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Evolution and development of brain sensory organs in molgulid ascidians

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SUMMARY The ascidian tadpole larva has two brain sensory organs containing melanocytes: the otolith, a gravity receptor, and the ocellus, part of a photoreceptor. One or both of these sensory organs are absent in molgulid ascidians. We show here that developmental changes leading to the loss of sensory pigment cells occur by different mechanisms in closely related molgulid species. Sensory pigment cells are formed through a bilateral determination pathway in which two or more precursor cells are specified as an equivalence group on each side of the embryo. The precursor cells subsequently converge at the midline after neurulation and undergo cell interactions that decide the fates of the otolith and ocellus. *Molgula occidentalis* and *M. oculata*, which exhibit a tadpole larva with an otolith but lacking an ocellus, have conserved the bilateral pigment cell determination pathway. Programmed cell death (PCD) is superimposed on this pathway late in development to eliminate the ocellus precursor and supernumerary pigment cells, which do not differentiate into either an otolith or ocellus. In contrast to molgulids with tadpole larvae, no pigment cell precursors are specified on either side of the *M. occulta* embryo, which forms a tailless (anural) larva lacking both sensory organs, suggesting that the bilateral pigment cell determination pathway has been lost. The bilateral pigment cell determination pathway and superimposed PCD can be restored in hybrids obtained by fertilizing *M. occulta* eggs with *M. oculata* sperm, indicating control by a zygotic process. We conclude that PCD plays an important role in the evolution and development of brain sensory organs in molgulid ascidians.

INTRODUCTION

The life or death of a cell can be a critical determinant of adult morphology. For example, programmed cell death (PCD) leads to tissue regression between the digits in vertebrate appendages, and its suppression may be responsible for webbed feet in ducks (Zou and Niswander 1996). Likewise, the absence of eyes in blind cavefish is due to PCD of the embryonic lens, which controls eye growth and development (Jeffery and Martasian 1998; Yamamoto and Jeffery 2000). Finally, many cells in *Caenorhabditis elegans* embryos are fated to die by apoptosis, which is responsible for differences in gonad morphology between nematode species (Felix and Sternberg 1996). Recently, PCD has been described during ascidian development (Chambon et al. 2002; Jeffery 2002) and is thought to be responsible for interspecific differences in larval morphology (Jeffery 2002).

Ascidians are characterized by a life cycle with swimming larval and sessile adult stages (Satoh 1994; Jeffery and Swalla 1997). The tadpole larva has been favored in developmental studies because of its rapid development, determinative cell lineage, low cell number, small genome, and distinct chordate features, including a notochord and dorsal central nervous system. Ascidian tadpoles consist of a head (or trunk) containing a brain, endoderm, and mesenchyme cells and a tail containing a nerve cord, notochord, and bands of striated muscle cells. The larva of some species consists of only 2500 cells, including 40 notochord cells, 40 muscle cells, and about 380 neural cells. The brain has two sensory organs containing melanocytes: the otolith, a gravity detector, and the ocellus, part of a photoreceptor (Dilly 1962, 1964; Tsuda et al. 2003).

Pigment sensory cells develop in a stereotypical pattern during ascidian embryogenesis. The otolith and ocellus arise from the paired a8.25 blastomeres, which are positioned bilaterally in the 110-cell embryo (Nishida 1987). Several steps are involved in pigment cell development. First, bilateral pigment cell precursors are induced from competent ectoderm by a basal fibroblast growth factor–like signal during the midgastrula stage (Nishida and Satoh 1989; Nishida 1991). These cells are not yet committed to a particular sensory cell fate, however, and have the potential to form either an otolith or ocellus. Second, the decision to become a specific pigment sensory cell type occurs after neurulation when the bilateral

precursor cells converge and interact along the dorsal midline of the developing tadpole (Nishida and Satoh 1989). We refer to this sequence of events as the bilateral pigment cell determination pathway.

The differentiation of the anterior pigment cell precursor as an otolith and the posterior cell into an ocellus appears to be controlled by a classic BMP/Chordin antagonism (Darras and Nishida 2001). During tailbud stage, pigment cell precursors synthesize tyrosinase, the key enzyme in the melanogenic pathway, and melanin granules are deposited after terminal differentiation (Whittaker 1966). Actually, four or more cells with tyrosinase activity are aligned in single file along the anteroposterior axis of the developing larval brain, although only the two posterior cells eventually differentiate into pigment sensory cells (Whittaker 1973; Nishida and Satoh 1989). The additional pigment cell precursors, which are known as supernumerary pigment cells, express tyrosinase but normally do not synthesize melanin, and their fate is unknown.

Although tadpole larvae of most ascidian species contain both an otolith and an ocellus, in some species the ocellus is greatly reduced (e.g., *Steyla*) or eliminated (e.g., *Molgula*) (Berrill 1950). Molgulid species with tadpole (urodele) larvae usually lack an ocellus (Berrill 1931) and are unable to detect changes in light intensity (Grave 1926). Moreover, most molgulids lacking a tadpole larvae (anural species) have lost both pigment sensory cells (Berrill 1931; Swalla and Jeffery 1990, 1991). The developmental mechanisms underlying these evolutionary changes have not been investigated.

Here we show that evolution and development of pigment sensory cells is controlled by different mechanisms in urodele and anural molgulids. The bilateral pigment cell determination pathway typical of ascidians with two pigment sensory cells is conserved in urodele molgulid species, but PCD late in development eliminates the ocellus precursor and supernumerary pigment cells, leaving only the otolith. In contrast, the bilateral pigment cell pathway is lost in an anural molgulid species, and the otolith, ocellus, and supernumerary pigment cells do not develop. The loss of this pathway, including superimposed PCD, can be reversed by interspecific hybridization with a urodele sister species, suggesting the involvement of a zygotic process. We conclude that PCD is an important factor in the evolution of the ascidian brain and that the mechanisms involved in pigment sensory cell development can diverge significantly in closely related molgulid species.

MATERIALS AND METHODS

Animals and embryos

Molgula occidentalis was purchased from Gulf Specimens, Inc. (Panacea, FL, USA) and maintained in Instant Ocean at the University of Maryland. *Molgula oculata* and *M. occulta* were dredged from sand flats at Pointe de Bloscon near Roscoff, France and maintained in running seawater at Station Biologique. Gametes were obtained and fertilized, and embryos were cultured in Millipore filtered seawater (MFSW) at 16–18°C, as described previously (Swalla and Jeffery 1990; Jeffery 2002). In these species, gastrulation occurred at about 4 h postfertilization (hpf), neurulation at 5 hpf, tail elongation between 5 and 10 hpf, and hatching between 10 and 12 hpf. *Molgula occulta* × *M. oculata* hybrids were prepared as described by Swalla and Jeffery (1990).

Blastomere lysis procedure

Molgula occidentalis two-cell embryos were diluted to a concentration of about 500 embryos/ml in MFSW. A 5-ml volume of embryos was placed in a 15-ml plastic centrifuge tube, and the tube was shaken vigorously by hand for 3 min. This manipulation resulted in the lysis of one or both blastomeres within the intact chorion. The embryos were transferred to a Syracuse dish, and those with one cleaving (living) and one lysed cell inside the chorion were selected manually and cultured until controls reached the mid to late tailbud stage.

Blastomere separation procedure

Beginning at about 15 min before first cleavage, *M. occidentalis* embryos were treated with 0.9% Pronase E (Sigma Chemical Co., St. Louis, MO, USA) for about 10 min and then washed extensively with MFSW supplemented with the protease-inhibitor leupeptin (Sigma) (Olsen and Jeffery 1997). The last wash was into Ca-free MFSW. At first cleavage, embryos lacking a chorion were selected by mouth pipet and expelled into cell wells containing an excess of Ca-free MFSW, which resulted in separation into individual blastomeres. The isolated blastomeres were cultured in MFSW until controls reached the late tailbud stage and then were assayed for tyrosinase.

Cytochalasin treatment

Embryos were suspended in MFSW containing 2 μg/ml cytochalasin B (Sigma) diluted from a 1-mg/ml stock solution in dimethyl sulfoxide. The embryos were cultured in MFSW containing cytochalasin B until controls reached the mid to late tailbud stage and then were assayed for tyrosinase or examined for melanin pigment deposition by microscopy. Controls were treated with MFSW containing 0.05% dimethyl sulfoxide.

Tyrosinase assay

Tyrosinase assays were carried out according to the method of Laidlaw (1932). Briefly, embryos were fixed for 30 min at 18°C in 5% formalin-MFSW, washed extensively in MFSW, and resuspended in phosphate-buffered (pH 7.4) L-3,4 dihydroxyphenylalanine. Cells with active tyrosinase showed oxidation of L-3,4 dihydroxyphenylalanine to melanin after incubation for about 2 h at 37°C.

PCD detection

PCD was detected by vital nuclear staining with SYTOX green or orange (Molecular Probes, Eugene, OR, USA) as described by Jeffery (2002). Living dechorionated embryos or larvae were exposed to 5 μM SYTOX for 5–10 min and examined by

fluorescence microscopy. Dying cells were detected by their bright green or red stained nuclei (Poot et al. 1997).

RESULTS

Multiple pigment cell precursors are present in *Molgula* embryos

Molgula occidentalis tadpoles contain an otolith but lack an ocellus (Fig. 1A). In nonmolgulid ascidians, four or more cells with the potential to develop into melanocytes are aligned along the midline of tailbud embryos (Whittaker 1966; Nishida and Satoh 1989), although only two of these cells eventually differentiate into melanocytes. Thus, we determined the number of pigment cell precursors in *M. occidentalis* tailbud embryos by assaying for tyrosinase, a specific marker for larval pigment cell precursors. Up to four tyrosinase-positive cells were observed along the dorsal midline of tailbud embryos, two weakly stained anterior cells and two strongly stained posterior cells (Fig. 1B). As in other ascidian species, the two posterior cells are likely to correspond to the otolith and ocellus melanocytes and the two anterior cells to supernumerary pigment cells. These results show that more pigment cell precursors are produced in molgulids than eventually differentiate into melanocytes.

Bilateral pigment cell precursors are present in *Molgula* embryos

We next determined the origin of sensory pigment cell precursors in *Molgula* embryos. In other ascidians, pigment cell precursors are specified bilaterally during the late cleavage stages. Are pigment cell precursors also specified bilaterally or is development of the future ocellus pigment cell suppressed on one side of molgulid embryos? This question was addressed in three different experiments.

The first two experiments capitalized on the fact that the ascidian embryo is bisected into two bilaterally symmetric halves by first cleavage. In the first experiment, one blastomere of an *M. occidentalis* two-cell embryo was killed and the living half-embryo assayed for tyrosinase-positive cells after controls reached the mid tailbud stage (Fig. 1C). In 78% of these cases ($n = 115$), the living half-embryo produced tyrosinase-positive cells (Fig. 1, D and E). In most cases, two tyrosinase-positive cells were present (Fig. 1D), although a few half-embryos contained up to four tyrosinase-positive cells (Fig. 1E). In the second experiment, *M. occidentalis* two-cell embryos were separated, the blastomeres cultured individually, and the resulting half-embryos assayed for tyrosinase-positive cells after controls reached the mid tailbud stage (Fig. 1F). In 84% of these cases ($n = 49$), up to two tyrosinase-positive cells were present in each pair of half-embryos (Fig. 1, G–J). These results are consistent with a bilateral origin of pigment cell precursors in the molgulid embryo.

In ascidians with two sensory pigment cells, bilateral specification of their precursors can occur after cytokinesis is inhibited with cytochalasin B during the late cleavage stages (Whittaker 1973; Nishida and Satoh 1989). Therefore, in the third experiment, 64- and 110-cell stage *M. occidentalis* embryos were treated with cytochalasin B, allowed to develop until controls reached the mid tailbud stage, and examined for melanized cells. The results showed that two melanin-containing cells developed on each side in most of these embryos (Fig. 2, A and B), showing that the bilateral pigment cell specification pathway is conserved in molgulid embryos.

Midline convergence restricts the potential for pigment cell development in *Molgula* embryos

Pigment cell precursors converge at the midline during neurulation and then undergo interactions that establish their fates (Nishida and Satoh 1989; Darras and Nishida 2001). To determine whether this method of determination also operates in molgulids, the cytochalasin B experiments were extended to later stages of *M. occidentalis* development. It was shown that four cells have the potential to develop into pigment cells when cytochalasin treatment was initiated before the early tailbud stage (Fig. 2, A–E). Afterward, the number of pigment cell precursors was reduced from four to two (Figs. 2F and 3G) and then to one: the otolith (Fig. 3E). However, even after treatment during the late tailbud stage, an hour before hatching, a few hatched larvae exhibited two pigment sensory cells, suggesting that restrictive interactions continue in some embryos throughout the period of tail development. When two pigment cells were present, they were aligned in single file along the midline of the hatched tadpole, in positions resembling that of the otolith and ocellus in nonmolgulid species. The anterior pigment cell contained a large melanin granule and the posterior pigment cell a small granule (Fig. 3G). The same results were obtained in another molgulid species, *M. oculata* (Fig. 2H). Despite its position, the posterior cell contained only a single melanin granule, not the multiple small granules characteristic of an ocellus in other ascidians (Nishida and Satoh 1989). The results indicate that the potential to develop bilateral pigment cells is restricted as their precursors meet and interact at the embryonic midline.

Undifferentiated pigment cell precursors undergo PCD in *Molgula* tadpoles

If molgulids have precursors to both brain pigment cells, what is the fate of the ocellus precursor during normal development? Furthermore, what is the fate of the supernumerary pigment cells, which are also conserved in molgulid embryos? We tested the possibility that the ocellus precursor and supernumerary pigment cells are eliminated by PCD (Chambon et al. 2002; Jeffery 2002).

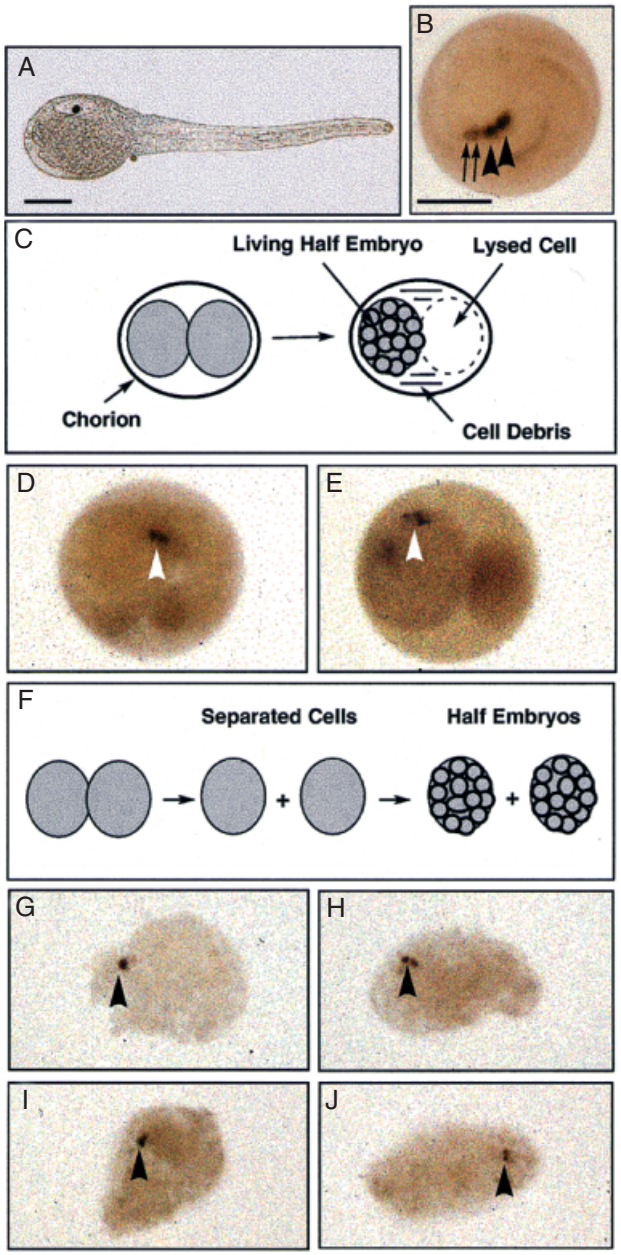


Fig. 1. Pigment cell development in *Molgula occidentalis*. (A) A tadpole larva showing a single otolith pigment cell. (B) A mid tailbud embryo showing four tyrosinase-positive cells in the developing brain, two strongly stained cells representing the otolith and ocellus precursors (arrowheads), and two weakly stained cells (arrows) representing supernumerary pigment cells. (C) Diagram showing method for obtaining a half-embryo by lysis of one blastomere at the two-cell stage. (D, E) Development of two (D) or four (E) pigment cells in a living half-embryo. (F) Diagram showing the method for obtaining two half-embryos by blastomere separation at the two-cell stage. (G–J) Development of pigment cells in half-embryos that developed from isolated blastomeres of a two-cell embryo. Pigment cells were determined by tyrosinase assay. Arrowheads: pigment cells. Scale bars in A and B, 50 μm. Magnification is the same in B, D, E, and G–J.

Hatched *M. occidentalis* larvae were treated with SYTOX (Poot et al. 1997; Jeffery 2002), a PCD indicator that selectively permeates the plasma membrane and stains the nuclear DNA of dying cells. As shown in Figure 3, A–D, in *M. occidentalis* tadpoles the differentiated otolith was closely associated with several SYTOX-stained cells. The apoptotic cells were present for only a short period in the swimming larva and were undetectable by the time of metamorphosis, presumably because they are removed by phagocytosis. As described previously (Jeffery 2002), other apoptotic cells were also detected by SYTOX in the anterior head and tail epidermis, although the few cells surrounding the otolith were always the first-appearing and most brightly stained cells in the larval head. SYTOX treatment at earlier stages of development did not reveal dying cells. The possibility that SYTOX did not stain early developmental stages because it cannot penetrate the chorion was excluded by inducing cell death via a 37°C heat shock and showing that staining was present in cell nuclei throughout the tailbud embryo (data not shown). The results indicate that all embryonic cells have the capacity to undergo PCD but only a subset of larval cells, particularly those located near the otolith, actually die before metamorphosis.

The presence of dead cells near the otolith suggested that the ocellus and supernumerary pigment cell precursors might be fated for PCD. To further explore this possibility, we treated late tailbud stage embryos with cytochalasin B, which generates identifiable pigment cell precursors along the midline, and subsequently stained the resulting hatched larvae with SYTOX (Fig. 3, E–H). As described above, cytochalasin results in some hatched larvae with a single pigmented otolith (Fig. 3E) and others with two morphologically distinct pigment cells aligned in the position of the otolith and ocellus (Fig. 3G). In tadpoles with a single differentiated pigment cell (otolith), we observed SYTOX staining in cells anterior and posterior to the otolith (Fig. 3F), in the positions of the supernumerary pigment cells and the ocellus, respectively. In tadpoles with two pigment cells, the dying cells were aligned in the anterior position of supernumerary pigment cells but not in the posterior position of the ocellus, presumably because the ocellus precursor had differentiated (Fig. 3H). These results suggest that the normal fate of the ocellus precursor and supernumerary pigment cells is PCD in molgulid ascidians.

The bilateral pigment cell determination pathway is lost in anural molgulids

In contrast to molgulid ascidians with tailed (or urodele) larvae, molgulids with tailless (or anural) larvae have lost both the ocellus and the otolith (Berrill 1931; Swalla and Jeffery 1990). Therefore, we asked whether the bilateral pigment cell determination pathway is conserved in an anural molgulid

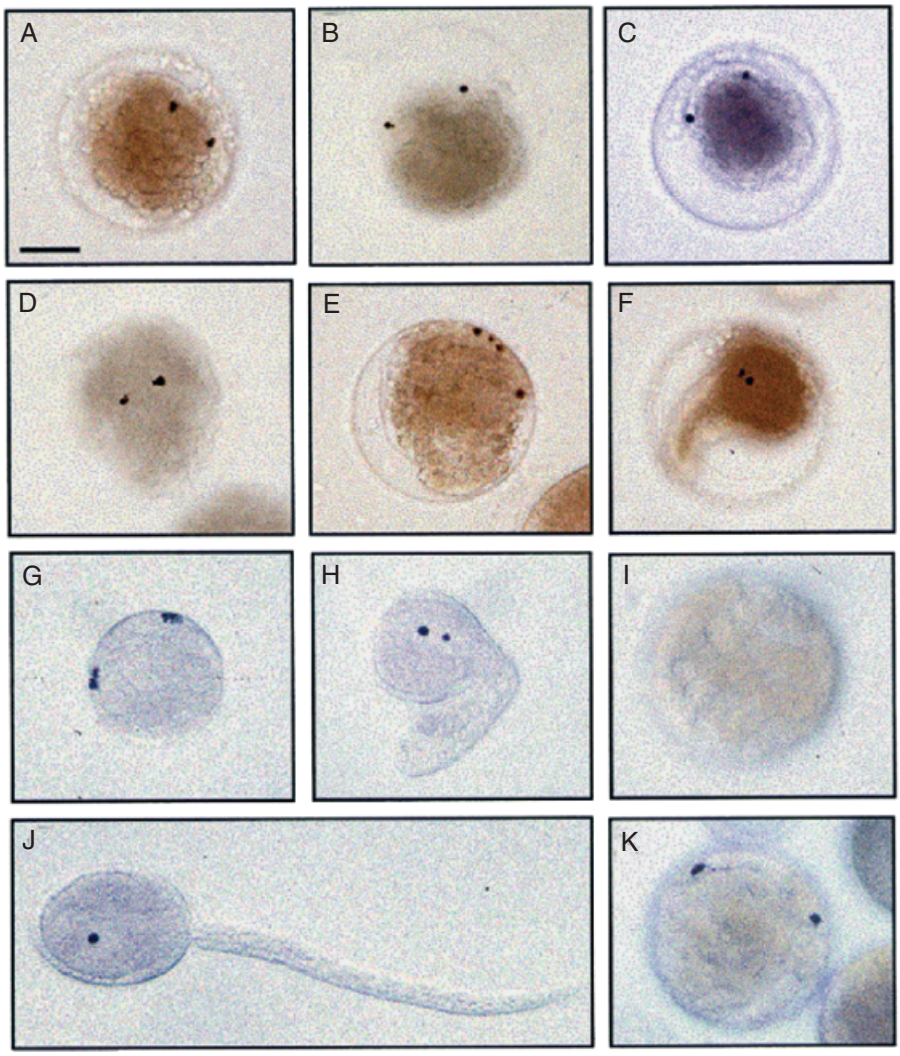
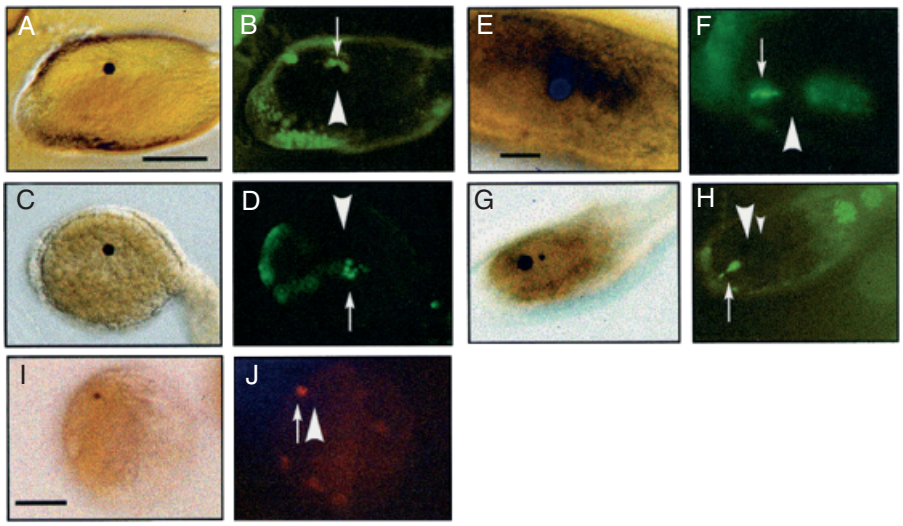


Fig. 2. Pigment cell development in cytochalasin B–treated molgulid embryos. (A–F) *Molgula occidentalis* embryos treated at the 64-cell (A), 110-cell (B), gastrula (C), neurula (D), and early tailbud (F) stages showing bilateral pigment cell development. Embryos arrested at the late tailbud stage are shown in Figure 3, E and G. (E) An embryo treated at the neurula stage and then compressed under a coverslip to demonstrate the presence of four individual pigment cells. (G–K) *Molgula oculata* (tailed species), *M. occulta* (tailless species), and *M. occulta* × *M. oculata* hybrid. Cytochalasin treatment progressively restricted the development of bilateral pigment cells in *M. oculata* (tailed species), as it did in *M. occidentalis*, another tailed species. Cytochalasin B treatment was initiated at the 110-cell (G), mid tailbud (H), and late tailbud (J) stages. (I) An *M. occulta* embryo treated with cytochalasin B at the 64-cell stage showing no pigment cell development. (K) An *M. occulta* × *M. oculata* hybrid embryo treated with cytochalasin B at the 64-cell stage showing restoration of bilateral pigment cell development. Pigment cell development was determined by melanin deposition. Scale bar in A, 50 μm. Magnification is the same in each frame.



species. The anural species *M. occulta* and its sister urodele species, *M. oculata*, were used in these experiments because they can be cross-hybridized to obtain information on the maternal or zygotic basis of changes in the determination system.

Treatment of *M. oculata* (tailed species) embryos with cytochalasin B at various stages of development yielded the same results (Fig. 2, G, H, and J) as those obtained with embryos of the urodele species *M. occidentalis* (Fig. 2, A–F). Bilateral precursor cells were capable of pigment cell differentiation after cytochalasin treatment from the 64-cell through the mid tailbud stages. Likewise, treatments beginning after the late tailbud stage resulted in hatched larvae with one or two pigment cells in the position of the otolith and ocellus. Curiously, two pigment cells, both with the otolith morphology, sometimes developed even in control *M. oculata* tadpoles (data not shown), which may be attributed to failure of embryos to achieve complete midline convergence in laboratory culture or to a natural developmental plasticity. We conclude that urodele species in different molgulid clades (e.g., *M. occidentalis* and *M. oculata*; Hadfield et al. 1995) have conserved the bilateral pigment cell determination pathway typical of other ascidians.

In contrast to *M. oculata*, cytochalasin B treatment of *M. occulta* (tailless species) embryos did not result in the appearance of pigment cells (Fig. 2I; data not shown). These results suggest that the bilateral pigment cell determination pathway is lost in this anural species.

Additional experiments were done to obtain more information about how the bilateral pigment cell determination system is lost in *M. occulta*. First, we considered the possibility that tyrosinase itself is lacking. Because tyrosinase-positive cells are also present in ascidian juveniles after metamorphosis (Swalla and Jeffery 1991), we asked whether these cells could be detected in *M. occulta* juveniles. Tyrosinase staining showed many cells capable of melanin synthesis scattered throughout *M. occulta* juveniles (Fig. 4A), indicating that the ability to produce tyrosinase has not been affected during tailless species evolution. Second, *M. occulta* embryos were treated with SYTOX to determine whether early PCD is responsible for eliminating pigment sensory precursors. Previous studies using TUNEL as a PCD indicator showed that many embryonic cells, including brain, epidermal, notochord, and muscle cells, initiate PCD in *M. occulta* embryos (Jeffery 2002). However, we did not detect SYTOX-stained cells in the dorsal anterior location of sensory pigment cell precursors in these embryos at any time prior to hatching (Fig. 4, C and D). In contrast, SYTOX-stained cells, presumably tail precursors that had completed apoptosis (Jeffery 2002), were detected in the posterior region of 8 hpf embryos (Fig. 4, B and C). The results indicate that loss of the bilateral pigment cell precursor system in *M. occulta* cannot be attributed to the absence of a functional tyrosinase gene or early cell death in the pigment sensory cell lineage.

Restoration of the bilateral pigment cell determination pathway in hybrids

Molgula occulta (tailed species) eggs fertilized with *M. oculata* (tailed species) sperm rescue some of the lost urodele features, including the otolith and tail (Swalla and Jeffery 1990). To determine whether bilateral pigment cells are restored as well, hybrid embryos were treated with cytochalasin B at different times in development. The results were the same as those obtained for urodele species (Fig. 2, A–H and J). Hybrid embryos treated with cytochalasin from the 64-cell through the neurula stage were able to develop bilateral pigment cells (Fig. 2K). Furthermore, when treatment began after the neurula stage, the number of differentiated pigment cells declined from four to one (Fig. 3I) or two. The results show that the bilateral pigment cell determination pathway can be restored in hybrids.

Does PCD restrict pigment sensory cell development in hybrid embryos, as it does in urodele molgulids? To answer this question, hybrid larvae were stained with SYTOX. The hybrid larvae showed one or two dying cells located near the otolith, which may represent the initially restored but dying ocellus and supernumerary pigment cell precursors (Fig. 3, I and J). As in urodele molgulids, the dying cells disappeared within an hour after hatching. Thus, both the bilateral pigment cell determination pathway and removal of pigment cell precursors by PCD can be restored in hybrid embryos. The results suggest that a zygotic process, including activation

Fig. 3. PCD in *Molgula occidentalis* tadpoles (A–H) and *M. occulta* × *M. oculata* hybrid larvae (I, J) determined by SYTOX staining. (A, C, E, G, I) Bright field images. (B, D, F, H, J) Fluorescence images of the same specimens. (A–D) Hatched larvae showing three (B) or four (D) brightly stained apoptotic cells (arrow) associated with the otolith (arrowhead). (E–H) Hatched larvae developed from an embryo arrested with cytochalasin B at the late tailbud stage that formed one (E, F) or two (G, H) pigment cells aligned along the dorsal midline of the brain. (E, F) Two or three apoptotic cells (arrow) aligned immediately anterior to the otolith (arrowhead) and several stained cells lying out of focus posterior to the otolith in a tadpole with an otolith but not an ocellus. (G, H) Two apoptotic cells aligned anterior to the otolith in a tadpole with an otolith and an ocellus. Scale bar in A, 50 μm; magnification is the same in A–D, G, and H. Scale bar in E, 20 μm; magnification is the same in E and F. (I, J) Apoptotic cells near the restored otolith in an *M. occulta* × *M. oculata* hybrid larva. Scale bar in I, 40 μm; magnification is the same in J.

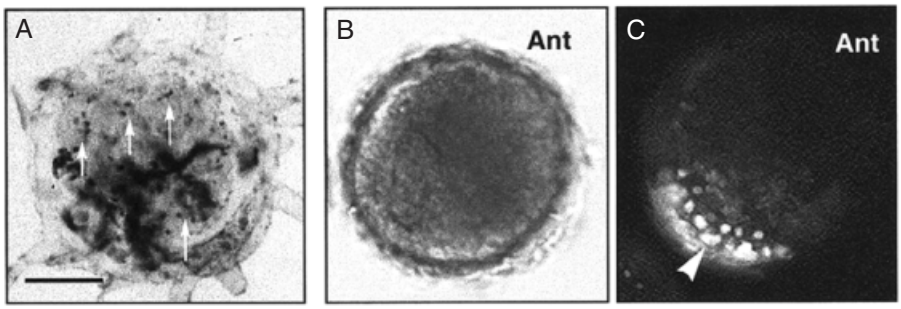


Fig. 4. (A) Tyrosinase-positive cells (arrows) are present in a *Molgula occulta* juvenile after metamorphosis. (B, C) Dorsal views of bright field (B) and fluorescence (C) images of the same SYTOX-treated 8 hpf *M. occulta* embryo. PCD was detected in the nuclei of posterior cells (arrowhead in C) but not in other parts of the embryo, including the anterior dorsal region where undifferentiated pigment cell precursors would be located. Scale bar, 50 μm. Magnification is the same in all frames.

of PCD, is involved in loss of the bilateral pigment cell determination pathway in anural molgulids.

DISCUSSION

We studied the developmental basis for evolutionary changes in pigment sensory cells in molgulid ascidians. Sensory pigment cell development was studied in three different molgulid species: *M. occidentalis* and *M. oculata*, which exhibit urodele larvae missing an ocellus, and *M. occulta*, the sister species of *M. oculata*, which has an anural larva that is lacking both the otolith and ocellus. In urodele molgulids, sensory pigment cells are initially specified by the same bilateral mechanism as in other ascidians, but the precursors of the ocellus and the supernumerary pigment cells are subsequently eliminated by PCD. In anural molgulids, no bilateral pigment cell precursors are specified during embryogenesis, suggesting that the conventional pigment cell determination pathway has been lost. Our results suggest that closely related molgulid species have evolved different mechanisms to remodel their brain sensory organs.

Conservation of the bilateral pigment cell determination system in urodele molgulids

Studies of the mechanisms of pigment cell development have been largely restricted to two ascidian species, *Ciona intestinalis* (Whittaker 1966, 1973) and *Halocynthia roretzi* (Nishida and Satoh 1989; Darras and Nishida 2001). These studies showed that larval pigment cells are determined by a bilateral mechanism: Two or more cells with the potential to develop into melanocytes are specified on either side of the embryo. During the neurula and tailbud stages, as the bilateral pigment cell precursors undergo convergence and meet at the midline, cell interactions involving a classic BMP-Chordin antagonism mediate differentiation of one bilateral cell into an otolith and the other into an ocellus. Two or more additional pigment cell precursors (supernumerary pigment cells) are also specified bilaterally during the cleavage period but do not develop into either an otolith or ocellus. *Ciona* and *Halocynthia* are members of diverse ascidian families, suggesting that the bilateral pigment cell determination pathway is likely to be conserved widely among ascidians. An exception, however, could be molgulids, in which one or both sensory pigment cells are absent. Here we show the bilateral pigment determination pathway is also conserved in urodele molgulids, although late modifications involving PCD are imposed on it to remove the ocellus and supernumerary pigment cells.

Because the otolith and ocellus are derived from precursor cells on each side of the embryo, there are two possibilities to explain the failure of ocellus differentiation in urodele molgulids. First, bilateral specification could be changed into unilateral specification: The potential for producing a pigment cell precursor on one side of the embryo could be lost. Second, bilateral specification could be conserved but another process that affects ocellus development could be superimposed on it. Our results suggest that bilateral specification is conserved in urodele molgulids but modified to eliminate the ocellus precursor later in development. Three independent lines of evidence support this conclusion. First, when one cell of a two-cell embryo is killed, the living half-embryo develops pigment cell precursors in more than 50% of the cases. Second, when blastomeres of a two-cell embryo are separated, both half-embryos have the capacity to produce pigment cell precursors. These results do not support the idea that the bilateral pathway is changed into a unilateral system. Third, cytochalasin B treatment, which blocks the movement of pigment cell precursors toward the midline, results in pigment cell differentiation on both sides of the embryo, directly demonstrating the ability of molgulid embryos to develop bilateral pigment cell precursors. Therefore, the results support a model in which the bilateral pigment cell

determination pathway is conserved in urodele molgulid ascidians.

Roles of midline convergence and PCD in loss of the ocellus

In nonmolgulids, the otolith and ocellus are part of an equivalence group in which one unilaterally derived cell, the prospective ocellus, is dominant and causes the other cell to differentiate into an otolith as a default fate (Nishida and Satoh 1989). The ability to exercise this mode of fate determination is based on the convergence and subsequent interaction of bilateral pigment cell precursors at the embryonic midline. Our results support the possibility that this part of the bilateral pigment cell determination system is also conserved in urodele molgulids. First, the prevention of midline convergence by treatment with cytochalasin before the postneurula stages leads to differentiation of pigment cells on both sides of the embryo. Second, cytochalasin treatment at postneurula stages shows gradual restriction in the number of pigment cell precursors that are able to differentiate into melanocytes. It is concluded that cell movements and interactions throughout the latter half of the embryonic development period are necessary to determine the final number and identity of brain pigment cells in molgulid tadpoles. In molgulids, the ocellus, the dominant cell of the equivalence group, does not differentiate, apparently permitting other pigment cell precursors to develop as otolith-like melanocytes.

Although the precise mechanisms through which cell interactions lead to ocellus regression remain to be elucidated, we show here that PCD has a critical role in its disappearance. Apoptotic PCD has recently been described in ascidian development and shows diversity in timing and extent between different species (Chambon et al. 2002; Jeffery 2002). In some species (e.g., *Ciona*), PCD begins after larval hatching (Chambon et al. 2002), whereas in others (e.g., molgulids), it is initiated precociously during larval development but its completion may be delayed until after hatching (Jeffery 2002). In anural molgulids, PCD is extensive, including some of the epidermal, neural, muscle, and notochord cells, whereas in other ascidian species, it is more restricted and occurs in some parts of the larval head and in the tail epidermis (Jeffery 2002). We show here using the PCD marker SYTOX that cells positioned near the otolith, which are likely to include the ocellus precursor as well as the supernumerary pigment cells (see below), die after hatching and are rapidly removed from the larva prior to metamorphosis. Although it is clear that ocellus PCD, based on the maintenance of plasma membrane integrity, is not complete until after hatching, it cannot be determined from our results at what time the cell death process is initiated during embryogenesis. Caspase and DNA fragmentation (TUNEL) assays, which define apoptotic events before the loss of plasma membrane integrity, are not effective here because of the extent of PCD in molgulid embryos (Jeffery 2002).

There are two possibilities for the relationship between PCD and ocellus differentiation. First, PCD may be causal and the reason the ocellus fails to differentiate. Second, PCD may be triggered secondarily by developmental arrest and inability of the precursor to differentiate into an ocellus. Whether cell death is the cause or effect of failed ocellus differentiation, our results demonstrate how PCD is imposed on the conserved pigment sensory determination pathway to eliminate the ocellus, the dominant member of the pigment sensory cell equivalence group.

Supernumerary pigment cells

Urodele ascidians exhibit only one or two sensory pigment cells but initially form up to four or more precursors with the potential to produce tyrosinase (Whittaker 1966; Nishida and Satoh 1989; Jeffery 1993). The supernumerary pigment cells arise bilaterally and may be sister cells of the otolith and ocellus. Our experiments provide new information about the development and fate of the supernumerary pigment cells.

Like the otolith and ocellus precursors, the supernumerary pigment cells are specified bilaterally in urodele molgulids as part of the conserved pigment cell determination pathway. The presence of supernumerary pigment cells is supported by the presence of four tyrosinase-positive cells along the dorsal midline of tailbud embryos and the detection of two or more pigment cell precursors on each side of cytochalasin-arrested embryos. Thus, as in other ascidians, the pigment cell determination system has the capacity to develop the full range of pigment cell precursors in urodele molgulids.

Our results also suggest a fate for the supernumerary pigment cells: They appear to die by PCD and do not contribute to the larva or adult. It is known that more cells are produced than survive in the vertebrate nervous system (Jacobson et al. 1997), the excess being removed by PCD. Thus, it appears that the production of excess cells that eventually die is also an important theme in the ascidian nervous system. The overproduction and elimination of neural cells appears to predate the origin of vertebrates within the chordates.

Bilateral pigment cell determination system is lost but capable of restoration in anural molgulids

Anural or tailless molgulids, which have lost both brain sensory organs as well as larval muscle and notochord (Jeffery 1997), represent an extreme situation in which to assess changes in the bilateral pigment cell determination pathway. Several lines of evidence suggest that this pathway has been lost in most anural species. First, tyrosinase-positive cells are not detectable during embryonic development (Swalla and

Jeffery 1990; Tagawa et al. 1997). However, *M. occulta* does have the ability to produce tyrosinase because enzyme activity appears in cells scattered throughout *M. occulta* juveniles, as it does in another anural species, *Bostrichobranchus digonas* (Swalla and Jeffery 1991). Therefore, failure to detect the pigment cell precursors in anural species is not due to tyrosinase loss of function. Second, cytochalasin B experiments, which provide direct evidence for the presence of the bilateral pigment determination pathway in *M. occidentalis* and *M. oculata*, do not support the presence of this system in *M. occulta*. Furthermore, we excluded the possibility that the bilateral pigment cell determination system is lost because the pigment sensory cell precursors undergo early PCD in *M. occulta*. We propose that early cell interactions responsible for determining neural developmental pattern and ultimately the pigmented sensory cells (Lemaire et al. 2002) may be modified in anural ascidians.

Interestingly, there is one known exception to the axiom that anural molgulids have lost their sensory pigment cells. Swalla and Jeffery (1991) showed that the anural ascidian *B. digonas*, like urodele molgulids, can develop several tyrosinase-positive cells along the embryonic midline and later a single otolith pigment cell. We suggest that this situation may be due to the presence of a vestigial pigment determination pathway in this anural species.

Molgula oculata and *M. occulta*, which represent urodele and anural sister species, respectively, provided the opportunity to obtain additional information about pigment cell determination via interspecific hybridization. Multiple pigment cell precursors and a differentiated otolith are restored in *M. occulta* × *M. oculata* hybrids, in which the *M. oculata* genome is expressed in *M. occulta* embryo cytoplasm (Swalla and Jeffery 1990; Jeffery and Swalla 1992). We showed that the pigment cell determination pathway typical of urodele molgulids, including bilateral differentiation of melanocytes and gradual restriction of developmental potential to form a single otolith, is also restored in these hybrids. The restoration in the interspecific cross is consistent with a zygotic basis for the absence of pigment cell determination in anural species.

Remarkably, not only bilateral pigment cell determination but also the removal of the ocellus and supernumerary pigment cells by PCD is restored in hybrids. In anural *B. digonas* embryos, the vestigial pigment cell also disappears before metamorphosis and is not present in the juvenile (Swalla and Jeffery 1991). These features argue that PCD may be an integral part of the bilateral pigment cell determination pathway and an important determinant of developmental diversity in ascidian brain sensory organs.

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