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The Embryonic Origin of Hydractinia I-Cell Development

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Abstract. While pluripotent stem cells, called i-cells, in Hydractinia play a critical role in regeneration and evolutionary biology, their embryonic origin remains unresolved. This study aims to trace i-cells back to specific progenitor cells and define their developmental trajectory within the embryo. Fate-mapping is combined with fluorescent in situ hybridization targeting Piwi mRNA to spatially localize i-cell emergence. Single-cell multi-omics Parallel-seq complements these data by profiling transcriptional and chromatin dynamics across embryogenesis. It is expected that Piwi expression will overlap with a specific labelled lineage, and through integrated analysis, the developmental path from progenitors to i-cells can be reconstructed, revealing key regulators of pluripotency.

Keywords: hydractinia, pluripotency, stem cell, i-cell, regeneration

1. Introduction: the unclear embryonic origin of pluripotent stem cells in hydractinia

1.1. Hydractinia is key models for regenerative biology

Cnidarians (corals, sea anemones, jellyfish, etc.) offer a unique perspective for animal evolution due to their complex genomes, regenerative capabilities, and sister-group relationships with bilaterally symmetrical animals [1]. Among cnidarians, hydractinia is a representative model animal for studying regeneration [2]. Their stem cells, called interstitial cells (i-cells), are pluripotent [3]. I-cells are marked by high levels of Piwi1, Piwi2, and Soxb1 expression [4]. They can differentiate into germ cells and somatic cells [3], and their already differentiated cells can be induced by aging factors and reprogrammed into i-cells [5]. Hydractinia symbiolongicarpus have been proven to have lifelong pluripotency. Their single adult i-cell can self-renewed and develop into a complete individual, so that they are able to carry out regenerative clonal growth like plants [3].

1.2. Unresolved questions and hypothesis regarding the embryonic origin of I-cells

The embryonic development of Hydractinia progresses through distinct stages: symmetric cleavages form a transient 16-cell blastula by ~2 h.p.f., gastrulation (completing before 20 h.p.f. according to Kraus et al., as evidenced by the end of protointestinal activity), and finally the formation of the planula larva between 24 and 72 h.p.f. The timeline of Hydractinia embryo development is illustrated in Figure 1. Unlike other animals with pluripotent stem cells, hydractinia only form ectoderm and endoderm in gastrula stage [6], which makes the developmental origin of i cells still unknown. So far, no research has clearly revealed the earliest population of I-cell progenitor cells in

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hydractinia embryogenesis. Some studies have found that i cells first appear in the endoderm of the embryo in the later stage of the blastoderm, and then migrate to the epidermis during metamorphosis, and persist in the adult [7]. However, the developmental pathways of these i cells before they differentiate into i cells remain unknown. Varley et al. proposed two hypotheses regarding the embryonic origin of I-cells: they might directly originate from pluripotent blastomeres and not attach to any germ layer during the formation of the progut embryo. Alternatively, they may originate from fixed endodermal cells and secondary acquisition of pluripotency [3].

1.3. Proposed experimental strategy and research value

To experimentally verify the embryonic origin of i-cells, this study will focus on the period from the 8-cell stage (approximately 1-2 h.p.f) to the 20 h.p.f stage before the emergence of I-cells. The cell development path will be visualized through fate mapping [8] combined with fluorescent in situ hybridization (FISH) and single-cell sequencing methods, as well as methods such as canonical correlation analysis (CCA) and Uniform Manifold Approximation and Projection (UMAP). This approach aims to trace i-cells back to a small group of specific precursor cells in the early stage of embryonic development. This will help further explore the development mechanism of pluripotent stem cells and understand the origin of this characteristic of pluripotent stem cells in the process of animal evolution [9].

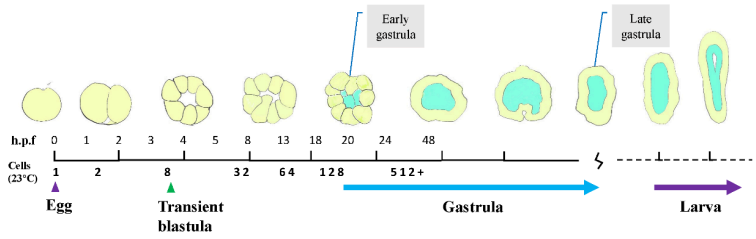


Figure 1. The timeline of hydractinia embryo development

The morphological stages corresponding to hydractinia embryos from 0 hours after fertilization (h.p.f) to 48 h.p.f.

2. Project description

This study aims to trace the embryonic origin of i-cells in Hydractinia. Fate-mapping is combined with fluorescent in situ hybridization to trace lineage origins and detect initial Piwi expression, alongside parallel single-nucleus scRNA-seq and scATAC-seq to profile transcriptomic and chromatin accessibility dynamics during embryonic development. Dimensionality reduction and trajectory inference methods such as Canonical Correlation Analysis, Uniform Manifold Approximation and Projection, and Slingshot/ Partition-based Graph Abstraction will reconstruct i-cell differentiation paths. It is hypothesized that i-cells derive from endoderm-committed progenitors at early gastrula, regulated by Wnt, Notch and other undiscovered signaling pathways. This work will elucidate the developmental mechanism of pluripotency in cnidarians, offering insights into the evolutionary origins of regenerative stem cells. The experimental workflow is summarized in Figure 2.

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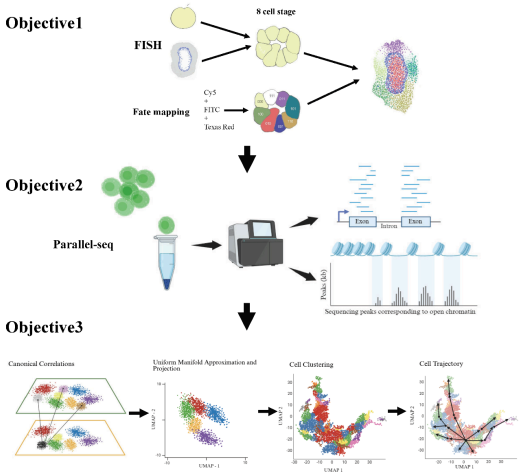


Figure 2. Experimental workflow for deciphering cell fate decisions in hydractinia embryogenesis

3. Objective1: location the origin of I-cells by fate-mapping and fluorescence in situ hybridization

3.1. Rationale

Hydractinia embryos enter the initial stage of gastrulation at 2 hours post-fertilization (h.p.f.) (8-cell stage), a time when i-cells are not yet present. By using fate-mapping to track the progeny of each cell, combined with fluorescence in situ hybridization (FISH) to locate the emergence of i-cells, the origin of i-cells can be traced back to specific cells in the early stages of embryonic development.

3.2. Method

3.2.1. Fate-mapping-constructing a cell lineage map via dye labeling

Dextran-conjugated dyes are inert, highly fluorescent molecules that stably persist in the cytoplasm [10]. In table 1, Three spectrally distinguishable dyes (green FITC, red Texas Red, and far-red Cy5) will be used in a coding scheme. A unique combination of these three dyes will be injected into each cell to label all 8 cells of the embryo. Several healthy embryos of Hydractinia symbiolongicarpus will be collected at the 8-cell stage. (Since dye injection may affect embryonic development, to ensure result reliability, embryos will be divided into experimental and control groups. Control groups will include uninjected and sham-injected embryos. Each group will have a sufficient number of embryos.) Using micromanipulation techniques, the eight distinct dye combinations will be injected into the cytoplasm of the eight cells individually. The injected embryos will be transferred to fresh culture medium and continue to develop under appropriate conditions [11,12]. The dye will be distributed to daughter cells upon cell division. Using confocal microscopy, the location, migration path, and ultimate tissue contribution of the progeny of each cell can be tracked throughout gastrulation.

3.2.2. Determining the emergence of I-cells via Fluorescence in Situ Hybridization (FISH)

FISH is a molecular biology technique used to detect the presence, distribution, and relative abundance of specific nucleic acid sequences in their original location within tissues, cells, or chromosomes [13]. Specific probes will be designed targeting the 3' UTR region of the Hydractinia

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Piwi gene (this region is often conserved and specific). Gene data was obtained from Schnitzler et al., 2024 and the Hydractinia Genome Project Portal (https://research.nhgri.nih.gov/hydractinia/). Templates will be prepared via PCR, followed by in vitro transcription to synthesize Digoxigenin (DIG)-labeled RNA probes. These fluorescently labeled, known-sequence, single-stranded nucleic acid probes will hybridize with complementary nucleic acid sequences in the sample, forming stable duplex structures. To verify the reliability of the experimental results, negative and positive controls will be included. The negative control will use a sense strand probe transcribed from the same Piwi gene template. Because the sense strand probe sequence is identical to the mRNA in the sample, it should not bind specifically; the absence of signal will confirm the experiment can proceed. The positive control will use the designed Piwi probe to detect tissue from adult Hydractinia. I-cells are enriched in adult Hydractinia, so observing strong positive signals in areas rich in i-cells, such as the stolons of the body wall, will demonstrate that the Piwi probe is effective and specific [13]. After confirming probe efficacy, starting from the 8-cell stage, confocal microscopy will be used to observe the in situ hybridization results hourly until active and stable fluorescence signals -- indicating the first expression of Piwi -- are detected. The time point and location of their appearance will be recorded. By comparing the location of the fluorescence signals from FISH with the color-coded regions from the fate-mapping map, the origin of i-cells within the embryo can be determined, identifying the specific parent blastomere or specific cluster of cells in the early embryo.

Table 1. Encode each cell using dye combinations (FITC, Texas Red and CY5)

Cell number	FITC	Texas Red	Cy5	Code
1	Injection	No injection	No injection	100
2	No injection	Injection	No injection	010
3	No injection	No injection	Injection	001
4	Injection	Injection	No injection	110
5	Injection	No injection	Injection	101
6	No injection	Injection	Injection	011
7	Injection	Injection	Injection	111
8	No injection	No injection	No injection	000

3.3. Expected outcome

The signal for the Piwi gene, representing i-cells, will most likely first appear during the gastrula stage or later. Regarding the spatial relationship of the signals, two possible outcomes are predicted: (1) The Piwi-positive cell population is almost entirely located within a region marked by a specific color. Other regions marked by early cells largely lack Piwi-positive cells. This would indicate that the progeny of one specific cell primarily or completely cover the area where Piwi is first expressed during subsequent development, supporting the view that i-cells have a specific and determined embryonic origin. (2) The Piwi-positive cell population appears within regions marked by the progeny of multiple early injected cells. No single early cell gives rise to all Piwi-positive cells. This would suggest that i-cells may originate from multiple different lineages early in embryonic development, or that their precursor cells are dispersed throughout the early embryo.

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4. Objective2: determining gene sequences in embryos at the single-cell level

4.1. Rationale

To validate the emergence of i-cells at the molecular level and to explore gene expression during the formation of i-cells in Hydractinia embryos, Parallel-seq, a technique enabling simultaneous snRNA-seq and scATAC-seq in single cells, will be used, combined with FISH. This approach will synchronously capture the transcriptome and chromatin accessibility of single cells in their original tissue locations across different stages of Hydractinia embryogenesis, providing a comprehensive molecular data foundation for subsequent analysis.

4.2. Method

4.2.1. Material preparation

Hydractinia symbiolongicarpus embryos will be collected at 19 stages from the 8-cell stage (~1-2 h.p.f.) to 20 h.p.f. (~524,288 - 1,048,576 cells) and the samples will be processed using Parallel-seq. It is noteworthy that by the 128-cell stage (~5-7 h.p.f.), ectodermal cells no longer contribute to the endoderm, and the two cell populations are separated. However, to construct the subsequent developmental trajectory, measurements will be continued until the germ layer commitment stage.

4.2.2. Using combinatorial indexing and droplet overloading to simultaneously measure chromatin accessibility and gene expression in the same single cell

Parallel-seq, developed by Tang et al., is a technique that simultaneously measures chromatin accessibility and gene expression in the same single cell. Its ultra-high throughput and low cost make it a powerful tool for deciphering cellular heterogeneity and gene regulatory relationships [14]. The principle involves using multiple rounds of barcoding to assign the same cell identity to both chromatin DNA and RNA molecules from the same cell. The core of this technology lies in using specific barcode combinations to uniquely identify each cell. First, in a 96-well plate, well-specific round 1 barcodes (BC1) are used to simultaneously label chromatin (cleaved by Tn5 transposase to mark open regions) and mRNA (via reverse transcription primers containing the same BC1 for cDNA synthesis), ensuring homologous molecules share the same origin. Next, cells are pooled and randomly redistributed into a new set of 96-well plates, where a round 2 barcode (BC2) is added via ligation. Finally, a droplet overloading strategy is employed on a microfluidic chip, where each droplet encapsulates approximately 10 cells, and a droplet-specific round 3 barcode (BC3) is added via linear amplification within the droplet. The sequencing data is then processed through a computational bioinformatics pipeline, using the cell barcode combination (BC1+BC2+BC3) to uniquely identify each cell, thereby generating a gene expression matrix and a chromatin accessibility matrix.

4.2.3. Data preprocessing of the obtained sequencing results

Raw sequencing data may contain low-quality cells and genes with extremely low expression. To reduce interference from such data in subsequent analyses, ScanPy will be used to process the single-cell sequencing results [15]. The raw output data from snRNA-seq (gene × cell count matrix) and scATAC-seq (peak × cell count matrix) will be loaded into ScanPy separately. Thresholds will be set based on the specific measured data, and functions will be used to filter out low-quality cells,

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such as those with too few or too many genes or peaks [16]. The scATAC-seq peak matrix will then be converted into a gene activity matrix by calculating the accessibility of gene promoters and nearby enhancer regions to estimate gene "activity", aligning its features with the "gene expression" matrix from RNA-seq.

4.3. Expected outcome

Through the above process, single-cell transcriptomes and chromatin accessibility profiles throughout Hydractinia embryonic development will be obtained. This will provide the foundation for subsequent cell clustering and the construction of a gene expression atlas for Hydractinia embryonic development.

5. Objective3: tracing the origin of I-cells by visualizing cell clustering and developmental trajectory

5.1. Rationale

The data from Objective 2 will be processed by employing a series of methods to partition it into distinct cell clusters, infer their identities, and then construct a dynamic developmental trajectory based on cellular similarities, presenting it visually. This will allow tracing i-cells back to specific precursor cells, deducing the differentiation path of i-cells, and resolving the dynamic processes during development, including which gene regulatory networks are sequentially activated during differentiation, ultimately answering the initial question: i-cells originate from progenitor cell y at stage x and evolve via trajectory z.

5.2. Method

5.2.1. Dimensionality reduction by using canonical correlation analysis

Canonical Correlation Analysis (CCA) can identify similar cell states across different stages and position them close together in a reduced-dimensional space [17]. As a dimensionality reduction technique, CCA is more suitable than Principal Component Analysis (PCA) for finding biological signals shared across datasets while suppressing dataset-specific noise [17]. The principle and computational method of CCA are described in detail in the study by Butler et al [18]. In this study, the data from Objective 2 will first be standardized: log normalization will be applied to the snRNA-seq gene expression matrix and log-CPM normalization to the gene activity matrix derived from scATAC-seq. Then, 500 highly variable genes will be selected from the snRNA-seq data and the corresponding gene activity values will be extracted from the scATAC-seq data. Following the method described in Butler et al., CCA calculation will be performed: both matrices will be standardized so that genes have a mean of 0 and variance of 1, the cross-covariance matrix K=X^TY will be computed, and truncated SVD decomposition will be performed on K to obtain the canonical correlation vectors (CCVs) for snRNA-seq and scATAC-seq. Multiple pairs of Canonical Correlation Vectors (CCVs) and canonical correlations (CCs) will be calculated. Based on the characteristics of the Hydractinia embryo data and referencing similar tissue-level data, the first N pairs of CCs corresponding to the CCVs will be selected as the dimensionality reduction result.

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5.2.2. Visualization by using uniform manifold approximation and projection

Uniform Manifold Approximation and Projection (UMAP) is a non-linear dimensionality reduction technique whose core goal is to preserve both the local and global structure of high-dimensional data in a low-dimensional space [19]. Using UMAP, the data reduced by CCA can be further projected into a 2-dimensional space. Compared to traditional non-linear dimensionality reduction methods such as t-SNE, UMAP better preserves the global data structure such as cell differentiation trajectories and relationships across clusters while retaining local clustering features, avoiding the loss of large-scale information [16]. The CCs matrix obtained from CCA will be input into the UMAP algorithm, the neighborhood relationships between cells in the CCs space will be computed, and then two UMAP coordinates will be calculated for each cell, resulting in a 2D scatter plot that intuitively displays the similarities and clustering relationships between cells.

5.2.3. Cell clustering-partitioning cells into different groups based on similarity in gene expression

Cells with similar expression profiles and chromatin accessibility will also be close in distance. The goal of clustering algorithms is to find these tightly grouped cell populations in space. By algorithmically partitioning cells into distinct clusters, which are visually represented as different cell groups on the UMAP dimensionality reduction plot, all the different cell states can be objectively defined. Specifically, the CCs obtained from CCA will be input, the K-Nearest Neighbors (KNN) for each cell will be found based on the Euclidean distance in the CCA space, and a cell relationship graph will be constructed. The Louvain algorithm will be used for clustering on this KNN graph [20]. These algorithms will identify densely connected cell groups within the graph and partition them into a cluster. Ultimately, each cell will be assigned a cluster label. These labels will be mapped to the UMAP plot from the previous step, using different colors for visualization, to observe the clustering effect.

5.2.4. Inferring cell trajectory

Potential developmental trajectories or cell fate decision processes will be inferred by reconstructing the paths of cells in the low-dimensional space. The dimensionality reduction results from CCA will still be used as cell coordinates. The algorithm for computation will be chosen based on the structure displayed on the UMAP plot: Slingshot will be used for simpler linear or branched structures [21]; Partition-based Graph Abstraction (PAGA) will be used for very complex multi-branch or cyclic structures [22]. The algorithms will connect cells in the low-dimensional space, construct one or multiple paths, and calculate a Pseudotime value for each cell on the path, representing its degree of development from the starting point, aiding in the understanding of the developmental trajectory.

5.2.5. Validating the developmental path

By comparing the fate-mapping data from Objective 1 with the developmental trajectory reconstructed in this study, it is expected that the computationally derived cell paths will be highly consistent with the experimentally observed cell fates, thereby validating the accuracy of the i-cell origin and the reliability of the trajectory. Ultimately, this study will provide an experimentally validated, high-resolution developmental trajectory model, revealing the dynamic process of i-cell differentiation from early progenitor cells and clarifying their origin and evolutionary path.

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5.3. Expected outcome

G. Plickert et al. first discovered i-cells in the embryo in the endodermal region [2]. Therefore, it is hypothesized that i-cells likely originate from an early endodermal lineage. Based on the current data analysis and the planned trajectory inference, it is predicted: i-cells most likely originate from a specific progenitor cell at the 8-cell stage destined to form the endoderm. This cell subsequently follows a specific differentiation path, gradually acquiring the characteristics of i-cells. At the molecular mechanism level, it is anticipated that the Wnt and Notch signaling pathways play key roles in the formation of i-cells. The Wnt pathway might play a leading role in body axis establishment and cell fate determination by regulating genes such as Brachyury, Nanos, and Vasa [23,24]; the Notch signal might be involved in regulating the differentiation of interstitial cell lineages, influencing the final fate of i-cells. Furthermore, it is expected to discover new regulatory factors, signaling pathways, or key genes such as Wnt3, Tcf, Brachyury that may have previously undiscovered functions in the regeneration process [2].

6. Conclusion

This study integrates fate mapping, FISH, single-cell multi-omics sequencing and other techniques to trace the embryonic origin of i-cells in Hydractinia. It aims to demonstrate that i-cells originate from specific endodermal blastomeres as early as the initial gastrula stage, following a distinct developmental trajectory marked by Piwi expression and the sequential activation of key regulatory genes. These findings are expected to provide direct experimental evidence supporting the hypothesis of a deterministic embryonic origin for pluripotent stem cells in cnidarians, rather than their secondary acquisition from differentiated somatic cell lineages.

These insights are anticipated to offer significant contributions to understanding the evolutionary conservation of pluripotency mechanisms and may inspire novel approaches in regenerative medicine, particularly regarding how stem cell populations are established and maintained. Furthermore, this work establishes Hydractinia as a powerful model for studying stem cell ontogeny within a comparative evolutionary context. However, this study has limitations, including its reliance on in vitro embryonic development and potential off-target effects in FISH probe design. Inferences about regulatory networks also largely depend on computational predictions that require functional validation.

Future work should include functional genetic experiments, such as CRISPR-Cas9-based knockout of candidate genes (e.g., Wnt3, Brachyury, Tcf), to validate their roles in i-cell specification. Long-term lineage tracing and single-cell metabolomic analyses could further elucidate the dynamic changes during i-cell maturation. Additionally, applying similar approaches to other cnidarian species would help determine whether the mechanisms discovered here are conserved across species. In summary, this study provides a foundational framework for understanding the embryonic origin of pluripotent stem cells in basal metazoans and opens new avenues for exploring the mechanisms of stem cell biology and evolutionary development.

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