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Localization and characterization of hematopoietic tissues in adult sea cucumber, *Apostichopus japonicus*

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ABSTRACT

Sea cucumber *Apostichopus japonicus* rely on the efficient innate immune mechanisms against invaders, in which the consumption and regeneration of coelomocytes take place at the same time. In the present study, histological features of putative hematopoietic tissues (HPTs) including the rete mirabile, the respiratory tree, the polian vesicle and the coelomic epithelium were characterized. The distribution of transcription factor GATA1 in coelomocytes and putative HPTs was examined by immunohistochemistry. In addition, cell proliferation using EdU labeling and coelomocyte distribution in different tissues using monoclonal antibody labeling were analyzed to further confirm the HPTs. The results showed that two homologs of GATA1 were detected with molecular weight of 43 and 90 kDa in coelomocytes, rete mirabile, respiratory tree and polian vesicle, whereas no signals were detected in the coelomic epithelium. A few cells were detected to be EdU-positive for coelomocytes, which accounted for approximately 9.5%. In the rete mirabile and the respiratory tree, the EdU signals were strong in cells of the tube wall. In the polian vesicle, numerous EdU-positive cells were detected in the cyst wall. In the coelomic epithelium, little EdU signaling was detected. Immunohistochemistry analysis by mAb 3F6 against *A. japonicus* coelomocytes showed that positive signals were observed in the tube wall of the rete mirabile, respiratory tree, cyst wall of the polian vesicle and in the coelomocyte antrum of coelomic epithelium. These results suggest that the rete mirabile, respiratory tree and polian vesicle are the HPTs of *A. japonicus*.

1. Introduction

The body cavity of echinoderms is filled with coelomic fluid, which bathes the internal organs and forms the fluid medium in which the coelomocytes are suspended [1]. Coelomocytes are the cell type involved in cellular immune functions, such as phagocytosis, encapsulation, and cytotoxicity. In addition, coelomocytes are indispensable in biodefense and wound healing in echinoderms [2].

Hematopoiesis is a complex process, by which different kinds of blood cells are formed and released from hematopoietic tissue (HPT) [3]. The coelomic epithelium, lining the dorsal part of the coelomic cavity throughout the animal, has been suggested as the most probable source of the coelomocytes in echinoderms [4]. However, the axial organ that comprises the junction of the perivisceral coelom and the hemal system, as well as the Tiedemann bodies, small paired organs located as pockets on the peristomal ring, have also been suggested as HPTs [5–7]. There has been no evidence regarding the possibility of direct self-replication of circulating coelomocytes.

Sea cucumbers are echinoderms from the class Holothuroidea. At present, the origin of coelomocytes in sea cucumbers is still in dispute. Hatanaka reported that coelomocytes of *Molpadia roretzi* originate in the hemal ring and associated vessels [8]. Prosser and Judson showed that coelomocytes of *Parastichopus californicus* might originate in the hemal vessel [9]. Hetzel reported the hemal system of holothurians (*Cucumaria miniata, Eupentacta quinquesemita, Psolus chitonoides* and *P. californicus*) might be regarded as lymphoid tissues that produce coelomocytes [10]. Endean indicated that homogeneous amebocytes (lymphocytes) originated from the lining epithelium of the respiratory trees in *Holothunia leucospilota*, which then migrated into the coelomic fluid, and differentiated into morula cells and possibly other coelomocyte types [11]. Smith inferred that coelomocytes might arise from polian vesicle epithelium in *Holothuria cinerascens* [12].

The taxonomy of the sea cucumber, *Apostichopus japonicus*, is as follows: Echinodermata, Holothuroidea, Aspidochirotida,

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Stichopodidae, and *Apostichopus*. *A. japonicus* is considered to be an economically important organism in East Asia due to its nutritional value and potential health benefits [13]. The classification of *A. japonicus* coelomocytes has not been established, and the composition of unique cell types is widely described among different researchers. Eliseikina and Magarlamov described nine types of coelomocytes in *A. japonicus*: progenitor cells, amoebocytes, vacuolated cells, small morula cells, morula cells of type I, type II and type III, crystal cells and vibratile cells [14]. Xing et al. distinguished six coelomocyte types in *A. japonicus*: lymphocytes, morula cells, amoebocytes, crystal cells, fusiform cells and vibratile cells [15]. In our previous study, coelomocytes of *A. japonicus* were classified into lymphoid cells, spherulocytes, amoebocytes, hyaline cells, fusiform cells and crystal cells [16]. However, the origin of coelomocytes in *A. japonicus* is still unknown.

The GATA transcription factor family contains evolutionarily conserved proteins that are of vital importance in regulating the development and differentiation of eukaryotic organisms, especially hematopoiesis. GATA1 transcription factor has been extensively characterized, indicating a crucial role in the development of erythroid cells, megakaryocytes, eosinophils, and mast cells [17–19]. In the crustacean *Pacifastacus leniusculus*, GATA binds to transglutaminase to regulate the release of hematopoietic stem cells into the hemolymph [20]. In the crab *Eriocheir sinensis*, GATA1 was involved in the development of certain hemocytes. GATA1 positive cells in the HPT were pro-hemocytes released into the hemolymph [21]. In the scallop *Chlamys farreri*, GATA1/2/3 has been identified and proven to have a conserved function in regulating hemocyte production. As a result, GATA1/2/3 was selected as a hematopoietic marker to explore potential developmental events in hematopoiesis during ontogenesis in scallops [22]. In the Pacific oyster *Crassotrea gigas*, the hematopoietic transcription factor Cg-GATA2/3 was primarily distributed in the immune organs, such as gill, hemocytes, and mantle. After Cg-GATA2/3 was interferenced by dsRNA, the mRNA expressions of the hemocyte-specific gene EcSOD and the hematopoietic transcription factor C-Myb were significantly down-regulated. As a result, the hemocyte renewal rates also decreased both in hemolymph and gill [23].

In the present study, the histological features of putative HPTs in *A. japonicus* were characterized, and the distribution of transcription factor GATA1 in putative HPTs was examined. In addition, cell proliferation using EdU labeling and coelomocyte distribution in different tissues using antibody labeling were analyzed to further confirm the HPTs of *A. japonicus*. The results from this study will be greatly helpful to further study the origin of coelomocytes and important immune regulatory mechanisms in *A. japonicus*.

2. Materials and methods

2.1. Sea cucumber and sample collection

The sea cucumber *A. japonicus* was collected from a local aquatic farm in Dalian, China. Animals weighing approximately 60 g were cultured at 17–19 °C in tanks for 7 days prior to experiments. Coelomocytes were collected according to the method described previously [16]. Briefly, coelomic fluid of *A. japonicus* was drawn from the right lateral side of the body and immediately diluted (1:1) in anti-aggregant modified Alsever's solution (27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 5.6) and immediately centrifuged at 800 × g, 4 °C for 10 min to harvest the coelomocytes. Then, the ventral surface of *A. japonicus* was opened longitudinally with a surgical knife. The putative HPTs of *A. japonicus* including the rete mirabile, respiratory tree, polian vesicle and coelomic epithelium were dissected and embedded in tissue freezing medium or fixed in 10% formalin, respectively.

2.2. Histological observation of putative HPTs

The putative HPTs fixed in 10% formalin were dehydrated in 80%, 95%, and 100% successive ethanol baths, and finally dehydrated twice in xylene before being embedded in paraffin wax, followed by preparation of sections (5 μm-thick) for mounting on slides. After deparaffination in xylene and rehydration in an ethanol series, serial sections were stained with hematoxylin and eosin (H & E) and observed under the light microscope.

2.3. Western-blot analysis of GATA1

After 12% SDS-PAGE, the protein samples of coelomocytes and putative HPTs from *A. japonicus* were electrophoretically transferred onto nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin (BSA) in PBS at 4 °C overnight, and incubated with anti-GATA1 antibody (BBI, diluted at 1:500) for 1 h at 37 °C, followed by three washes with PBST. Membranes were incubated with alkaline phosphatase conjugated goat-anti-rabbit IgG (Sigma, diluted at 1:4000) at 37 °C for 1 h, followed by three washes with PBST. Membranes were stained with the substrate solution containing nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at room temperature for 5 min. Mouse spleen was used as a positive control according to the anti-GATA1 antibody instruction.

2.4. Immunohistochemistry analysis of GATA1

Immunohistochemistry was done according to the method of Jia et al. [21] with some modifications. For the immunohistochemistry analysis of circulating coelomocytes, cell suspensions were added onto glass slides and incubated at room temperature in a wet chamber for 15 min, 4% PFA (paraformaldehyde diluted in PBS) was used to fix the slides at room temperature for 15 min. The slides were treated with 0.5% Triton® X-100 in PBS. After three washes with PBS slides were blocked with 3% BSA in PBS at 37 °C for 30 min. The supernatant was removed, and the slides were incubated with anti-GATA1 antibody (BBI, diluted at 1:100) as the primary antibody at 37 °C for 1 h. After three washes with PBS, the slides were incubated at 37 °C for 1 h in darkness with goat-anti-rabbit Ig conjugated with fluorescein isothiocyanate (Abclone, diluted at 1:100) as the second antibody. The Evans blue dye (Solarbio, 0.5%) was included as the counterstain. After three washes, the slides were mounted in antifade solution (Solarbio) and viewed under a fluorescence microscope (Leica). For immunohistochemistry analysis of putative HPTs, continuous 6 μm-thick frozen sections of each tissue were cut using a cryostat (CM1900, Leica) at −20 °C and transferred onto the glass slide, dried at room temperature, then fixed with 4% PFA for 15 min. After being blocked in 3% BSA, these sections were incubated with anti-GATA1 antibody, and goat-anti-rabbit Ig conjugated with fluorescein isothiocyanate successively. Mouse spleen was used as a positive control according to the anti-GATA1 antibody instruction.

2.5. Immunohistochemistry analysis using the monoclonal antibody against coelomocytes

In order to study the distribution of coelomocytes, circulating coelomocytes and putative HPTs were collected to prepare cell monolayers and cryosections as described above. Immunohistochemistry using a monoclonal antibody against coelomocytes was performed to localize coelomocytes according to the following procedures. Monolayers or cryosections were fixed with 4% PFA for 15 min, blocked in 3% BSA for 30 min, then overlaid with monoclonal antibody (mAb 3F6) against coelomocytes of *A. japonicus* [16] as the primary antibody and incubated at 37 °C for 45 min in a wet chamber. After three washes in PBS with 5 min intervals, the slides were incubated at 37 °C for 45 min in darkness with goat-anti-mouse Ig conjugated with fluorescein

isothiocyanate (Sigma, diluted at 1:256). After three washes and incubating with 4′, 6-Diamidino-2-Phenylindole (DAPI) to stain the nucleus, the slides were mounted in antifade solution and observed under a fluorescence microscope. Myeloma culture supernatant was used as a negative control.

2.6. Detection of DNA replication by EdU labeling

EdU labeling assay was performed as previously described [21]. Briefly, 200 μL 5-ethynyl-2′-deoxyuridine (EdU, RiboBio, 1 mg/mL in sterilized saline) was injected into the coelom of *A. japonicus* using a syringe. After 24 h, circulating coelomocytes and putative HPTs were collected to prepare cell monolayers and cryosections as described above.

For the detection of EdU, monolayers or cryosections were fixed in 4% PFA for 15 min, washed two times in PBS with 5 min intervals and 2 mg/mL glycine for 10 min, then 0.5% Triton® X-100 in PBS was used to treat the samples at room temperature for 10 min. After two times washing with PBS, EdU was detected using the Cell-Light™ Apollo® 567 Stain Kit (RiboBio, China) under fluorescence microscopy (Leica).

3. Results

3.1. Localization and histology of putative HPTs

The rete mirabile, respiratory tree, polian vesicle and coelomic epithelium were studied as the putative HPTs to investigate the origin of *A. japonicus* coelomocytes based on previous reports. The localization and histology of putative HPTs are shown in Fig. 1. The rete mirabile, a part of the blood system, is formed by much plexiform blood lumen. One end is connected to the intestine, and the other end is closely connected to the left branch of the respiratory tree, which together with the blood sinus form the blood system of *A. japonicus.* (Fig. 1-B). The vascular system is composed of the vascular epithelia, the basement membrane and the vascular lumen (Fig. 1-C). The respiratory tree, a unique respiratory organ of *A. japonicus*, is divided into two branches. It lies on either side of the intestine and consists of a series of narrow tubules branching from a common duct (Fig. 1-B). The respiratory tree is composed (from outside to inside) of mesothelium, muscular coat, hemocoel, endothelial cells and a center antrum (Fig. 1-D). The polian vesicle, part of water vascular system, is suspended in the coelom and connected with the ring canal of *A. japonicus.* It is a spindle-shaped sac filled with coelomic fluid encased by a thin wall (Fig. 1-A). The polian vesicle is composed (from outside to inside) of mesothelium, muscular coat, connective tissue and ciliated epithelium (Fig. 1-E). The body wall consists (from outside to inside) of the horny layer, epithelial lining, connective tissue, circular muscle, coelomocyte antrum and coelomic epithelium. The coelomic epithelium is a peritoneal structure with cilium in the innermost part of the body wall (Fig. 1-F).

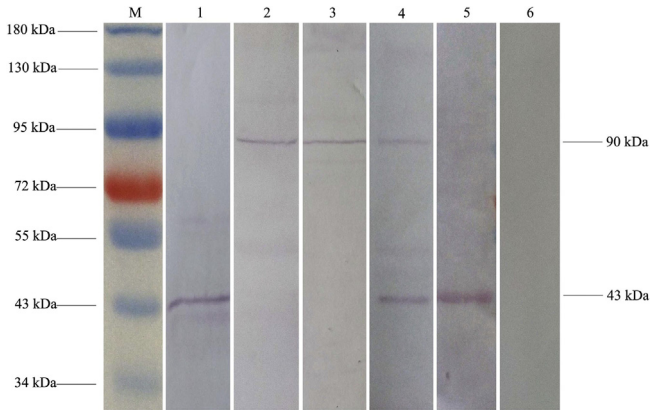

Fig. 2. Western blot analysis of GATA1. GATA1 was detected in coelomocytes, rete mirabile, respiratory tree and polian vesicle. Mouse spleen was used as a positive control. M: molecular marker; 1: mouse spleen; 2: coelomocytes; 3: rete mirabile; 4: respiratory tree; 5: polian vesicle; 6: coelomic epithelium.

3.2. Analysis of homologous proteins for GATA1

The distributions of hematopoietic transcription factor GATA1 in coelomocytes and putative HPTs were determined by western blotting. The results showed that there was a band of 90 kDa detected in coelomocytes and rete mirabile, a band of 43 kDa in the polian vesicle, two bands of 43 and 90 kDa in the respiratory tree and no band detected in the coelomic epithelium. Mouse spleen was used as a positive control with a band of 43 kDa (Fig. 2).

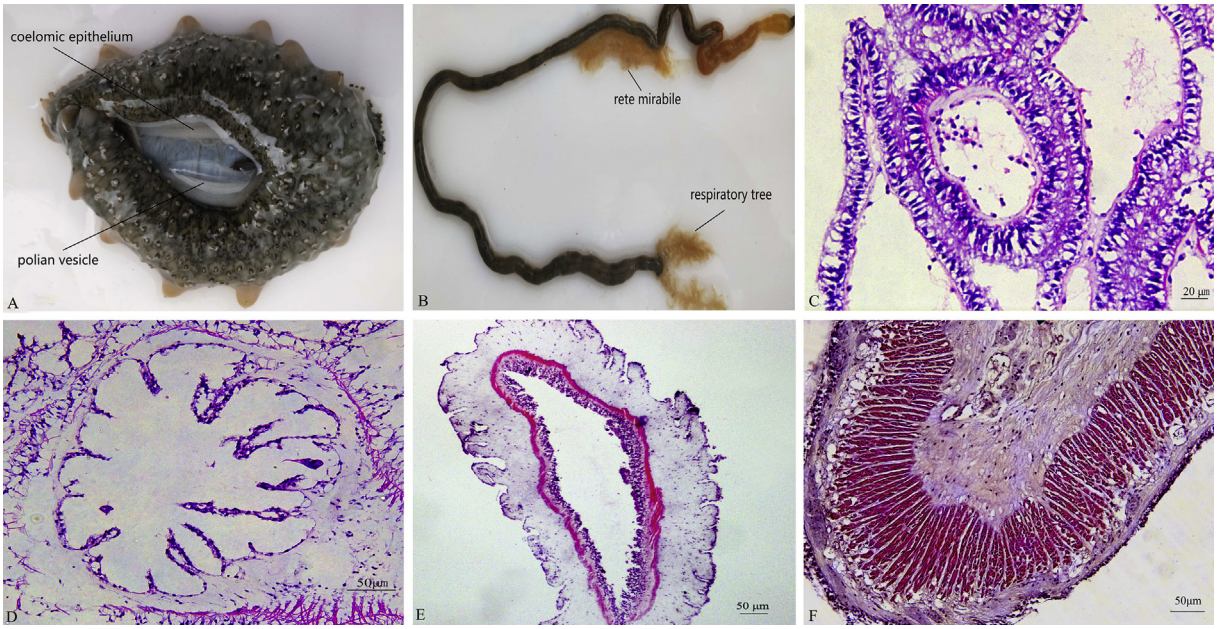

Fig. 1. Localization and histology of putative HPTs in the sea cucumber *A. japonicus.* A, B: Localization of putative HPTs in *A. japonicus.* C, D, E, and F: histology of rete mirabile, respiratory tree, polian vesicle and coelomic epithelium by hematoxylin and eosin (H&E) staining.

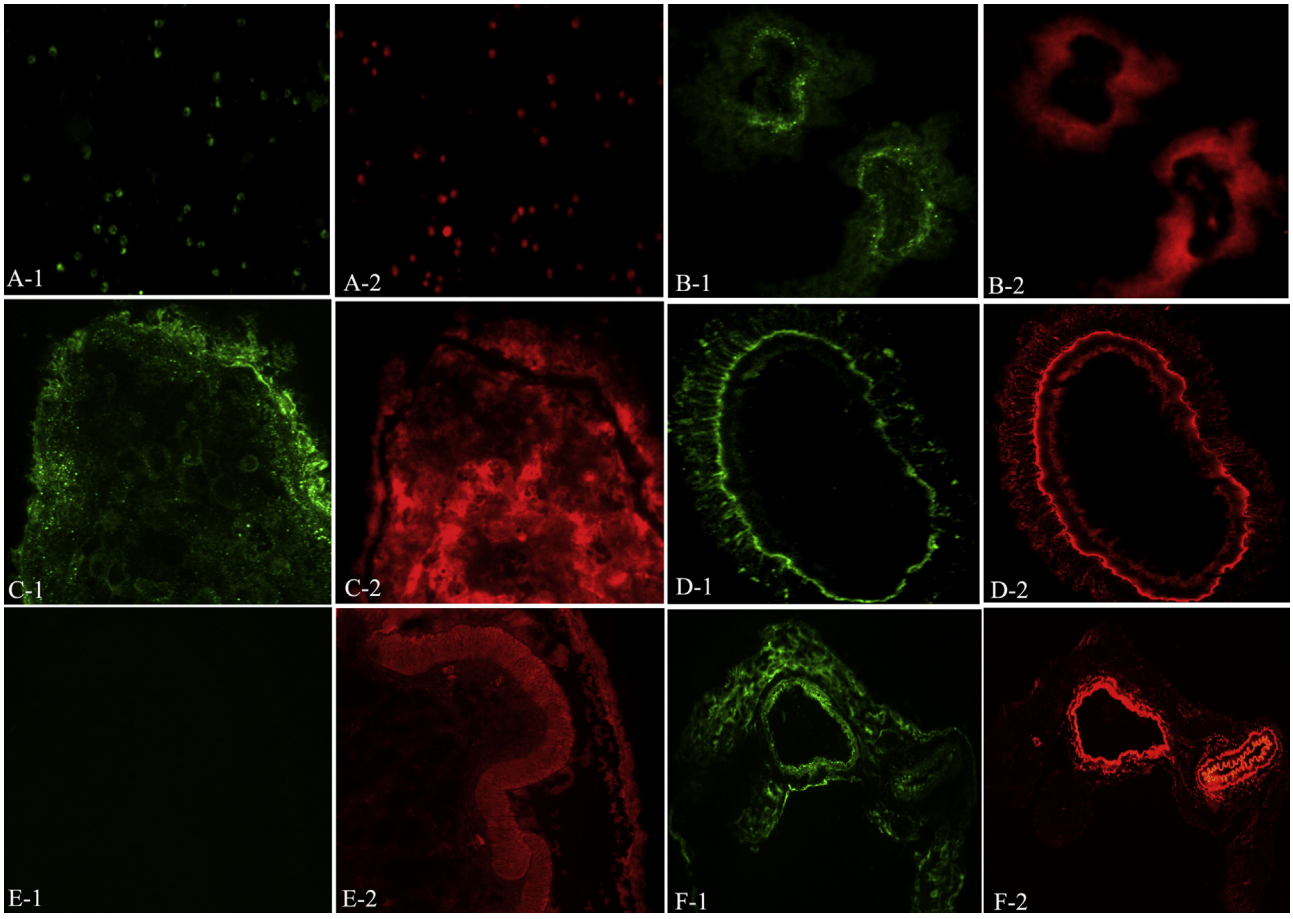

Fig. 3. Localization of GATA1 in coelomocytes and putative HPTs. GATA1 was visualized by fluorescein isothiocyanate-labeled goat-anti-rabbit antibody (green). Cells were stained with Evans blue dye (red). A: coelomocytes (× 40); B: rete mirabile (× 20); C: respiratory tree (× 20); D: polian vesicle (× 5); E: coelomic epithelium (× 5); F: mouse spleen (× 20). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Immunohistochemistry localization of GATA1

Immunohistochemistry was performed to detect the localization of GATA1, and the positive signal was green fluorescence. The signal of GATA1 was detected in coelomocytes, but only a few cells were GATA1-positive. In putative HPTs, GATA1 was detected in the cells which located at the tube wall of the rete mirabile and the respiratory tree, and the cyst wall of the polian vesicle. However, there was no signal detected in the coelomic epithelium. Mouse spleen was used as a positive control (Fig. 3).

3.4. Distribution of coelomocytes

MAb 3F6 against spherulocytes, a subtype of coelomocytes of *A. japonicus,* was produced previously by our laboratory, which recognized an antigen of molecular weight 136 kDa [16]. In this study, immunohistochemistry analysis using mAb 3F6 showed that positive signals were primarily observed in the tube wall of the rete mirabile and respiratory tree, the cyst wall of the polian vesicle, and the coelomocyte antrum of the coelomic epithelium (Fig. 4).

3.5. DNA replication in coelomocytes and putative HPTs

DNA replications in coelomocytes and putative HPTs were detected at 24 h after EdU injection. Only a few cells, approximately 9.5%, were detected to be EdU-positive for circulating coelomocytes. In the rete mirabile and respiratory tree, EdU signals showed strongly positive on the cells of the tube wall. Numerous EdU-positive cells were detected on the cyst wall of the polian vesicle. For the coelomic epithelium, little EdU signal was detected (Fig. 5).

4. Discussion

Hematopoiesis is a complex process, by which different kinds of blood cells are formed and released from HPT [3]. The process has been studied extensively in vertebrates, and blood cells can be distinguished according to their morphological and functional characteristics. In invertebrates, there are no oxygen-carrying erythrocytes nor lymphoid lineages cells involved in the adaptive immune defense. Thus, hematopoiesis in these organisms provides a good model to study the regulation mechanism of the hemocytes in an innate immune system [21].

Sea cucumbers, like other echinoderms, lack a developed blood circulatory system. Instead, they possess coelomic fluid that fills the coelomic cavity. The coelomic fluid has been known to serve in circulation and homeostasis [1]. Coelomocytes like hemocytes are involved in cellular immunity such as phagocytosis of pathogens, encapsulation, and cytotoxicity [2]. Since there is a turnover of coelomocytes in these processes, there is a need for rapid generation of several new coelomocytes during infection. However, the HPTs in sea cucumber are still in dispute due to the limitation of research technologies. Until now, some studies demonstrated that the hemal vessel, respiratory tree and polian vesicle could be considered as the probable origin of coelomocytes [8–12]. In the present study, HPTs of *A. japonicus* were investigated by immunochemistry analysis using an anti-GATA1 antibody

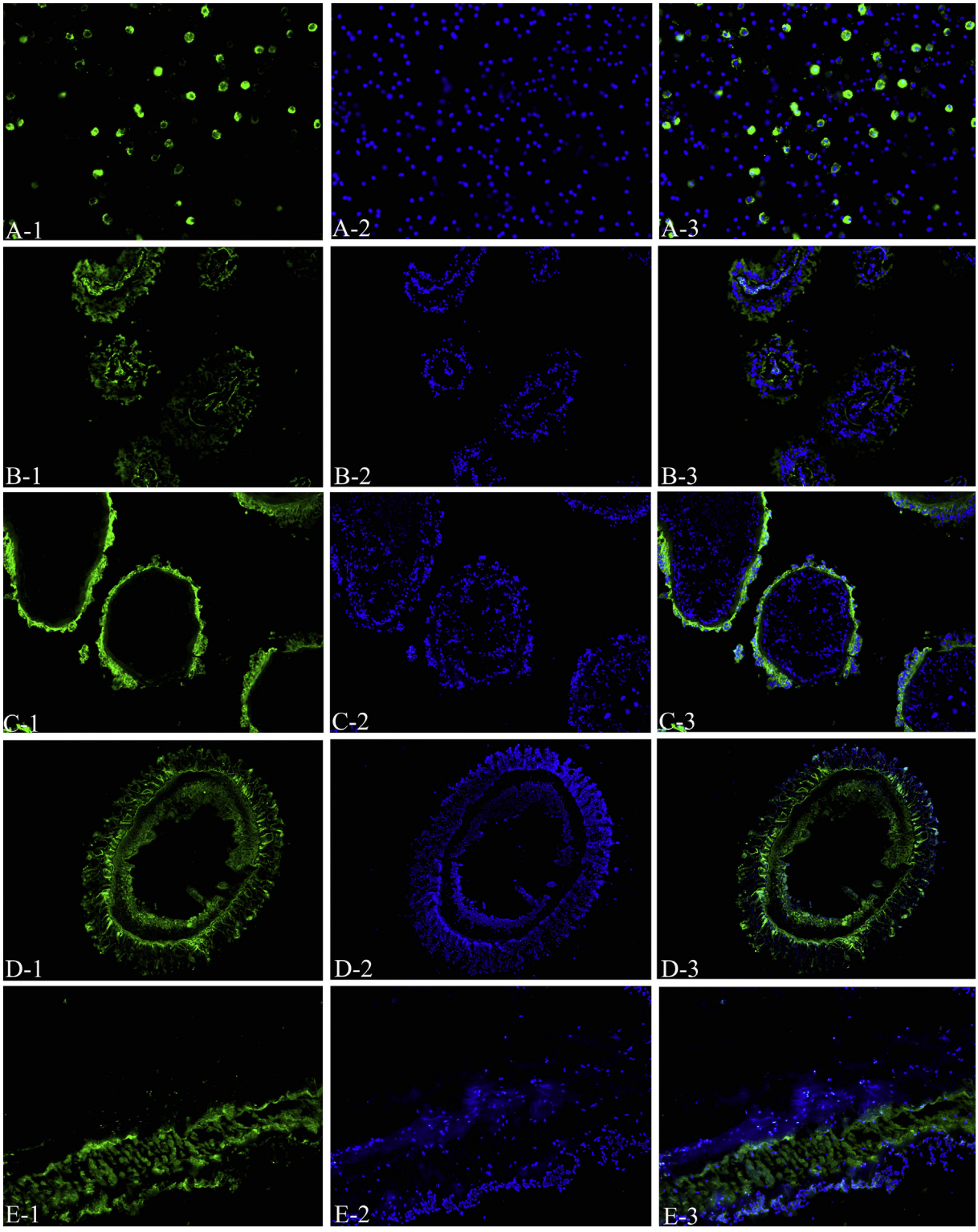

Fig. 4. Distribution of coelomocytes in rete mirabile, respiratory tree, polian vesicle and coelomic epithelium using the mAb 3F6 against coelomocytes. MAb labeling was visualized by fluorescein isothiocyanate-labeled goat-anti-mouse antibody (green). Cells were stained with DAPI (blue). A: coelomocytes (× 40); B: rete mirabile (× 20); C: respiratory tree (× 20); D: polian vesicle (× 5); E: coelomic epithelium (× 20). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and a mAb against coelomocytes, and by a DNA replication assay through EdU labeling. This combination of approaches will together be used to elucidate the localization and characterization of HPTs in sea cucumbers.

In humans, a cluster of differentiation (CD) antigens has been successfully used to characterize the functions of the different leukocyte subtypes. Monoclonal antibodies (mAbs) against these cell surface proteins, and the development of a common nomenclature system, have been of vital importance for the development of human/vertebrate immunology research since the 1980s. There were similar attempts in

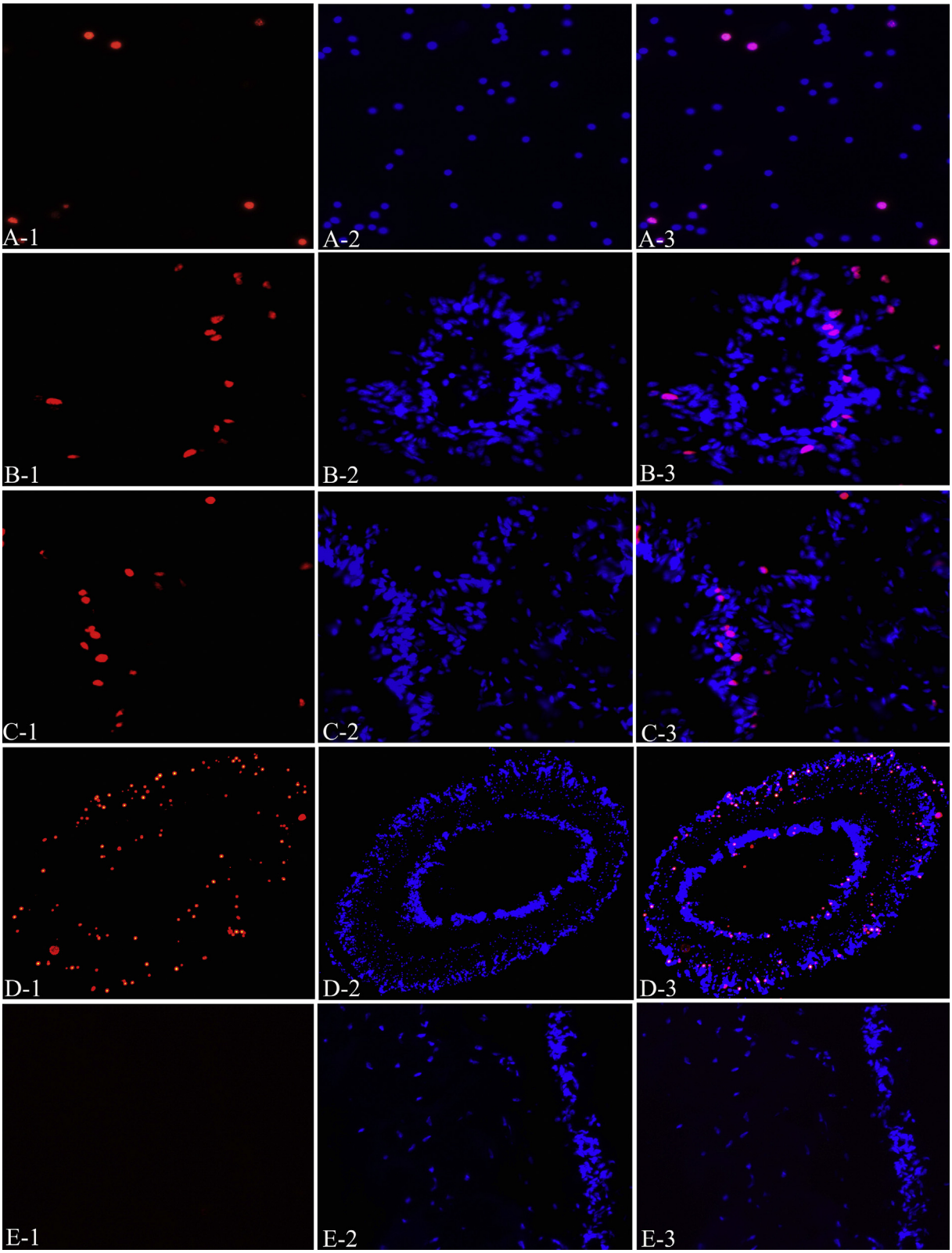

Fig. 5. Detection of DNA replication in coelomocytes, rete mirabile, respiratory tree, polian vesicle and coelomic epithelium by EdU. EdU-positive cells were stained in red and whole cells were stained with Hoechst 33342 in blue. A: coelomocytes (× 40); B: rete mirabile (× 20); C: respiratory tree (× 20); D: polian vesicle (× 5); E: coelomic epithelium (× 20). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the 1990s to characterize hemocyte types in crustaceans using mAbs [24]. For example, in the black tiger prawn *Penaeus monodon*, sixteen mAbs specific to hemocytes were obtained and could be divided into six groups. The common antigens recognized by the first and the second groups of antibodies in the hematopoietic tissue and the lymphoid organ may reflect relationships among these organs in the development of the sub-populations of granulocytes and semi-granulocytes. Hematopoietic tissue may be the site for hemocyte production, and the

lymphoid organ may be the site for further differentiation of at least two different lines of hemocytes [25]. In the present study, mAb 3F6 against coelomocytes of *A. japonicus* was used to study the distribution of coelomocytes in putative HPTs. The results showed that positive signals were observed in the tube wall of the rete mirabile, respiratory tree, cyst wall of polian vesicle and coelomocyte antrum of the coelomic epithelium. These results indicated an antigen of 136 kDa from coelomocytes that was distributed in all the putative HPTs examined.

Hematopoiesis is a process to continuously produce hemocytes, and the cells in HPT exhibit strong proliferative ability. Cell proliferation can be measured by BrdU or EdU labeling assays. The regeneration of hemocytes in Chinese mitten crab *E. sinensis* was observed by EdU labeling at 48 h after injection [21]. In the sea star, *Asterias rubens* (L.), the animals were pre-challenged with ConA-injection, before estimating proliferation with BrdU after 4 h [7]. In the present study, EdU signals could be observed on the tube wall of the rete mirabile and respiratory tree, and the cyst wall of the polian vesicle at 24 h after injection, which indicated that cell proliferation in the above tissues was more active than in the coelomic epithelium. The EdU signal was also detected in circulating coelomocytes, suggesting that the HPTs contained a large number of stem cells and the pro-coelomocytes that could be released into the coelomic fluid. A similar result was reported in *E. sinensis* [21].

Hematopoiesis is tightly regulated by numerous transcription factors. GATA is an important transcription factor family considered as a critical role during hematopoiesis in animals from Drosophila to mammals [26]. GATA1 is expressed in terminally differentiated erythroid cells, megakaryocytic cells, eosinophilic cells and mast cells, playing an essential role in regulating the development of these cells. In the present study, two homologs of human GATA1 were detected with a molecular weight of 43 and 90 kDa in coelomocytes, rete mirabile, respiratory tree and polian vesicle. It was suspected that there are different homologs of GATA1 during the maturation of coelomocytes. These results were similar to a report by Jia et al. showing that there were two bands of 30 and 25 kDa detected in HPT and hemocytes of *E. sinensis* [21]. Interestingly, there was a homolog of GATA1 in the respiratory tree and the polian vesicle with a molecular weight of 43 kDa that was in accord with GATA1 in mouse. The immunohistochemical results showed that homologs of human GATA1 were detected in some coelomocytes and in cells which were located at the tube wall of the rete mirabile, respiratory tree and polian vesicle. However, there was no signal detected in the coelomic epithelium. These results suggested that GATA1 might be involved in the development of some subtypes of coelomocytes. However, the specific cellular functions of GATA1 require further investigation.

In conclusion, the rete mirabile, respiratory tree and polian vesicle in *A. japonicus* were suggested as HPTs. Two homologs of the hematopoietic transcription factor GATA1 were detected with a molecular weight of 43 and 90 kDa in coelomocytes and HPTs. Future studies are needed to determine the relationship of coelomocytes and HPTs after stimulation of coelomocyte proliferation.

Acknowledgments

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