Lineage tracing of the bivalve shell field with special interest in the descendants of the 2d blastomere

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Lineage tracing of the bivalve shell field with special interest in the descendants of the 2d blastomere

Masakuni Mohri, Naoki Hashimoto and Hiroshi Wada*

Graduate School of Life and Environmental Sciences, University of Tsukuba,

Tsukuba 305-8572, Japan

*Author for Correspondence:

Tel&Fax:+81-29-853-4671

e-mail:hwada@biol.tsukuba.ac.jp
Abstract

By evolving bilaterally separated shell plates, bivalves acquired a unique body plan in which their soft tissues are completely protected by hard shell plates. In this unique body plan, mobility between the separated shell plates is provided by novel structures such as a ligament and adductor muscles. As a first step toward understanding how the bivalve body plan was established, we investigated the development of the separated shell plates and ligament. Over 100 years ago it was hypothesized that the development of separated shell plates is tightly linked with the unique cell cleavage (division) pattern of bivalves during development, wherein each bilateral daughter cell of the 2d descendant, 2d$^{1121}$, develops into one of the bilateral shell fields. In the present study we tested this hypothesis by tracing the cell lineages of the Japanese purple mussel Septifer virgatus. Although the shell fields were found to be exclusively derived from the bilateral descendant cells of 2d: 2d$^{11211}$ and 2d$^{11212}$, the descendants of these cells were not restricted to shell fields alone, nor were they confined to the left or right side of the shell field based on their lineage. Our study demonstrated that ligament cells are also derived from 2d$^{11211}$ and 2d$^{11212}$ indicating that the ligament cells
emerged as a subpopulation of shell field cells. This also suggests that the establishment of the novel developmental system for the ligament cells was critical for the evolution of the unique bodyplan of bivalves.
1. INTRODUCTION

Molluscs share several characteristic features, such as calcareous shells (or spicules) and a muscular foot. However, their body plans are highly variable, as demonstrated by the differences between the worm-like, shell-less Aplacophora and the highly motile Cephalopoda. With the development of bilaterally separated shell plates, bivalves evolved a unique body plan in which their soft tissue is completely protected by hard shell plates. The muscle system was rearranged to accommodate the evolution of this shell plate morphology, resulting in another evolutionary novelty, the adductor muscle, which controls the opening and closing of the shell plates. Determining how this unique bivalve body plan was achieved through the coordinated evolution of shell plate morphology and muscles is challenging. To address this question we first investigated how the bilaterally separated shell plates developed through the modification of shell development.

Over 100 years ago Lillie and Meisenheimer [1, 2] reported a pattern of spiral cleavage in bivalves. Most molluscan species exhibit spiral cleavage wherein the animal blastomeres are smaller than the vegetal blastomeres.
However, the dorsal vegetal blastomeres (1D) produce a larger animal blastomere (2d) in bivalves from the eight-cell stage to the 16-cell stage[3, 4]. This animal blastomere is thought to be the precursor of the shell field cells, which underlie shell plates[1, 2]. After four rounds of asymmetric cleavage, the largest blastomere (2d_{1121}) exhibits bilateral cleavage (Figure 1a-e), and the bilateral daughter cells were suggested to differentiate into the left and right shell field cells of their respective side[1]. This hypothesis suggests that development of the novel shell plate morphology was driven by a modification of the early cleavage pattern. However, it was based solely on microscopic observations and experimental validation is required through direct cell lineage tracing.

It is also notable that bivalves show a stereotypic pattern of spiral cleavage prior to the occurrence of bilateral cleavage. The largest blastomere, 2d, undergoes four rounds of asymmetric spiral cleavage prior to the bilateral cell division (Figure 1a-e). The first two rounds of asymmetric cleavage give rise to two smaller vegetal blastomeres, 2d^2 and 2d_{12}, and a larger animal blastomere, 2d_{11} (Figure 1a-b). When 2d_{11} divides the polarity is reversed, and a smaller animal blastomere (2d_{111}) and a larger vegetal blastomere (2d_{112}) are generated.
(Figure 1c). The cell size polarity is again reversed during the next cleavage of 2d\textsuperscript{112}, yielding a smaller vegetal blastomere (2d\textsuperscript{1122}) and a larger animal blastomere (2d\textsuperscript{1121}; Figure 1e). Blastomere 2d\textsuperscript{1121} then divides symmetrically to produce a left (2d\textsuperscript{11211}) and right daughter (2d\textsuperscript{11212})(Figure 1e).

In the present study we investigated how this series of cleavages is linked with the development of the unique morphology of bivalves. Focusing on the shell field precursors in bivalve embryos we traced the cell lineages of the early blastomeres with a fluorescent photoconversion technique using Kaede fluorescent protein[5].

2. MATERIALS AND METHODS

Adult specimens of the Japanese purple mussel Septifer virgatus (Wiegmann, 1837) were collected in Tsuyazaki, Fukuoka Prefecture, Japan. Induction of spawning and in vitro fertilization were performed as described in [4]. The handedness of spiral cleavage was unexpectedly reversed in the eggs from Tsuyazaki individuals compared with those from Kashima described in [4](dextral in Kashima [4] and sinistral in Tsuyazaki: this study), and we
confirmed that the direction of the spiral cleavage was reversed for all cleavages up to the bilateral cleavage of 2d for all specimens examined (Figure 1a–e, Table 1). This polymorphism in the handedness of spiral cleavage has been reported in another bivalve species *Dreissena polymorpha* [6].

mRNA for Kaede was transcribed from a pBluescript RN3 vector [7], and Kaede mRNA (3 μg/μl) was injected into fertilized eggs.

Kaede fluorescence can be irreversibly converted from green to red by irradiation with ultraviolet light. Photoconversion was performed using a confocal laser scanning microscope (CLSM. Zeiss LSM710, Germany) at a 405 nm wavelength. The laser was applied until we confirmed that sufficient photoconversion was induced. Among photoconverted embryos, about 20% showed abnormal morphology at the trochophore stage and were excluded from our analysis. Swimming larvae were immobilized prior to observation by fixing with 4% paraformaldehyde and observed by CLSM. The fluorescent signal could be observed up to 10 h after fixation. It should be noted that some converted cells appeared yellowish because unconverted green Kaede protein was translated.
from the injected mRNA even after photoconversion. Unmerged fluorescent
signals are shown in Figure S1.

3. RESULTS

To confirm that 2d blastomeres contribute shell field precursors, 2d blastomeres
were photoconverted at the nine-cell stage. Following photoconversion of a 2d
blastomere, the converted signal was widely detected in the dorsal region of the
post-trochal epidermis (Figure 2a-c). Importantly, all of the shell field cells were
labeled (Figure 2b, Table 1), indicating that the shell field cells were solely
derived from 2d descendants.

Prior to the occurrence of bilateral cleavage, 2d blastomeres undergo four
rounds of asymmetric cleavage to produce four micromeres (Figure 1a-d). These
micromeres were photoconverted after the bilateral cleavage of 2d\textsuperscript{1121} because
each blastomere is most easily identified at this stage of development.

At this stage, derivatives of 2d\textsuperscript{2} have already undergone two rounds of
cell division. We photoconverted all of the derivatives of 2d\textsuperscript{2} (Figure 2j). The
converted signal was detected in the left side of both the anterior and posterior of
the post-trochal epidermis in these larvae. Importantly, however, the signal was not detected in the shell field (Figure 2k, Table 1).

When 2d\textsuperscript{112} was photoconverted, the signal was observed on the right side of the anterior of the post-trochal epidermis, but no signal was detected in the shell field (Figure 2l-m, Table 1).

When the 2d\textsuperscript{111} micromere was photoconverted, the signal was detected in the anterior dorsal midline of the post-trochal epidermis (Figure 2o-p, Table 1).

When 2d\textsuperscript{1122} was labeled, the signal was detected in the posterior epidermis (Figure 2q-r, Table 1). No signal was detected in the shell field in either case (2d\textsuperscript{111} or 2d\textsuperscript{1122}).

We then photoconverted each bilateral daughter of 2d\textsuperscript{1121} to determine whether the bilateral shell fields differentiate according to the bilateral cleavage of 2d\textsuperscript{1121}. When 2d\textsuperscript{1121} (the left side daughter of 2d\textsuperscript{1121}) was photoconverted, the signal was detected not only in the shell field, but also in the surrounding epidermis (Figure 2d-f). Thus, even at this stage the developmental outcome is not restricted to the shell field cells. Importantly, the signal was detected not only in the left side of the shell field, but also in the right side (Figure 2e, Table 1).
Interestingly, the signal was biased toward the left posterior in all larvae.

Similarly, when 2d\textsuperscript{11211} (the right side daughter of 2d\textsuperscript{1121}) was photoconverted, the signal was observed in both the shell field and the surrounding epidermis (Figure 2g-i, Table 1). The signal in the shell field was biased toward the right anterior of the shell fields in all larvae.

Bivalve shell fields are bilaterally separated by ligament cells that develop along the dorsal midline (Fig. 1f, g, [3]). Differentiation of the ligament cells is clearly visible by specific upregulation of the \textit{chitin synthase (cs)} gene during the trochophore stage\cite{8}. Photoconversion indicated that all of the shell field cells are derived either from 2d\textsuperscript{11211} or 2d\textsuperscript{11212}. Based on \textit{dpp} expression noted in oyster embryos, Kin et al.\cite{3} suggested previously that ligament cells are derived from the descendants of 1d\textsuperscript{12} and 2d\textsuperscript{2}. Thus, we examined any possible contribution from the 1d cell lineage, and found that 1d develops into the anterior epidermis, including the prototroch (Figure S2, Table 1), but not into shell field. Thus we concluded that the ligament cells are only derived from 2d\textsuperscript{11211} and 2d\textsuperscript{11212}.
4. DISCUSSION

In the present study we found that all shell field precursors are derived from 2d\textsuperscript{1121}, although the developmental fate of 2d\textsuperscript{1121} is not restricted to the shell field cells alone. Importantly, the bilateral shell fields were not derived exclusively from the daughter cells of 2d\textsuperscript{1121} of each respective side. Instead, the derivatives of the daughter blastomeres contributed to both sides of the shell field by spreading across the midline (Figure 1f, g, 2e,h). Thus, our results did not support the classical hypothesis that the bilaterally separated shell plates of bivalves are derived from bilateral descendants of 2d[1]. It is notable that descendants of the 2d blastomere also show bilateral cell division in gastropods, as well as in annelids (e.g., [9-13]), and together with 4d, 2d was shown to demonstrate organizing activity in annelids[14]. So it is likely that the bilateral cell division of 2d descendants was established much earlier than the emergence of bivalves, possibly for the establishment of the bilateral body plan from the spiral cleavage[15]. The bilateral shell plates, however, might have evolved irrespective of bilateral cleavage.
The innovation of ligament cells in the dorsal midline of the shell field is critical for the unique body plan of bivalves[16]. Our lineage tracing indicates that ligament cells differentiate from the 2d lineage of cells just like other shell field cells (Figure 1f, g, 2d-i), indicating that the ligament cells emerged as a subpopulation of shell field cells. The ligament cells are specifically marked by the upregulation of cs [8], and the expression of dpp earlier than cs [3]. Prior to shell field invagination, dpp is also expressed in cells abutting the shell field midline, both anteriorly and posteriorly [3]. Although we demonstrated that these dpp positive cells (1d12 and 2d9) do not differentiate into either shell field cells or ligament cells, it is still possible that dpp plays an inductive role in ligament differentiation. Functional studies of bivalve dpp may advance our understanding of the evolution of the unique bivalve body plan.

Innovation of the ligament provided mobility between the separated shell plates of bivalves, and thus it may have accompanied the evolution of adductor muscles to open and close the shells. Elucidation of the developmental mechanism of ligament cells may provide a clue to understanding how the
innovation of the ligament and that of adductor muscles are linked during evolution.

Ethics: Research was carried out according to the university’s guideline.

Data accessibility: The datasets supporting this article have been uploaded as part of the supplementary material.

Author’s contribution: All authors contributed to the design of the study, collection of data and writing of the article. All authors approve the final version of this manuscript and agree to be held accountable for all aspects of the work performed.

Competing interests: We declare we have no competing interests.

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References


Figure legends

Figure 1. Schematic illustration of the cleavage pattern and cell lineage mapping of 2d descendants.

(a-e) Schematic illustration of the cleavage pattern of 2d descendants. (f) Summary of the cell lineage mapping of the 2d descendants in trochophore larva. Dorsal views, anterior to the top. (g) Tree diagram of the cell lineage and developmental fate of 2d descendants.

Figure 2. Cell lineage tracing of 2d and descendant blastomeres.

(a-c) Cell lineage of 2d. Kaede was converted at 9 cell stage (a: view from right side), and the fate of 2d was observed at trochophore stage (b-c). (d-r) Cell lineage of 2d$^{11211}$ (d-f), 2d$^{11212}$ (g-i), 2d$^{2}$ (j-k), 2d$^{12}$ (l-m), 2d$^{111}$ (o-p) and 2d$^{1122}$ (q-r). Noted that some converted cells appeared yellowish because unconverted green Kaede protein was translated from the injected mRNA even after photoconversion.

Shell field boundary is indicated by broken line. Anterior to the top except for (o) in which ventral to the top. Scale bars: 50µm.
(a) (b) (c) (d) (e) (f) (g)

- **prototroch**
- **shell field**
- **ligament**

Diagram illustrating the development of the prototroch and shell field. The labels indicate different regions and stages of development, including anterior dorsal post-trochal epidermis, right-posterior shell field and epidermis, left-anterior shell field and epidermis, right-posterior post-trochal epidermis, left-anterior dorsal post-trochal epidermis, and right-anterior and right-posterior dorsal post-trochal epidermis.
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