Shell field morphogenesis in the polyplacophoran mollusk Acanthochitona rubrolineata

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Abstract

Background

The polyplacophoran mollusks (chitons) possess serially arranged shell plates. This feature is unique among mollusks and believed to be essential to explore the evolution of mollusks as well as their shells. Previous studies revealed several cell populations in the dorsal epithelium (shell field) of polyplacophoran larvae and their roles in the formation of shell plates. Nevertheless, they provide limited molecular information, and shell field morphogenesis remains largely uninvestigated.

Results

In the present study, we investigated the shell field development in the chiton *Acanthochitona rubrolineata* based on morphological characteristics and molecular patterns. A total of four types of tissue could be recognized from the shell field of *A. rubrolineata*. The shell field comprised not only the centrally located, alternatively arranged plate fields and ridges, but also the tissues surrounding them, which were the precursors of the girdle and we termed as the shell field margin. The shell field margin exhibited a concentric organization composed of two imaginary circles, and spicules were only developed in the outer circle. Dynamic *en grailed* expression and F-actin (filamentous actin) distributions revealed relatively complicated morphogenesis of the shell field. The repeated units (plate fields and ridges) were gradually established in the shell field, seemingly different from the manners used in the segmentation of *Drosophila* or vertebrates. The seven repeated ridges also experienced different modes of ontogenesis from each other. In the shell field margin, the presumptive spicule-formation cells exhibited different patterns of F-actin aggregations with the ongoing of their specification.

Conclusions

These results reveal the details concerning the structure of polyplacophoran shell field as well as its morphogenesis. They would contribute to exploring the mechanisms of polyplacophoran shell development and molluscan shell evolution.

Background

Polyplacophora, whose extant members are called chitons, represents a unique lineage of mollusks. In particular, polyplacophorans possess eight shell plates aligned in a serial pattern along the anteroposterior axis [1] (Fig. 1a). This characteristic is emphasized due to its uniqueness in shell plate number compared to conchiferan mollusks (mostly having one or two shells, e.g., bivalves and gastropods) and its apparent (albeit limited) similarities with segmentation in animals such as arthropods, annelids and vertebrates [2–5]. In addition, polyplacophorans also develop spicules (or sclerites/scales) in the tissue surrounding shell plates (girdle). As another type of mineralized structures,
spicules are proposed to be evolutionarily and structurally related to shell plates and are informative to infer molluscan evolution [6–8].

In development, seven serially arranged units of tissues are formed on the dorsal epithelium of polyplacophoran larva, which then produce seven shell plates after metamorphosis [9–11]; the eighth shell plate forms later. Each of the seven units that will secrete a shell plate is termed as a plate field [2, 6, 11]. We follow this terminology and further suggest that as in conchiferans, the total larval tissue of polyplacophorans that are related to shell formation should be termed as the shell field. This is not an evolutionary definition but is a developmental/functional one. We think the common terminology of the larval shell-formation tissues in conchiferans and polyplacophorans would ensure better comparisons among these shelled mollusks. As shown below, we indeed found taking all shell-formation tissue as a whole was important in certain contexts, e.g., those concerning shell formation mechanisms or evolutionary issues.

The polyplacophoran shell fields contain distinct cell populations, with different roles in the formation of shell plates and the connective tissues [6, 11]. On the molecular level, it has been revealed a number of genes showing striped expression in the shell field, including the well-accepted molluscan shell-formation gene **engrailed** [12], the key developmental genes **hox** [13–15] and others [16, 17]. Nevertheless, it is largely unknown that which gene is expressed in particular cell types. It seems the only exception is **engrailed**, which is expressed in cells not involved in shell plate secretion [12]. While the fates of cell populations inside the shell field are generally determined [6, 11], additional molecular information is required to elucidate the roles of related cells.

Given the distinct cell populations in different compositions of the shell field [6, 11], it is necessary to further ask how these cells are specified and how they are organized into the characteristic pattern. Nevertheless, current knowledge regarding shell field morphogenesis is limited except for a few morphological observations [9–11]. As mentioned above, calcified shell plates are only developed after metamorphosis and there is merely a shell field in larval stages [4, 11] (Fig. 1b). Meanwhile, a recognizable shell field is yet not formed in the newly hatched larva of some species [10]. These facts indicate that shell field morphogenesis occurs during the period between hatching and metamorphosis (Fig. 1b). Indeed, the appearances of the shell field continuously change with larval development in **Chiton olivaceus** [4], and gene expression in the shell field exhibits dynamic patterns during polyplacophoran larval development [13, 14, 16].

The morphogenesis of shell field establishes the framework for subsequent shell plate secretion, and thus investigations of this process would be essential to understand polyplacophoran shell formation. Moreover, given that development often provides important evolutionary implications, investigating the early development of polyplacophoran shell field may also provide clues to explore evolutionary issues such as the origin of shell plates, the evolutionary relationships among the sclerotized structures of mollusks/spiralians, and even the evolution of animal segmentation [5, 7, 8, 18]. To explore more information about polyplacophoran shell development, in the present study, we investigated the shell field
morphogenesis in the chiton *Acanthochitona rubrolineata*. The results revealed details regarding the molecular patterns of different tissues inside the shell field, as well as those concerning their morphogenesis. These results add to the knowledge of polyplacophoran shell development and provide insights into molluscan shell evolution.

**Results**

**General development of A. rubrolineata**

The oocyte of *A. rubrolineata* was enclosed by an egg hull with extensive protrusions (Fig. 2a). After fertilization, zygotes experienced two rounds of equal cleavage followed by characterized spiral cleavage (Fig. 2b-e). Embryonic development occurred in the egg hull and only larval cilia (prototroch) could be discriminated during this period (Fig. 2b-e). Beginning at 8.5 hours post fertilization (hpf), the early trophophore larva hatched by breaking through the egg hull (Fig. 2g-h). The times of hatching varied for different individuals (could be later than 10 hpf), but we did not observe evident changes in developmental rates between these individuals. Some individuals could not hatch due to abnormal development, and they could remain alive in the egg hull for several days.

At around 24 hpf, two larval eyes emerged in ventral lateral tissues of the posttrochal region (Fig. 2i), contrasting to the pretrochal eyes of conchiferan mollusks. Characteristic larval structures including a ventral foot anlagen and a dorsal shell field also developed (Fig. 2j). The subsequent larval development showed minor morphological changes under ordinary microscopy, except that the larval body gradually got elongated and somewhat flattened. Beginning at as early as 60 hpf, the larvae could metamorphose when proper substrates were supplied (Fig. 2k). However, if no inductive clues were available, they could remain as swimming larvae till at least 7 days post fertilization without losing the capacity to metamorphose. During metamorphosis, the larva greatly flattened its body and lost the prototroch. Spicules and seven shell plates were developed in marginal and central regions of the dorsal epithelium, respectively; the larval eyes were retained (Fig. 2l). Juveniles were morphologically similar with an adult chiton, despite the lack of the eighth shell plate (Fig. 2l).

Based on the aforementioned results, we concluded that very early larvae did not possess a recognizable shell field in *A. rubrolineata*, and the shell field was fully developed at 48 hpf (Fig. 2j). We thus used samples between 12 and 48 hpf in subsequent analyses.

**Shell field morphogenesis: morphological changes**

We first explored morphological changes using scanning electron microscopy (SEM). The early larvae at 12–16 hpf showed no recognizable dorsal structures (Fig. 3a-c, f-h). At 18 hpf, the cilia in the pretrochal region expanded compared to earlier larvae, leaving a non-ciliated area recognizable (Fig. 3d, d’). This region was subsequently revealed to be a part of the shell field. Beginning at 22 hpf, tissues inside in this region started to exhibit varied morphological characteristics (Fig. 3k-I). The inner tissues adjacent to the
prototroch showed small pores on their surface (also observed in most posttrochal tissues; Fig. 3k'). At 36 and 48 hpf, these inner tissues developed irregularly distributed, shallow depressions on their surface (Fig. 3m’-n’). At the same time, the outer tissues in the pretrochal region, although showing no morphological characteristics in earlier stages, developed tiny protrusions (Fig. 3m’-n’). This morphological difference indicates the formation of two cell populations inside the pretrochal region.

The lateral cilia in the posttrochal region could be discerned at 14 hpf (Fig. 3g). Very close to these cilia were shell field tissues, which, however, showed no morphological characteristics in early larvae (Fig. 3h’-j’, o’-p’). At 36 and 48 hpf, this part of tissues developed tiny superficial protrusions similar to those of the outer tissues in the pretrochal region (Fig. 3q’-r’, compare to Fig. 3m’-n’). Moreover, these larval tissues with tiny protrusions overall formed an imaginary circle enclosing the central region (Fig. 3m’-n’, q’-r’). Inside to these tissues, we revealed an additional imaginary circle containing similar cell types (see below). These two imaginary circles together exhibited a concentric organization (Fig. 3m’-n’, q’-r’) and were the precursor tissues of girdle. We term these larval tissues as the shell field margin, with the outer circle as the peripheral margin and the inner circle as the proximate margin (shaded regions in Fig. 3m’-n’, q’-r’). The peripheral margin generally overlapped with the spicule-formation region during metamorphosis (compare Fig. 3m with Fig. 2k), suggesting that it contained spicule-formation cells.

Specification in the central region of the shell field became evident at around 22 hpf, when alternative bulges and grooves were discernable (Fig. 3o’). Soon at 24 hpf, most bulges and grooves were well developed (lacking the last ones; Fig. 3l, and compare to Fig. 4k). The bulges and grooves did not emerge at the same time and their numbers could vary among samples collected at the same time point, indicating rapid formation. These bulges and grooves showed minor changes in later larvae (36–48 hpf; Fig. 3m-n). According to previous reports, only the grooves will produce shell plates, and they are designated plate fields [6, 11], while the bulges are called (intersegmental) ridges [11].

**F-actin dynamics during shell field morphogenesis**

As SEM revealed continuous morphogenetic changes during the development of the shell field, we investigated the dynamics of filamentous actin (F-actin) to explore more details. In particular, CLSM 3D projections were carefully prepared to avoid influences from strong staining in larval muscles (supplemental figure S1; see Methods). F-actin was generally evenly distributed in the dorsal epithelium of early larvae (Fig. 4a-c), despite random concentrations without a clear pattern. Beginning at 16 hpf, the pretrochal tissues adjacent to the prototroch became slightly depressed, and this depression sustained till at least 22 hpf (yellow dashed curves in Fig. 4g-j). This depression indicated the development of the shell field margin. Nevertheless, it was till 20 hpf when F-actin was aggregated in this region (Fig. 4d, i). The F-actin aggregations became further evident at 22 hpf, and simultaneously, comparable aggregations became detectable in posttrochal cells (Fig. 4e, j). They continued to enhance at 24 hpf and outlined the specifying peripheral margin (Fig. 4k, n).
At the same time, weak but steady F-actin stripes were detected in the developing plate fields (except the last one that was not developed yet; Fig. 4k), separating by ridges devoid of such F-actin patterns. In the proximate margin, no characteristic F-actin distribution patterns were detected, but this region could be roughly determined under CLSM as a gap between the peripheral margin and the developing ridges/plate fields (Fig. 4n). Together, all four subregions of the shell field were recognizable at 24 hpf, indicating that the larval shell field was generally established at this stage (Fig. 4k, n).

F-actin aggregations in the peripheral margin showed “spotted” patterns initially (from the apical view; Fig. 4q), which then transited into tiny “circles” (36 and 48 hpf; Fig. 4r-s). CLSM 3D projections revealed that they were actually “tubes” that inserted deeply inside the larval body (Fig. 5a). From semithin sections, similar tube-like structures could be recognized in this region (Fig. 5b-c), and we interpret these structures correspond to the F-actin tubes under CLSM. Interestingly, it was revealed that each tube was derived from a single cell with a deep nucleus (Fig. 5b-c). This type of cells were alternatively arranged with other cell types with more superficial nuclei (Fig. 5b-c), consistent with the alternative arrangement of cell populations with and without F-actin aggregations under CLSM (supplemental figure S2a-d). The cells showing F-actin aggregations may contribute to spicule formation (see below).

In the central region, F-actin stripes became further stronger with ontogenesis (Fig. 4l-m), indicating continuous development of plate fields. From longitude semithin sections, the plate field cells exhibited deeply located nuclei, contrasting with the more superficial nuclei of ridge cells (Fig. 5d). CLSM optical sections revealed similar results, and further demonstrated that although having deep nuclei, the apical sides of plate field cells were actually exposed to the surface (supplemental figure S2e-i).

**engrailed expression**

We finally investigated the expression of the well-known shell-formation gene *engrailed* [12]. In *A. rubrolineata*, *engrailed* expression was detected in both dorsal and ventral tissues. We previously revealed that the dorsal proportion of *engrailed* expression was related to shell development [15].

Dorsal expression of *engrailed* could be detected at 12 and 14 hpf, which was scattered distributed in both pretrochal and posttrochal regions and it was difficult to conclude a clear pattern (Fig. 6a). A notable fact is that evident *engrailed* expression was detected in several cells very adjacent to the prototroch (Fig. 6a-b). Major changes were observed at 16 hpf, when *engrailed* expression was evidently enhanced and covered a relatively large area of dorsal epithelium (Fig. 6d). More importantly, beginning at this stage, the dorsal *engrailed* expression showed a trend of striped expression (Fig. 6d). At 18 and 20 hpf, the striped expression of *engrailed* became much more clear and seven stripes were ultimately established in the posttrochal region later (Fig. 6e-f). It could be determined that the expression was in intersegmental ridges, and this expression pattern was sustained in subsequent development (Fig. 6k, m).

Interestingly, these seven stripes of *engrailed* expression showed different modes of ontogenesis from each other. The two most anterior ones, stripes 1 and 2, were formed individually. They were recognizable since 16 hpf and gradually established from 16 to 20 hpf (Fig. 6d-f). In contrast, the other five stripes
were derived from two relatively broad expression regions. The first region was relatively faint at 16 hpf (Fig. 6d), and became stronger and split into two stripes (stripes 3 and 4) at 18 hpf (Fig. 6e). The second, more posterior region was somewhat intensely stained at 16 hpf (Fig. 6d), and gradually transited into three stripes in subsequent development (stripes 5–7; Fig. 6e-f). Moreover, the two *engrailed* expression regions also showed a trend of bilateral pattern along the dorsal midline, which was even detectable after the full development of five stripes (Fig. 6d-g). This is somewhat consistent with the fact that we frequently observed a middle depression throughout the posterior ridges of some larvae under SEM (supplemental figure S3).

In addition to the striped expression in the central region, a pretrochal stripe of *engrailed* expression was gradually established in early larvae (arrows in Fig. 6d-h). In the posttrochal region, the left and right marginal cells adjacent to the ridges also began to show *engrailed* expression at 24 hpf, and became evident later (dashed arrows in Fig. 6i, l, n). Ultimately, *engrailed* expression became discernable in cells posterior to the seventh ridge, and it connected the left and right marginal expression (the lower arrow in Fig. 6m). All aforementioned *engrailed* expression formed an imaginary circle, corresponding to the proximate margin.

A close look revealed that the cells right adjacent to the prototroch, both pretrochally and posttrochally, all expressed *engrailed* (Fig. 6p-q), indicating they were not involved in shell plate formation. This is consistent with the observation under DIC microscopy that although small granular structures could be observed in plate fields, they were never observed in more anterior cells adjacent to the prototroch (Fig. 6q').

**Discussion**

Polyplacophoran shell development is evolutionarily important to understand the evolution of mollusks as well as their shells [2, 3, 6, 7, 18, 19]. Previous studies pay primary attention to the formation of shell plates, which actually represents relatively late stages of shell development [9, 11, 12], leaving shell field morphogenesis largely elusive. Moreover, while previous research revealed the various cell types inside the shell field and their behaviors during shell formation [2, 6, 11], efforts are required to explore the molecular aspects. Some genes are reported to be expressed in the shell field [13–17], but their correlations with particular cell types remain largely unknown.

In the present study, we investigated morphological and molecular changes during shell field morphogenesis in the chiton *A. rubrolineata*. Previous studies reported an recognizable shell field [11] or no shell field development [10] in newly hatching larvae. We found that in *A. rubrolineata*, neither a morphologically discernable shell field, nor molecular evidence indicating its existence, could be detected in newly hatched larvae, suggesting that shell field morphogenesis occurred totally in larval stages. This feature makes *A. rubrolineata* particularly useful to study shell field morphogenesis. Combining the results of multiple approaches, we revealed relatively complicated developmental events during the process. The different patterns of F-actin aggregations in peripheral margin and plate fields, coupled with
enlarged expression in proximate margin and ridges, comprise the molecular patterns of different parts of the larval shell field. Schematic diagrams summarizing our findings are shown in Fig. 7.

Development of the central region: plate fields and ridges

The serial arrangement of chiton shell plates attracts attention from researchers [6, 9–11]. In A. rubrolineata, we observed characteristic seven plate fields that were alternatively arranged with seven intersegmental ridges, as the situation of all other polyplacophorans investigated. The ontogenesis of these two types of tissues are tightly correlated, indicating that they are modulated by the same regulatory signals.

One notable fact is that the plate fields exhibited significant invagination in A. rubrolineata, which can be reflected by much deeper nuclei compared to those of ridge cells (Fig. 5d); a similar feature is also reported in Lepidochitona cinera [6]. In contrast, the cells of plate fields and ridges are distributed in comparable depths in Ischnochiton rissoa and Lepidochitona caverna [11, 12], indicating a different situation with much reduced invagination of plate fields. Accordingly, very different morphological characteristics of enlarged positive cells are revealed in A. rubrolineata (Fig. 6o) and L. caverna [12] (Fig. 3F of the study). We cannot determine whether these different degrees of invagination are caused by variations among species or developmental stages. Indeed, evident shape changes were reported in the cells of plate fields during shell plate genesis [11]. However, given that invagination has been a common process in molluscan shell development and should affect shell formation [2, 20, 21], the apparent different degrees of plate field invagination in polyplacophorans are worthy of further investigations.

Kniprath identified four cell types (1–4) in the central region of the shell field in I. rissoa, including types 3 and 4 in the plate field producing the shell plates and types 1 and 2 in the intersegmental ridges that generate connective tissues between shell plates [11]. Similar results are revealed in L. caverna, and types 1 and 2 cells are further demonstrated to express enlarged [12]. Our results are consistent with these reports and further revealed that F-actin aggregations marked plate fields, i.e., types 3 and 4 cells. However, we could not determine whether they marked a single or both types of these cells.

The plate fields and ridges became morphologically detectable in a very short term in A. rubrolineata (between 22 and 24 hpf), making it difficult to explore the details of their morphogenesis. We thus traced the dynamics of related molecules to reveal more details. The enlarged expression was continuously changing before the formation of morphologically discriminable ridges (16–24 hpf), suggesting relatively complicated development. The key characteristic of enlarged expression is that it initially showed no clear patterns (12–14 hpf), and gradually transited into striped pattern later (16–24 hpf). This mode of development seems to be different from the formation of striped gene expression of segmentation genes in vertebrates or Drosophila, indicating that the serial ridges (and plate fields) may be formed through different manners from those used in the segmentation of vertebrates or Drosophila (segmentation clock or simultaneous formation of segments [22]).
We concluded several additional characteristics regarding *engrailed* expression in ridges (and thus the development of ridge and plate field). First, different stripes of *engrailed* expression experienced varied modes of changes. In particular, stripes 1 and 2 formed individually, but other five stripes were derived from two expression areas, each of which transited into two (no. 3–4) or three (no. 5–7) stripes later. These different modes of genesis should provide useful clues to explore the underlying regulatory mechanisms (e.g., lateral inhibition). Second, the formation of these seven stripes of *engrailed* generally follow an anterior-to-posterior pattern (Fig. 6d-g). This pattern, as well as the fact that the expression levels of *engrailed* were apparently higher in posterior ridges (Fig. 6g-h), may indicate the involvement of a morphogen with graded activities along the anterior-posterior axis. Lastly, the two posterior *engrailed* expression regions showed a bilateral pattern in relatively early stages. This may indicate the existence of a central signaling center along the dorsal midline.

Unlike the dynamic *engrailed* expression, we observed very limited changes in the F-actin aggregations of the developing shell field. The aggregation of F-actin in this region was only detected after the formation of morphologically detectable plate fields (24 hpf). It then enhanced with the invagination of plate fields (compare Fig. 4n and o), indicating a role of actomyosin networks in this invagination event. Moreover, although we did not detect molecular markers for the plate fields in earlier stages (before 24 hpf), they may be revealed in future investigations.

**The chiton shell field comprises a margin region**

The girdle (or perinotum) of adult chiton encircles the shell plates and develops spicules on it. It is proposed that the epithelium of the girdle is not so different from that producing the shell plates [6]. From this perspective, it is reasonable to take girdle development as a part of shell formation. On the other hand, although the girdle precursor was noticed previously, it was frequently paid less attention than the plate fields [11, 12]. Our results clearly revealed that the girdle precursor of *A. rubrolineata* experienced complicated development comparable to ridge/plate fields and that it contained different cell populations that arranged in a regular pattern. Since spicules are essential to explore molluscan shell origin [6–8], we propose to emphasize the girdle precursor and suggest a specific term for it. Here we picked “shell field margin” for simplicity and clarity. This term should be used to describe the larval tissue of polyplacophorans that 1) will develop to girdle after metamorphosis, and 2) contains cells of certain degrees of specification related to girdle development. The shell field margin of *A. rubrolineata* comprised two cell populations forming concentric imaginary circles, namely, the peripheral and proximal margin (Figs. 3 and 7). As also shown in *Middendorffia caprearum* [11], it was separated into pretrochal and posttrochal parts by the prototroch.

The peripheral margin likely contained spicule-formation tissues that exhibited superficial tiny protrusions and F-actin aggregations (“spots” or “tubes” at different stages) but lacked *engrailed* expression. This is consistent with the previous observation that only the larval girdle without *engrailed* expression develop spicules [12]. It was reported that the polyplacophoran spicule was formed within a deep invagination of a papillar cell, which had a collar formed by its apical part [6]. We found that the F-actin “tubes” in the peripheral margin were deeply inserted into the larval body and were likely derived from a single cell
(Fig. 5a-c), indicating they should correspond to the apical collars of spicule-formation cells mentioned previously. Moreover, although the spicule-formation cells already showed superficial tiny protrusions when the F-actin "tubes" emerged, there was no morphological characteristics for these cells in the earlier stage (24 hpf), and they can be only recognized based on the “spotted” F-actin patterns. This fact can facilitate identifying spicule-formation genes during the early specification of spicule-formation cells.

The demonstration of the correlation between characteristic F-actin aggregations and spicule formation would contribute to several evolutionary issues concerning aculiferan spicules. First, as two representatives of extant aculiferan mollusks, the common feature of polyplacophorans and aplacophorans is the ability to develop spicules, and thus spicule development should contain important information of aculiferan evolution. Although almost the same process of spicule formation is suggested in two aculiferan clades [6], molecular evidence is required to certify this notion. Current researches concerning aplacophoran spicule development reveal relatively few molecular data [23–26], and it would be intriguing to explore whether aplacophoran spicule formation also exhibited similar F-actin dynamics as we revealed. Second, some researchers speculate molluscan spicules to have the common origin with the chaetae/setae of annelids and brachiopods, thereby being greatly informative to infer spiralian evolution [8]. The potential homology of these sclerotized structures has been explored based on the fine structure of the sac that generate them as well as related genes/molecules [27]. However, from current reports, we could not discern specific F-actin aggregations in annelid or brachiopod chaetal sacs [28–30]. On the other hand, as we have shown in supplemental figure S1, such a correlation may be relatively subtle and could be masked by the strong staining in muscular tissues. Further CLSM analysis with a high resolution and devoid of influences from muscles may contribute to exploring this issue. Investigating the expression and function of chaeta-formation genes in *A. rubrolineata* is also required.

Consistent with the report in *L. caverna* [12], we found circular expression of *engrailed* corresponding to the gap between the peripheral margin (marked by F-actin aggregations) and the central region. We interpreted these circularly organized cells to be a part of the shell field margin, and termed them as the proximate margin. Although the roles of these cells are not fully elucidated, it is reported that thy are not involved in spicule development and contain some secretory cells [11, 12]. Moreover, when taking the *engrailed*-expressing tissue in the shell field as a whole, it is attempting to propose that the *engrailed* expression seems to define the boundary of shell plates: that in the proximal margin demarcates the entire region bearing all shell plates, and the expression in ridges delineates each plate. A similar idea regarding the role of *engrailed* in determining developmental boundaries was proposed previously [12].

The common expression of *engrailed* in intersegmental ridges and proximate margin indicates similarities between related cells. This is consistent with the report that type 1 and 2 cells are distributed in both ridges and girdle of *I. rissoa* [11]. Similarly, common F-actin aggregations were revealed in presumptive spicule-formation cells in the peripheral margin and those of the plate fields (despite the different patterns), indicating some common features between these sclerotization-related cells. It is also notable that in both the shell field margin and the central region, the tissues showing *engrailed* expression and F-actin aggregations are adjacent to each other but never intermixed.
Pretrochal contribution to shell plate formation?

If accepting the development of girdle to be a part of shell development, the involvement of pretrochal tissues in shell development could be confirmed by the fact that the shell field margin contains both pretrochal and posttrochal tissues [11, 12]. However, this speculation does not contribute to the debate concerning whether the pretrochal region contribute to the formation of the first shell plate [9, 11]. Since we did not investigate shell formation in metamorphosis, our results provide no direct evidence to this question. However, if shell plates are indeed secreted by the cells of plate fields, our results seem to indicate no pretrochal contribution to shell plate formation in *A. rubrolineata*. We found that the tissues right adjacent to the prototroch, in both pretrochal and posttrochal regions, all expressed *engrailed* (Figs. 5d and 6o-p) and thus should not be involved in shell plate formation. The first plate field was distributed posteriorly to these tissues, indicating the first shell plate should be posttrochal.

Molluscan shell evolution: shell fields, shell plates and spicules

The shell field represents a key node of shell development, and it should provide useful clues to explore essential questions concerning molluscan shell evolution, i.e., whether conchiferan and polyplacophoran shell plates are homologous [3, 6, 19]. Conchiferan shell development has been investigated in various lineages, especially with the aid of molecular markers such as gene expression and endogenous enzyme activities [20, 31–37]. It is clear that conchiferan shell fields show a concentric (“rosette”) pattern, with different cell populations distributed in varied distances from the center. More importantly, the shell plate is only formed in the central region of the shell field [2, 20, 37–40]. These features are also revealed in the polyplacophoran *A. rubrolineata*. Despite interrupted by the prototroch, the shell field margin enclosed the central region where the (seven) shell plates would develop. The shell field margin also showed a comparable concentric organization, comprising two circles of F-actin “spots/tubes” (the peripheral margin) and *engrailed* expression (the proximate margin).

The common expression of *engrailed* in the shell field margin is another shared feature between polyplacophorans and conchiferans [12, 15, 34, 36, 41]. Similarly, comparable expression of *pax2/5/8* is observed in the shell field margin of the chiton *Acanthochitona crinita* [16] and the margin of larval mantle in a gastropod [42]. Therefore, despite the very different shell plates they produce, a key feature between polyplacophoran and conchiferan shell fields is the existence of a shell field margin and the common expression of particular genes in the tissue, while the shell plate(s) will develop in the central region they surround.

However, it is still too early to say that the aforementioned data support the homology of polyplacophoran and conchiferan shell fields. Researchers revealed different cell lineages for polyplacophoran and conchiferan shell fields, arguing against such homology [3, 19, 43–45]. In fact, the common feature of aculiferan mollusks is the development of spicules but not shell plates. If assuming that a shell plate could be evolved when multiple spicule-formation cells join together (amalgamation) [6,
it is more favorable to speculate that the common organization of polyplacophoran and conchiferan shell field may be the prerequisite of placing the sclerotization cells in the central region, which underpins the common evolution of shell plates in these two lineages. The highly similar shell fields themselves, however, may be the result of convergent evolution.

Given the well supported phylogeny of mollusks comprising of Aculifera and Conchifera [46, 47], polyplacophorans are more evolutionarily close to aplacophorans than conchiferans. Thus, the similarities between the shell field of polyplacophorans and conchiferans bring an intriguing question, that is, whether spicule-formation tissues in aplacophorans share some common characteristics with polyplacophoran (and conchiferan) shell fields? Due to the lack of shell plates, a shell field does not exist in aplacophorans. Nevertheless, here we call the larval epithelium related to spicule development as the aplacophoran shell field for simplicity, given the accepted homology between aplacophoran and polyplacophoran spicules. Seven rows of sclerotization cells/spicules in the dorsal larval epithelium of two aplacophorans [23, 26] resemble the seven serially arranged plate fields in polyplacophorans, suggesting shared features of aculiferan shell fields. However, it is not known whether aplacophoran shell fields also possess a margin with a concentric organization. Another line of evidence is that gbx is common expressed in the epithelium responsible for spicule/shell development in a aplacophoran, a polyplacophoran and a bivalve conchiferan [17].

Taken together, it could be concluded that some common features of shell fields are revealed among aculiferans, between polyplacophorans and conchiferans, or between aculiferans and conchiferans, but they are far from sufficient to certify any issues regarding the homology of shell fields, that of shell plates, or that of shell plates and spicules. Compared to the extensive researches in conchiferans, studies on the development of aculiferan sclerotized structures are limited, and further investigations are required to make better comparisons to explore molluscan (shell) origin and evolution.

Lastly, it is notable that the development of the eighth shell plate represents another aspect of polyplacophoran shell development. Nevertheless, it occurs after metamorphosis and thus was not investigated in the present study. Given that the polyplacophoran shell field contains only the anlagen for the first seven shell plates, other manners of development may be employed for the eighth one. This would be an interesting issue for future studies.

Conclusions

Four types of tissues were recognized from the shell field of the polyplacophoran mollusk A. rubrolineata, namely, the peripheral and proximate margins showing a concentric organization and the centrally located, alternatively arranged plate fields and ridges. Molecular patterns are revealed for each type of tissues, including common F-actin aggregations in the peripheral margin and the plate fields (with different patterns), and engrailed expression in the proximate margin and the ridges. These results provide a detailed description regarding the structure of the polyplacophoran shell field on both molecular and cellular levels. The dynamics of different molecules revealed the ontogenesis of related tissues and
provide clues for exploring the underlying regulatory mechanisms. Further studies are required to elucidate the roles of each type of cells, as well as the mechanisms modulating their specifications and organizations, which will help understand the formation and evolution of polyplacophoran shells and spicules.

Methods

Animals and larval culture

The adults of *A. rubrolineata* (Lischke, 1873) were collected from intertidal rocks in Qingdao, China. After transferred to the lab, each individual was placed in a 100-ml plastic cup filled with fresh seawater. In reproductive seasons (June to August), a proportion of individuals spawned within approximately three hours after the transferring, and each type of gametes was thus collected. Sperm was added to oocyte suspension for artificial fertilization, and zygotes were cultured in filtered seawater (FSW) at 25°C in an incubator. The developmental stages were referred to as hours post fertilization (hpf).

Trochophore larvae hatched after 8.5 hpf. A few abnormal larvae were neglected in most circumstances; when their numbers could not be neglected, healthy larvae were collected from the upper half of the water column at around 10 hpf. The larvae older than 60 hpf could be induced to metamorphose by supplying plastic sheets coated with the algae collected from the rock that their parents inhabited. We found that the time points to start metamorphosis were very different for different individuals. This prevented us from investigating the development during and after metamorphosis in details, but indeed allowed us to collect a few such samples. Live samples at varied developmental stages were recorded using an Olympus CKX53 inverted microscope.

At desired developmental stages, the larvae were anesthetized by adding 1 M MgCl$_2$, fixed in 4% paraformaldehyde (PFA) (1×PBS, 100 mM EDTA, 0.1% Tween-20, pH 7.4) or 2.5% glutaraldehyde, and stored in methanol or PBSTw (1× PBS, 0.1% Tween-20, pH 7.4), as described previously [37].

Scanning electron microscopy

Samples were gradually dehydrated to ethanol, and successively transferred to a mixture of ethanol and isopentyl acetate (v/v = 1:1, once) and isopentyl acetate (twice). They were then submitted to critical-point drying (with liquid CO$_2$), coated by gold, and observed using a scanning electron microscope (Hitachi S-3400N).

Semithin sectioning

Samples fixed by glutaraldehyde and stored in PBSTw were stained with hematoxylin and eosin before subsequent manipulations. After washed with PBSTw, stained samples were gradually dehydrated to ethanol and successively transferred to acetone and resin (EPON 812). After curing, samples embedded in resin was sectioned into 1 µm sections. Although we stained the samples with two dyes, we found eosin staining was washed out in subsequent treatments, the samples were thus only stained dark blue.
Phalloidin staining

Fixed samples stored in PBSTw were successively treated with PBSTx (1X PBS plus 0.5% TritonX-100) and 0.1% BSA in PBSTx for 5min each. Then the samples were stained in 0.1μM TRITC (tetramethylrhodamine)-conjugated phalloidin (Solarbio, cat. no. CA1610) at 4°C overnight. After washing with PBSTw, the samples were mounted in glycerol and observed under a confocal laser scanning microscope (ZEISS LSM 710).

In confocal laser scanning microscopy (CLSM), Z-stack projections were applied in most circumstances. When doing this, the optical sections were carefully selected to not include the deep sections containing very strong staining in the larval muscle. This strategy helped reveal very important structures such as the F-actin aggregations in plate fields (supplemental figure S1).

Whole mount in situ hybridization

Whole mount in situ hybridization using a probe targeting the shell-formation gene *engrailed* was performed as described previously [15]. The stained samples were mounted in glycerol and observed under a Nikon 80i microscope.

Abbreviations

hpf
hours post fertilization
F-actin
filamentous actin
SEM
scanning electron microscopy
CLSM
Confocal laser scanning microscope
FSW
filtered seawater
PFA
paraformaldehyde.

Declarations

Ethics approval and consent to participate

This study does not involve endangered invertebrates. According to the national regulation (Fisheries Law of the People's Republic of China), no permission is required to collect the animals and no formal ethics approval is required for this study.

Consent for publication
Not applicable.

Availability of data and materials

All data generated during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

PH and BL designed the study. YX performed experiments. YX and PH captured microscopy images. YX, PH and BL analyzed the data. PH wrote the first draft of the manuscript with inputs from YX. All authors contributed to subsequent revisions. All authors read and approved the final manuscript.

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Figures
Figure 1

Polyplacophoran shell plates and their development. **a.** An adult *A. rubrolineata*; dorsal view with the anterior to the top. The eight serially arranged shell plates (arrows) are encircled by the girdle (double arrow). **b** A generalized scheme depicting polyplacophoran shell ontogenesis. In some species, the newly hatched trochophore larva does not have a recognizable shell field. After metamorphosis, seven calcified shell plates emerge in the juvenile (arrows) and spicules develop in the girdle (the double arrow). Developmental stages are referred to *A. rubrolineata.*
Figure 2

General development of A. rubrolineata. An egg hull (eh) can be recognized before the hatching of the trochophore larva (a-g). In b, the zygote is dividing. Larval eyes emerge at around 24 hpf (arrowheads in i), and are sustained after metamorphosis (arrowheads in k-l). In j, the shell field (sf) showing serially arranged repeated units could be clearly recognized. Spicules (double arrows in k and l) start to develop during metamorphosis and seven shell plates could be observed in juveniles (numbers in l). pt, prototroch. Bars represent 50 μm.
Figure 3

**Larval development under SEM.** Dorsal and lateral views are shown, with the anterior to the top. For larvae after 16 hpf, panels c’-r’ show the details of pretrochal and posttrochal regions. Note that for some stages they are not the same larvae as those in c-r. The pretrochal region of the shell field is indicated by arrows in d-e and k-n. The small pore in the cells adjacent to the prototroch are highlighted by the arrowhead in k’. In larvae after 24 hpf, alternative bulges and grooves are discernable (indicated by numbers in l-n). Stars in l indicates that the seventh ridge, the seventh plate field and proximate margin are not developed (compare to Fig. 4k). Periferal margin (pem) and proximate margin (prm) exhibit different morphological characters after 36 hpf, and are indicated by brown and green shadows, respectively (m’, n’, o’ and r’). lc, lateral cilia; pt, prototroch. Bars represent 50 μm.
**Figure 4**

**F-actin dynamics during morphogenesis of the shell field.** The presumptive shell field tissues in the pretrochal region becomes invaginated at 16 hpf (dashed line in g). F-actin starts to aggregate in this region at 20 hpf (arrow in d and i), which then spreads to the posttrochal region (arrows in e and j). These aggregations show “spot” patterns at 24 hpf and “circled” patterns at 36 and 48 hpf (arrows in k-m; more details are shown in q-s). Plate fields and ridges are formed in the central region at 24 hpf (k). Stars in k
indicates that the seventh ridge, the seventh plate field and proximate margin are not detectable. The proximate margin is recognizable between the peripheral margin and the central region (in the posttrochal region) or between the peripheral margin and prototroch (in the pretrochal region) (kp). ri, ridge; pf, plate field; pem, peripheral margin; prm, proximate margin; pt, prototroch. Bars represent 50 μm.

**Figure 5**

**Details of the peripheral margin and the plate fields.** a-c. The tube-like structure of the presumptive spicule formation cells (arrows) in the peripheral shell field margin (pem). a. CLSM 3D projection showing F-actin “tubes” (arrows). b-c. A semithin section cross the peripheral margin showing similar structures (arrows). c is a magnified image corresponding to the area indicated by the box in b, in which the two recognizable tubes are highlighted by dashed lines. d. A longitude section showing the posttrochal region of a 48-hpf larvae, with anterior to the right and dorsal on the top. Plate fields (arrowheads) and ridges (arrows) can be recognized. Note that the cells adjacent to the prototroch (pt) are not invaginated, indicating that the first plate field does not reach this distance. Bars represent 20 μm.
Figure 6

Expression of the shell formation gene *engrailed* during early development of *A. rubrolineata*. Dorsal and lateral views are shown, with the anterior on the top. In lateral views (i, l, n, o), dorsal is to the right. Striped expression in the posttrochal region (ridges) is indicated by numbers (1-7). Black arrows in d-o indicate the expression in the proximate margin that encircles the central region showing striped expression. Expression of *engrailed* with no evident correlation with shell field development is indicated by white crosses. Particular morphological characteristics could be recognized in the peripheral margin (gray double arrows in k-o), which should correspond to the tiny protrusions under SEM (compare to Fig. 3q’). Panels p and p’ show the *engrailed* expression adjacent to the prototroch, in which several positive cells right adjacent to the prototroch are highlighted by white arrows. Panel q’ corresponds to the region enclosed by the black box in q, which shows granular structures in plate fields but not in cells adjacent to the prototroch. pf, plate field; prm, proximate margin; pt, prototroch. Bars represent 50 μm.
Figure 7

Schematic diagrams showing shell field morphogenesis in *A. rubrolineata*. Different parts of the shell field can be recognized based on gene expression patterns or aggregated F-actin. The organization of the shell field is generally established at 24 hpf, including the shell field margin comprising the peripheral and proximate margins and the central region showing alternatively arranged plate fields and ridges.

**Supplementary Files**

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