Fish and Shellfish Immunology 88 (2019) 28–35

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Directed differentiation of granular cells from crayfish hematopoietic tissue cells

Fang Li[a,b,*], Limei Xu[a,b], Xuan Hui[a], Wanzhen Huang[a], Feng Yang[a,b,c,**]

[a] Key Laboratory of Marine Genetic Resources, State Key Laboratory Breeding Base of Marine Genetic Resources, Third Institute of Oceanography, Ministry of Natural Resources, Xiamen, 361005, China

[b] Fujian Key Laboratory of Marine Genetic Resources, Xiamen, 361005, China

[c] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, PR China

ARTICLE INFO

Keywords:
Hematopoiesis
Granular cell
Differentiation
Hematopoietic tissue cell
Crayfish

ABSTRACT

Hemocytes are the major immune cells of crustaceans. New hemocyte production is required throughout the life cycle of these animals to maintain a functional immune system. The mechanism of crustacean hematopoiesis has just begun to be understood and new methods are needed for the investigation of this process. Here we report the directed differentiation of granular cells (GCs) from the hematopoietic tissue (HPT) cells of *Cherax quadricarinatus in vitro*. We started by providing the cultured HPT cells with different additives to induce possible differentiation. We found that crayfish muscle extract greatly promoted the physical status of the cells and induced the formation of refractile cytoplasmic granules. The transcription of marker genes and the production of functional prophenoloxidase further confirmed the formation of mature GCs. In our experiments, young GCs usually started to develop in ~2 weeks post induction and over 60% of the cells became mature within 3–4 weeks. This is the first time that the fully differentiation of crustacean hemocytes is accomplished *in vitro*. It provides a powerful tool for in-depth study of crustacean hematopoiesis.

1. Introduction

Hemocytes are the major immune cells of crustaceans, participating in melanization, phagocytosis, encapsulation, nodulation, coagulation, and the production antimicrobial peptides [1]. There are three morphological types of circulating hemocytes in crustaceans, the granular cells (GCs), semigranular cells (SGCs) and hyaline cells. In crayfish, GCs and SGCs are the dominant types of circulating hemocytes, which normally represent 15–35% and 65–85% of the population, respectively [1–3]. Hematopoiesis is required throughout the life cycle of these animals, to routinely renew the circulating hemocytes and replenish the loss of hemocytes during immune responses or injury [1,4].

Compared with the hematopoiesis in vertebrates which has been intensively studied, much less is known about the blood cell formation in invertebrates. Over the past years, progress has been made to understand the hematopoiesis of crustaceans, and the research was mainly done in fresh water crayfish. The hematopoiesis tissue (HPT) of crayfish is a thin sheet of structure located at the dorsal side of stomach, which contains lobules packed with progenitors of the blood lineages. Previous research suggests that there are five morphological types of cells in crayfish HPT. Type 1 and type 2 cells are proliferating cells that can differentiate into both GCs and SGCs. Type 3 and 4 cells are precursors of GCs, while type 5 cells are precursors of SGCs [5]. Young hemocytes are believed to develop in HPT, and then become mature as they are released into circulation.

Astakine 1 (AST1), a homolog of vertebrate prokineticin, was found to act as a key regulator for homeostasis. As a hematopoietic growth factor, AST1 directly stimulates the proliferation of HPT cells and promotes SGC lineage differentiation [6]. It also enhances the release of new hemocytes by inactivating transglutaminase (TGase) and repressing the cross linking of extracellular matrix proteins [7,8]. Moreover, AST1 is able to prevent apoptosis of HPT cells and hemocytes by up-regulating the expression of crustacean hematopoietic factor [9]. In addition, molecules like serotonin [10] and β-thymosins [11] were found to regulate crayfish hemotopoiesis possibly through AST1. By contrast, Astakine 2 (AST2), a paralog of AST1, does not induce the

Abbreviations: GC, granular cell; SGC, semigranular cell; HPT, hematopoietic tissue; dpi, days post induction; AST1, Astakine 1; AST2, Astakine 2; TGase, transglutaminase; ME, muscle tissue extract; NE, nerve tissue extract; TEM, transmission electronic microscopy; proPO, prophenoloxidase; PO, phenoloxidase

* Corresponding author. Third Institute of Oceanography, Ministry of Natural Resources, 184# Daxue Road, Xiamen, 361005, China.

** Corresponding author. Third Institute of Oceanography, Ministry of Natural Resources, 184# Daxue Road, Xiamen, 361005, China.

E-mail addresses: lifang@tio.org.cn (F. Li), yangfeng@tio.org.cn (F. Yang).

https://doi.org/10.1016/j.fsi.2019.02.054
Received 30 November 2018; Received in revised form 25 January 2019; Accepted 25 February 2019
Available online 28 February 2019

proliferation of crayfish HPT cells. Instead, it may be involved in GC maturation and circadian regulation of hematopoiesis [12–14].

In most of the studies concerning crayfish hematopoiesis, the production of new hemocytes was evaluated by the variation in total hemocyte number and the percentages of GCs and SGCs. However, the increase of circulating hemocyte may not solely result from new hemocyte production. Replenishment from sessile hemocytes can also play a role [15–18]. On the other hand, primary cultured HPT cells has been developed as a powerful model system to study hematopoiesis [19]. Yet, fully differentiation of these cultured precursors into mature hemocytes has not been reported. In most cases, the differentiation of HPT cells was judged by the spreading (or migration) of cultured cells or by the expression of marker genes. A method to direct differentiation of hematopoietic stem cells into mature hemocytes is required for in depth study of crustacean hematopoiesis.

In this study, we showed that crayfish muscle extract (ME) was able to direct GC differentiation from HPT cells. This is the first time that the complete differentiation of crustacean hemocytes is accomplished *in vitro*. It not only provides a powerful tool to study hematopoiesis, but also presents new ideas for crustacean blood cell formation.

2. Material and methods

2.1. Animals

Crayfish *Cherax quadricarinatus* were purchased from Zhangzhou Yuansentai Agriculture Technology Co. Ltd. Healthy inter-molt males ~55 ± 5 g in weight were used in the experiments. They were maintained in fresh water at 25 °C and fed daily with commercial diet.

2.2. Preparation of crayfish muscle tissue extract (ME) and nerve tissue extract (NE)

Five grams of muscle tissue or nerve tissue was collected from healthy *C. quadricarinatus* and homogenized on ice in 100 ml of Leibovitz's L-15 medium. The homogenate was centrifuged at 12000 g for 10 min at 4 °C, and the supernatant was heated at 56 °C for 30 min. The sediment resulted from heating was removed by centrifugation and the supernatant was sterilized by filter through a 0.22 μm filter. The extracts were stored at −80 °C before use.

2.3. Preparation of cell-free crayfish plasma

Crayfish were prechilled on ice for 10 min and the hemolymph was withdrawn with a cold syringe. The hemolymph was centrifuged at 1000 g for 5 min at 2 °C, and the supernatant (the plasma) was collected. The plasma was further diluted in L-15 medium to make a 5% stock solution. After heated at 56 °C for 30 min, this solution was passed through a 0.22 μm filter and stored at −80 °C.

2.4. Primary culture of crayfish HPT cells and induction of differentiation

HPT cells were isolated and grown as previously described [20,21]. In brief, the HPTs were incubated with 0.1% collagenase (type I and IV) (Sigma) in CPBS (10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 μM $CaCl_2$, 10 μM $MnCl_2$, and 2.7 μM KCl) at 30 °C for 30 min to dissociate HPT cells. After rinsing with L-15 medium, the cells were collected by centrifugation at 300*g* for 5 min at room temperature and re-suspended in L-15 medium. The remaining tissue mass was removed by passing the suspension through a cell strainer (70 μm). The cells were then seeded in 48-well plates at a density of 4×10^5/well and grown at 27 °C. Twenty-four hours later, the medium was replaced by fresh L-15 medium, L-15 plus 1% FBS, or L-15 plus 1% FBS supplied with different additives. The final concentrations of the additives in the culture medium were: 20% ME, 20% NE or 1% plasma. Three wells of cells were used for each group and half of the culture medium was changed every 7 days. The experiment was repeated for three time with different preparations of HPT cells and tissue extract. The cells were observed daily under an optical microscope and images were taken every 7 days.

2.5. Estimation of cell survival rate

On day 0 and day 28 post ME induction, Hoechst 33342 (Sigma-Aldrich) was added to the culture to a final concentration of 2 μg/ml, and incubated at room temperature for 30 min. Cells were observed under a Leica fluorescent microscope. For each well, images were randomly taken for five visual fields using a 20× objective, and the number of cells was counted using ImageJ software (https://imagej.nih.gov/ij/index.html). Three wells of cells were examined at each time point. The survival rate was estimated using the formula: survival rate = average number of cells per field on day 28/average number of cells per field on day 0.

2.6. Purification of GCs and SGCs

GCs and SGCs were purified from circulating hemocytes by density gradient centrifugation as previously described [22]. In brief, hemolymph were taken using a syringe preload with equal volume of prechilled anticoagulant solution (26 mM sodium citrate, 30 mM citric acid, 100 mM glucose, 140 mM NaCl, pH 5.8). Hemocytes were collected by centrifugation at 350*g* for 3 min at 4 °C. Cells were re-suspended in anticoagulant solution at a concentration of 2.5×10^4/μl. Hemocyte suspension (200 μl) was loaded onto a prechilled Percoll density gradient containing (from bottom to top) 0.2 ml 100% Percoll (GE health care), 2 ml 65% Percoll, and 1 ml 20% Percoll. The cells were separated by centrifugation at 450*g* for 30 min at room temperature. SGCs formed a layer on the interface of 20% and 65% Percoll, while GCs formed a layer on the interface of 65% and 100% Percoll. Each layer was collected and diluted with 6–10 volume of anticoagulant solution, and the cells were harvested by centrifuged at 350 g for 5 min. The purity of the cells was determined by flow cytometry and microscopy analysis [3].

2.7. Transmission electronic microscopy (TEM)

Cells were directly fixed in-well with 2.5% glutaraldehyde for 2 h at 4 °C and collected by scraping and centrifugation. The samples were post-fixed in 1% osmium tetroxide for 4 h. After dehydration in ethanol, the specimens were embedded in Epon resin, polymerized at 60 °C for 24 h, and ultrasectioned. The slices were stained with 2% uranyl acetate and 0.4% lead citrate sequentially, and visualized with a transmission electron microscope (JEM-1230, JEOL).

2.8. RNA isolation and RT-PCR analysis

Total RNA was isolated using SV Total RNA Isolation System (Promega). Total hemocytes (THC), purified GCs and SGCs were pelleted by centrifugation, and lysed with the RNA lysis buffer provided in the kit. Primary culture cells were lysed directly in-well after removal of culture medium. Total RNA was extracted as instructed, and genomic DNA contamination was eliminated by DNase treatment. First strand cDNA was synthesis using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) with oligo $dT_{(18)}$ primer, and the genes of interest were detected by PCR with primers listed in Supplementary Table 1. The reaction was performed for 31 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/50 s, after an initiate denaturing of 94 °C/3 min. The final extension was accomplished for 5 min at 72 °C. The PCR products were analyzed by electrophoresis. For each experiment, 1×10^6 of THC, GCs, and SGCs were used. The cultured cells in three wells of 48-well plate cells were lysed and pooled at each time point. The experiment was repeated for 3 times.

2.9. SDS-PAGE and western blotting

For cultured cells, after removal of culture medium, the cells were washed once with PBS, and lysed in-well with SDS-loading buffer. For hemocytes, purified SGCs and GCs, cells were collected as described in 2.6 and lysed with SDS-loading buffer. The lysates were boiled for 10 min and centrifuged at 10000 g for 5 min at 4 °C to remove cell debris. The protein samples were separated on 7% SDS-PAGE gel and stained with Coomassie brilliant blue. For Western blotting, proteins separated on SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Millipore). The blotting was performed using an iBind Western blotting system (Thermofisher). The membrane was probed with anti-prophenoloxidas (proPO) polyclonal antibody (see below) and anti-α-tubulin monoclonal antibody (Sigma), followed by incubating with alkaline phosphatase-conjugated goat anti-mouse (or anti-rabbit) IgG. The signal was detected using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate substrate (Roche). The experiment was repeated.

The rabbit polyclonal antibody specific for *C. quadricarinatus* proPO was produced by Shanghai Immune Biotech Ltd. (China), using the mixture of two peptides (FNFCGCGWPQHMLLPRG and YPDKRPMGFPFDR) derived from *C. quadricarinatus* proPO (GenBank no. AFD61667.1) as the antigen.

2.10. Detection of phenoloxidase (PO) activity

The PO activity was detected as described previously [23,24] with some modification. In brief, cultured HPT cells were fix with 2.5% glutaraldehyde in PBS for 15 min and wash twice for 10 min in 55 mM sodium phosphate buffer (PB) pH 6.8 containing 0.3% Triton X-100. After three washes with PB, the cells were incubated in 5 mM L-dopa, 5% sucrose in PB for 7 h at 27 °C. Hemocytes, purified GCs and SGCs were seeded as described before [25], and the PO activity was measured. The experiment was repeated.

3. Results

3.1. ME induces differentiation of GCs from cultured HPT cells

Crayfish HPT contains progenitors for SGCs and GCs, and the successfully culture of HPT cells *in vitro* provides a useful tool to study hematopoiesis [19]. However, fully development of these progenitors into mature hemocytes *in vitro* has not yet been reported. In our previous work, we found that HPT cells could survive in L-15 medium containing 1% FBS for 2–3 weeks (data not shown). Here to induce hemocyte differentiation and maturation from cultured HPT cells, different additives, including 20% of ME, 20% of NE, and 1% of plasma, were added to the medium. The concentrations of the additives were chosen according to previous reports [26–29] and our preliminary observation (data not shown). We found that the supplement of either plasma or NE had a negative effect on the survival of HPT cells. Most of the cells in these two groups died within 21 days (Fig. 1, row 4 and 5). In contrast, the addition of ME greatly improved the physical status of the cells, prolonging their survival time from 2 to 3 weeks to over a month (Fig. 1, row 3, Supplementary Fig. 1). Interestingly, small refractile granules appeared in the cytoplasm of some cells at ~14 days post induction (dpi). The size of granules, the number of granules per cell, and the ratio of cells containing granules increased with time. At 21–28 dpi the refractile granules were observed in over 60% of the cells which were very similar in morphology with GCs. The average survival rate of culture cells was estimated to be ~70% at 28 dpi by cell staining and counting. In our experiment, some cells could survive for 2 months, when they were flat and looked just identical to mature GCs (Supplemental Fig. 1). Besides, there were some cells in the culture that did not develop into GCs. These cells spread a lot and became very long and thin. They connected to each other and built up a network-like structure (Supplementary Fig. 2). We currently don't know what type of cells they are. To confirm this observation, the experiment was repeated for three times with HPT cells and tissue extract prepared from different animals, and similar results were obtained. To optimize the induction condition, ME was added to the culture at a final concentration of 5%, 10%, 20%, 30%, 50%, and 100%, separately. It turned out that the supplement of 20% and 30% ME gave the best result (data not shown). Therefore, 20% ME was used in the following experiments.

The ME-induced cells were further analyzed by TEM, and the formation of GCs were investigated. The results showed that the un-induced HPT cells had a large nuclear/cytoplasmic ratio with no or few granules in the cytoplasm (Fig. 2 A). With the progress of induction, the nuclear/cytoplasmic ratio decreased and electronic density cytoplasmic granules gradually appeared in the cells (Fig. 2 B and C). At 28 dpi, the cells containing granules (Fig. 2 C) were very similar to mature GCs (Fig. 2 D). The size of cytoplasmic granules from 30 GCs developed in the culture (at 28 dpi) and 30 GCs from the circulation were measured (over 700 granules were analyzed for each group). We found that the average diameter of the granules in GCs developed in our system (1.04 μm) was comparable to that of the granules in circulating GCs (1.26 μm). Based on these morphological evidences, we speculate that ME efficiently promotes the differentiation of HPT cells along the GC lineage.

3.2. The transcription of marker genes in ME-induced cells

The transcription of marker genes for mature hemocytes (prophenoloxidase, proPO), GCs (superoxide dismutase, SOD), and undifferentiated HPT cells (proliferation cell nuclear antigen, PCNA) [2,20] were analyzed by RT-PCR (Fig. 3). THC, purified SGCs and GCs were used as controls. We found that the transcription level of PCNA was high in un-induced HPT cells, and greatly reduced after ME induction. No PCNA expression was detected in THC, GCs, or SGCs. In contrast, low level of SOD transcription was detected in both THC and GCs, but not in SGCs, un-induced HPT cells, or induced HPT cells. Moreover, the transcription of proPO was detected in THC, GCs, SGCs, and ME-induced HPT cells, but not in un-induced cells. The changes in the transcription profile of marker genes suggest the development of mature hemocytes from HPT cells.

3.3. The GCs derived from culture HPT cell produce functional proPO

To see the change in protein expression profile during the differentiation process, lysates of the culture cells were collected at different stages and analyzed by SDS-PAGE (Fig. 4 A). Based on our result, the amount of two major proteins (~110 kDa and ~30 kDa) of HPT cells steadily decreased with the progress of induction. A band of 70–100 kDa emerged at 7 dpi, and increased over time. No obvious change was found in the cells grown in medium without ME supply. The 70–100 kDa protein appeared during induction is similar in size with proPO, an enzyme that is mainly stored in mature GCs [4,5,24] (Fig. S3). Therefore, the cell lysates were further analyzed by Western blotting with anti-proPO antibody (Fig. 4 B, Fig. S3) and the protein was confirmed to be proPO. No signal was detected when pre-immune serum was used. The amount of proPO in the induced cells at 56 dpi was comparable to that in the mature hemocytes.

proPO is the key enzyme of melanization, which was mainly expressed and stored in GCs [4,5,20,24,30] (Fig. S3). PO is the active form of proPO and its activity can be measured by using L-dopa as the substrate [23,24]. When hemocytes (total hemocyte, or purified SGCs and GCs) were fixed and incubated with L-dopa for 7 h (Fig. 5 D, E, F), all the GCs turned black, implying a strong PO activity. In contrast, SGCs only exhibited a very weak (or no) PO activity. Therefore, PO activity can be used as a “marker” for the maturation of GCs. To confirm the differentiation of GCs from HPT cells, we further measured the PO activity in these cells. No PO activity was detected in un-induced cells

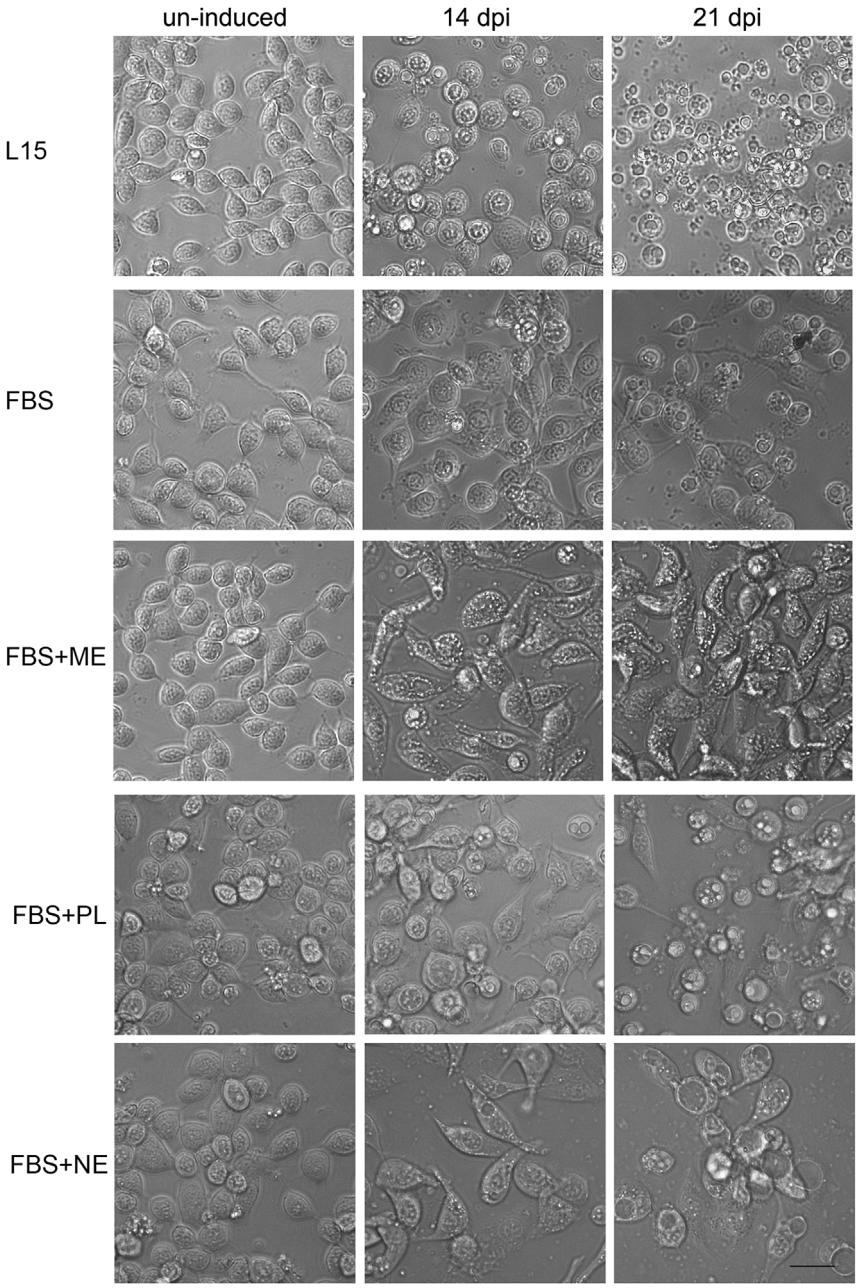

Fig. 1. Primary cultured HPT cells supplied with different additives. Crayfish HPT cells were grown in L15 medium (row 1), L15 + 1% FBS (row 2), L15 + 1% FBS + 20% ME (row 3), L15 + 1%FBS + 1% plasma (row 4), or L15 + 1%FBS + 20% NE (row 5). Every 7 days, images of the cells were taken and half of the culture medium was changed. Bar, 20 μm.

(Fig. 5A), a low level of PO activity was detected in some cell with granules at 14 dpi, while much stronger activity was detected at 28 dpi (Fig. 5B and C). The intensity of PO activity in the GCs developed *in vitro* varied from cell to cell, suggesting that they might in different stage of differentiation. The enzyme activity in a considerable portion of ME-induced cells at 28 dpi was comparable to that in circulating GCs. These data further support the successful formation of mature GCs in our experiment.

4. Discussion

In this study, we tried to induce differentiation of HPT cells *in vitro* by adding different additives to the culture medium. It has been shown that several regulators for hematopoiesis, such as AST1 [12], AST2 [13], thymosin [11], and serotonin [10], are present in the hemolymph and brain tissue. In addition, muscle extract is often used as a supplement in primary cell cultures [27,29,31], and it has been shown to induce differentiation of neural stem cells in mammals [28,32]. Therefore, we used plasma, ME and NE as our candidates to induce possible hemocyte differentiation.

We demonstrated that ME could induce efficient differentiation of GCs from cultured HPT cells. Hemocytes of crustaceans are classified based mostly on morphological features, especially the number and size of cytoplasmic granules [3]. In our experiments, the development of GCs was determined by light microscopy (Fig. 1) and TEM analysis (Fig. 2). The formation of large refractile cytoplasmic granules were seen in ME-induced HPT cells. The size of the granules and the number of granules per cell were similar to those in circulating GCs at ~28 dpi.

Additionally, the expression of marker genes was analyzed. Hemocyte-lineage marker genes have been identified in fresh water

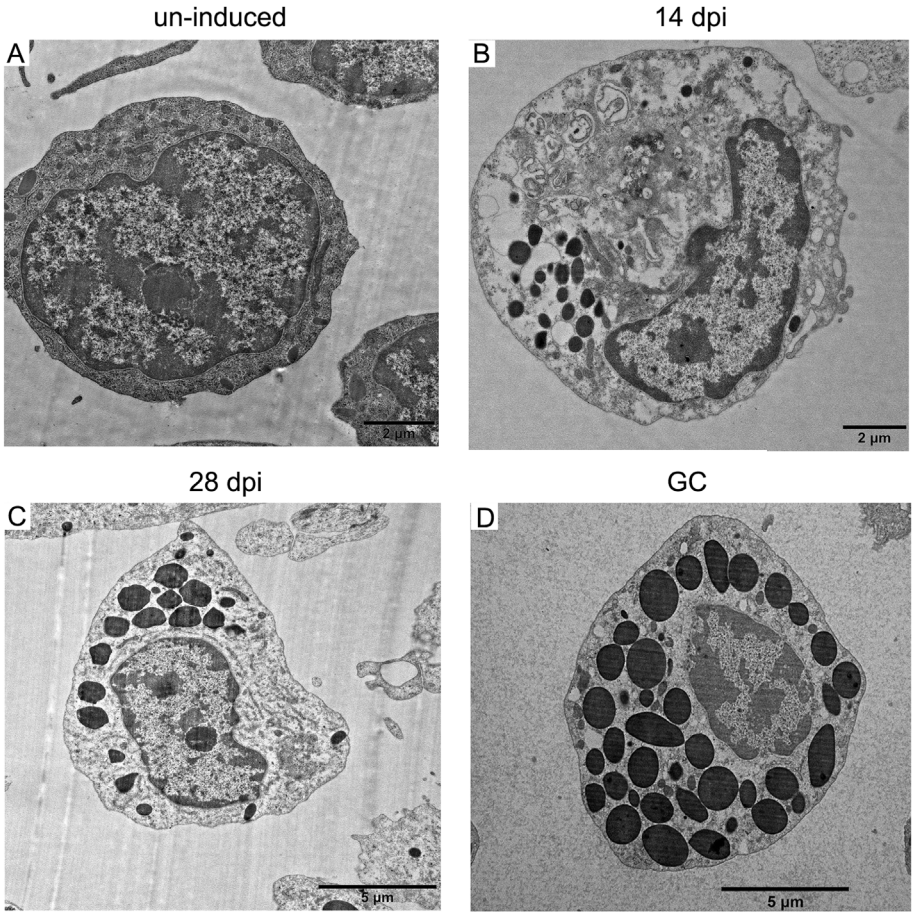

Fig. 2. Ultra-structural analysis of the cells induced by ME. HPT cells grown in L15 medium containing 1% FBS and 20% ME were collected at different time post induction. The samples were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, sequentially. After dehydration in ethanol, the specimens were embedded in Epon resin and ultrasectioned. The slices were stained with 2% uranyl acetate and 0.4% lead citrate, and observed using a transmission electron microscope. GCs collected from the circulation was used as a control. A. Un-induced; B. 14 dpi; C. 28 dpi; D. GC.

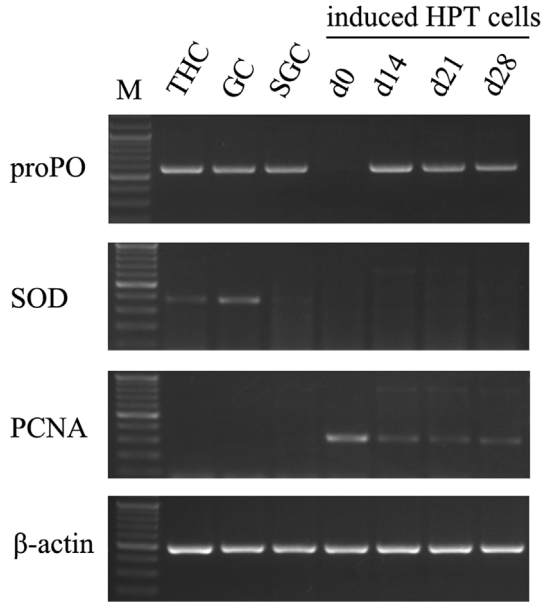

Fig. 3. RT-PCR analysis of marker gene expression. Total RNA was isolated from total hemocytes (THC), purified GCs, SGCs, and cultured HPT cells at different time post ME-induction. Genomic DNA contamination was eliminated by DNase treatment. First strand cDNA was synthesis with oligo $dT_{(18)}$ primer, and the marker genes were detected by PCR. β-actin was used as a internal control. The PCR products were analyzed by electrophoresis. For each experiment, 1×10^6 of THC, GCs, and SGCs were used. The cultured cells in three wells of 48-well plate cells were lysed and pooled at each time point. The experiment was repeated for 3 times.

crayfish *Pacifastacus leniusculus*, which include PCNA (specific for HPT cells); a two-domain Kazal proteinase inhibitor (KPI) (specific for SGCs), SOD (specific for GCs), and proPO (specific for circulating hemocytes, mainly expressed and stored in GCs) [2,5,20]. We failed to amplified KPI gene from *C. quadricarinatus*. Therefore, only PCNA, SOD and proPO were analyzed in this study. As shown in Fig. 3, the expression of PCNA, the marker gene for undifferentiated cells, significantly decreased in ME-induced cells, while the expression of proPO, the marker gene for mature hemocytes greatly increased. The shift of gene transcription profile indicated that the cultured cells did undergo differentiation and maturation. However, no obvious difference in the transcription level of proPO in SGCs and GCs could be seen by RT-PCR.

Although both SGCs and GCs transcribed proPO, we found that the enzyme protein was mainly stored in GCs (Fig. S3). This is in agreement with several previous reports [4,5,24]. In addition, GCs exhibited a very high PO activity, while SGCs exhibited no or very low PO activity (Fig. 5 E and F). Therefore, the PO activity can be used as a biochemical marker for the maturation of GCs. Further analysis indicated that the amount of proPO protein in ME-induced cells increased with time (Fig. 4). At 28 dpi, a large portion of the cells produced functional proPO that was comparable to circulating GCs (Fig. 5 C), confirming the formation of mature GCs. However, since not all the cultured cells differentiated into GCs, and the cells differentiated along the GC lineage were at various stages, the amount of proPO protein in ME-induced cells was not as much as that in equal amount of mature GCs at 28 dpi (Fig. S3).

We've noticed that SOD, a marker gene of GCs [2], was not detected in ME-induced cells within 28 days of induction. In our experimental animals, the transcription level of SOD was relatively low (Fig. 3). It is likely that SOD is not constitutively expressed in GCs. As an immune-

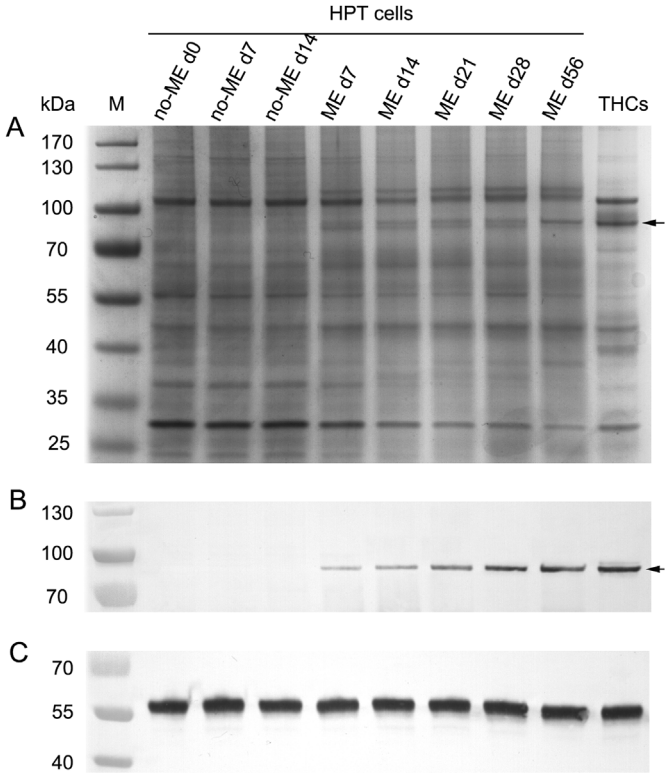

Fig. 4. SDS-PAGE and Western blotting analysis of the cells induced by ME. The HPT cells grow in culture medium with (ME) or without ME (no ME) were collected at different time point and lysed with SDS-loading buffer. The protein samples were separated on SDS-PAGE gel and stained with Coomassie brilliant blue (A). For Western blotting analysis, the proteins were transferred to a polyvinylidene difluoride membrane and probed with anti-porPO monoclonal antibody (B) and anti-α-tubulin monoclonal antibody (C). The proPO bands are indicated with arrows.

related gene, it may be induced during pathogen infection as previously reported [33–35].

Taken together, the morphological and biochemical evidences demonstrate that ME can induce GC differentiation form HPT cells. This is the first time that the differentiation and maturation of crustacean hemocytes are accomplished *in vitro*. The formation of GCs could be observed at ~14 dpi, and > 60% of the cells differentiated into mature GCs at 21–28 dpi (Fig. 1). Convincing results were obtained from different experiments, with different preparations of HPT cells and tissue extracts. Therefore, we offer a simple and reliable system to study the mechanism of crustacean hematopoiesis *in vitro*, which will greatly facilitate the identification of regulatory factors that are critical for this procedure. Moreover, crayfish hemocytes are comparable to the cells of the myeloid lineage in vertebrates, considering their function in innate immunity and blood clotting. Thus this model system can also be used to study the regulation of cells involved in innate immunity from an evolutionary point of view.

The successful induction of GC differentiation also raises several interesting questions. The first question is what molecule (s) directs the differentiation towards GC lineage. Because addition of crayfish NE or plasma hastened the death of cultured cells (Fig. 1), we speculate that the molecule(s) directing GC formation may mainly exist in muscle tissue, rather than in nerve tissue or hemolymph. The known factors involved in crayfish hematopoiesis, including AST1 [6], ASK2 [12], crustacean hematopoietic factor [9], thymosines [11], TGase [7], clotting protein [36], exist in hemolymph, and some of them, like clotting protein and TGase are quite abundant. In addition, a hematopoietic related molecule, serotonin [10], is abundant in the nerve system. Therefore, they may not be the factor(s) that function in our system. As reported in vertebrates, hormones or growth factors present in the muscle tissue may play a role [28,32,37,38]. It will be very interesting to identify this unknown hematopoiesis regulator from muscle extract in the future.

Moreover, the procedure of GC differentiation and maturation in crayfish is obscure so far. It has been reported in *P. leniusculus* that HPT may contain common stem cells of both SGCs and GCs, and precursor cells specific for GCs or SGCs, but the percentages of these cells have not yet been determined [5]. GCs is certainly not present in HPT, which have been proved by previous researches [5,19,20] and our own observation. It was described in several literature that the GCs become mature as they are released to the circulation. But the intermediate stage of GC maturation in the circulation is unknown. How long it takes and how it is regulated remained unclear. The only experimental

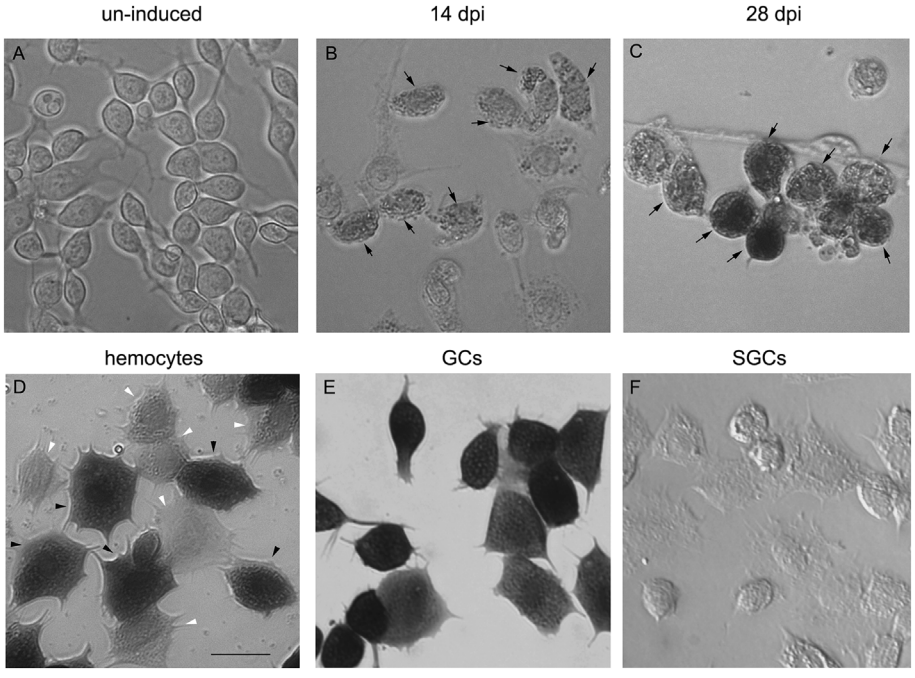

Fig. 5. Analysis of PO activity in ME-induced cells. At different time post ME induction, HPT cells were fix in 2.5% glutaraldehyde and penetrated in 0.3% Triton X-100. To measure PO activity, the cells were incubated in 5 mM L-dopa for 7 h and observed under a microscope (A, B, C). The cells with PO activity are indicated with black arrows. Total circulating hemocytes (D), purified GCs (E), and SGCs (F) were used as controls. GCs are indicated with black arrow heads and SGCs are indicated with white arrow heads (B, C, and D). Bar, 20 μm.

evidence we can find was provided by Söderhäll et al. [20]. They showed that when crayfish were injected with BrdU, the first labeled cells found in the circulation were all SGCs, whereas labeled GCs appeared in a few days. In our experiment, ~70% of cultured HPT cells survived till 28 dpi, and over 60% of them differentiated into GCs (Figs. 1 and 2). It suggests that a large majority of the progenitors in HPT have GC potential, and it take at least 21 days for them to differentiate into mature GCs. Notably, the HPT cells were undistinguishable from SGCs in the early stage of induction, before the formation of cytoplasmic granules. Therefore, it is likely that the so call GCs and SGCs in the circulation are not actually two types of mature hemocytes. At least some of them may represent different developmental stages of the GC lineage. How many cells in the circulation are in this intermediate stage, and whether they conduct any immune function, remain to be explored. The transdifferentiation of hemocyte from one type to another occurs in both vertebrates and drosophila. In vertebrates, monocytes located at the site of infection can undergo further differentiation into macrophages [39]. In drosophila, plasmatocytes are able to develop into either lamellocytes [15] or crystal cells [40]. The existence of an intermediate stages between pluripotent and fully differentiated hemocytes has been suggested in drosophila, and the cells in the intermediate stage are immune functional. For now, we don't know whether it is a similar case in crustacean, and it will be very interesting to look into it.

Conflicts of interest

The authors declare that they have no competing interests.

Authors' contributions

FL and FY conceived and designed the experiments; FL performed the differentiation assay, marker gene transcription, and the proPO activity analysis. LX performed Western blotting analysis and participate in cell culture. XH performed TEM analysis. WH participate in Western blotting analysis. FL, and FY drafted the manuscript. All authors read and approved the final manuscript.

Fundings

This work was supported by the National Natural Science Foundation of China [grant number 31672675] and the China Agriculture Research System [grant number CARS-48].

Acknowledgements

We thank Dr. Jianming Chen for his valuable discussion.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.054.

References

[1] I. Soderhall, Crustacean hematopoiesis, Dev. Comp. Immunol. 58 (2016) 129–141.
[2] C. Wu, I. Soderhall, Y.A. Kim, H. Liu, K. Soderhall, Hemocyte-lineage marker proteins in a crustacean, the freshwater crayfish, Pacifastacus leniusculus, Proteomics 8 (2008) 4226–4235.
[3] F. Li, X. Chang, L. Xu, F. Yang, Different roles of crayfish hemocytes in the uptake of foreign particles, Fish Shellfish Immunol. 77 (2018) 112–119.
[4] P. Jiravanichpaisal, B.L. Lee, K. Soderhall, Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization, Immunobiology 211 (2006) 213–236.
[5] X. Lin, I. Soderhall, Crustacean hematopoiesis and the astakine cytokines, Blood 117 (2011) 6417–6424.
[6] I. Soderhall, Y.A. Kim, P. Jiravanichpaisal, S.Y. Lee, K. Soderhall, An ancient role for a prokineticin domain in invertebrate hematopoiesis, J. Immunol. 174 (2005) 6153–6160.
[7] X. Lin, K. Soderhall, I. Soderhall, Transglutaminase activity in the hematopoietic tissue of a crustacean, Pacifastacus leniusculus, importance in hemocyte homeostasis, BMC Immunol. 9 (2008) 58.
[8] R. Sirikharin, K. Junkunlo, K. Soderhall, I. Soderhall, Role of astakine1 in regulating transglutaminase activity, Dev. Comp. Immunol. 76 (2017) 77–82.
[9] X. Lin, K. Soderhall, I. Soderhall, Invertebrate hematopoiesis: an astakine-dependent novel hematopoietic factor, J. Immunol. 186 (2011) 2073–2079.
[10] C. Noonin, Involvement of Serotonin in crayfish hematopoiesis, Dev. Comp. Immunol. 86 (2018) 189–195.
[11] N. Saelee, C. Noonin, B. Nupan, K. Junkunlo, A. Phongdara, X. Lin, et al., beta-thymosins and hemocyte homeostasis in a crustacean, PLoS One 8 (2013) e60974.
[12] X. Lin, M. Novotny, K. Soderhall, I. Soderhall, Ancient cytokines, the role of astakines as hematopoietic growth factors, J. Biol. Chem. 285 (2010) 28577–28586.
[13] A. Watthanasurorot, N. Saelee, A. Phongdara, S. Roytrakul, P. Jiravanichpaisal, K. Soderhall, et al., Astakine 2–the dark knight linking melatonin to circadian regulation in crustaceans, PLoS Genet. 9 (2013) e1003361.
[14] A. Watthanasurorot, K. Soderhall, P. Jiravanichpaisal, I. Soderhall, An ancient cytokine, astakine, mediates circadian regulation of invertebrate hematopoiesis, Cell. Mol. Life Sci. 68 (2011) 315–323.
[15] R. Markus, B. Laurinyecz, E. Kurucz, V. Honti, I. Bajusz, B. Sipos, et al., Sessile hemocytes as a hematopoietic compartment in Drosophila melanogaster, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 4805–4809.
[16] K. Koiwai, H. Kondo, I. Hirono, The immune functions of sessile hemocytes in three organs of kuruma shrimp Marsupenaeus japonicus differ from those of circulating hemocytes, Fish Shellfish Immunol. 78 (2018) 109–113.
[17] A. Accorsi, E. Ottaviani, D. Malagoli, Effects of repeated hemolymph withdrawals on the hemocyte populations and hematopoiesis in Pomacea canaliculata, Fish Shellfish Immunol. 38 (2014) 56–64.
[18] L.T. Sigle, J.F. Hillyer, Mosquito hemocytes associate with circulatory structures that support intracardiac retrograde hemolymph flow, Front. Physiol. 9 (2018) 1187.
[19] I. Soderhall, Recent advances in crayfish hematopoietic stem cell culture: a model for studies of hemocyte differentiation and immunity, Cytotechnology 65 (2013) 691–695.
[20] I. Soderhall, E. Bangyeekhun, S. Mayo, K. Soderhall, Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of Pacifastacus leniusculus, Dev. Comp. Immunol. 27 (2003) 661–672.
[21] J. Huang, F. Li, J. Wu, F. Yang, White spot syndrome virus enters crayfish hematopoietic tissue cells via clathrin-mediated endocytosis, Virology 486 (2015) 35–43.
[22] R. Fu, F. Li, F. Yang, Separation of hemocytes of Cherax quadricarinatus by Percoll discontinuous density gradient centrifugation, J. Fish. China 43 (4) (2019), https://doi.org/10.11964/jfc.20180511286.
[23] C. Noonin, X. Lin, P. Jiravanichpaisal, K. Soderhall, I. Soderhall, Invertebrate hematopoiesis: an anterior proliferation center as a link between the hematopoietic tissue and the brain, Stem Cell. Dev. 21 (2012) 3173–3186.
[24] P.G. Giulianini, M. Bierti, S. Lorenzon, S. Battistella, E.A. Ferrero, Ultrastructural and functional characterization of circulating hemocytes from the freshwater crayfish Astacus leptodactylus: cell types and their role after in vivo artificial non-self challenge, Micron 38 (2007) 49–57.
[25] J. Wu, F. Li, J. Huang, L. Xu, F. Yang, Crayfish hematopoietic tissue cells but not hemocytes are permissive for white spot syndrome virus replication, Fish Shellfish Immunol. 43 (2015) 67–74.
[26] X. Xu, H. Duan, Y. Shi, S. Xie, Z. Song, S. Jin, et al., Development of a primary culture system for haematopoietic tissue cells from Cherax quadricarinatus and an exploration of transfection methods, Dev. Comp. Immunol. 88 (2018) 45–54.
[27] A.L. Mulford, F. Lyng, C. Mothersill, B. Austin, Development and characterization of primary cell cultures from the hematopoietic tissues of the Dublin Bay prawn, Nephrops norvegicus, Methods Cell Sci. 22 (2000) 265–275.
[28] J.X. Jiang, R.C. Choi, N.L. Siow, H.H. Lee, D.C. Wan, K.W. Tsim, Muscle induces neuronal expression of acetylcholinesterase in neuron-muscle co-culture: transcriptional regulation mediated by cAMP-dependent signaling, J. Biol. Chem. 278 (2003) 45435–45444.
[29] S.K. George, A.K. Dhar, An improved method of cell culture system from eye stalk, hepatopancreas, muscle, ovary, and hemocytes of Penaeus vannamei, In Vitro Cell. Dev. Biol. Anim. 46 (2010) 801–810.
[30] S. Sricharoen, J.J. Kim, S. Tunkijjanukij, I. Soderhall, Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish, Dev. Comp. Immunol. 29 (2005) 1017–1031.
[31] S.N. Chen, C.S. Wang, Establishment of cell culture systems from penaeid shrimp and their susceptibility to white spot disease and yellow head viruses, Methods Cell Sci. 21 (1999) 199–206.
[32] T.T. Wang, A.H. Jing, X.Y. Luo, M. Li, Y. Kang, X.L. Zou, et al., Neural stem cells: isolation and differentiation into cholinergic neurons, Neuroreport 17 (2006) 1433–1436.
[33] Y.-T. Liu, C.-I. Chang, J.-R. Hseu, K.-F. Liu, J.-M. Tsai, Immune responses of prophenoloxidase and cytosolic manganese superoxide dismutase in the freshwater crayfish Cherax quadricarinatus against a virus and bacterium, Mol. Immunol. 56 (2013) 72–80.
[34] M.N. Hung, R. Shiomi, R. Nozaki, H. Kondo, I. Hirono, Identification of novel copper/zinc superoxide dismutase (Cu/ZnSOD) genes in kuruma shrimp Marsupenaeus japonicus, Fish Shellfish Immunol. 40 (2014) 472–477.
[35] G.A. Gomez-Anduro, F. Ascencio-Valle, A.B. Peregrino-Uriarte, A. Campa-Cordova, G. Yepiz-Plascencia, Cytosolic manganese superoxide dismutase genes from the white shrimp Litopenaeus vannamei are differentially expressed in response to lipopolysaccharides, white spot virus and during ontogeny, Comp. Biochem. Physiol.

B Biochem. Mol. Biol. 162 (2012) 120–125.
[36] K. Junkunlo, K. Soderhall, I. Soderhall, Clotting protein - an extracellular matrix (ECM) protein involved in crustacean hematopoiesis, Dev. Comp. Immunol. 78 (2018) 132–140.
[37] J.L. McManaman, F.G. Crawford, Skeletal muscle proteins stimulate cholinergic differentiation of human neuroblastoma cells, J. Neurochem. 57 (1991) 258–266.
[38] R. Tatsumi, J.E. Anderson, C.J. Nevoret, O. Halevy, R.E. Allen, HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells, Dev. Biol. 194 (1998) 114–128.
[39] V. Hartenstein, Blood cells and blood cell development in the animal kingdom, Annu. Rev. Cell Dev. Biol. 22 (2006) 677–712.
[40] A.B. Leitao, E. Sucena, Drosophila sessile hemocyte clusters are true hematopoietic tissues that regulate larval blood cell differentiation, eLife 4 (2015).