

Reviewed Preprint

v1 • May 11, 2026

Not revised

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liushk@ouc.edu.cnqili66@ouc.edu.cn**Competing interests:** No competing interests declared**Funding:** See [page 19](#)**Reviewing editor:** Ariel Chipman, The Hebrew University of Jerusalem, Israel

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Ancestral secretory programs underlie the evolution of morphological innovations across Spiralia

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eLife Assessment

This manuscript examines the evolution of molluscan shells using single-cell analyses of the adult mantle of *Crassostrea gigas* and compares these data with previous datasets from embryonic and larval stages of this species and other spiralian. The authors provide **important** support for a scenario in which secretory cells are broadly conserved across spiralian, and the incorporation of lineage-restricted genes contributes to the evolution of molluscan shells. While some of the conclusions of the authors are **convincing**, many aspects of the manuscript remain **incomplete** and could be improved, especially aspects of cell-type classification and validation.

<https://doi.org/10.7554/eLife.111168.1.sa4>

Abstract

Understanding how morphological innovation arise from ancestral genetic and cellular systems remains a major challenge in evolutionary biology. Molluscan shells represent one of the most diverse morphological novelties within Spiralia and have been central to the ecological and evolutionary diversification of molluscs. However, the cellular basis of shell formation and the evolutionary origin of shell-forming cell types remain poorly understood. Here, we present a single-cell transcriptomic atlas of the Pacific oyster (*Crassostrea gigas*) mantle and show that molluscan shell-forming cell types are evolutionarily recent innovations built upon ancestral epithelial secretory systems of spiralian. We identify five spatially segregated shell-forming epithelial cell types, and demonstrate that larval and adult shell-forming cells are developmentally independent and characterized by transcriptomes enriched for evolutionarily young genes. Cross-species cell-type comparisons further reveal that oyster shell-related genes are embedded within conserved epithelial and secretory programs across spiralian. Together, we propose that a substantial genetic foundation for shell formation was already present in the last common ancestor of Spiralia, and that molluscan shell diversity arose through repeated co-option of ancestral genetic programs coupled with novel genes. Our study provides a framework for understanding how ancient cellular architectures can be repeatedly reconfigured to generate morphological novelty during evolution.

Introduction

Morphological innovations are lineage-restricted traits that occur in specific taxa but are absent in outgroups or serially homologous structures, and they represent a major driver of evolutionary diversification and ecological expansion (Erwin 2021 [↗](#)). Despite their evolutionary importance, the mechanisms by which novel structures arise from pre-existing genetic and developmental systems remain poorly understood (Moczek 2008 [↗](#); Wagner and Lynch 2010 [↗](#); Carscadden et al. 2023 [↗](#)). While genetic mutations and gene duplication provide raw material for evolutionary

change, a central question concerns how such variation is reorganized within cellular systems to produce new functions and, ultimately, novel morphological traits (Xia et al. 2025 [↗](#)). Therefore, identifying the cellular and regulatory substrates that mediate the emergence of evolutionary novelty is critical for linking genetic variation to phenotypic innovation (Arendt et al. 2016 [↗](#); Tanay and Seb -Pedr s 2021 [↗](#); Parker and Pennell 2025 [↗](#)).

Spiralia is one of the most diverse bilaterian clades, encompassing molluscs, annelids, flatworms, and brachiopods, and related phyla that exhibit remarkable diversity in body plans, developmental strategies, and biomineralized structures (Laumer et al. 2015 [↗](#); Piovani and Marl taz 2023 [↗](#); Sleight 2023 [↗](#)). A striking feature of spiralian evolution is the repeated emergence of hardened extracellular structures, such as molluscan carbonate shells, annelid chaetae and tubes, and brachiopod phosphate shells (Wernstrom et al. 2022 [↗](#); Bai et al. 2026 [↗](#)). Although these structures perform similar protective and structural roles, they differ markedly in composition, developmental origin, and morphology. This raised the question of whether they share common cellular and regulatory origins or instead evolved independently through convergent recruitment of distinct molecular mechanisms. The extensive diversity and recurrent evolution of these systems also provide an ideal framework for investigating the cellular and regulatory bases of morphological novelty.

Among these structures, molluscan shell represents a prominent example of evolutionary innovation and have contributed substantially to the ecological success and morphological diversification of molluscs (Wanninger and Wollesen 2019 [↗](#); Chen et al. 2025 [↗](#); Bai et al. 2026 [↗](#)). Shell formation is orchestrated by the mantle, a specialized secretory epithelium that deposits mineral and organic components through highly coordinated cellular processes (Clark et al. 2020 [↗](#)). Increasing evidences have indicated that this process is governed by a conserved regulatory architecture coupled with rapidly evolving effector genes, a combination that supports extensive morphological diversity in shell structure and composition (Aguilera et al. 2017 [↗](#); Zhao et al. 2018 [↗](#); Yarra et al. 2021 [↗](#); Bai et al. 2026 [↗](#)). However, the cellular origin and evolutionary history of shell-forming cell types remain unclear. In particular, it is unknown whether molluscan shell-secreting cells represent lineage-specific cellular inventions or derive from ancestral cellular systems shared across Spiralia.

Recent advances in single-cell transcriptomics have enabled the classification of cell types beyond traditional histological models and quantitative comparisons of cell-type repertoires across species (Zhong et al. 2025 [↗](#)), which provide unprecedented resolution for dissecting the cellular basis of novel phenotypes across animal evolution (Li et al. 2025 [↗](#); Piovani et al. 2025 [↗](#); Tommasini et al. 2025 [↗](#); Xiong et al. 2025 [↗](#)). These methods offer a powerful framework for understanding the gene expression programs and cellular organization underlying morphological novelties. Here, we investigate single-cell transcriptomics, developmental comparisons, phylostratigraphy, and cross-species cell-type alignment to investigate the cellular and evolutionary origins of molluscan shell formation in the Pacific oyster (*Crassostrea gigas*). We construct a comprehensive single-cell atlas of the adult mantle, identify spatially distinct shell-forming epithelial cell populations, and compare them with secretory cell types across representative spiralian. By linking gene expression profiles with evolutionary gene-age analyses and interspecific transcriptomic alignments at cell level, we reveal that molluscan shell-forming cells represent lineage-specific innovations built upon deeply conserved epithelial and secretory programs of Spiralia. Our study supports a model in which morphological innovations arise through repeated co-option and diversification of ancestral cellular architectures, coupled with the integration of lineage-specific genes.

Results

A single-cell atlas of the oyster mantle reveals spatially distinct shell-forming cell types

To explore the cellular and molecular characteristics underlying molluscan shell formation, we performed the single-cell RNA sequencing (scRNA-seq) on the mantle of *C. gigas*. After stringent quality control and doublet removal, we obtained transcriptomes from 37,547 cells and identified 35 cell clusters by applying the uniform manifold approximation and projection (UMAP) method for the dimensionality reduction (Figure 1A [↗](#); Supplementary file 1 [↗](#)). These clusters were annotated based on previously established marker genes (Piovani et al. 2023 [↗](#); De La Forest Divonne et al. 2025 [↗](#)) and published *in situ* hybridization (ISH) data (Takahashi et al. 2012 [↗](#); Foulon et al. 2018 [↗](#); Li et al. 2021 [↗](#); Zhu et al. 2021 [↗](#); Min et al. 2022 [↗](#); Bai et al. 2023 [↗](#); Bai et al. 2026 [↗](#); Ren et al. 2026 [↗](#)), which were consolidated into major cell types such as shell-secreting epithelium cells (SECs), hemocytes, myocytes, cilia, neurons, vesicular connective tissue cells, and proliferative cells (Figure 1B [↗](#); Supplementary file 1 [↗](#)). Among them, five SEC types were distinguished by the differential expression of biomineralization genes, including *rbp4b*, *Gigasin2*, *LamG3*, *sleB*, and *Tyr* (Figure 1C [↗](#); Supplementary file 2 [↗](#)). Spatial mapping of representative SEC markers revealed distinct regional distributions along the mantle epithelium. The SEC1 marker (*rbp4b*) stained the middle and outer pallial regions of the outer epithelium, whereas a marker shared by SEC1 and SEC2 (*Gigasin2*) was broadly expressed across the outer epithelial zone. The SEC3 marker (*LamG3*) was expressed along both the outer and inner surfaces of the outer fold near the periostracal groove, which is involved in periostracum formation. In contrast, the SEC4 (*sleB*) and SEC5 (*Tyr*) markers were restricted to the middle pallial zone of the outer epithelium and the inner surface of the outer fold, respectively. These expression patterns are likely associated with distinct biomineralization processes, corresponding to the formation of different shell layers and microstructures (Marie et al. 2012 [↗](#); Shimizu et al. 2022 [↗](#); Bai et al. 2023 [↗](#)). This result indicates a high degree of functional compartmentalization within the shell-forming epithelium.

To reconstruct differentiation pathways of shell-forming cell types, we performed the pseudotime trajectory analysis on the five SECs together with proliferative cells (Figure 1D [↗](#)). Using proliferative cells as the root, we inferred two major differentiation paths (Figure 1E [↗](#)). In the predominant path, proliferative cells transitioned through an intermediate SEC3_27 (cluster 27 within SEC3) state and then SEC1, before branching into SEC2 and ultimately SEC5, suggesting a stepwise progression toward terminally differentiated shell-forming cells. In a second path, proliferative cells directly diverged into SEC2 and subsequently to SEC3_29 (cluster 29 within SEC3), representing an alternative differentiation route within the mantle epithelium. The SEC4 was excluded from these trajectories, likely reflecting the limited number of cells in this cluster or a specialized functional state that is not captured along the major differentiation pathway. Density distribution along pseudotime confirmed that SEC3_27 and SEC1 are enriched at early stages, SEC2 spans intermediate pseudotime, and SEC5 and SEC3_29 occupy terminal positions (Figure 1F [↗](#)). Together, these analyses indicate that mantle shell formation is driven by progenitor-like SEC populations that diversify into spatially segregated secretory cell types (Figure 1G [↗](#)).

Larval and adult shell-forming cells are developmentally independent and rapidly evolving

During molluscan development, larval and adult shells exhibit striking differences in shapes, microstructures and shell matrix protein (SMP) repertoires (McDougall and Degnan 2018 [↗](#); Zhao et al. 2018 [↗](#); Cavallo et al. 2022 [↗](#)). We thus reasoned whether adult mantle SECs correspond to shell-forming cell populations that are present in early development. To identify the shell-forming cell types at early larval stages, we re-analyzed publicly available scRNA-seq datasets from gastrula and trochophore stages of *C. gigas*, and integrated them into a unified single-cell atlas comprising 27,910 embryonic and larval cells (Figure 2A [↗](#)). Notably, four larval shell gland cell

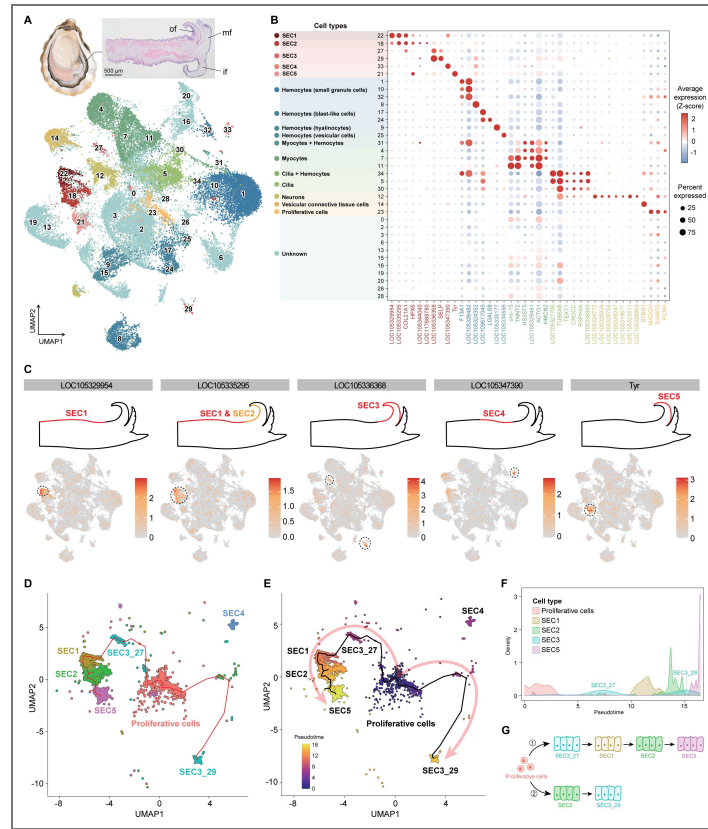


Figure 1. A single-cell atlas of the oyster mantle and spatial-temporal distribution of shell-forming cells.

(A) UMAP visualization of mantle cells, with clusters colored according to their cell identities. A schematic illustration of the internal anatomy of the oyster and H&E staining of the mantle are shown at the top. Abbreviations: of, outer fold; mf, middle fold; if, inner fold. (B) Dot plot of marker gene expression for each cell cluster shown in (A). Marker genes were retrieved from published studies (Supplementary file 1). Clusters lacking characterized functional marker genes were labeled as “Unknown” or classified as mixed cell populations. (C) Spatial distribution of the five SEC types across mantle regions, as indicated by their marker genes. Top: schematic cross-sections of the mantle showing the expression locations of five representative marker genes corresponding to the SEC types. SEC1 and SEC3-5 are highlighted in red, and SEC2 in yellow. Bottom: expression plots of the same SEC marker genes. (D) UMAP visualization of six major cell types used for pseudotime analysis. Red lines indicate the inferred trajectories. SEC3_27 and SEC3_29 represent clusters 27 and 29 within the SEC3 type, respectively. (E) Pseudotime trajectories of SEC and proliferative cell populations. Proliferative cells were used as the origin for the pseudotime analysis. Red arrows indicate predicted differentiation paths, and black lines show the inferred trajectories. (F) Density distribution of the five SEC types on the pseudotime trajectory. (G) Schematic summary of inferred differentiation trajectories from proliferative cells to SEC populations. Two major paths were identified: Path 1, proliferative cells → SEC3_27 → SEC1 → SEC2 → SEC5; and Path 2, proliferative cells → SEC2 → SEC3_29.

types were identified, spatially coincided with the dorsal shell field in larvae (Figure 2A). From gastrula to trochophore, the proportion of proliferative cells markedly decreased, while shell gland cells nearly doubled (Figure 2B and C; Supplementary file 3), reflecting a burst of differentiation activity consistent with larval shell formation. These observations suggest a tightly regulated developmental program driving the specification and expansion of shell-forming cells at early larval stages.

We further analyzed the expression patterns of oyster SMP genes at both bulk (Zhang et al. 2012; Lian et al. 2025) and single-cell levels (Figure 2-figure supplement 1). Larval SMP genes were predominantly expressed in shell gland cells but were barely detected in SECs (figure supplement 1A-C). In contrast, adult SMP genes mostly showed no expression in shell gland cells and were highly expressed in SECs (figure supplement 1D-F). Comparative analysis further demonstrated minimal overlap between genes specifically enriched in larval shell glands and those enriched in adult SECs, with no significant correlation between their expression profiles across cell types (Figures 2D; Figure 2-figure supplement 2; Supplementary file 4). Moreover, the GO enrichment of highly expressed genes revealed largely distinct functional profiles between larval shell glands and adult SECs, with only a small set of shared GO terms related to translation and RNA binding (Figure 2E; Supplementary file 5). These findings indicate that larval and adult shells are secreted by developmentally independent cell populations that deploy largely stage-specific gene repertoires.

Accordingly, our phylostratigraphic analyses of single-cell transcriptomes revealed that both larval shell glands and adult SECs exhibited the younger transcriptome ages, defined as a relative enrichment of expressed genes with more recent evolutionary origins inferred from phylostratigraphy, compared to many other cell types in larvae and adult tissues (Figures 3, Figure 3-figure supplement 1). In contrast, by extending this approach to the bulk level, a peak of young gene expression was observed during the trochophore stage of *C. gigas* (Figure 3-figure supplement 1C), whereas adult shells exhibited among the oldest transcriptome profiles and the digestive gland exhibited among the youngest ones (Figure 3-figure supplement 1D). Molluscan trochophore larvae and mantle show transcriptomes enriched for evolutionarily young genes, underlying shell formation and diversity (Wu et al. 2019; Wang et al. 2020). The transcriptome age index (TAI) observed in bulk RNA-seq analyses likely reflected artifacts caused by cellular heterogeneity, as suggested in a previous study on trochophore larvae (Piovani et al. 2023). Biomineralization genes involved in shell formation rapidly evolve and includes a high proportion of lineage-specific genes (Jackson et al. 2006; Kocot et al. 2016; Aguilera et al. 2017), which may contribute to more rapid evolution of shell-forming cells than other cell types. This molecular evolution may have facilitated the co-option of biomineralization pathways and novel effector genes involved in shell formation, thereby promoting the emergence of lineage-specific shell architectures (Arivalagan et al. 2017; Cavallo et al. 2022). These results suggest that both larval and adult shell-forming cells are evolutionarily recent innovations and evolved independently, each recruiting distinct sets of novel genes into shell formation.

Conserved spiralian epithelial and secretory cell types underlie molluscan shell-forming innovation

To trace the evolutionary origins of molluscan shell-forming cells, we performed cross-species cell-type comparisons between *C. gigas* and four representative spiralian, including the annelid *Pristina leidy*, the flatworms *Dugesia japonica* and *Schmidtea mediterranea*, and the chaetognath *Paraspadella gotoi* (Figures 4A-D, Figure 4-figure supplement 1). We found that several SEC types showed transcriptional similarity to chaetal cells in *P. leidy* and *P. gotoi*, which are responsible for secreting chitinous chaetae. Similar affinities were observed with secretory cells in both flatworms, as well as with epidermal cells in *P. gotoi*. These alignments suggest that molluscan shell-forming cells likely share a common ancestral function related to secretion and epithelial matrix production, consistent with their secretory roles in biomineralization. We further investigated the regulatory basis underlying these similarities by transcription factor (TF) genes highly expressed in the oyster SECs. We found that orthologs of *CEBPD*, *PBX1*, *TCF4*, *PAX5*, *cpb-2*,

Figure 2. Distinct shell-forming cell types between larval and adult stages in *C. gigas*.

(A) UMAP visualization of integrated single-cell transcriptomes from gastrula and trochophore stages. Different colors indicate the distribution of shell gland cell populations in the schematic of the trochophore dorsal side (top left). (B) Separate UMAP plots of gastrula (left) and trochophore (right) cells. (C) Proportions of major cell types from gastrula (left) to trochophore (right). (D) Pairwise comparison of marker-gene overlap of larval (gastrula and trochophore) and adult (mantle) cell types. For each pair, the box is shaded by statistical significance ($-\log_{10}$ scale of P values by hypergeometric test). Shell-forming cell types are highlighted by grey backgrounds, and dashed boxes indicate the comparisons between larval and adult shell-forming cell types. (E) Scatter plot showing significantly enriched GO terms (q value < 0.01 , FDR-adjusted) for marker genes of SEC (blue) and shell gland (purple) cell types. GO terms shared between the two cell types are highlighted in green.

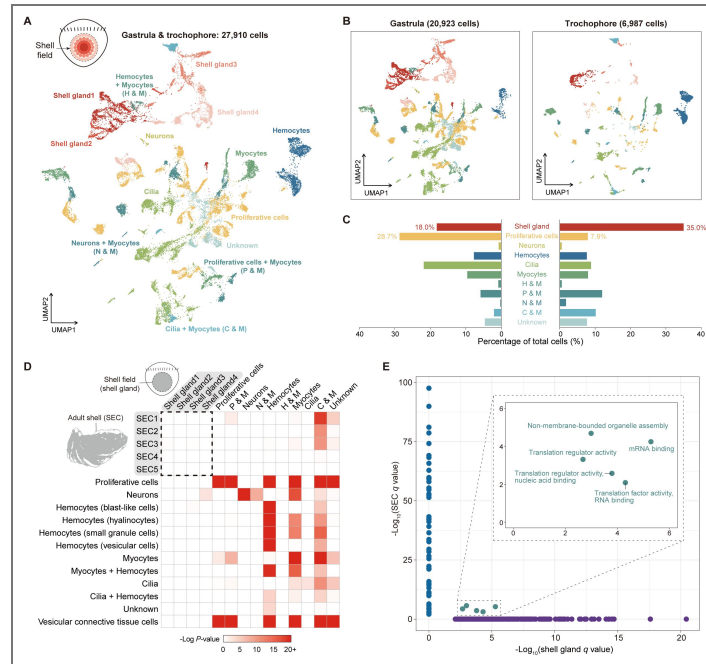
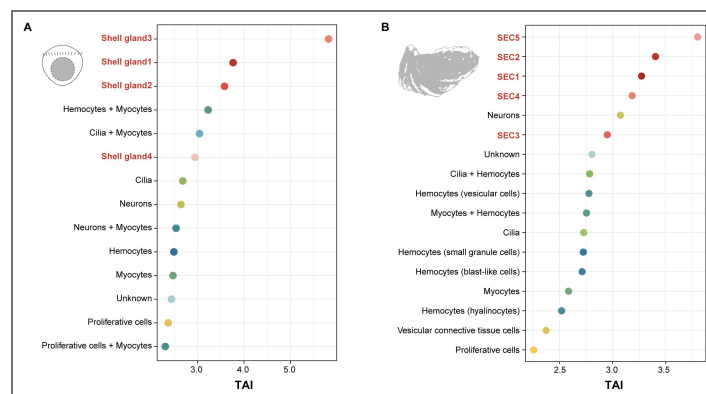


Figure 3. Transcriptome age indices (TAI) for larval (gastrula and trochophore) (A) and adult (mantle) (B) cell types.

Lower TAI values correspond to “older” gene ages. Shell-forming cell types are highlighted in red.



MEIS1, *RUNX1*, and *CREB3L2* were also expressed in the secretory or epidermal cells of other spiralian (Figures 4E [↗](#)). The shared TF repertoire reflects a conserved regulatory backbone for secretory and epithelial programs that contribute to the emergence of molluscan shells, and has been repeatedly co-opted for secretory and epithelial systems across Spiralia. In contrast, TFs including *HELT* and *EVX1* were restricted to oyster SECs but not expressed in aligned cell types in other species, suggesting that lineage-specific regulators may contribute to the unique features of molluscan shell formation.

To assess how lineage-specific gene novelties are integrated into these conserved frameworks, we further analyzed phylostrata enrichment across major mantle cell types. Lineage-specific bursts of gene novelties were detected within SECs, along the spiralian phylogeny, especially within Autobranchia, Pteriomorphia, Ostreidae, and *Crassostrea* (Figure 5A [↗](#)). Therefore, the progressive integration of lineage-specific novelties of effector genes directly involved in shell formation, built upon conserved genetic programs from the spiralian ancestor, may shape the diversification of shell-forming cell types and the complexity of molluscan shells. Notably, we found multiple cell types, including myocytes, neurons, and proliferative cells, aligned between oyster and other spiralian species (Figure 4A-D [↗](#)). These conserved cell populations are likely to be homologous across Spiralia (Piovani et al. 2025 [↗](#)), as supported by the shared expression patterns of orthologous genes (Figure 5B [↗](#); Supplementary file 6 [↗](#)). Moreover, oyster SECs share six co-expressed genes with *P. leydi* chaetal cells and eight with flatworm secretory cells, and share over a hundred co-expressed genes with the chaetal and epithelial cells of *P. gotoi*, reflecting a deeply conserved spiralian secretory program that trace back to an ancestral origin. This represents the ancient genetic modules repeatedly redeployed across lineages to generate morphological novelties. Taken together, our findings support a model in which molluscan shell-forming cell types emerged through innovation within an ancestral spiralian epithelial or secretory cell-type framework, through the stepwise integration of lineage-specific effectors and regulatory factors.

Discussion

Spiralia is an extremely species-rich clade that diverged from the other major protostome lineage Ecdysozoa during the early Cambrian period, approximately 550 million years ago (Peterson et al. 2004 [↗](#)). Since then, spiralian have undergone remarkable diversification of body plans, developmental modes, and biomineralized structures. Many of these morphological novelties are characterized by lineage-specific hardened structures, including molluscan carbonate shells, annelid and chaetognath chaetae, and brachiopod calcium phosphate shells (Figure 5C [↗](#)), raising the question of whether they arose convergently or share deep cellular and regulatory origins (Piovani and Marlétaz 2023 [↗](#); Sleight 2023 [↗](#)). By resolving the cell-type composition responsible for oyster shell formation and integrating developmental, evolutionary and cross-species comparative analyses, our study reveals that molluscan shell formation represents a recent innovation within a conserved epithelial and secretory cell-type repertoire across Spiralia. Several SEC types (SEC2, SEC3, and SEC4) show strong transcriptional affinities to secretory or epithelial cell types in other spiralian, whereas SEC1 and SEC5 exhibit relatively weak cross-species alignments and deploy particularly young transcriptomes. This observation is consistent with the viewpoint that cellular novelties often emerge through the recombination or redeployment of conserved gene expression programs, while subsequently integrating rapidly evolving effector repertoires (Parker and Pennell 2025 [↗](#)). Pseudotime trajectory analyses identified SEC5 as a terminally differentiated population associated with rapidly evolving secretomes, further supporting that mantle shell-forming cells comprise a mosaic of ancestrally related secretory cell types and more recently derived effector cell types that have diversified within molluscs (Jackson et al. 2006 [↗](#); Kocot et al. 2016 [↗](#)). Distinct cellular zones within the mantle epithelium are dedicated to secreting specific organic matrices and controlling mineral polymorphs through differential gene expression (Herlitzte et al. 2018 [↗](#); Sleight et al. 2020 [↗](#); Shimizu et al. 2022 [↗](#); Bai et al. 2023 [↗](#)). The pronounced compartmentalization of SECs along the mantle epithelium suggests that region-specific modification of an ancestrally homogeneous epithelium enabled the emergence of distinct biomineralizing modules that pattern shell architecture.

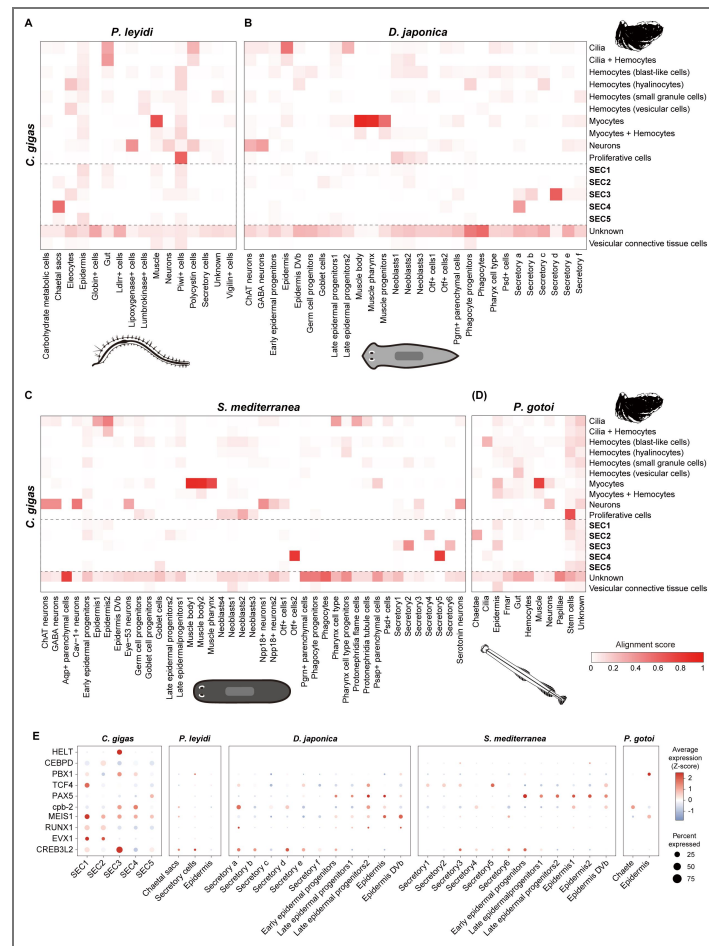


Figure 4. Cross-species cell-type alignment between adult *C. gigas* and other spiralian.

(A-D) Pairwise SAMap alignments between *C. gigas* and (A) the annelid *P. leidy*, (B) the flatworm *D. japonica*, (C) the flatworm *S. mediterranea*, and (D) the chaetognath *P. gotoi*. The scale bar represents the SAMap alignment score, defined as the average number of mutual nearest cross-species neighbors for each cell relative to the maximum possible number. Dashed boxes highlight the alignment with SECs of *C. gigas*. (E) Dot plots showing the expression of highly expressed TF genes in SECs, as well as their orthologous expression patterns in aligned cell types of other spiralian.

Similarly to adult mantle, the larval shell gland also exhibits a modular organization comprising distinct cell populations (Liu et al. 2020 [↗](#); Piovani et al. 2023 [↗](#)). However, no significant transcriptional correlation was observed between larval shell-gland cells and adult SECs, consistent with the previous report that larval and adult shells rely on largely distinct gene repertoires (Zhao et al. 2018 [↗](#)). Both larval and adult shell-forming systems process young transcriptomes enriched for lineage-specific genes, indicating that the evolution of larval and adult shell formation involved multiple, independent rounds of effector gene recruitment. These findings support a life-stage modularity model in which conserved regulatory modules are repeatedly combined with novel effector sets to generate stage-specific innovations within a single lineage (Bai et al. 2026 [↗](#)). A recent study reported that a stem cell niche within the adult scallop shells expressed several larval SMP genes and are involved in shell formation, which were identified through sequence homology with oyster SMPs (Lian et al. 2025 [↗](#)). Nonetheless, these SMP genes are highly expressed during the umbo larva stages and subsequent developmental stages in the oyster and scallop (Lian et al. 2025 [↗](#)), and may represent a subset of conserved proliferative or stem cell-related genes broadly shared among bivalves, rather than the complete suite of SMPs in scallop shells. It is likely that a large number of lineage-specific SMPs in scallops remain unidentified, exhibiting stage-specific expression patterns for larval and adult shell formation. Further in-depth studies across a broader range of molluscan species, integrating SMP identification by mass spectrometry and cell-type annotation with lineage-specific biomineralization genes, will be essential for resolving these lineage- and stage-specific biomineralization programs.

Molluscan shell-forming cells possess the genetic framework necessary for the secretory program of other hardened structures across Spiralia, supporting the existence of a conserved genetic toolkit involved in the secretion of hardened parts (Sun et al. 2020 [↗](#); Sun et al. 2021 [↗](#); Wernstrom et al. 2022 [↗](#); Barrera Grijalba et al. 2025 [↗](#)). Many TF genes highly expressed in SECs are also co-expressed in secretory, epithelial or chaetal cells of other spiralian, indicating that the regulatory backbone enabling hardened structures predates the appearance of biomineralized shells. Transcriptional homology is likely reflected in conserved regulatory architectures, such as core TF networks, cis-regulatory elements and signaling molecules, which provide a stable framework for the recruitment of effector genes and facilitate the emergence of novel pathways and cell-type specializations (Piovani and Marlétaz 2023 [↗](#); Sleight 2023 [↗](#); Li et al. 2024 [↗](#)). The diversification of SECs fits this paradigm, where conserved secretory programs were progressively modified through TF co-option and genetic innovation, giving rise to distinct biomineralizing cell populations with specialized function of shell formation. Thus, the genetic foundation for spiralian morphological innovations was already established in their LCA, which not only comprised conserved cell types such as muscle, neurons, and stem cells, but also provided the framework from which novel cell types subsequently diversified in each lineage (Figure 5C [↗](#)). Our findings suggested that morphological innovations arise through modifications of conserved cell-type architectures rather than *de novo* invention of entirely novel cellular systems. Furthermore, these innovations may be governed by the presence and activation of regulatory elements that control gene expression (Martin-Zamora et al. 2023 [↗](#); Parey et al. 2024 [↗](#); Li et al. 2025 [↗](#); Piovani et al. 2025 [↗](#)). Future integrative approaches such as single-cell ATAC-seq, by mapping open chromatin regions, cis-regulatory elements, and TF binding landscapes at cellular resolution, will enable cross-species reconstruction of regulatory networks and provide more definitive evidence for cell type homology and conserved regulatory programs underlying evolutionary innovations.

More broadly, an unresolved question is whether brachiopod shells and chaetae share the common evolutionary origins with the molluscan carbonate shells and annelid chitinous chaetae (Figure 5C [↗](#)). Although convergent evolution of effector gene repertoires is well documented in biomineralization (Jackson et al. 2010 [↗](#); Luo et al. 2015 [↗](#); Murdock 2020 [↗](#); Sleight 2023 [↗](#)), our results suggest that the spiralian hardened structures, including molluscan shells and chaetae in annelids and chaetognaths, may trace back to an ancestral secretory cell-type framework. Fossil and comparative evidences further indicated that brachiopod and molluscan skeletons repeatedly evolved through the co-option of organic, often chitinous, precursors rather than from a biomineralized common ancestor (Murdock 2020 [↗](#)). Nonetheless, future single-cell transcriptomic

studies of brachiopods will be crucial to determine whether these hardened structures are derived from conserved ancestral cell types or represent independent evolutionary acquisitions. Another related question concerns whether the ancestral mollusc possessed chaetae, as implied by the Cambrian fossil *Pelagiella exigua*, which potentially exhibited both chaetae and a mineralized shell (Thomas et al. 2020 [↗](#)). Although genomic resources from *Pelagiella* remain inaccessible, our findings indicate that extant molluscs retained components of the ancestral genetic architecture required for chaetal formation, which may have been secondarily co-opted, modified or lost during molluscan evolution. Together, these considerations highlight the need for integrative cellular and regulatory comparisons across spiralian phyla to reconstruct the ancestral toolkit from which diverse hardened structures evolved.

In summary, our study provides a cellular and evolutionary framework for understanding the interplay between ancient epithelial-secretory gene expression programs and lineage-specific effector diversification underlies the evolution of molluscan shells. By tracing the genetic and regulatory connections between molluscan shells and other hardened structures across Spiralia, our findings highlight a general principle that evolutionary novelties emerge from the combinations, redeployments, and modifications of deeply conserved cellular programs, rather than from the invention of entirely novel cellular substrates. These advances provide insights into reconstructing the ancestral spiralian cell-type repertoire, revealing hidden ancestral traits, and distinguishing which morphological features were inherited from the LCA of animals and which evolved multiple times independently.

Materials and methods

scRNA-seq library preparation and sequencing

The mantle tissue was collected from an adult oyster obtained from the oyster farm in Laizhou (Shandong Province, China) and washed three times with ice-cold 1×PBS at 4 °C. The cleaned tissue was cut into ~ 0.5 mm² fragments, washed again with 1×PBS, and digested on a shaker at 37 °C until complete dissociation. The suspension was filtered through a 40 μm strainer, and centrifuged at 300 g for 5 min at 4 °C. After removing the supernatant, cells were washed, centrifuged and resuspended with ice-cold 1×PBS. Cell concentration and viability were assessed using a cell counter, and the suspension was adjusted to 700-1200 cells/μL with >85% viability and <15% aggregation. Prepared cells were loaded onto the BMKMANU C1000 chip for single-cell library preparation.

A single-cell RNA-seq library was prepared using the BMKMANU DG1000 GEM Kit and Droplet Generation Equipment (BMKMANU) following the standard manufacturer protocol. In brief, single-cell suspensions were resuspended in PBS containing 0.04% BSA and loaded onto the BMKMANU C1000 Chip to generate single-cell gel beads in the emulsion (GEMs). Captured cells were lysed within individual GEMs, and released RNA was barcoded during reverse transcription. Amplified cDNA was used to construct the sequencing library, whose quality was assessed using Qubit 4.0 (Thermo Fisher Scientific) and Agilent 2100 (Agilent Technologies). Subsequently, the library was sequenced on the Illumina NovaSeq™ X Plus platform, yielding 150 bp paired-end reads.

Processing of scRNA-seq data

Raw sequencing data from the adult mantle tissue were processed with dealFQ v.1.3 (<http://www.biomarker.com.cn/zhizao/tools> [↗](#)) and then aligned to the *C. gigas* genome (GCF_902806645.1) (Penalosa et al. 2021 [↗](#)) using CellRanger v.9.0.1 (<https://github.com/10XGenomics/cellranger> [↗](#)). The raw count matrix was generated and imported into Seurat v.5.3.0 (Stuart et al. 2019 [↗](#)) for downstream analysis. Genes expressed in fewer than three cells and cells expressing fewer than 300 genes were removed. Low-quality cells were further filtered based on feature counts, total UMI counts, and mitochondrial gene percentage using median ± 3×MAD thresholds. Filtered data were normalized using SCTransform (Choudhary and Satija 2022 [↗](#)), and cell-cycle effects were regressed out using custom S-phase and G2/M-phase

gene sets, identified by best-reciprocal hits with MMseqs2 v.17.b804f (Steinegger and Söding 2017) between *C. gigas* and *Homo sapiens*. Dimensionality reduction was performed using PCA, followed by UMAP visualization based on the K-nearest-neighbor graph (Hao et al. 2024). Clustering was performed across multiple resolutions, an optimal resolution of 1.2 was selected based on Clustree analysis (Zappia and Oshlack 2018). Doublets were identified and removed using DoubletFinder (McGinnis et al. 2019). Following clustering, marker genes for each cell population were determined using the FindAllMarkers function in Seurat. Cell populations were annotated by integrating marker gene expression patterns with previously published *in situ* hybridization data (Takahashi et al. 2012; Foulon et al. 2018; Li et al. 2021; Zhu et al. 2021; Min et al. 2022; Bai et al. 2023; Bai et al. 2026; Ren et al. 2026) and single-cell transcriptomic datasets (Piovani et al. 2023; De La Forest Divonne et al. 2025) in oysters (Supplementary file 2). The R package Monocle3 v.1.4.26 (Cao et al. 2019) was used for pseudotime analysis to construct single-cell trajectories.

Embryonic and larval single-cell transcriptomes of *C. gigas* were obtained from published datasets (NCBI Bioproject accessions PRJNA909306 and PRJNA967338), and processed following the same general pipeline. After quality control and SCTransform normalization (Choudhary and Satija 2022), datasets from different developmental stages and biological replicates were integrated using Harmony to correct for batch effects (Korsunsky et al. 2019). Dimension reduction, clustering, and marker gene identification were performed as described above, with an optimal resolution of 2.0. Cell types were annotated by integrating marker gene expression with ISH and scRNA-seq data from previous studies (Liu et al. 2020; Li et al. 2021; Zhu et al. 2021; Piovani et al. 2023). To assess transcriptomic similarity between embryonic/larval and adult mantle cell types in *C. gigas*, we employed multiple complementary approaches, including Pearson and Spearman correlation of average gene expression, Jensen-Shannon divergence (JSD) to quantify overall expression distribution similarity, Jaccard indices to evaluate overlap of marker genes, and hypergeometric tests to determine statistical significance of shared marker gene sets.

Expression profiling of SMP genes

Larval and adult SMPs of *C. gigas* were identified by best-hit BLAST searches against the *C. gigas* genome database, using the previously reported sets of *C. gigas* SMPs (Zhao et al. 2018). Bulk RNA-seq datasets encompassing 37 developmental stages and 11 major tissues/organs of *C. gigas* were retrieved from published studies (Zhang et al. 2012; Lian et al. 2025). Raw reads were quality-filtered using fastp v.0.23.4 (Chen 2023) and gene expression (Transcripts per million, TPM) was quantified with salmon v.1.10.1 (Patro et al. 2017) using the reference transcript sequences. Expression matrices were normalized using the Trimmed Mean of M-values (TMM) method implemented in the Trinity utility scripts (Haas et al. 2013).

Functional annotation and enrichment analysis

Protein-coding genes of *C. gigas* were represented by their longest isoforms, and were functionally annotated using emapper v.2.1.12 against the eggNOG 5.0 database (Huerta-Cepas et al. 2019). GO enrichment analysis was performed using the R package clusterProfiler v.4.10.1 (Xu et al. 2024), against all expressed genes annotated by eggNOG database (Huerta-Cepas et al. 2019) as the background. GO terms with FDR-adjusted significance ($q < 0.01$) were considered statistically enriched.

Phylostratigraphy analysis

Gene age was estimated using the GenERA v.1.4.2 (Barrera-Redondo et al. 2023), which implements genomic phylostratigraphy (Domazet-Lošo et al. 2007) by searching homologs in the NCBI non-redundant (NR) database (Release 2024_01_01) and mapping them onto the NCBI Taxonomy to assign an evolutionary origin to each gene. To quantify evolutionary signatures in expression, we calculated the transcriptome age index (TAI) using the R package myTAI (Drost et al. 2018), based on log-transformed average expression per cluster obtained with the AverageExpression function of Seurat. Phylostrata enrichment in cell type-specific marker genes

was assessed with a hypergeometric test, comparing the observed distribution across phylostrata with that of the globally expressed gene set. The TAI contribution of each phylostratum for larval stages and adult tissues was calculated as previously described (Wang et al. 2020 [↗](#)).

Cross-species cell-type comparison

We re-analyzed the single-cell transcriptomes of the annelid worm *P. leidyi* (Alvarez-Campos et al. 2024 [↗](#)) and the chaetognath *P. gotoi* (Piovani et al. 2025 [↗](#)) using the same processing pipeline applied to the oyster mantle dataset. Pre-processed count matrices from the published study were retrieved for the flatworms *D. japonica* and *S. mediterranea* (Garcia-Castro et al. 2021 [↗](#)). Seurat objects were generated according to the original cell annotation.

SAMap v.1.0.15 (Tarashansky et al. 2021 [↗](#)) was applied between the *C. gigas* scRNA-seq dataset with datasets from *P. leidyi* (Alvarez-Campos et al. 2024 [↗](#)), *D. japonica* (Garcia-Castro et al. 2021 [↗](#)), *S. mediterranea* (Garcia-Castro et al. 2021 [↗](#)), and *P. gotoi* (Piovani et al. 2025 [↗](#)). Count matrices exported from Seurat in h5ad format were loaded into a SAMap object, and a BLAST-based homology graph was generated. Alignment scores between cell types were computed using the `get_mapping_scores` function, and conserved gene pairs were identified with GenePairFinder using a threshold of 0.10.

Figure supplements

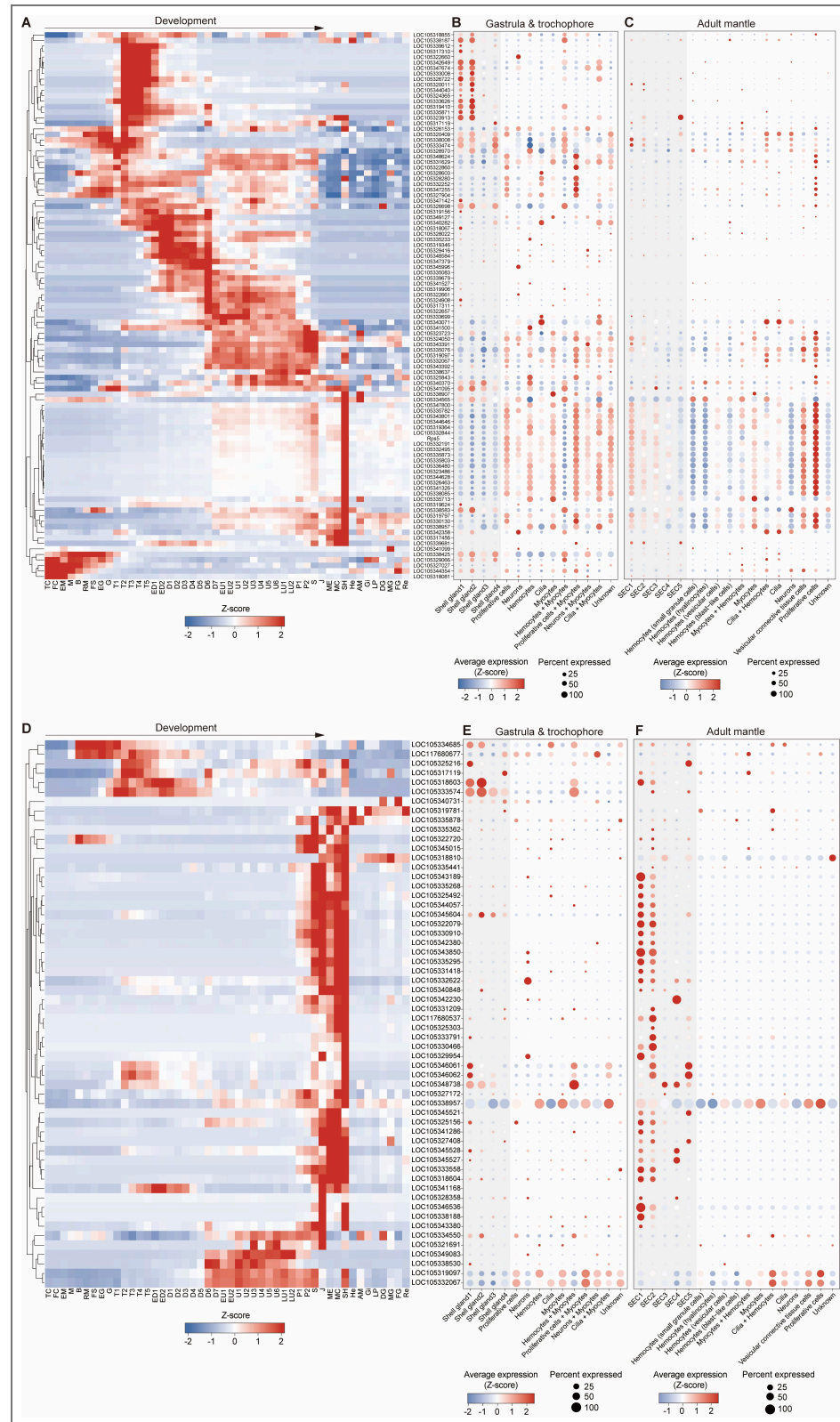


Figure 2-figure supplement 1. Expression patterns of larval and adult shell matrix protein (SMP) genes in *C. gigas*. (A, D) Heatmaps showing the expression dynamics of larval (A) and adult (D) SMP genes across developmental stages and adult tissues. (B, E) Dot plots showing the expression of larval (B) and adult (E) SMP genes in each cell type from single-cell transcriptomic data of the adult mantle. Shell-forming cell types are

highlighted with gray backgrounds. (C, F) Dot plots showing the expression of larval (C) and adult (F) SMP genes in each cell type from single-cell transcriptomic data of the gastrula and trochophore stages. Shell-forming cell types are highlighted with gray backgrounds. Abbreviations: TC, two-cell; FC, four-cell; EM, early morula; M, morula; B, blastula; RM, rotary movement; FS, free swimming; EG, early gastrula; G, gastrula; T1-T5, trochophore stages; ED1-ED2, early D-shape larva stages; D1-D7, D-shape larva stages; EU1-EU2, early umbo stages; U1-U6, umbo stages; LU1-LU2, late umbo stages; P1-P2, pediveliger stages; S, spat; J, juvenile; ME, mantle edge; MC, mantle center; SH, shell; He, hemolymph; AM, adductor muscle; Gi, gill; LP, labial palp; DG, digestive gland; MG, male gonad; FG, female gonad; Re, remaining tissues; SEC: Shell-secreting epithelial cells.

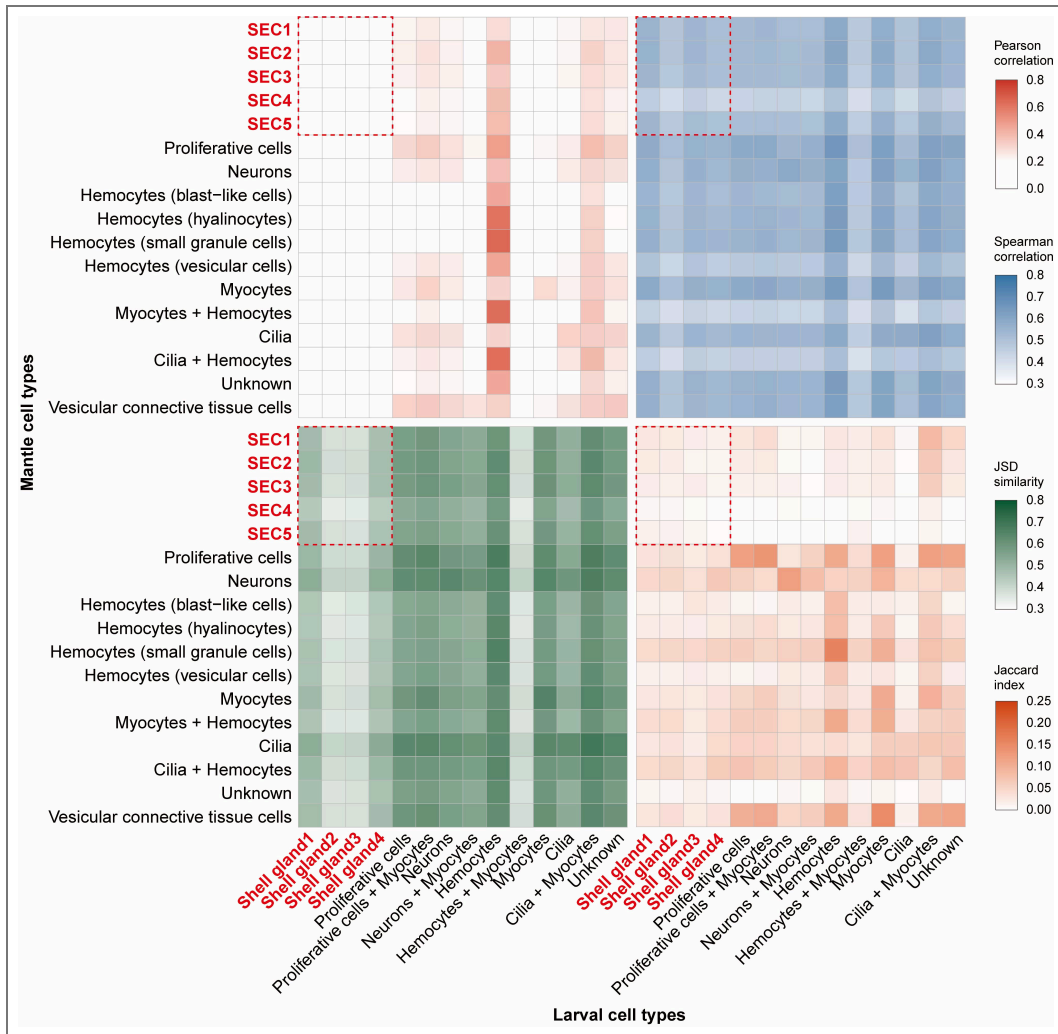


Figure 2-figure supplement 2. Pairwise comparisons of cell types between larval (gastrula and trochophore) and adult (mantle) single-cell transcriptomes based on Pearson correlation (upper left), Spearman correlation (upper right), Jensen-Shannon distance (JSD) (lower left), and Jaccard index (lower right).

Shell-forming cell types are marked in red. Red dashed boxes indicate the comparisons between larval and adult shell-forming cell types. Abbreviations: SEC, shell-secreting epithelial cells.

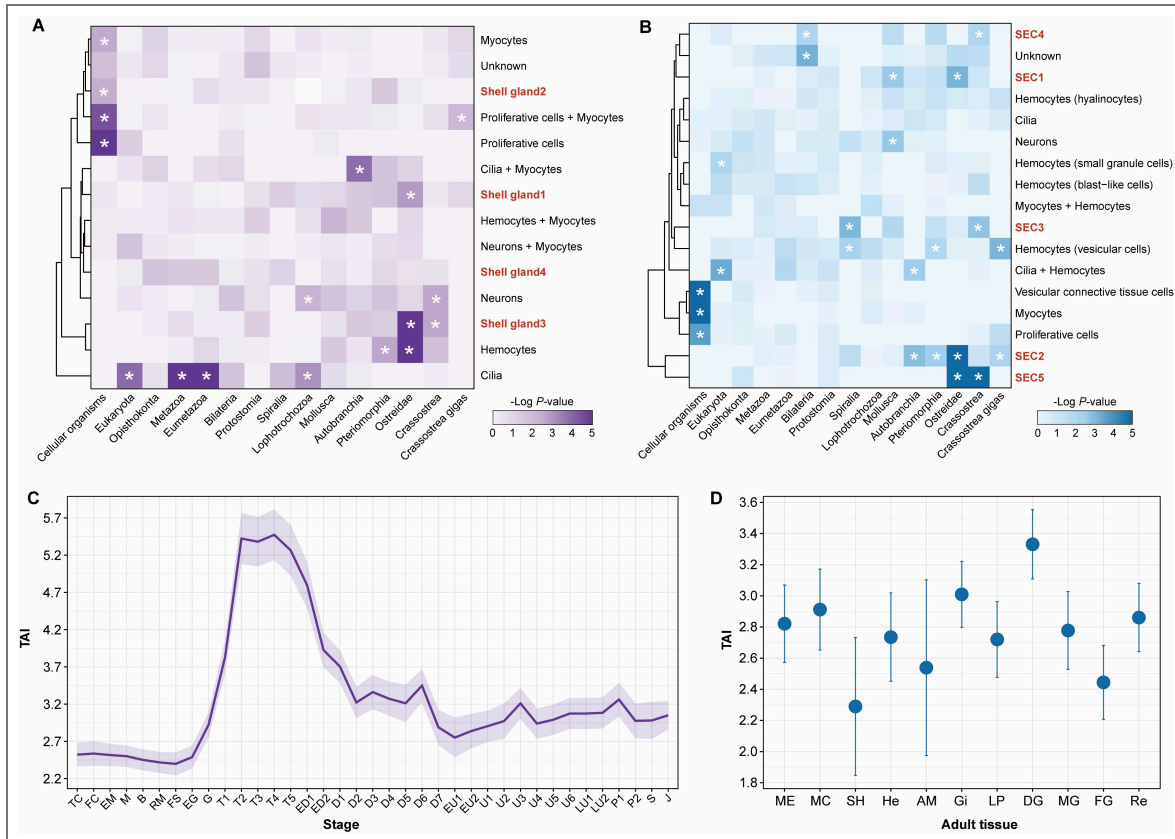


Figure 3-figure supplement 1. Gene age analyses for different developmental stages and adult tissues of *C. gigas*.

(A, B) Hypergeometric enrichment of genes that originated at successive phylostrata in larval (A) and adult (B) cell types of oysters. Shell-forming cell types are marked in red. Asterisks indicate significant enrichment ($P < 0.01$). (C, D) Transcriptome Age Index (TAI) across oyster ontogeny (C) and adult tissues (D), computed using published bulk transcriptomes. Shaded areas indicate \pm standard error. Abbreviations are as in Figure 2-figure supplement 2.

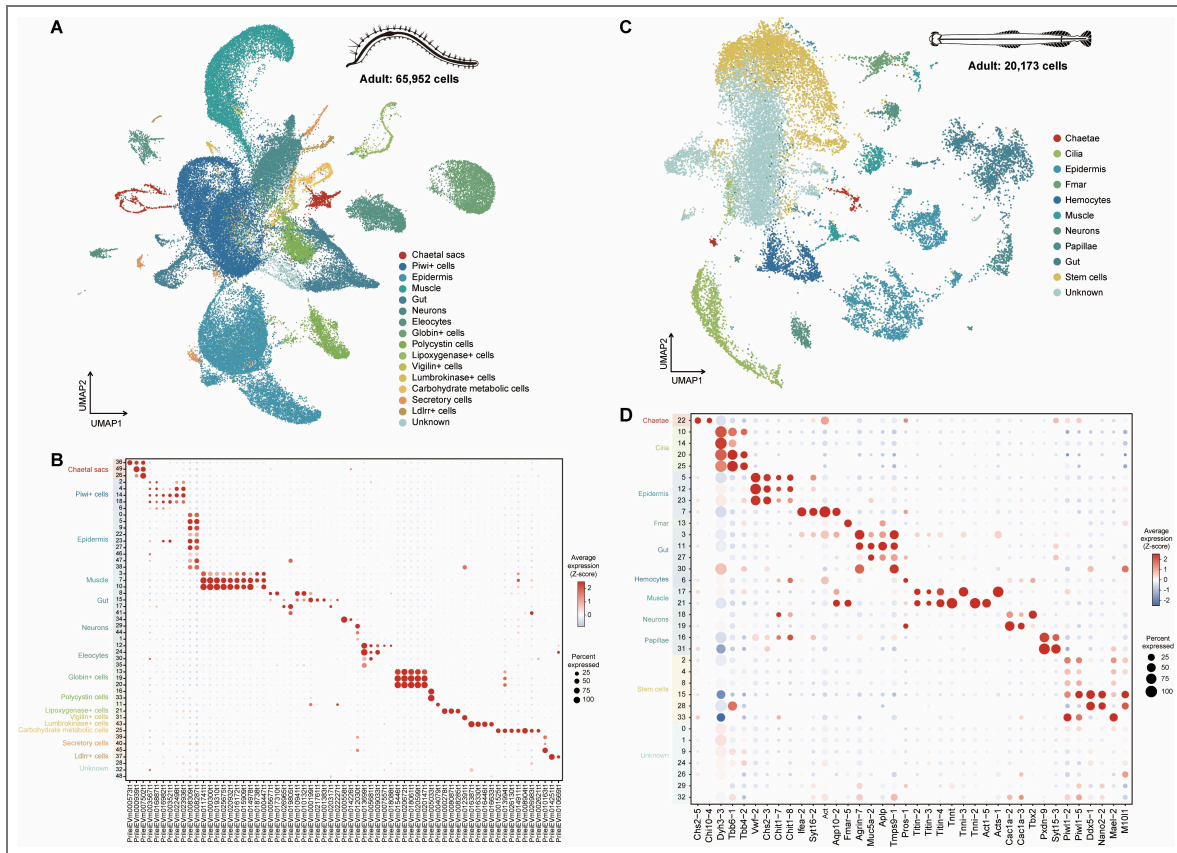


Figure 4-figure supplement 1. Re-analyses of cell atlases of two spiralian species.

(A, C) Two-dimensional uniform manifold approximation and projection (UMAP) visualizations of cell clusters in the annelid worm *P. leidy* (A) and the chaetognath *P. gotoi* (C). (B, D) Dot plots showing the expression of selected cell-type marker genes for each cluster in *P. leidy* (B) and *P. gotoi* (D).

Data availability

The scRNA-seq data generated in the current study have been deposited in the NCBI database under BioProject accession number PRJNA1338802. All original code has been deposited on GitHub at <https://github.com/StevenBai97/Shell-cell-type-innovations>.

Acknowledgements

This work was supported by National Natural Science Foundation of China (W2411019), Blue Seed Industry Innovation Project of Qingdao Institute of Blue Seed Industry (QDLYY-2024001), and Shandong Province (2025LZGC036).

Additional information

Author contributions

Yitian Bai, Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft; Kunyin Jiang, Sample collection, Data generation; Hong Yu, Resources, Methodology; Lingfeng Kong, Resources, Methodology; Shaojun Du, Funding acquisition, Writing – review and editing; Shikai Liu, Conceptualization, Supervision, Resources, Writing – review and editing; Qi Li, Conceptualization, Supervision, Funding acquisition, Resources, Writing – review and editing.

Funding

Funder	Grant reference number	Author
MOST National Natural Science Foundation of China (NSFC)	W2411019	Qi Li

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Additional files

Supplementary file 1. [↗](#) The proportion of cell clusters in adult mantle of *C. gigas*.

Supplementary file 2. [↗](#) Marker genes for cell type annotation in larval (gastrula and trochophore) and adult (mantle) stages of *C. gigas*.

Supplementary file 3. [↗](#) Proportions of cell types in gastrula and trochophore stages of *C. gigas*.

Supplementary file 4. [↗](#) Pairwise counts of overlapping marker gene between larval (gastrula and trochophore) and adult (mantle) cell types. Numbers in parentheses are *P* values from a hypergeometric test. Cells with a red background indicate significant correlation ($P < 0.01$).

Supplementary file 5. [↗](#) GO enrichment analyses of marker genes in the shell gland and SEC cell types of *C. gigas* (FDR-adjusted significance, q value < 0.01).

Supplementary file 6. [↗](#) Cross-species co-expressed genes between similar cell types of *C. gigas* and other spiralian.

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Peer reviews

Reviewer #1 (Public review):

Summary:

This manuscript examines the evolution of molluscan shells using single-cell analyses of the adult mantle of *Crassostrea gigas* and compares these data with previous datasets from embryonic and larval stages of this species and other spiralian. The authors provide support for a scenario in which secretory cells are broadly conserved across spiralian, and the incorporation of lineage-restricted genes contributes to the evolution of molluscan shells.

Strengths:

High-quality datasets for mantle tissue in *Crassostrea gigas* and thorough comparisons with existing datasets for this species and other spiralian. Balanced discussion.

Weaknesses:

No major weaknesses. The analyses follow fairly standard approaches in the field that have been previously applied and developed in similar systems.

<https://doi.org/10.7554/eLife.111168.1.sa3>

Reviewer #2 (Public review):

Summary:

Bai *et al.* present in their study three single-cell RNA seq datasets derived from gastrulae, trochophores, and adults of the bivalve *Crassostrea gigas*. While a dataset on the oyster trochophore has already been published previously (Piovani *et al.* 2023), the gastrula and adult datasets have not been published yet. The authors conclude that cell types secreting the oyster shell valves use a genetic repertoire that is also used by epithelial and secretory cell types of very different spiralian, such as annelids, chaetognaths and flatworms.

Strengths:

The study provides new single-cell datasets from multiple developmental stages of an oyster, offering a valuable resource for the field. It takes a broad comparative approach using state-of-the-art techniques across diverse animal groups and addresses an important question regarding the origin and evolution of shell-forming cell types.

Weaknesses & suggestions to improve the manuscript:

(1) Validation of cell types

Cell type identities are not convincingly validated. Although the authors cite previous studies (l. 92), the referenced marker genes are largely not used, and the cited works do not provide sufficient spatial validation. Without *in situ* data, the inferred locations of cell types (e.g. Figure 2A) are not supported. Spatial validation of marker genes (e.g. via HCR) is essential,

particularly for a study addressing shell field evolution. In addition, the gastrula dataset is not meaningfully analyzed, and its inclusion remains unclear.

(2) Robustness of cell type classification

Several proposed cell types may not represent distinct entities (not individuated) but rather reflect over-clustering. Marker genes are often not specific and are shared across clusters (e.g. Sec1/Sec2), making it difficult to distinguish cell types reliably.

(3) Comparative analysis of secretory cells

The comparative framework is not sufficiently supported. Secretory cells are highly diverse, and without proper validation, their comparison across taxa is not meaningful. The transcription factor analysis is limited, as only a few genes are shared and many are inconsistently expressed (Figure 3E). The conclusion of a conserved regulatory program across spiralian is therefore overstated.

(4) Clarity and interpretation of results

Results are at times difficult to follow and remain superficial. Marker genes are insufficiently annotated (especially for *Crassostrea*), and comparisons across taxa lack functional interpretation. Unvalidated and heterogeneous cell types are grouped together, and transcriptional similarities are overinterpreted. Overall, key conclusions are not adequately supported by the presented data.

<https://doi.org/10.7554/eLife.111168.1.sa2>

Reviewer #3 (Public review):

Summary:

This manuscript by Bai et al. reports single-cell transcriptomics of the oyster mantle to elucidate the respective contributions of ancient conserved programmes and lineage-specific genes to the origin of the molluscan shell. The authors compare their dataset with other oyster larval datasets as well as data from other organisms (annelids, chaetognaths) and find evidence of evolutionary conservation and functional similarity with secretory cell types. They also observe that cells involved in secreting the larval skeleton express predominantly recent genes, whereas the adult skeleton-secreting programme is evolutionarily more conserved.

Strengths:

The manuscript is well written and clearly presented, and the results are interesting, particularly the distinction between larval and adult skeleton secretion, which is placed in a thoughtful evolutionary context.

Weaknesses:

(1) My main concern is that the authors rely primarily on previous studies for the experimental and functional characterisation of the identified cell types. The cited papers (Piovani, 2023 and de la Forest Divonne et al., 2025) deal with distinct stages or tissues (larvae and hemocytes, respectively), which limits their direct relevance. The authors also cite other papers for in situ expression data; it would be helpful to summarise somewhere (e.g. in a table) which genes have been experimentally characterised and what their expression domains are, or alternatively to provide HCR or in situ staining on the mantle. For instance, what is the rationale for the claim that proliferative cells give rise to the mantle? The trajectory inference approach used (Monocle) would likely yield a similar result regardless of the reference cell type, so additional justification is needed.

(2) More broadly, I find that the functional properties of the identified cell types and their relationship to the expressed genes deserve more detailed discussion. For example, at L100, several genes are mentioned, but their functional roles are not discussed. Similarly, the basis for annotating the proliferative cells is not explained. How was gene orthology assessed? Throughout the manuscript, vertebrate-style gene names are used without explicitly establishing orthology status in oyster, which should be addressed.

(3) More detail is needed on the methods and quality control for the single-cell data. The authors should clarify that the platform used (BMKMANU) is a droplet-based technology comparable in principle to Drop-seq. BMKMANU is not widely used in the field. How does it compare to 10x Genomics in terms of sensitivity and cell recovery? The authors appear to use the 10x Chromium cellranger pipeline for data analysis, which suggests compatibility, but this should be stated explicitly. Additionally, no information is provided on the number of sequencing runs or biological replicates, nor on how reproducible the results are across samples.

(4) A limitation of the phylostratigraphic analysis is that it is restricted to mantle tissue, making it difficult to place the results in a whole-organism context. How do the age profiles of mantle-expressed genes compare to those of more evolutionarily conserved tissues, such as the nervous system? I appreciate the methodological and experimental constraints, but this is a genuine limitation of the study. The authors could at least discuss it explicitly, and ideally consider generating a broader single-cell atlas of the oyster to provide this comparative baseline.

(5) Have the authors considered the potential importance of lineage-specific gene duplication? It is well established that spiralian, including oysters, have undergone extensive lineage-specific duplication of transcription factors such as homeobox genes, and many structural shell-associated proteins may similarly have been duplicated. This could be relevant to interpreting both the phylostratigraphic results and the expansion of secretory gene families.

<https://doi.org/10.7554/eLife.111168.1.sa1>

Author response:

Public Reviews:

Reviewer #1 (Public review):

Summary:

*This manuscript examines the evolution of molluscan shells using single-cell analyses of the adult mantle of *Crassostrea gigas* and compares these data with previous datasets from embryonic and larval stages of this species and other spiralian. The authors provide support for a scenario in which secretory cells are broadly conserved across*

spiralian, and the incorporation of lineage-restricted genes contributes to the evolution of molluscan shells.

Strengths:

*High-quality datasets for mantle tissue in *Crassostrea gigas* and thorough comparisons with existing datasets for this species and other spiralian. Balanced discussion.*

Weaknesses:

No major weaknesses. The analyses follow fairly standard approaches in the field that have been previously applied and developed in similar systems.

We thank the reviewer for the positive evaluation of our work. We are encouraged that the reviewer finds our conclusions balanced and the analyses appropriate. Although no major concerns were raised, we will incorporate clarifications and improvements prompted by the other reviewers to further strengthen the manuscript.

Reviewer #2 (Public review):

Weaknesses:

(1) Validation of cell types

Cell type identities are not convincingly validated. Although the authors cite previous studies (l. 92), the referenced marker genes are largely not used, and the cited works do not provide sufficient spatial validation. Without in situ data, the inferred locations of cell types (e.g. Figure 2A) are not supported. Spatial validation of marker genes (e.g. via HCR) is essential, particularly for a study addressing shell field evolution. In addition, the gastrula dataset is not meaningfully analyzed, and its inclusion remains unclear.

We thank the reviewer for this important comment regarding cell type validation. In the previous version of the manuscript, we provided a detailed compilation of referenced marker genes from previous studies in Supplementary File 2. It is possible that, due to an incorrect or unclear reference in the main text, this information was not readily accessible. We will correct and clarify these citations in the revised manuscript to ensure that these resources are clearly presented.

We agree that spatial validation would provide important support for cell type identities. In the revised version, we will strengthen this aspect by selecting more specific marker genes for each SEC cluster and performing fluorescence in situ hybridisation (FISH) to validate their spatial localization.

Regarding the gastrula dataset, our original intention was to investigate the developmental transition of shell gland-related cell populations from gastrula to trochophore stages. However, following the reviewer's suggestion and considering the limited interpretability of the gastrula dataset in its current form, we agree that its inclusion does not substantially strengthen the study. We therefore plan to remove the gastrula dataset from the revised manuscript, and instead focus on the trochophore stage as a representative developmental stage for larval shell formation, enabling a clearer comparison between larval and adult shell-forming cell populations. We note that this change does not affect the main conclusions of the study. In addition, we will curate a refined set of experimentally supported marker genes, and provide an updated supplementary table summarizing detailed information, including cell type annotations, literature sources, and experimental validation methods.

(2) Robustness of cell type classification

Several proposed cell types may not represent distinct entities (not individuated) but rather reflect over-clustering. Marker genes are often not specific and are shared across clusters (e.g. Sec1/Sec2), making it difficult to distinguish cell types reliably.

In the revised manuscript, we will refine marker gene selection by prioritizing genes with higher specificity and stronger discriminatory power to improve the robustness of cell type identification. To further support cell identity assignment, we will select representative marker genes for SEC clusters and perform FISH to validate their spatial localization. These revisions will lead to a more robust and conservative interpretation of cell populations.

(3) Comparative analysis of secretory cells

The comparative framework is not sufficiently supported. Secretory cells are highly diverse, and without proper validation, their comparison across taxa is not meaningful. The transcription factor analysis is limited, as only a few genes are shared and many are inconsistently expressed (Figure 3E). The conclusion of a conserved regulatory program across spiralian is therefore overstated.

We agree that secretory cell types are highly diverse across spiralian and that cross-species comparisons require careful interpretation. In the revised manuscript, we will adopt a more cautious framework, highlight partial conservation of regulatory program alongside functional convergence in secretory processes. We also will strengthen the comparative framework by integrating functional annotations, which may provide complementary support beyond individual gene overlaps. Importantly, we will improve the reliability of oyster SEC annotations through FISH-based spatial validation, thereby increasing confidence in cross-species comparisons. These revisions will provide a more balanced and biologically grounded interpretation of secretory cell evolution across spiralian.

(4) Clarity and interpretation of results

Results are at times difficult to follow and remain superficial. Marker genes are insufficiently annotated (especially for Crassostrea), and comparisons across taxa lack functional interpretation. Unvalidated and heterogeneous cell types are grouped together, and transcriptional similarities are overinterpreted. Overall, key conclusions are not adequately supported by the presented data.

In the revised manuscript, we will re-evaluate marker gene annotations to ensure support from existing experimental evidence. For SEC populations, we will validate representative markers using FISH. We will also expand the functional annotation of marker genes and strengthen cross-species comparisons. In addition, we will substantially revise the Results and Discussion sections to improve clarity and depth, reduce overinterpretation of transcriptional similarities, and ensure that all conclusions are more tightly aligned with the strength of the supporting evidence.

Reviewer #3 (Public review):

Weaknesses:

(1) My main concern is that the authors rely primarily on previous studies for the experimental and functional characterisation of the identified cell types. The cited papers (Piovani, 2023 and de la Forest Divonne et al., 2025) deal with distinct stages or tissues (larvae and hemocytes, respectively), which limits their direct relevance. The authors also cite other papers for in situ expression data; it would be helpful to summarise somewhere (e.g. in a table) which genes have been experimentally characterised and what their expression domains are, or alternatively to provide HCR or in situ staining on the mantle. For instance, what is the rationale for the claim that proliferative cells give

rise to the mantle? The trajectory inference approach used (Monocle) would likely yield a similar result regardless of the reference cell type, so additional justification is needed.

We agree that our reliance on previous studies for functional and experimental characterization requires clearer justification and integration. In the revised manuscript, we will compile a new supplementary table summarizing marker genes with available experimental validation, including their associated cell types, literature sources, and experimental methods. For SEC populations, we will select representative marker genes and perform FISH to validate their spatial localization, thereby providing independent support for cell identity.

Regarding trajectory inference, we agree that methods such as Monocle are sensitive to assumptions. We will clarify the rationale for root cell selection, test alternative root assignments to assess robustness, and revise our interpretation to avoid strong lineage claims. Rather than stating that proliferative cells give rise to mantle cells, we will describe the observed trajectory as being consistent with a potential developmental relationship, while acknowledging that this does not constitute direct evidence of lineage progression.

(2) More broadly, I find that the functional properties of the identified cell types and their relationship to the expressed genes deserve more detailed discussion. For example, at L100, several genes are mentioned, but their functional roles are not discussed. Similarly, the basis for annotating the proliferative cells is not explained. How was gene orthology assessed? Throughout the manuscript, vertebrate-style gene names are used without explicitly establishing orthology status in oyster, which should be addressed.

We thank the reviewer for this important comment. In the revised manuscript, we will expand the functional interpretation of key genes by incorporating available literature and, where possible, functional annotations. We will also clarify the basis for cell type annotation and explicitly describe the criteria used, including for proliferative cell populations (e.g. cell proliferation-associated markers).

Regarding gene annotation, gene names in oyster were assigned based on sequence similarity searches against the eggNOG database. In the revised manuscript, we will provide a comprehensive supplementary table linking gene IDs to their annotations, along with the corresponding database sources. In addition, we will clearly describe how orthology relationships were assessed, including the methods and criteria used (e.g. sequence similarity searches and orthology databases). Throughout the revised manuscript, we will ensure that the use of vertebrate-style gene names is accompanied by appropriate annotation information and does not imply unsupported one-to-one orthology relationships.

(3) More detail is needed on the methods and quality control for the single-cell data. The authors should clarify that the platform used (BMKMANU) is a droplet-based technology comparable in principle to Drop-seq. BMKMANU is not widely used in the field. How does it compare to 10x Genomics in terms of sensitivity and cell recovery? The authors appear to use the 10x Chromium cellranger pipeline for data analysis, which suggests compatibility, but this should be stated explicitly. Additionally, no information is provided on the number of sequencing runs or biological replicates, nor on how reproducible the results are across samples.

In the revised manuscript, we will expand the Methods section to provide a clearer and more detailed description of the experimental and analytical procedures. BMKMANU is a droplet-based single-cell RNA-seq platform, conceptually comparable to Drop-seq and similar in principle to 10x Chromium. We will also explicitly state that the data generated are compatible with the Cell Ranger pipeline, which was used for downstream processing and analysis. Although BMKMANU is less widely used than 10x Genomics platforms, it has been successfully applied in several recent studies (e.g. Li et al., 2024:

<https://doi.org/10.1007/s11427-023-2548-3>; <https://doi.org/10.1038/s41559-025-02642-6>; <https://doi.org/10.1038/s41467-024-46780-0>), demonstrating its applicability for single-cell transcriptomic analyses across different biological systems. Regarding platform performance, based on technical information provided by the manufacturer, BMKMANU shows comparable sensitivity and cell capture efficiency to 10x Genomics platforms (<http://www.biomarker.com.cn/zhizao/dg1000danxibao>). In this study, the mantle sample was obtained from a single individual oyster and processed in a single sequencing run, without batch effects introduced by multiple runs. We will clearly state this in the revised manuscript. In addition, we will provide detailed quality control metrics, including the number of cells retained, gene detection rates, and filtering criteria.

(4) A limitation of the phylostratigraphic analysis is that it is restricted to mantle tissue, making it difficult to place the results in a whole-organism context. How do the age profiles of mantle-expressed genes compare to those of more evolutionarily conserved tissues, such as the nervous system? I appreciate the methodological and experimental constraints, but this is a genuine limitation of the study. The authors could at least discuss it explicitly, and ideally consider generating a broader single-cell atlas of the oyster to provide this comparative baseline.

We agree that restricting the phylostratigraphic analysis to mantle tissue represents a limitation when attempting to place our findings in a whole-organism evolutionary context. In the revised manuscript, we will explicitly acknowledge this limitation and expand the Discussion to address how gene age profiles in mantle tissue may differ from those in more evolutionarily conserved tissues. In particular, we will clarify that the enrichment of younger, lineage-specific genes observed in shell-forming cells may reflect tissue-specific functional specialization, and therefore should not be directly generalized to other cell types.

We acknowledge that a broader single-cell atlas spanning multiple tissues would provide an important comparative baseline for interpreting gene age patterns across the organism. While generating such a dataset is beyond the scope of the present study, we will highlight this as an important direction for future research.

(5) Have the authors considered the potential importance of lineage-specific gene duplication? It is well established that spiralian, including oysters, have undergone extensive lineage-specific duplication of transcription factors such as homeobox genes, and many structural shell-associated proteins may similarly have been duplicated. This could be relevant to interpreting both the phylostratigraphic results and the expansion of secretory gene families.

We thank the reviewer for this insightful suggestion. Lineage-specific gene duplication is likely to play an important role in shaping both transcription factor repertoires and shell-associated gene families in spiralian, including oysters. In the revised manuscript, we will incorporate a discussion of lineage-specific duplication, particularly in relation to transcription factors and biomineralization-related proteins. We will also, where feasible, explore its potential contribution to our observations and highlight how such duplications may drive the expansion and diversification of secretory gene families.

<https://doi.org/10.7554/eLife.111168.1.sa0>