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Effects of lithium on pigmentation in the embryonic zebrafish (*Brachydanio rerio*)

Eun-Jung Jin, Giselle Thibaudeau *

Department of Biological Sciences, 130 Harned Hall, Lee Blvd, Mississippi State University, Mississippi State, MS 39762, USA

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Abstract

Pigment cell precursors of the embryonic zebrafish give rise to melanophores, xanthophores and/or iridophores. Cell signaling mechanisms related to the development of pigmentation remain obscure. In order to examine the mechanisms involved in pigment cell signaling, we treated zebrafish embryos with various activators and inhibitors of signaling pathways. Among those chemicals tested, LiCl and LiCl/forskolin had a stimulatory effect on pigmentation, most notable in the melanophore population. We propose that the inositol phosphate (IP) pathway, is involved in pigment pattern formation in zebrafish through its involvement in the: (1) differentiation/proliferation of melanophores; (2) dispersion of melanosomes; and/or (3) synthesis/deposition of melanin. To discern at what level pigmentation was being effected we: (1) counted the number of melanophores in control and experimental animals 5 days after treatment; (2) measured tyrosinase activity and melanin content; and (3) employed immunoblotting techniques with anti-tyrosine-related protein-2 and anti-melanocyte-specific gene-1 as melanophore-specific markers. Although gross pigmentation increased dramatically in LiCl- and LiCl/forskolin treated embryos, the effect on pigmentation was not due to an increase in the proliferation of melanophores, but was possibly through an increase in melanin synthesis and/or deposition. Collectively, results from these studies suggest the involvement of an IP-signaling pathway in the stimulation of pigmentation in embryonic zebrafish through the synthesis/deposition of melanin within the neural crest-derived melanophores.

Keywords: Signal transduction; Pigment; Melanophore; Melanocyte-specific gene-1; Tyrosine-related protein-2; Tyrosinase; Melanin

1. Introduction

Essential steps involved in the development of the vertebrate embryo are achieved through proper functioning of coordinated inductive mechanisms that mediate regional specialization of cells. The molecular basis of such mechanisms are only partially understood and it is likely that a key role is played by receptor-mediated signal transduction processes which induce region-specific gene expression.

During vertebrate development, cells of the embryonic neural crest undergo a progressive series of developmental restrictions, and are the progenitors of many adult cell types, including sympathetic and parasympathetic sensory neurons, Schwann cells, connective tissue cells, and all pigment cells of the body [1,2].

Fish possess three types of pigment-producing cells, melanophores (containing melanin), xantho-

* Corresponding author. Fax: +1 (601) 325-7939; E-mail: giselle@ra.msstate.edu

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phores (containing yellow pigment), and iridophores (containing reflecting platelets). It is the specific developmental sequence that dictates the location and timing of chromatophore differentiation [3]. Furthermore, the deposition of the various pigments into organelles, the distribution of these organelles within the cell, and the distribution and organization of the three pigment cell types elaborate the many colors and patterns seen in amphibians and fish.

Although the embryonic neural crest, which gives rise to all of the pigment cells of the skin, is one of the best cell models to study patterning, cell-signaling mechanisms of the chromatophores remain unclear. Cyclic AMP has been found to inhibit neural crest-derived adrenergic neuron development in vitro [4] and this pathway is known to play an important role in the control of melanoblast and melanocyte numbers during cell migration and the establishment of pattern in the epidermis [5–10]. Likewise, this signal transduction system has been shown to play a role in the proliferation of melanoma cells, a derived mutant pigment-cell line [11], human keratinocytes [12], and human melanocytes [13]. Bertolotto et al. [14] suggested that cAMP-elevating agents led to a stimulation of tyrosinase activity in melanoma cells.

Lithium, an inhibitor of inositol phosphate suppresses the recycling of inositol, causing the accumulation of various forms of inositol phosphate (e.g. Ins(1)P, Ins(3)P, Ins(4)P, Ins(1,4)P_2, Ins(1,3,4)P_3) [15,16]. Although it is known that all these forms of inositol phosphate can act as biological second messengers, the mechanisms of their actions remain unclear.

Chang and Meng [17] investigated the involvement of inositol phosphate (IP) cell-signaling pathways in early development of the zebrafish. They reported that cytokinesis-associated Ca^{2+} signals occurring at cell cleavage sites arise mainly from internal stores of calcium rather than from free calcium in the zebrafish. It has also been suggested that intracellular cAMP, Ca^{2+}, or both are responsible for movement of pigment organelles (aggregation/dispersion) within chromatophores. Examples include a requirement for cAMP-dependent protein kinase for organelle dispersion in *Carassius* xanthophores [18], CaM-activated protein phosphatase for aggregation in *Tilapia* melanophores [19], and an elevation of intracellular Ca^{2+} for aggregation of erythrophores in *Holocentrus* [20].

Evaluation of factors affecting pigmentation has often been performed via examination of the melanin content or tyrosinase activity in melanocytes or melanoma cells. Many substances affecting melanin formation in normal melanocytes and melanoma cells have been found. Examples include hydroquinone, linoleic acid, kojic acid, retinoic acid [21], endothelins [22], theophylline [23], and melanocyte-stimulating hormone [24].

In this investigation, we examined tyrosinase activity, melanin content and the expression of two melanophore precursor-specific markers, melanocyte-specific gene-1 (MSG-1) [25], and tyrosine-related protein-2 (TRP-2) [26] in embryonic zebrafish (*Brachydanio rerio*) after treatment with various chemicals known to affect cell signaling. We propose that the IP pathway, which is stimulated by LiCl is involved in enhancing pigmentation in the zebrafish embryo. This enhancement could be due to a stimulation of proliferation or differentiation of melanophores, dispersion of melanosomes throughout the cell and/or synthesis or deposition of melanin into melanosomes. These potential mechanisms leading to altered pigmentation are discussed.

2. Materials and methods

2.1. Embryos and treatments

Zebrafish eggs were spawned in the laboratory, collected, placed in embryo medium [27] supplemented with antibiotics and raised until they reached the appropriate stage of development. Twenty-four hours after fertilization, embryos were treated with 50 mM LiCl, 1 μM forskolin, 50 mM LiCl/1 μM forskolin, 50 mM LiCl/1 μM EGTA (a calcium chelator), 50 mM LiCl/5 μM U73122 (a phospholipase-C inhibitor), 50 mM LiCl/10 μM W-7 (a calmodulin antagonist) or 50 mM LiCl/2 μM 5-*N*,*N*-(hexamethylene)-amiloride (a Na^+/H^+ antiporter inhibitor). Controls consisted of sibling embryos raised in a similar manner, but receiving no treatment, NaCl substituted for LiCl experimentals and DMSO for forskolin and inhibitor experimentals as they were dissolved in DMSO, or NaCl and DMSO for LiCl/forskolin experimentals. Each treatment and the corresponding control series was repeated in six wells of

a 24-well tissue culture plate with each well containing five embryos. The entire experiment was run with four different spawnings. All embryos were monitored daily for signs of altered development and/or changes in pigmentation. Embryos were collected 5 days after treatment and washed with several changes of Tris-buffered saline (TBS) to remove any excess chemical in the medium. Melanophores were counted in control and experimental embryos 5 days after treatment. For melanophore counts all embryos were anesthetized in MS222/embryo medium and positioned on agar-coated plates containing embryo medium. The number of melanophores occupying an area which covered from the eyes to the anterior extent of the yolk sac was recorded. Data analysis was done using the Student's *t*-test ($\alpha = 0.05$). All embryos were then prepared for protein collection.

2.2. Measurement of melanin content

The melanin content was measured by the method of Hedley et al. [28]. In brief, 5 days post-treatment, embryos were digested in up to 0.5 ml of 1 M NaOH. The following day, the OD values were determined at 414 nm. A standard curve of synthetic melanin was run to calculate the melanin content for each control and each treated sample. Treated embryos were compared to appropriate controls (plotted as 100%) and reported as percentages of control samples. Protein quantification was determined by the BCA assay.

2.3. Tyrosinase assay

Tyrosinase activity was assessed by the method of Tomita et al. [29]. Treated embryos were washed

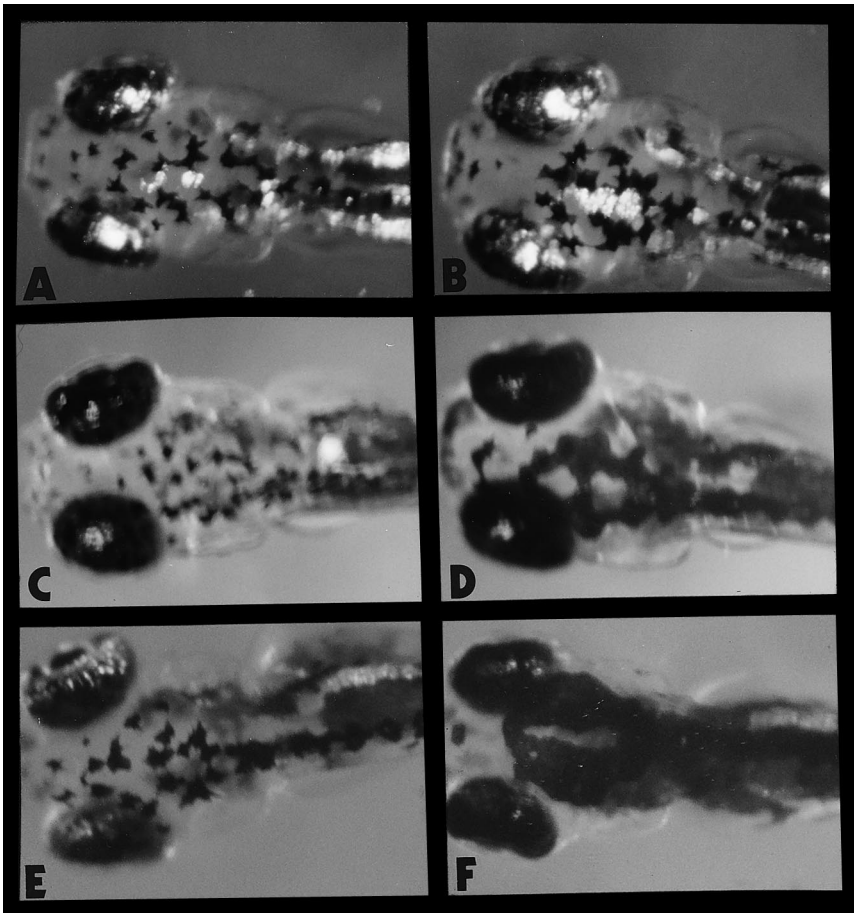

Fig. 1. Representative photos of zebrafish embryos 5 days after treatment with forskolin (B), LiCl (D), or LiCl/forskolin (F), compared to appropriate controls (A,C,E) for each as described in Section 2.

with TBS and lysed in 1% Triton X-100/TBS with agitation. Five microliters of 10 mM L-DOPA was added to each tube of treated-embryo lysate. Following incubation at 37°C for 30 min, the absorbance was measured at 475 nm. Data were standardized with mushroom tyrosinase to calculate tyrosinase activity for each control and each treated sample. Tyrosinase activity in samples from treated embryos were compared to appropriate controls (plotted as 100%) and reported as percentages of control samples. Protein quantification was determined by the BCA assay.

2.4. Immunoblotting

Five days after treatment, embryos were homogenized in 0.5% pre-condensed Triton X-114/TBS and proteins were extracted into membrane and cytosolic fractions. Protein fractions were then subjected to Western-blotting techniques. Proteins were separated on a 10 or 7% acrylamide gel and then transferred to PVDF membrane and used for immunodetection with MSG-1 and TRP-2 antibodies. MSG-1 (gift of Dr. Shioda, Massachusetts General Hospital Cancer Center) was used in a 1:3000 dilution, and TRP-2 (gift of Dr. Hearing, National Institutes of Health) was used in a 1:2000 dilution. After a 1-h incubation at 37°C, membranes were washed in several changes of Tween-20/TBS. Membranes were then incubated with a peroxidase-conjugated secondary antibody and washed as before. Detection was carried out using enhanced chemiluminescence (Armersham, IL).

3. Results

In order to examine signal transduction events, embryos were treated with LiCl (an inositol-phosphatase inhibitor), forskolin (a PKA activator), a combination of LiCl/EGTA (a calcium chelator), LiCl/forskolin, LiCl/U73122 (a phospholipase-C inhibitor), or LiCl/W-7 (a calmodulin antagonist). Among the signaling-related chemicals tested, LiCl and LiCl/forskolin demonstrated the most dramatic effects on pigmentation (Fig. 1D,F). Gross pigmentation increased when embryos were treated in this manner. Forskolin alone had no observable effect on

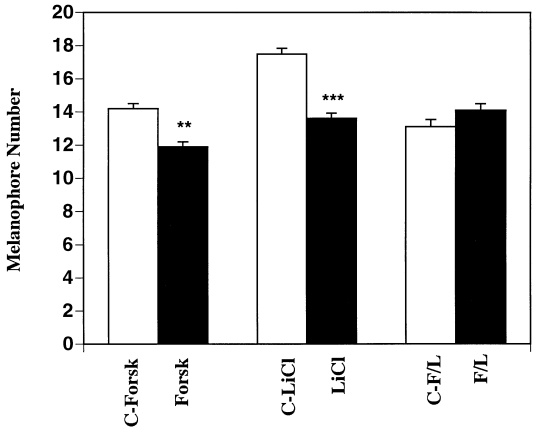

Fig. 2. Representative melanophore counts taken from the head region of control and treated zebrafish embryos. Embryos were treated with LiCl, forskolin (Forsk), or LiCl/forskolin (F/L) 24-h postfertilization and counts were taken 5 days after treatment. □, Controls; ■, treated. $^{**}P < 0.01$, $^{***}P < 0.001$.

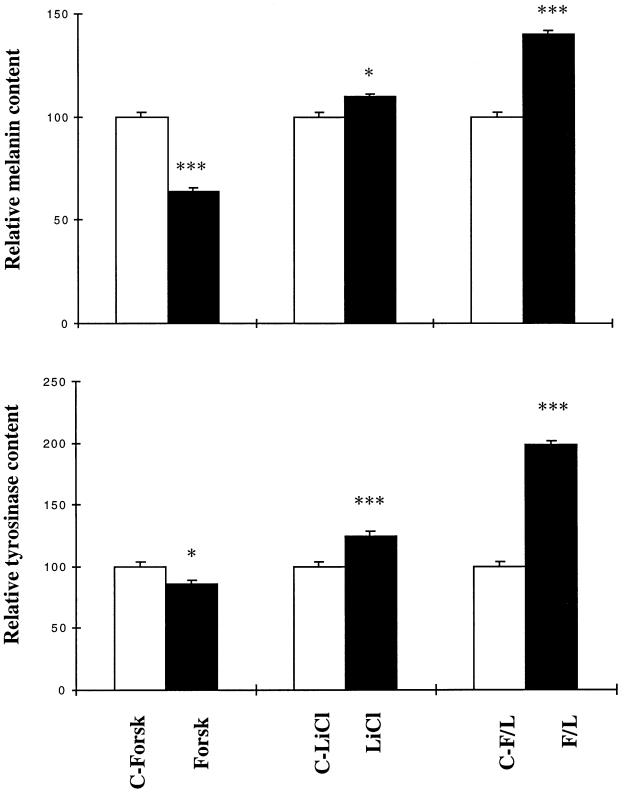

Fig. 3. Melanin content (top) and tyrosinase activity (bottom) in samples collected 5 days after treatment with forskolin, LiCl, or forskolin/LiCl (F/L). Results are expressed as percentages of control samples. Appropriate controls are plotted as 100%. □, Controls; ■, treated. $^{*}P < 0.05$, $^{***}P < 0.0005$.

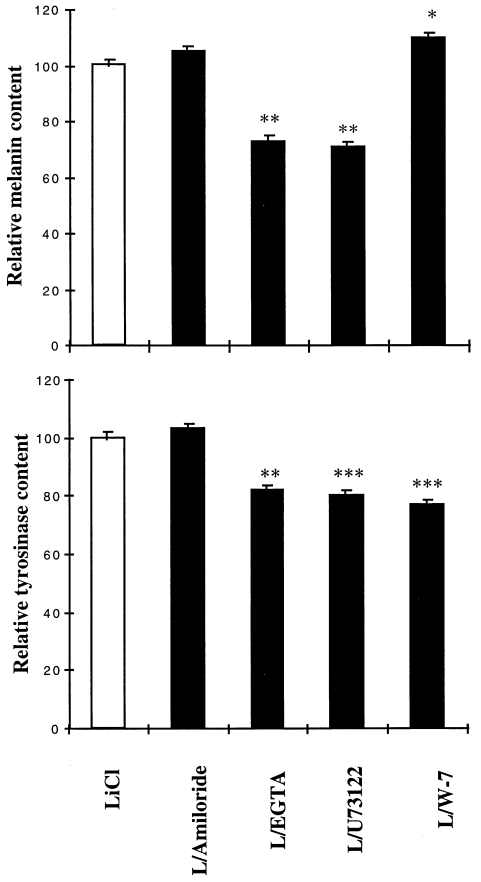

Fig. 4. Melanin content (top) and tyrosinase activity (bottom) in samples collected 5 days after treatment with LiCl/5-(*N*,*N*-hexamethylene)-amiloride, LiCl/EGTA, LiCl/U73122, or LiCl/W-7. Results are expressed as percentages of LiCl alone samples. LiCl treatment alone is plotted as 100%. □, LiCl; ■, treated. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

any aspect of melanophore proliferation/differentiation (Fig. 1B) when compared to controls (Fig. 1A).

In order to differentiate between the various potential sources of this increase in pigmentation we counted melanophore numbers and subsequently did melanin content and tyrosinase activity measurements, and used melanophore-protein-specific antibodies in Western-blotting techniques. Comparison of melanophore number in treated and control embryos would indicate whether the increase in dark pigmentation after administration of chemicals was due to an increase in proliferation and/or differentiation of melanophores. Over the course of these experiments, the melanophores of 50 embryos from each control group and each treatment group were counted.

Melanophore number was decreased by day 5 after treatment when 24-h postfertilization embryos were exposed to LiCl or forskolin. However, when embryos were exposed to LiCl/forskolin simultaneously no effect on the number of melanophores was observed (Fig. 2).

To identify other possible mechanisms responsible for the pigmentation induced by LiCl, melanin content, tyrosinase activity, and the expression of MSG-1 and TRP-2 were examined. Tyrosinase is known to be the rate-limiting enzyme in the process of melanin synthesis and MSG-1 and TRP-2 are melanocyte precursor-specific proteins expressed along the pathway of melanin synthesis. When embryos were treated with LiCl or LiCl/forskolin, melanin content increased. The most significant increase resulted from a combined treatment of LiCl/forskolin (Fig. 3, top). Tyrosinase activity also increased dramatically when embryos were treated with LiCl/forskolin and compared to controls (Fig. 3, bottom). However treatment with forskolin alone resulted in a decrease in melanin content and tyrosinase activity (Fig. 3, top and bottom). Compared to LiCl alone, the combination of various inhibitors with LiCl, e.g. EGTA or U73122, resulted in a decrease in melanin content and tyrosinase activity. Na^+/H^+ antiporter inhibitor in combination with LiCl had no significant effect on melanin content or tyrosinase activity (Fig. 4, top and bottom).

Western immunoblotting was carried out using an anti-MSG-1 antibody and an anti-TRP-2 antibody, as melanophore-specific markers. MSG-1 is a nuclear

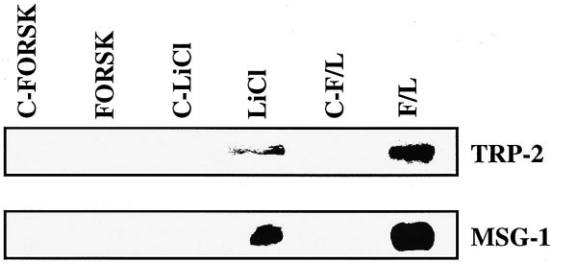

Fig. 5. Immunoblot analysis of MSG-1 and TRP-2 expression in zebrafish embryos. Embryos were treated 24-h postfertilization. Protein was collected 5 days after treatment and subjected to SDS-PAGE followed by immunoblotting using anti-MSG-1 and anti-TRP-2. Antibody binding was visualized by ECL as described in Section 2. Lanes and treatment groups: C-FORSK, forskolin control; FORSK, forskolin; C-LiCl, LiCl control; LiCl, LiCl; C-F/L, LiCl/forskolin control; F/L, LiCl/forskolin.

protein [19], and TRP-2 is a cytoplasmic protein [20]. Expression of MSG-1 increased in LiCl-treated embryos and increased to a greater degree in LiCl/forskolin-treated embryos (Fig. 5). A similar pattern resulted for TRP-2 expression (Fig. 5). MSG-1 and TRP-2 expression was not detected in embryos treated with forskolin alone. Interestingly, although melanophore number was not affected in LiCl/forskolin treated embryos, melanin content, tyrosinase activity, and MSG-1 and TRP-2 expression increased.

4. Discussion

Treatment of zebrafish embryos with LiCl and LiCl/forskolin caused a dramatic increase in the appearance of pigmentation, specifically in the melanin-producing melanophores. Counts of differentiated pigment cells revealed no change in melanophore number between LiCl/forskolin-treated and control embryos. However the melanophore number in embryos treated with either LiCl or forskolin alone decreased significantly. An increase in proliferation of melanophores did not seem a likely cause of the increase in the blackness of the treated embryos. LiCl and LiCl/forskolin treatment did, however, cause an increase in melanin content, tyrosinase activity, and the expression of MSG-1 and TRP-2, with the combined treatment having a more dramatic effect than LiCl alone. Melanin content and tyrosinase activity were dramatically decreased when embryos were treated with LiCl/EGTA or LiCl/U73122. Collectively, data suggests an important role for Ca^{2+} in the pigmentation in the embryonic zebrafish through an increase in: (1) differentiation of melanophores; (2) increase in melanin synthesis; or (3) increase in tyrosinase activity. Interestingly, simultaneous treatment of LiCl and W-7 affected melanin content and tyrosinase activity quite differently. That is, melanin content increased, but tyrosinase activity decreased in response to LiCl/W-7 (Fig. 4). Mitsunari et al. [30] found similar results using hydroquinone on human melanocytes. They suggested that melanin content was increased through mechanisms other than the induction of tyrosinase activity.

Western-blot analyses using MSG-1 and TRP-2 antibodies, indicated an increase in expression of these melanophore-specific proteins. The novel gene MSG-1 has been found to be expressed at high levels in B16-F cells, a highly pigmented murine melanoma cell line [25]. Nuclear localization of MSG-1 protein has been confirmed in B16-F cells, normal human epidermal melanocytes, and primary culture murine epidermal melanocytes using immunofluorescent staining [25], but little is known of its cellular functions. Melanocyte stimulating hormone (α-MSH) is also known to stimulate pigmentation. Abdel-Malek et al. [26] suggested that the increases in tyrosinase, TRP-1, and TRP-2 typically induced by α-MSH are due to post-transcriptional modifications of these tyrosinase family members. These conclusions result from the lack of significant changes seen in transcript levels for the three-melanogenic proteins. An increase in such melanophore-specific proteins in the study presented here do not imply anything about an increase in the dispersion of melanosomes throughout individual melanophores. It is possible that the increase in expression represents an increase in the synthesis or deposition of melanin into melanosomes, thereby suggesting a role in the differentiation of melanophores. Ultimately, the observed result in any of these scenarios is an enhancement of gross pigmentation.

Results from the studies described here have led us to propose several aspects of the signaling pathway involved in the development of the neural crest-derived melanophore in the zebrafish. We suggest that the IP pathway that eventually causes the release of intracellular Ca^{2+} is related to pigmentation by way of its involvement in the synthesis of melanin or its deposition of melanin into melanosomes. Various signaling pathways potentially affected directly and/or indirectly by lithium include, protein-kinase-A, protein-kinase C, tyrosine kinase, and/or glycogen synthase kinase-3 (GSK). Interestingly, lithium-induced inhibition of GSK has been implicated in the teratogenic effects of lithium on development [31,32]. However, we believe data presented here support a prominent role for LiCl in the inhibition of inositol phosphatase and subsequent accumulation of IP_3. Inositol phosphate would then lead to the release of intracellular Ca^{2+} and activation of Ca^{2+}/CaM kinase and/or a phosphorylation cascade of Ca^{2+}-binding proteins. This cascade of events then leads to the melanophore response; melanin synthesis is

increased and/or differentiation is affected. Other studies are underway to address more detailed questions of the various players in the pigment cell signaling pathway in the embryonic zebrafish during pigment pattern formation.

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