Abstract: Pigmentation, a natural mechanism, plays an important role in photo-
protecting larvae and embryos of sea urchins from harmful impacts of solar
radiation, hypoxia, pathogens, metals and toxicants and might be useful as a
marker of environmental stresses. The use of sea urchin embryos and gametes in
testing developmental and production effects has been successfully developed by
a number of laboratories worldwide. The objective of this study was to find the
maximal expression level of the genes encoding enzymes expressed in pigment
cells throughout the development of Scaphechinus mirabilis and in cell cultures of
this sand dollar. Two genes related to different gene families (pks and sult) were
selected for analysis in pigmentation, and their expression level was evaluated by
quantitative real-time PCR. The naphthoquinoid pigments of echinoderms and
related compounds form a new class of highly effective antioxidants of the phenol
type, exhibiting high bactericidal, algicidal, hypotonic and psychotropic activity.
Studying marine invertebrate stem cells and primarily differentiation processes
and growth regulation may open novel biotechnological avenues such as new
applications including basic research in translational medicine.

1. Introduction

Marine organisms are known to possess various compounds with significant
and valuable biotechnological potential for the pharmaceutical industry (Martins et al.
2014). Protostomes (Porifera, Cnidaria) and some deuterostomes (Echinodermata)
have been reported to contain very high concentrations of bioactive compounds, many
of which are not found in terrestrial organisms. In particular, sand dollar pigment
cells are the source of organic naphthoquinone pigments, known as potent antioxidant
substances (Koltsova et al. 1981). It has previously been reported that naphthoquinone
pigments from purple sea urchins (marked with various phenolic hydroxyl groups)
demonstrated the antioxidant ability to depress lipid peroxidation, driving purple
sea urchins as an original and natural source of antioxidants. Moreover, the sea
urchin naphthoquinone pigment response may manifest via an increased antioxidant
activity (Vasileva et al. 2020) due to carotenoids. The pigment–protein complex seems
to have appeared 2–3 billion years ago in primitive purple photosynthetic bacteria. Later, the resulting strong electronic donor became the naphthoquinoid pigment (Sakuragi et al. 2005).

Pigmentation, a natural mechanism, plays an important role in photo-protecting the larvae and embryos of sea urchins from harmful impacts of solar radiation, hypoxia, pathogens, metals and toxicants (Pinsino and Matranga 2015; Calestani and Wessel 2018). Pigmentation might be useful as a marker of environmental stresses in adult and larval individuals. In fungi, the biosynthesis of naphthoquinone pigments is an important response to stress exposure, as shown by Medentsev et al. (2005). In sea urchins, the main pigments produced in normal conditions and in response to different types of stress are also naphthoquinone pigments. For example, there are echinochrome A and some spinochromes in the cytoplasm of sand dollar pigment cells. One of the cellular defense mechanisms in sea urchins is the activation of specific coelomocytes—red spherule cells. These specific coelomocytes participate in recognizing and neutralizing pathogens. Only polyketide synthase (pks) and some flavin-containing monooxygenases (fmos) have been previously reported to have a role in the biosynthesis of naphthoquinone pigments of Strongylocentrotus purpuratus, a sea urchin (Perillo et al. 2020; Wessel et al. 2020). The sea urchin pks gene encodes an enzyme important in echinochrome synthesis. Other functions of pigment cells can be connected with their immune system (Smith et al. 2018; McClay et al. 2020). It is possible that a sulfotransferase gene (sult) is necessary for some enzymes also participating in naphthohynoid synthesis (Ageenko et al. 2011; Ageenko et al. 2014).

The chemical synthesis of naphthoquinones was reported in 1985, but commercial applications have been hampered by the toxicity of some synthesized substances, as demonstrated Klotz et al. (2014). Echinochrome A has been reported to usually be produced in sand dollar pigment cells, while the spinochromes are synthesized in cells of several sea urchin species (Koltsova et al. 1981; Ageenko et al. 2014). To protect sea urchins and their habitat from over-exploitation, some authors have developed in vitro approaches for the induction of pigment differentiation through gene transfection in embryonic cell cultures of two echinoderms, the sand dollar Scaphechinus mirabilis and Strongylocentrotus intermedius. After two-month cultivation, the cells of sand dollar embryos transfected with plasmid DNA containing the yeast gal4 produced naphthoquinone pigments with an absorbance spectrum similar to the echinochrome spectrum in vivo. A new in vitro technology that does not consider gene transfection into embryos of sea urchins was developed, supported by sea urchins’ coelomic fluid components (Ageenko et al. 2011).

The pks and sult expressions were evaluated by quantitative real-time PCR (qRT-PCR) in order to identify an association with the biosynthesis of naphthoquinone pigments in the sand dollar S. mirabilis. Peak expression levels of pks and sult in sand dollar embryos were detected at the blastula and gastrula stages. In vitro, sand dollar
pigment cell numbers were higher when cultured in sea urchin coelomic fluids than in seawater.

Currently, the knowledge on the growth actor genes expressed in the tissues of marine invertebrates is meager. For vertebrates, the key genes regulating the stem state of cells and ensuring a high level of proliferation of embryonic stem cells in culture are, mainly, nanog and oct-4. The mechanism of the realization of stem cell programming for the toti- and pluripotential states is determined by the key genes regulating “stemness”. Some authors previously discovered one of the conserved genes, SpOct, in the sea urchin *S. purpuratus*. In addition, a homologue of the pluripotent gene nanog was found in the genome of sea urchins, with clear expression at the mesenchymal blastula stage (the beginning of gastrulation). This nanog gene exhibited 64% homology and 44.7% identity in amino acid residues, further revealing high similarities with the mouse brain-specific homeobox gene *bsx* (80.8% homology, 61.7% identity; Odintsova 2009). The aim of this article was to describe naphthoquinoid pigments of sand dollars, obtained in vivo and in vitro for practical application.

2. Methods

2.1. Collection of Biological Material

Adult sand dollars (*S. mirabilis*) were collected from Vostok Bay (Sea of Japan, Russia) throughout the breeding season (at the beginning–middle of August) and were maintained in running aerated seawater aquaria at 17 °C for 1–3 days. There are different groups of Echinoderms: sea urchins, sea stars, holothurians and sea lilies. In terms of body shape, sea urchins (*Echinoidea*) are divided into two types: regular (spherical) and irregular (flat and heart-shaped) sea urchins. The irregular, flat sea urchin *S. mirabilis* (sand dollar) is one of the widespread representatives of shallow-water benthos. The larvae at 48 hpf (hours post-fertilization) were fed daily with *Isochrysis galbana* (100,000 cells mL⁻¹). The larvae of the mesenchymal blastula (14 hpf) were collected on a 30 μm nylon mesh and cell cultures were obtained, as described (Ageenko et al. 2014). The coelomic fluids from intact or injured (after needle pricks around Aristotle’s lantern) adult sea urchins were named normal coelomic fluid (CFₙ) or wounded coelomic fluid (CFₕ), respectively. SW was used as a control medium. All culture media (SW and the coelomic fluids) were supplemented with 2% fetal calf serum and gentamicin (40 mg·L⁻¹). All reagents were purchased from Sigma-Aldrich Co. LLC (USA). We used CF of the wounded sea urchins and CF of normal sea urchins, but not CF of sand dollars because it is very difficult to collect the required amount of CF. In our first experiments, we tested all sand dollar CFs: the effect was the same (data not shown).
2.2. RNA Isolation

Total RNAs from sand dollar gametes, embryos and larvae, as well as from their cultivated embryonic cells, were extracted using Yellow Solve reagent (Clonogen, Russia) followed by DNase I treatment (Sileks, Russia) to remove genomic DNA. The first strand of cDNA was synthesized using 1.5 μg of total RNA as a template with the Reverse Transcription System (Sileks) in a 50 μL reaction volume. The PCR reactions were conducted using an iCycler thermocycler (Bio-Rad Laboratories, Minneapolis/Saint Paul, Minnesota, USA) under the following conditions: one cycle of 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 50 °C and 35 s at 72 °C, with a final extension cycle of 10 min at 72 °C.

2.3. Quantitative Real-Time PCR (q-RT-PCR)

Q-RT-PCR (iCycler thermocycler equipped with the iQ5 Multicolor q-RT-PCR detection system; Bio-Rad Laboratories) was performed using an established protocol (Ageenko et al. 2011). cDNAs were amplified in 20 μL of the reaction mixture containing 1× TaqMan Buffer, 2.5 mM MgCl₂, 250 μM of each deoxynucleotide, 1U Taq DNA polymerase, 0.5–2 μL cDNA samples and 0.25 μM of each primer and probe (Real-Time PCR Kit, Syntol, Russia). Amplification conditions: one 2 min cycle at 95 °C followed by 50 cycles of 10 s at 95 °C and 25 s at 62 °C. Results were analyzed with the iQ5 Optical System Software v.2.0 and presented in relative units. The S. mirabilis actin gene (GenBank accession number DQ222227) and ubiquitin gene (PRJEB33560) were used as endogenous controls. Results were summarized from five independent experiments, each with three technical replicates in relative units. The primer and TaqMan probe used in q-RT-PCR, namely, 5′CTT CGC CAG CCC ATG ATC AAC3′ and 5′ACT CGC CCA CGT CAC CAT CT3′, were developed for expression analysis of the pks gene. The primer 5′GAT CTT CGC TGG CAA GCA GCT3′ and TaqMan probe 5′CCT TCT GGA TGT TGT AGT CGG ACA3′ were used for expression analysis of the sult gene (Ageenko et al. 2011; Kiselev et al. 2013).

3. Results

3.1. Pks and Sult Expression Profiles in Sand Dollar Gametes and throughout Development

We chose the sand dollar S. mirabilis from available species of regular and irregular Echinoids because the sand dollar embryos and larvae contain many pigment cells in their body (Figure 1). The gene expression profiles for the two genes tested, pks and sult, were different in gametes and throughout development.
Figure 1. Sand dollar *Scaphechinus mirabilis* pluteus larva. Arrows show the pigment cells. Bar 20 µm. Source: Graphic by author Natalya V. Ageenko.

We detected trace levels of *pks* and *sult* transcripts in spermatozoids and unfertilized eggs. The highest *pks* level of expression was observed at the gastrula stage (Figure 2A), while the maximum *sult* level was found at the earlier blastula stage (Figure 2B). Then, at the prism stage, the level of *pks* expression fell by more than three times but increased in the pluteus larvae, without reaching the previous peak gastrula levels. In contrast, the level of *sult* expression fell after gastrulation and equally increased in abundance at the prism and pluteus stages but also did not reach the level of *sult* expression at the blastula stage.
Figure 2. Expression of \textit{pks} and \textit{sult} in vivo: in unfertilized eggs (Egg), spermatozoids (Sp), embryos and larvae of the sand dollar \textit{Scaphechinus mirabilis} (A,B) and \textit{Strongylocentrotus intermedius} (C,D) at various stages of development: blastula, 12 h post-fertilization (Bl); gastrula, 24 hpf (Gl); prism, 34 hpf (Pr); and pluteus, 72 hpf (Pl). * \(p < 0.05\); ** \(p < 0.01\). Mean ± SD, five biological replicates, each with three technical replicates in relative units. One-way analysis of variance (ANOVA) followed by Tukey’s pairwise comparison test Significant at \(p < 0.05\). Y-axis—relative units (r.u.). Source: Graphics 2A,B by author Natalya V. Ageenko; graphics 2C,D from Ageenko et al. (2011).

3.2. Pigment Differentiation in Cell Culture

We found significant differences in the pigment gene expression profiling for the embryonic sand dollar cells cultivated for four days under various culture media (Figure 3). Three media were employed to test the effects of various culture conditions on the sand dollar pigment differentiation under in vitro conditions (Figure 3): SW, and the coelomic fluids of normal and wounded sea urchins. The appearance of sand dollar cells cultivated for 4 days is presented in Figure 3A–C. In the blastula-derived cultures, pigment cells were detected in all media tested at all cultivations. Pigment cell numbers (Figure 3D) were associated with the coelomic fluids tested, and we
revealed a >2-fold increase in pigment cell numbers in the CF\textsubscript{w} as compared to the CF\textsubscript{n} after 4 days of cultivation. After 10 days of cultivation, the pigment cell numbers drastically reduced in all tested media.

Figure 3. Pigment cells of the sand dollar \textit{Scaphechinus mirabilis}. (A–C) Embryonic pigment cells in a blastula-derived cell culture of the sea urchin \textit{S. mirabilis} cultivated for 4 days (bar 10 µm). The cells were cultivated in seawater (A); the coelomic fluid of intact sea urchins (B); and the coelomic fluid of injured sea urchins (C). (D) Cellular dynamics of the sand dollar pigment cells cultivated in the three culture media (SW, CF\textsubscript{n} and CF\textsubscript{w}) over 4–10 days of incubation. In total, >500 cells were counted for each studied culture medium. (E,F) Expression of two genes associated with the biosynthesis of naphthoquinone pigments in sand dollar cells maintained under various culture media (SW, CF\textsubscript{n} and CF\textsubscript{w}) over four days: E. The \textit{pks} expression level; F. the \textit{sult} expression level. Bars represent the mean ± SD, five biological replicates, each with three technical ones. One-way ANOVA followed by Tukey’s pairwise comparison test. Significant at \(p < 0.05\). Source: Graphics by author Natalya V. Ageenko.

The expression of \textit{pks} in sand dollar cells cultivated in the coelomic fluids was increased when compared with cells cultivated in SW, and the expression levels in CF\textsubscript{w}-cultivated cells were significantly (>2-fold) higher than those in cells cultivated in SW (Figure 3E). These results coincide with our data about the number of pigment cells cultivated in the various culture media. The expression profile of \textit{sult} (Figure 3F) had a similar trend to that of the \textit{pks} expression profile.
4. Discussion

Kominami and colleagues reported that sand dollar larvae contain one of the largest amounts of pigment cells compared with larvae of all other tested species of Echinoids (Kominami and Takata 2002). In contrast to our in vivo data obtained previously for the sea urchin *S. intermedius* (Ageenko et al. 2011), in the sand dollar *S. mirabilus*, the timing of *pks* and *sult* expressions differed between the blastula and gastrula stages, indicating the specificity of pigment cell appearance in these two echinoid species. Yet, we confirm previous results on the effects of the culture medium composition on the appearance rates of echinoid pigment cell precursors (Ageenko et al. 2014). As shown in this study, pigment differentiation in cultured sand dollar cells grown in coelomic fluids intensified when compared with cells grown in SW. The distinct changes in the proportion of pigment cells under the CFw versus the CFn conditions could be explained by the specific components of coelomic fluids: alternations in protein compositions of coelomic fluids after sea urchin injury compared to control (uninjured) animals and the considerable shift in the absorption maxima for some proteins were previously reported (Ageenko et al. 2014).

However, there is an alternative explanation for these effects, which is connected with carbonic anhydrases (CAs). CA is a participant in the calcification process in numerous invertebrates. Recently, very low concentrations of a specific inhibitor of biomineralization and a potent inhibitor of CAs, acetazolamide, have been found to inhibit pigment cell precursor differentiation, as well as the production of echinochrome in echinoid larvae (Zito et al. 2015). The authors suggest that some isoforms of CAs might be implicated in the production of echinochrome, providing plausible support for the impact of acetazolamide on the pigment cell number in the sea urchin larvae. Currently, the roles of CAs in echinoid larval pigment cell formation are still elusive, requiring further study and analysis.

Numerous endeavors focusing on the development of immortal cell lines from a wide range of marine invertebrate species have been reported, but all have been unsuccessful (Cai and Zhang 2014). Thus, we could not use any echinoid cell line. In this study, q-RT-PCR and cell culture applications were used for the quantitative assessment of pigment cell precursor differentiation in sand dollar primary cell cultures. We revealed that the maximum level of pigment differentiation was reached when the cells of the sand dollar *S. mirabilus* were cultivated in CFw. The same has previously been reported for *S. intermedius* cultivated cells, and pigment cell numbers were higher when cultured in sea urchin coelomic fluids than in seawater (Ageenko et al. 2014).

In vivo, the highest level of pigment expression in sand dollar embryos (the Sea of Japan, Russia) was observed at the blastula and gastrula stages. In vitro, genes of interest are also significantly expressed in blastula-derived cell cultures, confirming that primary embryonic cell cultures are suitable models for in vitro
investigation of pigment differentiation. Further, the employed assay has emerged as a valuable tool for naphthoquinone pigment assessment throughout development and in cell cultures of these sand dollars. The findings contribute to the understanding of the pigment biology of Echinoid cells (Calestani and Wessel 2018; Perillo et al. 2020; Wessel et al. 2020) and create opportunities for the commercial production of natural antioxidants of marine origin. The naphthoquinoid pigments of sea urchins are a promising source for the production of drugs with various pharmacological activities (Lebedev et al. 2005). The use of aqueous solutions of sodium salts of naphthoquinone—echinochrome A in experiments to study the level of emission of the cardiac isoenzyme creatine phosphokinase in the coronary effluent showed a decrease in the size of the necrosis zone (Elyakov et al. 1999b). Thus, echinochrome A was found to have some ophthalmological and cardioprotective properties (Elyakov et al. 1999a, 1999b). Based on the data obtained, new effective drugs with unique therapeutic properties, such as “Histochrome for cardiology” and “Histochrome for ophthalmology”, have been developed.

**Author Contributions:** A.N.V. and O.N.A.: conceptualization, methodology, visualization, investigation, original draft preparation, writing—reviewing and editing. In addition, they took part in the experiments with sand dollar embryos and larvae. A.N.V. and K.K.V.: software, validation. They performed q-RT-PCR experiments and analyzed the obtained data.

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