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Research Highlights

Proliferating cells in suborbital tissue drive eye migration in flatfish

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► We found that eye migration in flatfish is caused by cell proliferation in the suborbital tissue of blind side. ► We corrected the misunderstanding that eye migration is driven by the cranial asymmetry. ► We found that cranial asymmetry depends on the eye migration. ► Eye-symmetric flatfish with a bottom-dwelling lifestyle were first time produced artificially.
Proliferating cells in suborbital tissue drive eye migration in flatfish

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\textbf{A B S T R A C T}

The left/right asymmetry of adult flatfishes (Pleuronectiformes) is remarkable given the external body symmetry of the larval fish. The best-known change is the migration of their eyes: one eye migrates from one side to the other. Two extinct primitive pleuronectiformes with incomplete orbital migration have again attracted public attention to the mechanism of eye migration, a subject of speculation and research for over a century. Cranial asymmetry is currently believed to be responsible for eye migration. Contrary to that hypothesis, we show here that the initial migration of the eye is caused by cell proliferation in the suborbital tissue of the blind side and that the twist of frontal bone is dependent on eye migration. The inhibition of cell proliferation in the suborbital area of the blind side by microinjected colchicine was able to prevent eye migration and, thereafter, cranial asymmetry in juvenile Solea senegalensis (right sideness, Soleidae), Cynoglossus semilaevis (left sideness, Cynoglossidae), and Paralichthys olivaceus (left sideness, Paralichthyidae) with a bottom-dwelling lifestyle. Our results correct the current misunderstanding that eye migration is driven by cranial asymmetry and simplify the explanation for broken left/right eye-symmetry. Our findings should help to focus the search on eye migration-related genes associated with cell proliferation. Finally, a novel model is proposed in this research which provides a reasonable explanation for differences in the migrating eye between, and sometimes within, different species of flatfish and which should aid in our overall understanding of eye migration in the ontogenesis and evolution of Pleuronectiformes.

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on the observation of bone morphological changes, it is difficult to ascertain initial timing difference between eye migration and cranial asymmetry. Moreover, research of Schreiber (2006) has indicated that asymmetrical skull development alone is insufficient for eye migration in Southern flounder (Paralichthys lethostigma L.). Dense cell population of fibroblasts ventral to the eye in Atlantic halibut, Hippoglossus hippoglossus, was observed by Sæle et al. (2006) and postulated to be related to eye migration. Based on our understanding of the basic physical principles, we proposed a hypothesis that the force generated from the suborbital area should be the most efficient in pushing the eye upwards, and such a force might be generated by hyper-proliferating cells.

**Materials and methods**

**Fish maintenance**

Larvae *Solea senegalensis* (Soleidae), *Cynoglossus semilaevis* (Cynoglossidae), and *Paralichthys olivaceus* (Paralichthyidae) were obtained from Dahua Fisheries Farm (Laizhou, Shandong, China), Mingbo Fisheries Farm (Laizhou, Shandong, China), and the Central Experiment Station of Chinese Academy of Fisheries Sciences (Beidaihe, Hebei, China), respectively. Then, larvae were transported to the laboratory of College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China, and reared according to the references (Dinis et al., 1999; Bao et al., 2005; Liu et al., 2008). Larvae were fed with live brine shrimp (Artemia) nauplii through the end of metamorphosis. The normal metamorphic stages were defined as the following: pre-metamorphosis (the stage prior to the start of eye migration); Stage E (the eye begins to migrate); Stage F (the migrating eye visible from the ocular side); Stage G (the upper edge of the migrating eye beyond the dorsal margin); Stage H (the upper edge of the migrating eye beyond the dorsal mid-line); Stage I (entire migrating eye past the dorsal mid-line) (Minami, 1982). Under this definition, the days after hatch (DAH) corresponding metamorphosis stages were listed in Table 1.

**Cytogenetic analysis of whole-mount in situ cell proliferation**

Larvae were treated with 0.1% 5′-bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) for 8 h, then 20 larvae were fixed in 4% paraformaldehyde (PFA) overnight and stored in methanol at 4 °C after paraformaldehyde (PFA) overnight and stored in methanol at 4 °C after

**Fig. 1.** Left/right asymmetrical distribution of the proliferating cells in suborbital tissue before eye initial movement (pre-metamorphosis stage). The number of proliferating cells can be compared between two rectangle areas (suborbital areas) of both sides of *Solea senegalensis* (right sideness, Soleidae) (panel A), *Cynoglossus semilaevis* (left sideness, Cynoglossidae) (panel B), or *Paralichthys olivaceus* (left sideness, Paralichthyidae) (panel C). Each rectangle area for all three species has the same size. More proliferating cells were found in the suborbital area of blind side than that of ocular side. Proliferating cells in suborbital area of blind side were significantly more than that of ocular side. Panel A, *S. senegalensis*: (A1), ocular view; (A2), enlarged view of rectangle area in A1; (A3), blind view; (A4), enlarged view of rectangle area in A3. Panel B, *C. semilaevis*: (B1), blind view; (B2), blind side; (B3), ocular view; (B4), enlarged view of rectangle area in B3. Panel C, *P. olivaceus*: (C1), blind view; (C2), enlarged view of rectangle area in C1; (C3), ocular view; (C4), enlarged view of rectangle area in C3. (D), comparison of proliferating cell number in suborbital area between ocular side and blind side; values are means ± SD, n = 3, different letters at top of columns indicate significant differences (P<0.05). dm indicates dorsal mid-line; le, lateral ethmoid. Dash arrow shows movable eye; arrow, signal of cell proliferation. Bar, 500 μm.
anesthetized with 0.1 μg/μl tricaine methanesulfonate (MS 222). The larvae
with no BrdU labeling were treated as negative controls. Whole-
mount preparations were analyzed for BrdU immunohistochemistry
according to techniques developed by Byrd and Brunfes (2001) and
modified. Briefly, following fixation, larvae were rinsed in 0.1 M
phosphate-buffered saline (PBS), pH 7.4. Then, the fish were bleached
with 3% hydrogen peroxide in 25 mM NaOH under strong light, and
transferred to 2 M HCl at 37 °C for 90 min for DNA denaturation. After
neutralization with 0.1 M sodium borate for 20 min, the fish were rinsed
3 times with 0.5% Triton X-100 in PBST for 20 min each. Proteinase K
(67 μg/ml in PBST) was used to digest the fish at 37 °C for 30–60 min
(depends on the size of fish, smaller fish are more sensitive to proteinase
K); then, digestion was terminated by 100 mM glycine for 20 min. The
larvae then were rinsed twice with 0.5% Triton X-100 in PBST for 20 min
each. Nonspecific binding was blocked by using 5% goat serum, 1% BSA,
and 0.5% Triton X-100 in PBST at 37 °C for 90 min. The larvae were
incubated with mouse Monoclonal Anti-BrdU antibody (Sigma-Aldrich)
at 4 °C overnight. After washing several times with PBST containing
0.5% Triton X-100, the larvae were incubated with HRP conjugated goat
anti-mouse secondary antibody (Immunology Consultants Laboratory,
Newberg, OR) at 4 °C overnight. Through rinses with PBST and PBS,
larvae were placed in 3-amin-9-ethylcarbazole (AEC) (Amresco, Solon,
OH) solution until the appropriate staining density was attained. The
AEC stock solution was prepared by dissolving 10 mg AEC in 1 ml N,N-
dimethylformamide (Amresco). For the actual development, 1 ml of this
AEC stock solution was freshly diluted into 30 ml of 0.1 M sodium-
acetae buffer (pH 5.0), and mixed with 15 ml H2O2. Whole-mount
specimens were observed and photographed under a dissecting
microscope SZX7 (Olympus, Tokyo, Japan) and documented using
Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). Since
BrdU was incorporated into DNA during the synthesis-phase of the cell
cycle, the detected signal was only limited in the cell nucleus and hence
allows us to count the proliferating cells exactly. Based on the signals on
the photograph, the proliferating cells in suborbital areas from each
species were counted. Selected specimens were embedded with
embedding medium (Leica Instruments, Germany). Eight micrometer
frozen tissue sections were cut in the transverse planes to localize
precisely signal sites of proliferation and were photographed by the
above microscope with Q16242 digital camera (QImaging, Surrey, BC,
Canada).

Fig. 2. The signal of cell proliferation locating in the skin of suborbital areas at pre-metamorphosis stage. Panel A, section view across both eyes in Solea senegalensis. Cell proliferation in the skin of suborbital area (A1) and along ongoing migration route between two eyes (A2). Panel B, section view across both eyes in Cynoglossus semilaevis. Panel C, section view across both eyes in Paralichthys olivaceus. Arrow shows signal of cell proliferation. L indicates left side; R, right side; bar, 100 μm.

Microinjection with colchicine

To test the role of cell proliferation in eye migration, colchicine, an inhibitor of cell proliferation, was microinjected into the suborbital area of blind side at several days early before the putative initial eye migration (Table 2). The primary result from pre-test showed that colchicine (Sigma-Aldrich) at concentration of 500 μg/ml could kill the larvae *P. olivaceus* in 36 h. Finally, the concentration of 50 μg/ml colchicine was used in all formal experiments in this research. Appropriate 50 nl of colchicine at 50 μg/ml in 0.75% NaCl solution was injected into the suborbital area of anesthetized larvae. Same amount of 0.75% NaCl solution was injected as control. The injected larvae were reared in 2 L tank with same rearing condition as described above. Detailed information of colchicine injection was listed in Table 2. Several 19 DAH *S. senegalensis*, 25 DAH *C. semilaevis*, and 29 DAH *P. olivaceus* after colchicine injection were treated with 0.1% BrdU for another 8 h for detection of cell proliferation.

Skeletal staining and photography

Larvae and juvenile were fixed in 4% paraformaldehyde for at least 24 h. Alcian blue 8GX (A3157; Amresco) was used for cartilage staining, and alizarin red S (A5533; Amresco) was for bone staining. Detailed staining procedures followed the reference (Gavaia et al., 1999). Samplers were observed under a dissecting microscope SZX7 (Olympus) and documented by using Image-Pro Plus 6.0 software (Media Cybernetics). The scale on each photograph was autogenerated by Image-Pro Plus 6.0 software based on magnification.

Statistics

Significance of difference between two groups was tested by paired t-test. SPSS 13.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. Difference was considered significant if *P*<0.05. Data were reported as mean ± SD.

Results

Left/right asymmetrical distribution of the proliferating cells in suborbital tissue before eye initial migration

Using whole-mount *in situ* BrdU detection, we examined cell proliferation during eye migration of three species of flatfish, *S. senegalensis*, *C. semilaevis*, and *P. olivaceus*. At the pre-metamorphosis stage of initial upward eye migration, cell proliferation was found in the suborbital areas on both sides and the dorsal mid-line surface along the head between two eyes (Fig. 1). In *S. senegalensis*, more proliferating cells on the blind side (left side) were found in the suborbital area than that on the ocular side (right side; Fig. 1A). Similar result was observed in *C. semilaevis* (Fig. 1B) and *P. olivaceus* (Fig. 1C). Further analysis showed that these left/right differences in *S. senegalensis*, *C. semilaevis*, and *P. olivaceus* were statistically significant (Fig. 1D, *P*<0.05). In the frontal cartilage, regarded previously as the

Fig. 3. Cells proliferating in the suborbital tissue at climax of metamorphosis. Panel A, *Solea senegalensis* at 16 DAH. (A1), ocular view; (A2), blind view. Panel B, *Cynoglossus semilaevis* at 20 DAH. (B1), blind view; (B2), ocular view. Panel C, *Paralichthys olivaceus* at 24 DAH. (C1), blind view; (C2), ocular view. More proliferating cells are shown by arrows in the suborbital area of migrating eye. Bar, 500 μm.

source of the original force initiating eye migration, there was no cell proliferation signal found. Whereas in the lateral ethmoid, which was thought previously to push eye migration at later metamorphic stage through asymmetrical enlargement on both sides, there existed a strong signal of cell proliferation (Fig. 1A1, A3, B1, B3, C1, C3). The signal of cell proliferation located in the skin of suborbital area and along the head between two eyes in *S. senegalensis*, *C. semilaevis*, and *P. olivaceus* (Fig. 2). The phenotype of proliferating cell could not be discerned in the frozen tissue sections in this research. After initiation of eye migration, the cells in suborbital tissue on both sides and the

Fig. 4. Eye migration stopped by colchicine through microinjecting into the suborbital area of blind side. (A), the suborbital area of blind side where colchicine was microinjected. (B), the percentage of various *Solea senegalensis* with eye stopped in different locations at age of 21 DAH. (C), the percentage of various *Cynoglossus semilaevis* with eye stopped in different locations at age of 25 DAH. (D), the percentage of various *Paralichthys olivaceus* with eye stopped in different location at age of 30 DAH. Gray column, injected with colchicine; black column, injected with 0.75% NaCl solution (control). Metamorphic stages shown with E (the eye begins to migrate), F (the migrating eye visible from the ocular side), G (the upper edge of the migrating eye beyond the dorsal margin), H (the upper edge of the migrating eye beyond the dorsal mid-line), and I (entire migrating eye past the dorsal mid-line) on abscissa axis. (E1), proliferating cells of 19 DAH *Solea senegalensis* after colchicine microinjected into suborbital area of blind side; rectangle shows suborbital area. (E2), enlarged view of rectangle area in E1. (F1), proliferating cells of normal larvae at stage E (the eye begins to migrate); rectangle shows suborbital area of blind side (B1) and its enlarged view (B2). Panel C, proliferating cells of 29 DAH *P. olivaceus* after colchicine microinjected into suborbital area of blind side. Rectangle shows the suborbital area (C1) and its enlarged view (C2). Panel D, proliferating cells of normal larvae *P. olivaceus* at stage E. Rectangle shows the suborbital area of blind side (D1) and its enlarged view (D2). Bar, 500 μm.

Fig. 5. Cell proliferation inhibited by colchicine through microinjecting into the suborbital area of blind side in *Cynoglossus semilaevis* and *Paralichthys olivaceus*. Panel A, proliferating cells of 25 DAH *C. semilaevis* after colchicine microinjected into suborbital area of blind side. Rectangle shows the suborbital area (A1) and its enlarged view (A2). Panel B, proliferating cells of normal larvae *C. semilaevis* at stage E (the eye begins to migrate). Rectangle shows suborbital area of blind side (B1) and its enlarged view (B2). Panel C, proliferating cells of 29 DAH *P. olivaceus* after colchicine microinjected into suborbital area of blind side. Rectangle shows the suborbital area (C1) and its enlarged view (C2). Panel D, proliferating cells of normal larvae *P. olivaceus* at stage E. Rectangle shows the suborbital area of blind side (D1) and its enlarged view (D2). Bar, 500 μm.

dorsal surface along the head between the two eyes continue proliferating in *S. senegalensis*, *C. semilaevis*, and *P. olivaceus* (Fig. 3).

Eye migration stopped by the inhibitor of cell proliferation microinjected into the suborbital area of blind side

To determine the role of cell proliferation of suborbital tissue in initial eye migration, colchicine, a widely used inhibitor of cell proliferation, was microinjected two to three times into the suborbital area of the blind side four, three, or two days before initial eye migration in *S. senegalensis*, *C. semilaevis*, or *P. olivaceus*, respectively (Table 2, Fig. 4A). Eye migration could be stopped by injected colchicine. In *S. senegalensis*, while the eye on blind side in all larvae without colchicine injection successfully migrated past the dorsal mid-line (Stage I, 21 DAH), there were no larvae with eye migration past the dorsal mid-line (Stage I) in the colchicine microinjected group, and 17 (68%) individuals were at stage E with no observable movement of the eye on blind (left) side (Fig. 4B). Similar results were observed in *C. semilaevis* (Fig. 4C) and *P. olivaceus* (Fig. 4D). Moreover, while eye migration was partially or completely inhibited by microinjection of colchicine in the suborbital area of the blind side, all larvae went through metamorphosis successfully and developed into juveniles with a bottom-dwelling lifestyle with the exception of abnormal eye location. This result suggests that inhibition of eye migration was specific to interference with proliferating cells in suborbital tissue and not due to a general developmental disruption caused by colchicine injection. The cell proliferation in the suborbital area of the blind side was evidently inhibited by microinjected colchicine. Since colchicine inhibited cell mitosis through inhibiting microtubule polymerization by binding to tubulin, not through inhibiting DNA synthesis, the signal of cell proliferation from BrdU labeling still could be detected. However, the number of proliferating cells did not increase. Compared with normal larvae at stage E (the eye begins to migrate), less proliferating cells were found in the suborbital area of blind side in *S. senegalensis* injected with colchicine (Fig. 4E, F). Further analysis showed that the difference was statistically significant (Fig. 4G, *P* < 0.05). Similar inhibition of cell proliferation by microinjected colchicine in *C. semilaevis* and *P. olivaceus* are shown in Fig. 5. Representative individuals of *S. senegalensis*, *C. semilaevis*, and *P. olivaceus* with eye migration blocked completely by microinjected colchicine are shown in Fig. 6. Representative individuals with incomplete eye migration caused by microinjected colchicine are shown in Fig. 7.

Twist of frontal bones depending on eye migration

The juvenile with symmetrical eyes resulting from colchicine injection in this research provided an opportunity to determine the

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**Fig. 6.** Representative individuals with eye migration caused by microinjected colchicine. Panel A, *Solea senegalensis*. (A1), ocular view; (A2), blind view. Panel B, *Cynoglossus semilaevis*. (B1), blind view; (B2), ocular view. Panel C, *Paralichthys olivaceus*. (C1), blind view; (C2), ocular view. Bars in panels A and B, 1 mm; bars in panel C, 0.5 mm.

**Fig. 7.** Representative individuals with incomplete eye migration caused by microinjected colchicine. Panel A, *Solea senegalensis*. (A1), ocular view; (A2), blind view. Panel B, *Cynoglossus semilaevis*. (B1), blind view; (B2), ocular view. Panel C, *Paralichthys olivaceus*. (C1), blind view; (C2), ocular view. Bars in panels A and B, 1 mm; bars in panel C, 0.5 mm.
chronological sequence of eye migration and cranial asymmetry. In these flatfishes with symmetrical eyes, the skull kept left/right symmetry (Fig. 8). The twist of frontal cartilage was not observed in the juvenile S. senegalensis with symmetrical eyes. The lateral ethmoids on both sides were the similar size (Fig. 8A). Similar development of skull bones was observed in colchicine-treated C. semilaevis and P. olivaceus with symmetrical eyes (Fig. 8B, C). The observation provided here indicates that cranial asymmetry should depend on the eye migration in normal metamorphosing flatfish.

Discussion

What kind of tissue drives eye migration during metamorphosis is one of the most important issues in better understanding the genetic mechanism behind eye-asymmetry in flatfish. Sæle et al. (2006) first time observed the dense cell population of fibroblasts in the suborbital tissue in H. hippoglossus and postulated to be related to eye migration. In this study, we observed the proliferating cells in the suborbital tissue of S. senegalensis, C. semilaevis, and P. olivaceus, suggesting that the proliferating cells might be fibroblast within the suborbital connective tissue as observed in H. hippoglossus by Sæle et al. (2006). Moreover, the inhibition of this proliferation with colchicine provides strong evidence that the initial migration of the eye was caused by cell proliferation in the suborbital tissue of the blind side in three different species from families of Pleuronectiformes. In addition, the result in this study showed that the twist of frontal bone, which was thought to be primary reason for eye migration, is dependent on eye migration. It is also the first time in the world to produce eye-symmetrical or incomplete orbital migration juvenile S. senegalensis, C. semilaevis, and P. olivaceus with a bottom-dwelling lifestyle. Based on all the findings in this study, we propose a novel model here to explain eye migration in flatfish (Fig. 9).

Before eye migration initiated, the cells located in suborbital tissue (skin) and along the route of ongoing eye migration begin to proliferate (Fig. 9A). More proliferating cells exist in the suborbital tissue of the blind side than that of the ocular side. Left/right asymmetrical distribution of proliferating cells in suborbital tissue may correspond to “stronger” and “feeble” eye identified in an early hypothesis proposed by Giard (1877) in which he suggested that one eye was “stronger” causing it to rotate towards the “feeble” eye. The presence of uneven amount of proliferating cells exerting uneven pressure on two eyes could explain why the migrating eye differs between, and even within, species of flatfish. Generally, only one of the eyes will be able to be pushed upwards by overcrowded proliferating cells in the suborbital tissue. Once the movable eye receives enough pushing force from proliferating cells in its suborbital area to overcome the main counteracting force from the other eye, it starts migrating upwards (Fig. 9A). During migration, the migrating eye receives an additional pushing force from more proliferating cells in its enlarging suborbital area. Meanwhile, the counteracting force is becoming larger and larger as the two eyes become closer and closer (Fig. 9B). When the migrating eye reaches the place where pushing and counteracting forces are equalized, it finally stops migration (Fig. 9C). During the process of eye migration, once the eye starts migrating upwards, the cartilage of the skull closest to the route of the migrating eye (frontal cartilage and/or supraorbital cartilage in some species) begins facing the pushing force from the migrating eye and, in response, begins twisting towards another side.

Fig. 8. Left/right asymmetry of skull in the colchicine-treated flatfish with symmetrical eyes. There was no twist found in frontal bones (f), and lateral ethmoids (le) on both sides of head were similar size in the juvenile with symmetrical eyes. Panel A, 21 DAH symmetrical-eyed Solea senegalensis resulting from injected colchicine. (A1), ocular view; (A2), blind view; (A3), ocular view of skull; (A4), blind view of skull. Panel B, 60 DAH symmetrical-eyed Cynoglossus semilaevis resulting from colchicine. (B1), blind view; (B2), ocular view; (B3), blind view of skull; (B4), ocular view of skull. Panel C, 38 DAH symmetrical-eyed Paralichthys olivaceus resulting from colchicine. (C1), blind view; (C2), ocular view; (C3), blind view of skull; (C4), ocular view of skull. Calculated bone is red and cartilage is blue. e indicates eye; f, frontal cartilage/bone; le, lateral ethmoid. Arrow shows the bone on the ocular side. Bars in A3 and A4, 0.5 mm; bars in others, 1.0 mm.
Moreover, the lateral ethmoid on the blind side will enlarge more quickly than that on the ocular side because of the additional space left by the migrating eye.

Based on the above model, eye migration in Pleurostomes that varies from one species to another, and within the same species, and various observed natural mutant flatfish with different eye locations, such as reversed-eye, symmetrical eyes, and both eyes on the top of the head (Schreiber, 2006; Wagemans et al., 1998), are easily understandable. Mutant forms may be the result of abnormal competition between both sides of suborbital areas in the form of cell proliferation.

Our results presented in this study suggest that eye migration is caused by cell proliferation in suborbital tissue and correct the misunderstanding that eye migration is driven by the cranial asymmetry. These findings are important to simplify the explanation for broken left/right symmetry of eyes and will be of great help in identifying eye migration-related genes associated with cell proliferation. Obviously, the proposed model in this research will help us to understand eye migration in the ontogenesis and evolution of Pleurostomes.

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