 that were expressed differentially during pearl sac development. RT-PCR data showed ubiquitous expression of these stem cell marker genes in P. fucata, which further proposed their cell proliferation-related functions in different tissues. Additionally, qPCR results demonstrated that all these genes were highly expressed in mantle tissue, suggesting their potential role in the proliferation of the mantle epithelium into pearl sac. Furthermore, PAX2 and FZD1 were expressed higher in mantle compared to other tissues such as gonad and muscle.

2. Gene expression profiles at different stages of pearl formation during 3 months pearl culture

More than 200 molluscan biomineralization-related genes that contribute to the formation of shell and pearl have been identified till date. In this study, we screened out 192 genes likely involved in pearl biomineralization by blast search against a list of reference biomineralization genes prepared beforehand. It has been clearly defined that, the biomineralization genes are being secreted by the pearl sac developed from donor mantle graft, not by the host gonad tissue. So, the identified biomineralization-related genes in our study were expressed in the pearl sac, i.e., in the donor mantle epithelium.

Though the mantle tissue is primarily responsible for shell/pearl biomineralization, recent studies have also been reported that oyster hemocytes can mediate shell biomineralization by secreting and transporting $CaCO_3$ crystals to the site of mineralization. Therefore, the interaction observed between donor mantle graft and surrounding host hemocytes immediately after grafting is very essential for the proper development of pearl sac and pearl.

Principal component analysis (PCA) precisely elucidates that the mineralization process during the first 3 months of culture is regulated differently. Further hierarchical clustering of 192 biomineralization-related genes showed clearly different expression profiles between the earlier (before 1 week) and later stages (1 week to 3 months) of pearl grafting. Detailed expression analysis of the major SMPs demonstrated that most of the prismatic layer forming SMPs were first up-regulated and then gradually down-regulated, indicating their involvement in the development of pearl sac and the onset of pearl mineralization. Most of the nacreous layer forming SMPs were up-regulated after the formation of pearl sac with the highest expression at 1 month, suggesting the completion of the nacreous layer formation. Nacrein, MSI7 and shematrin involved in both layer formation were highly expressed during 0 h – 24 h, down-regulated up to 1 week and then up-regulated again after maturation of pearl sac. Actually, these SMPs control and mediate the CaCO3 crystal formation. However, the genes highly expressed in the pearl sac may not be highly expressed in the mantle pallium. Therefore, the expression profiling of the SMPs can be used as a marker of the shell and pearl formation.

3. Microstructural characterization of pearl layers recapitulates the mineralization sequence of pearl

Clear morphological differences were observed among the pearls obtained at 1 month and 3 months of culture. Surface examination of 1 month pearls revealed the variation in the initial mineralization among the pearls. Moreover, the nacre deposition at the early stage of pearl formation was not uniform throughout the surroundings of a given pearl. But at 3 months, the pearl surface became smoother and more regular with a pearl luster.

Scanning electron microscopy (SEM) demonstrated that an initial organic layer was deposited onto the nucleus surface before the secretion of prismatic and nacreous layers. But, the thickness of the organic material layer was variable among different pearls and even in different parts of the same pearl. Thus the initial mineralization of pearl is not simply the reappearance of the nacreous structures, rather it is more complex. The metabolic changes that occur in the mantle epithelial cells during its differentiation into a pearl sac may result in the formation of a new mineralizing sequence that is comparable to the structure of the shell. However, the prismatic layer of pearl is more diversified compared to the regular brick-wall like structures of nacre that develops later on it. Unlike the canonical mollusk shell, prismatic layer in pearl was composed of both aragonite and calcite prisms, organic materials and some unknown compounds.

The study recapitulates the mineralization sequence of pearl, where a heterogeneous prismatic layer is secreted first and followed by nacreous layer. Additionally, SEM imaging confirmed the deposition of nacreous layer around the nucleus by 1 month that we predicted from our gene expression study.

4. CRISPR/Cas9 mediated gene editing in *P. fucata*

We tried to establish a suitable method of gene editing in pearl oyster using high-throughput CRISPR/Cas9 system. Here, we showed that direct injection of Cas9 protein and appropriate sgRNA into the adductor muscle of adult oyster can induce noticeable mutation in desired gene. DNA sequencing results from two representative mutants indicated a large deletion (45 bp) on the targeted gene, nacrein. We got 3 mutant

oysters among 4 injected with sgRNA-Cas9 complex. The notable success rate suggests that, this tool can function in pearl oyster in vivo through a simple but efficient approach of direct injection.

This is the first and a preliminary trial of CRISPR-mediated gene alteration in bivalve mollusk. Therefore, further study is needed to make the method more appropriate.

Conclusion

The findings of the present study conclude two consecutive stages during the 3 months pearl culture. One is the initiation of pearl sac formation as part of the wound healing process in response to the oyster defense mechanism (before 1 week post grafting). Another is the maturation of pearl sac and deposition of organic matrices on the bare nucleus (2 week to 3 months). We figured out the key genes including some stem cell marker genes engaged in proliferation and differentiation of mantle epithelial cells into pearl sac. We also described the notable immune genes and pathways that provide insight into the increased understanding of the host immune reaction in response to accepting a graft.

The expression pattern of the key genes involved in the development of pearl sac and pearl elucidated that immune and cell proliferation related-genes were mostly enriched during earlier stages (before 2 weeks), whereas biomineralization genes were expressed in later stages (2 weeks to 3 months) of pearl grafting. The expression profiling of 192 biomineralization genes indicates that first 3 months of pearl biogenesis are very crucial when the pearl sac forms and secretes significant amount of nacre for making a lustrous pearl. Microstructural characterization of pearls explains the order of mineralization where a periostracum-like layer is secreted first before the deposition of the heterogeneous prismatic layer and the outermost nacreous layer onto the nucleus. CRISPR/Cas9 mediated gene editing suggests that it can be a simple but efficient tool for gene editing in pearl oyster towards improving the quality of cultured pearl.

The improved understanding of the molecular mechanisms underlying the formation of pearl sac and pearl obtained from this study will provide a basis for future research towards upgrading the pearl culture practice and pearl quality. The study also gives some valuable information for identifying the functional genes implicated for pearl sac formation. However, further functional analyses are needed to verify the functions of the identified stem cell marker genes in pearl sac development.