okomeduzy mutations affect neuronal patterning in the zebrafish retina and reveal cell-cell interactions of the retinal neuroepithelial sheet

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Accepted 22 December 1998; published on WWW 15 February 1999

SUMMARY
Mutations of the okomeduzy (ome) locus cause drastic neuronal patterning defect in the zebrafish retina. The precise, stratified appearance of the wild-type retina is absent in the mutants. Despite the lack of lamination, at least seven retinal cell types differentiate in okomeduzy. The ome phenotype is already expressed in the retinal neuroepithelium affecting morphology of the neuroepithelial cells. Our experiments indicate that previously unknown cell-cell interactions are involved in development of the retinal neuroepithelial sheet. In genetically mosaic animals, cell-cell interactions are sufficient to rescue the phenotype of okomeduzy retinal neuroepithelial cells. These cell-cell interactions may play a critical role in the patterning events that lead to differentiation of distinct neuronal laminae in the vertebrate retina.

Key words: Retina, Mutant, Pattern, okomeduzy, ome, Zebrafish, Neuroepithelium

INTRODUCTION
Organization of neurons in the vertebrate retina displays remarkable precision. Three major neuronal laminae are obvious on histological sections through the retina: the photoreceptor cell layer, the inner nuclear layer and the ganglion cell layer. The inner nuclear layer is further subdivided into the horizontal, bipolar and amacrine cell layers. The inner nuclear, the photoreceptor and the ganglion cell layers are separated from each other by laminae consisting of neuronal projections: the outer and inner plexiform layers (Cajal, 1893; Rodieck, 1973; Dowling, 1987). Within individual layers, cells display specific orientations. For example, the photoreceptor outer segments adjoin the pigmented epithelium whereas the photoreceptor synaptic termini contact the outer plexiform layer. The major cell types of the retina and their organization experienced remarkably little change throughout the vertebrate evolution. Retinal cells are organized in the same set of layers in organisms as diverse as humans and teleost fish (Muller, 1857; Cajal, 1893).

During embryogenesis, the vertebrate neural retina originates from a neuroepithelial sheet of cells. The retinal neuroepithelium consist of mitotically active, multipotent cells (Turner and Cepko, 1987; Holt et al., 1988; Wets and Fraser, 1988). In the course of neurogenesis, the neuroepithelial cells exit the cell cycle and acquire one of seven major cell fates – six neuronal and one glial (Altshuler et al., 1991). Individual cell types assume specific positions in the retina. The genetic mechanisms responsible for directing cells to specific locations remain largely unknown.

Several research approaches led to the identification of a handful of molecules with possible roles in retinal lamination. Targeted gene knockouts of at least three loci, N-CAM, MARCKS and HES-1, lead to lamination defects. N-CAM, one of the most studied adhesion molecules, produces surprisingly limited defects in the central nervous system. In the retina, targeted disruption of the N-CAM gene leads to a threefold increase of cell nuclei in the plexiform layers (Tomasiewicz, 1993). MARCKS knockout leads to more significant changes; although, in this case, the lamination defect may be limited to the inner retina (Stumpo et al., 1995). Finally, HES-1 knockout results in disruption of the photoreceptor cell layer and formation of abnormal clusters of the photoreceptor cells known as rosettes (Tomita et al., 1996). This phenotype is accompanied by extensive cell death, suggesting that the patterning defect of the photoreceptor cells may be a secondary consequence of massive cell death in the inner nuclear layer. In addition to the knockout experiments, earlier studies performed on tissue explants identified other factors potentially involved in the retinal patterning. Blocking experiments with antibodies directed to N-cadherin result in neuronal patterning defects in the retina and brain (Matsunaga et al., 1988; Ganzler-Odenthal and Redies, 1998). In the retina, mostly the outer layers are affected (Matsunaga et al., 1988).
Similar studies revealed that the B2 antigen may be involved in formation of the plexiform layers (Ohta et al., 1992). The role of N-cadherin in neuronal lamination could be further tested in knockout experiments. Such studies are likely to be complicated, however, because the N-cadherin knockout results in early embryonic lethality making it difficult to study the role of this gene in the retinal neurogenesis (Radice et al., 1997).

The zebrafish model system offers a unique opportunity to study the genetic basis of early vertebrate development. Two large scale and several smaller mutagenesis screens have been performed in zebrafish in recent years, leading to identification of nearly two thousand mutations affecting numerous aspects of zebrafish development (Driever et al., 1996; Haffter et al., 1996). In addition to the genetic attributes of the system, the zebrafish embryo is very well suited for studies of retinal development. In the central retina, neurogenesis is essentially completed by 60 h.p.f. (hours postfertilization) (Nawrocki, 1985) and the major neuronal laminae are very well differentiated by 72 h.p.f. The larval eyes are large and easily accessible to experimental manipulations. As judged by the startle and the optokinetic responses, the zebrafish larvae are able to see by 4 d.p.f. (days postfertilization) (Clark, 1981; Easter and Nicola, 1996). These characteristics make the zebrafish larva uniquely suited for identification of genes involved in development of the vertebrate visual system. Recent genetic screens led to the discovery of numerous mutants affecting development of the zebrafish eye (Baier et al., 1996; Malicki et al., 1996; Fadool et al., 1997). Among them, a group of identified mutations result in a drastic disruption of the neuronal pattern in the retina (Malicki et al., 1996). These mutations provide a unique opportunity to gain insight into the genetic basis of neuronal patterning in the vertebrate eye. In this work, we provide developmental analysis of the neuronal patterning defect in the zebrafish mutant oko meduzy. The precise, wild-type lamination of the retina is drastically disorganized in this mutant. Despite the loss of neuronal pattern, all cell types analyzed so far are specified in the oko meduzy retinae. The neuronal patterning defect is preceded by loss of normal morphology and integrity of the retinal neuroepithelial cells. As revealed by mosaic analysis of the oko meduzy neuroepithelial phenotype, cell-cell interactions are involved in development of the retinal neuroepithelial sheet.

MATERIALS AND METHODS

Strains of zebrafish
Four alleles affecting the oko meduzy locus were recovered in the course of a large-scale chemical mutagenesis screen in zebrafish: ome<sup>n08</sup>, ome<sup>n289</sup>, ome<sup>n298</sup> and ome<sup>n320</sup> (Malicki et al., 1996). All isolated alleles are recessive, fully penetrant and produce indistinguishable phenotypes. The phenotype of transheterozygotes is not distinguishable from homozygous phenotypes. With exception of the initial phenotypic analysis and DiI labeling, which were performed both on ome<sup>n289</sup> and ome<sup>n08</sup>, all experiments were done on the ome<sup>n08</sup> allele.

In situ hybridization and staining protocols
Some of the protocols were published previously by the following authors: AB131 staining (Sandell et al., 1994), Zn-5 staining (Trevorrow et al., 1990), and in situ hybridization (Oxtoby and Jowett, 1993). In situ hybridization and Zn-5 staining were performed on whole embryos. After hybridization, embryos were dehydrated, embedded in JB-4 resin (Polysciences, Inc.) and sectioned for further analysis. In all whole-mount protocols, development of pigmentation was inhibited by adding 0.003% 1-phenyl-2-thiourea (Sigma Inc.) to the embryo storage medium.

Staining with anti-gamma-tubulin antibody (gift from Pavel Draber) and anti-carbonic anhydrase antibody (gift from Paul Linser) were performed on frozen sections. Animals were fixed in 4% paraformaldehyde (pH 7.5) for 3 hours, infiltrated in 30% (w/v) sucrose in PBS overnight and embedded in TBS tissue freezing medium (Polysciences, Inc.). 20 μm sections were prepared on a Leica cryostat, collected on Superfrost Plus slides (Fisher Scientific Inc.), heated to 80°C for 15 seconds, and stored at room temp for 30-90 minutes. For anti-gamma-tubulin staining sections were rehydrated in PBS (5 minutes), acetone treated (5 minutes, −20°C), washed in PBS (3 times, 5 minutes each), treated with blocking solution (10% normal goat serum, 0.5% Triton X-100 in PBS, 1 hour), and washed again. The primary antibody was applied in PBS with the addition of Triton X-100 (overnight, 4°C). Sections were washed in PBS (3 times, 5 minutes each), stained with the secondary FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Inc., 1 hour at room temperature), washed as previously and mounted in glycerol. The staining pattern was analyzed using MRC-600 (Bio-Rad Inc.) and TCS 4D (Leica Inc.) confocal microscopes. Staining with the anti-carbonic anhydrase antibody was performed as above without the acetone treatment.

For phalloidin staining, frozen sections were prepared as above, washed in PBS (5 minutes), incubated with FITC-conjugated phalloidin (Sigma Inc., 0.1 mg/l, 15 minutes), washed with PBS (3 times, 5 minutes each), mounted in glycerol and viewed using a confocal microscope.

To detect M-phase nuclei, embryos were embedded in JB-4 resin (Polysciences, Inc.) and sectioned at 2.5 μm. Sections were collected on microscope slides and immersed in Hoescht 33258 (Molecular Probes Inc., 1 μg/ml in PBS) for 15 minutes, washed in PBS for 1 hour, mounted in glycerol and viewed under Axioptot microscope (Zeiss, Inc.) using u.v. illumination.

Dil labeling
For the DiI application, development of pigmentation was inhibited by adding 0.003% 1-phenyl-2-thiourea (Sigma Inc.) to the embryo storage medium. At 60 h.p.f., animals were collected and fixed in 4% paraformaldehyde in PBS (pH 7.5) for at least 2 hours. The DiI crystals (Molecular Probes Inc.) were inserted into the optic tectum using a glass needle. Embryos were left overnight at room temperature and analyzed using MR 600 confocal microscope (Bio-Rad Inc.).

Electron microscopy
Embryos were fixed using a mix of paraformaldehyde and glutaraldehyde for 2 hours (4% and 2%, respectively, in 75 mM phosphate buffer, pH 7.3, on ice), washed in ice-cold phosphate buffer and fixed in osmium tetroxide for 2 hours (2% in 50 mM phosphate buffer, on ice). Subsequently, they were washed in ice-cold maleate buffer (50 mM, pH 5.9), stained in uranyl acetate for 2 hours (2% in maleate buffer, room temperature), washed in maleate buffer again and dehydrated in ethanol/maleate buffer series. Dehydrated embryos were infiltrated in Epon/proplylene oxide series, embedded in Epon (Polysciences Inc.) and thin sectioned. Sections were analyzed using Phillips CM10 microscope.

Blastomere transplantations
Mosaic zebrafish were generated as described previously (Ho and Kane, 1990; Westerfield, 1994). Approximately 20-50 blastomeres were transplanted at the late blastula stage from donor to host embryos. Donor embryos were labeled at 2- to 8-cell stage with a mix
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of Texas Red- and biotin-conjugated dextran in the 1 to 9 ratio (Molecular Probes, Inc.; 10% w/v in 0.2 M KCl). Mosaic embryos were fixed at stages prm 23 to pectoral ridge (Kimmel et al., 1995) in 4% paraformaldehyde in PBS (pH 7.5). The phenotypes of hosts and donors were evaluated at the time of fixation.

Two series of transplantation experiments were performed. In the first series, after transplantation, embryos were fixed, washed in PBS (5 minutes), acetone treated (7 minutes, −20°C), washed in PBS (3 times, 5 minutes each) and incubated in blocking solution (2% normal goat serum, 1% Triton X-100, 1% DMSO, 20 minutes at room temperature). In the subsequent steps, detection of the donor-derived cells was performed using avidin-biotin-HRP complex and 3,3'-diaminobenzidine (DAB) substrate as recommended by the manufacturer (Vector Laboratories, Inc.). DAB-stained embryos were embedded in JB-4 resin (Polysciences, Inc.) and sectioned at 5-7 μm. Sections were collected on glass slides and photographed using a video camera. Images were stored on a Panasonic Optical Disc Recorder using AxoVideo 2.0 software (Axon Instruments, Inc.). By following individual cell clones on photographs of consecutive sections, we were able to determine whether they extend to the vitreal and the ventricular surfaces of the neuroepithelium. Due to gaps in collections of sections, we were not able to determine whether some clones contact the ventricular surface. These clones were excluded from analysis. Examples of cell clones that were shown not to extend to the ventricular surface are indicated with arrows (Fig. 5B,C). A donor-derived clone was scored as having no ventricular contact only if all its cells did not extend to the ventricular surface. This scoring method most likely underestimated the number of cells which do not extend to the ventricular surface. Complete photographic evidence is available upon request.

In the second series of experiments, after transplantation donor-derived cells were analyzed on frozen sections. Sections were prepared as described above and stained with a mix of anti-gamma-tubulin mouse monoclonal antibody (gift of Pavel Draber) and Texas Red-conjugated avidin (Jackson ImmunoResearch Inc.) as described above for the anti-gamma-tubulin staining. Stained sections were prepared as described above and stained with a mix of anti-gamma-tubulin mouse monoclonal antibody (gift of Pavel Draber) and Texas Red-conjugated avidin (Jackson ImmunoResearch Inc.) as described above for the anti-gamma-tubulin staining. Stained sections were mounted in glycerol and analyzed under a confocal microscope using FITC and Texas Red detection channels. Position of centrosomes was determined using the FITC channel. In parallel, morphology of the donor-derived cells was evaluated using the Texas Red channel. To determine cell morphology, we collected Z-series of 6-15 confocal images for each of the donor-derived clones. This approach allowed us to determine which centrosomes belonged to particular donor-derived clones. Each of the Z-series was projected on a single plane (Fig. 6). Some bleed-through was evident from the red to the green channel. Green signal due to the bleed-through was diffuse and could be distinguished from centrosome labeling. Complete set of confocal data is available upon request.

RESULTS

oko meduzy affects pattern but not specification of several cell types in the zebrafish retina

The oko meduzy (ome) locus is involved in the cellular organization of the zebrafish retina (Malicki et al., 1996). Four alleles have been characterized: ome<sup>98</sup>, ome<sup>289</sup>, ome<sup>298</sup> and ome<sup>320</sup>. All four produce recessive, fully penetrant phenotypes of similar strength. The oko meduzy mutations cause striking disorganization of the zebrafish retina. Neurons in the mutant retina do not form layers and their projections congregate in chaotically distributed patches (Fig. 1A,B). To investigate whether individual cell types are present in the oko meduzy retina and how they are localized, we performed labeling experiments using cell-type-specific antibodies and RNA probes. We analyzed three out of the six major neuronal cell classes known in the vertebrate retina (Cajal, 1893; Rodieck, 1973; Dowling, 1987): ganglion cells, amacrine cells and four types of photoreceptors. In addition, we characterized the phenotype of the Muller glia. To visualize individual cell types, we used the following antibodies and RNA probes: Zn-5 antibody to detect ganglion cells (Trevorrow et al., 1990; Fig. 1C,D), anti-GABA antibody to detect amacrine cells (Sandell et al., 1994; Fig. 1E,F), anti-carbonic anhydrase antibody to detect Muller glia (Linser and Moscona, 1984; Linser et al., 1985; Fig. 1G,H), and four opsin probes to detect different types of photoreceptor cells: blue opsin, red opsin, rod opsin, and UV opsin (Johnson et al., 1993; Robinson et al., 1995; Hisatomi et al., 1996; Fig. 1I,J,M-P; UV opsin not shown). The cell types detected with these probes are present in oko meduzy but are localized to abnormal positions (Fig. 1D,F,H,J,N,P). On the basis of these experiments, we conclude that the majority of cell types in the oko meduzy retina are specified but do not localize to their proper positions.

In the mutant retinas, some of the ectopically localized cells appear to be well differentiated. We evaluated the morphology of ectopic blue, red and rod opsin-expressing photoreceptor cells. On randomly oriented sections, approximately one fourth (27/105) of these cells are characterized by long axes twice the size of the short axes (Fig. 2, arrows in A and B). Such elongated appearance is characteristic of differentiated wild-type photoreceptor cells. The majority of them accumulate opsin mRNA asymmetrically, mostly in one pole of the cell (Fig. 2A,B). To further evaluate differentiation of ectopic cells in the mutant retina, we inserted Dil crystals into the optic tectum of wild-type and mutant individuals. In the wild type, application of Dil into the optic tectum results in a specific labeling of the ganglion cell layer (Fig. 1K). In the mutant, the Dil-labeled cells are not localized in a single layer but instead scatter throughout the retina (Fig. 1L). This experiment shows that at least some of the ectopic ganglion cells display a high degree of differentiation and send their axonal projections to the optic tectum. In addition to the position of neurons, oko meduzy may affect cell fate decisions during neurogenesis. On sections through the mutant retinae stained with an anti-GABA antibody, the number of GABA-positive cells appears to be consistently higher than in the wild type.

Although all analyzed cell types were disorganized, the degree of disorganization varied. For example, a significant number of the ganglion cells remained in the normal position next to the vitreal surface of the retina (Fig. 1D, 2D). In contrast, only a small fraction of the photoreceptor cells remained in contact with ventricular surface (Fig. 1J,N,P). Another interesting property of the ectopic ganglion cells in the mutant is their tendency to form aggregates, suggesting that they preferentially adhere to each other (arrows in Figs 1D, 2C,D). Axons of ectopic ganglion cells also stain with Zn-5 and sometimes are seen to form fascicles in abnormal locations. We have not observed fascicles exiting the eye in places other than the normal exit point of the optic nerve.

The oko meduzy embryos display defective circulation and abnormally curved body axis (Malicki et al., 1996). In addition, the lens of mutant embryos appears to be consistently smaller than in the wild type. Lens differentiation, however, appears to proceed normally. By histological criteria, the lens epithelium, cortex and nucleus appear normal at 3
To evaluate whether patterning defects are present outside the retina, in other parts of the central nervous system, we performed staining experiments with antibodies that label primary motor neurons (Zn-1; Eisen et al., 1990), primary sensory neurons (anti-HNK-1; Metcalfe et al., 1990) and reticulospinal neurons (anti-acetylated tubulin; Piperno and Fuller, 1985). These experiments did not reveal any obvious patterning defects (data not shown). Likewise, apart from a decreased volume of the ventricles, we did not see any obvious patterning abnormalities on histological sections through the brain (data not shown).

**oko meduzy specifically affects the retinal neuroepithelial sheet**

Before the appearance of postmitotic cells, the neural retina consists of a single neuroepithelial sheet. The apical (ventricular) surface of this sheet is in contact with the pigmented epithelium, whereas the basal (vitreal) surface is located on its outside rim, presumably the amacrine cells. (F) In *oko meduzy*, GABA-positive cells and plexiform patches are scattered throughout the retina. (G) An anti-carbonic anhydrase antibody recognizes the Muller glia. In wild-type retina, the cell bodies of these cells localize to the inner nuclear layer while their vitreal and ventricular processes span the entire thickness of the retina. (H) In *oko meduzy*, the cell bodies of carbonic anhydrase-positive cells are scattered. (I) In situ hybridization with the red opsin mRNA probe. Red light sensitive photoreceptor cells localize to the photoreceptor cell layer in wild-type retina. (J) In the *oko meduzy* mutant retina, almost all red opsin-positive cells are present in abnormal positions. The photoreceptor cell layer is absent. (K) In wild-type embryos, the DiI application to the optic tectum specifically labels the ganglion cell layer. (L) In the mutant, the DiI-labeled cells are scattered throughout the retina. (M) In situ hybridization with the blue opsin mRNA probe. Blue opsin-expressing cells localize to abnormal positions. (O) In situ hybridization with the rod opsin mRNA probe. Rods localize to the photoreceptor cell layer in wild-type retina. (P) In the *oko meduzy* mutant retina, almost all rod opsin-positive cells are present in abnormal positions. The photoreceptor cell layer is absent. (K,L) Confocal images (dorsal view) of whole eyes at 60 h.p.f.; (A-D,I-J,M-P) transverse sections through embryonic retinae at 3 d.p.f.; (E,F) sections of unoriented embryonic retinae at 5 d.p.f. Arrowheads and ‘le’ indicate the lens. Pigmentation of embryos shown in C,D,I-P was inhibited with 1-phenyl-2-thiourea (PTU). One scale bar is provided for each pair of panels comparing wild-type and mutant phenotypes. In all panels, scale bars equal 100 μm.
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**Fig. 2.** Details of the ganglion and the photoreceptor cell distribution in the *oko meduzy* retina. (A,B) Position of the photoreceptor cells at 72 h.p.f as visualized with the blue opsin (A) and the red opsin (B) in situ probes. The ectopic photoreceptors do not form rosettes and accumulate opsin mRNA in one pole of the cell. Some opsin-expressing cells (arrows) display elongated appearance characteristic of the differentiated photoreceptor cells. (C,D) Position of ganglion cells as visualized at 72 h.p.f. with Zn-5 antibody. The ectopic ganglion cells aggregate (arrows). All panels show transverse sections. Dorsal side is up and lens is to the left. Scale bars equal 25 μm in A and B; 50 μm in C and D.

**Fig. 3.** Localization of the apical markers in wild-type and *oko meduzy* retinal neuroepithelial sheet. (A) Section through wild-type retinal neuroepithelium stained with the DNA-binding dye, Hoechst 33258. Chromatin of the M-phase nuclei has granular appearance. In wild-type retinae, the M-phase nuclei are localized at the ventricular (apical) surface of the retinal neuroepithelium (red arrows). (B) Section through the *oko meduzy* retinal neuroepithelium stained with Hoechst 33258. The M-phase nuclei assume ectopic positions away from the ventricular surface (red arrows). (C) Section through the wild-type brain neuroepithelium stained with the DNA-binding dye, Hoechst 33258. The M-phase nuclei are present at the ventricular surface of the neuroepithelial sheet (red arrows). (D) Section through the *oko meduzy* brain neuroepithelium stained with Hoechst 33258. As in the wild type, the M-phase nuclei are present at the ventricular surface of the neuroepithelial sheet (red arrows). (E) Position of centrosomes visualized with an anti-gamma-tubulin antibody. Centrosomes are visible as small green dots. In wild-type retina, centrosomes are localized in a narrow region at the ventricular (apical) surface. (F) In the *oko meduzy* retinal neuroepithelium, centrosomes are localized in abnormal positions, away from the ventricular surface. (G) Percentages of ectopically localized M-phase nuclei in wild-type and *oko meduzy* eye neuroepithelium. At 30 and 38 h.p.f., respectively, 63% (110/175) and 84% (182/217) of the M-phase nuclei occupy ectopic positions (away from the ventricular surface) in the mutant. Ectopic mitoses in the wild type are infrequent: 2% (3/131) and 4% (6/149) at 30 and 38 h.p.f., respectively. (H) Percentages of ectopically localized nuclei in wild-type and *oko meduzy* brain neuroepithelium. Both in the wild type and in the mutant, the M-phase nuclei are localized at the ventricular surface. At 30 h.p.f., 2% (4/267) and 3% (5/198) of the M-phase nuclei were localized away from the ventricular surface in the wild type and mutant, respectively. In E and F, ‘le’ indicates the lens. Arrowheads in A through F indicate the ventricular (apical) surface of the eye (A,B,E,F) or the brain (C,D) neuroepithelia. Arrows in E and F indicate the ventricular surface of the brain neuroepithelium. Red arrows in A through D indicate M-phase nuclei. In G and H, the vertical axis indicates percentages of the ectopic M-phase nuclei. A through D show transverse sections; E and F unoriented sections. Sections were prepared at 36 h.p.f. One scale bar is provided for each pair of panels comparing wild-type and mutant phenotypes. Scale bars equal 25 μm in A, 15 μm in C, 50 μm in E.
directed towards the lens. Like all epithelia, the pseudostratified epithelium of the developing central nervous system displays distinct polarity evident in the arrangement of organelles and cytoskeletal elements. For example, in polarized epithelial cells, the centrosomes are localized near the apical pole, the Golgi apparatus is positioned basal to the centrosomes but apical to the nucleus, the microtubules extend along the long cell axis and have their plus ends directed towards the basal surface (Cajal, 1914; Hinds and Ruffett, 1971; Bacallao et al., 1989; Gilbert et al., 1991). The apical pole is also marked by junctional complexes, junction-associated actin microfilaments, microvilli and cilia (Farquhar and Palade, 1963; Hinds and Ruffett, 1971; Geiger et al., 1983; Svoboda and O’Shea, 1987). The neuroepithelial cells display characteristic mitotic behavior. Throughout most of the cell cycle, they have elongated shape and contact both the basal and the apical surface. M-phase cells, however, lose their basal process and round up at the apical surface, leading to the

**Fig. 4.** Position of adherens junctions in wild-type and mutant neuroepithelia. (A) Actin distribution visualized with fluorophore-conjugated phalloidin. In wild-type retinal neuroepithelium, actin microfilaments are distributed throughout the entire cytoplasm. Dense foci (arrow) are associated with the ventricular (apical) cell termini (arrowheads). (B) Actin foci (arrow) localize away from the ventricular surface (arrowheads) in oko meduzy. (C) Higher magnification of apical actin foci (arrows) in wild-type retinal neuroepithelial sheet. (D) In oko meduzy, actin foci (arrows) localize away from the ventricular surface (arrowheads). (E,F) In the brain neuroepithelium of both the wild type and the mutant, actin foci (arrows) localize to the ventricular surface (arrowheads). (G) Electron micrograph of the wild-type eye neuroepithelium. The termini of the retinal neuroepithelial cells adjacent to the pigmented epithelial cell (pe) are connected by cell junctions (arrows). Arrowhead indicates a pigment granule. (H) Electron micrograph of the oko meduzy retinal neuroepithelium. The cell junctions indicated with arrows are positioned at least two nuclear diameters from the ventricular surface. (I) Electron micrograph of the wild-type brain neuroepithelium. Cell junctions (arrows) are present at the ventricular surface. (J) Electron micrograph of the oko meduzy brain neuroepithelium. Cell junctions (arrows) are present at the ventricular surface. Ectopic cell junctions are not present. Arrowheads in A through F indicate the ventricular surface. All tissue samples were collected at 30 h.p.f. One scale bar is provided for each pair of panels comparing wild-type and mutant phenotypes. Scale bar equals 25 μm in A, 10 μm in C, 25 μm in E, 1 μm in G and I.
characteristic apical localization of the M-phase nuclei (Sauer, 1935).

To determine whether the retinal neuroepithelial cells are affected in the *oko meduzy* mutant animals, we investigated localization of apical markers in the early retinae. To localize the M-phase nuclei in the neuroepithelial sheet, we stained sections of the mutant retinae with the DNA-binding dye Hoechst 33258. Hoechst staining allows one to distinguish the M-phase nuclei because their chromatin is condensed and has a granular appearance. As expected in the wild-type retina, almost all M-phase nuclei occupy ventricular positions (Fig. 3A, red arrows) whereas, in the mutant, the majority of M-phase nuclei occupy ectopic positions, away from the ventricular surface (Fig. 3B). At 30 h.p.f., only 2% (3/131) of the M-phase nuclei are positioned away from the ventricular surface in the wild-type retina. In contrast, 63% (110/175) of the dividing nuclei are localized away from the ventricular surface in the mutant (Fig. 3G). Similarly, at 38 h.p.f, only 4% (6/149) of nuclei are away from the ventricular surface in the wild type, and the majority, 84% (182/217), in the mutant (Fig. 3G). In these experiments, all M-phase nuclei positioned more than one nuclear diameter away from the ventricular surface were considered ectopic.

To further evaluate the nature of the neuroepithelial defect, we determined position of centrosomes in this tissue using an antibody to gamma-tubulin (gift from Pavel Draber). Frozen sections were prepared from wild-type and mutant retinae, stained with anti-gamma-tubulin antibody and analyzed under a confocal microscope. Confocal images were collected from multiple planes of focus and projected on a single plane. All centrosomes positioned away from the ventricular surface at a distance exceeding 25% of the total thickness of the neuroepithelial sheet were considered ectopic. In wild-type animals, less than 1% of centrosomes (>1,000) of the retinal neuroepithelial cells are localized away from the ventricular surface (Fig. 3E). On the contrary, in *oko meduzy* more than 80% (>1,000) of centrosomes are positioned away from the ventricular surface (Fig. 3F). These observations are based on analysis of multiple sections from 20 wild-type and 20 mutant retinae. Centrosomes of the retinal margin were not taken into account. Interestingly, the ectopic centrosomes are often, but not always, localized in one region, approximately one third of the way across the thickness of the neuroepithelium towards the basal surface. These results show that the *oko meduzy* defect precedes neurogenesis and already affects the neuroepithelial cells.

We decided to investigate whether a similar phenotype is present in other *oko meduzy* CNS neuroepithelia. Analysis of sections through the forebrain, midbrain and hindbrain indicated that in the mutant, M-phase nuclei occupy ventricular positions (arrows in Fig. 3D). For example, at 30 h.p.f. both in the wild-type and the mutant brain almost all M-phase nuclei localize to the ventricular surface. Only 2% (4/257) and 3% (5/198) of the M-phase nuclei appear to localize away from the ventricular surface in the wild-type and mutant brain, respectively (Fig. 3H). Likewise, the centrosomes of the mutant brain neuroepithelium are positioned in the wild-type, ventricular positions (arrow in Fig. 3F). These observations indicate that development of the eye and brain neuroepithelium is at least partially controlled by different genetic mechanisms.

Another indicator of normal morphology of the neuroepithelial cells is the position of adherens junctions. Adherens junctions are associated with bundles of actin microfilaments (Geiger et al., 1983; Volberg et al., 1986). To detect actin microfilaments within the neuroepithelial cells, we stained frozen sections of wild-type and mutant embryos with fluorescent-conjugated phalloidin. Staining was analyzed by confocal microscopy. In wild-type neuroepithelial cells, actin microfilaments, although present throughout the cytoplasm, form distinct foci at the apical surface (Fig. 4A,C,E). These foci most likely correspond to the adherens junctions. They are either very infrequent or entirely absent in the basolateral region of the neuroepithelial cells. In the *oko meduzy* retina neuroepithelium, very few actin foci are present at the ventricular surface. Instead, the vast majority of them are found ectopically (arrows in Fig. 4B,D). In contrast to the retina, in the *oko meduzy* brain neuroepithelium, actin foci are distributed in the same way as in the wild type (arrow in Fig. 4F). These observations suggest that the adherens junctions are specifically affected in the *oko meduzy* retinal neuroepithelium. We confirmed these results by electron microscopy. On electron micrographs, the junctional complexes appear as membrane-associated densities located close to the ventricular lumen of the retina and brain (Hinds and Ruffett, 1971; Hinds and Hinds, 1974; arrows in Fig. 4G,I). In *oko meduzy*, the junctional complexes of the retina are seldom localized in their normal ventricular position. Instead, clusters of junctional complexes are localized ectopically (arrows in Fig. 4H). Within these ectopic clusters, cell junctions appear to be randomly oriented. The junctional complexes of the brain neuroepithelium are somewhat more extensive than in the wild type but do not occur in ectopic locations (arrows in Fig. 4J).

These results provide further evidence that the *oko meduzy* phenotype is largely specific to the retinal neuroepithelial sheet.

**Cell-cell interactions are involved in elaboration of normal morphology and alignment of the retinal neuroepithelial cells**

Our observations that the retinal neuroepithelium is affected in *oko meduzy* suggest that this mutation affects morphology of the neuroepithelial cells. In wild-type neuroepithelium, throughout the cell cycle, cells remain attached to each other at the ventricular surface through junctional complexes (Hinds and Ruffett, 1971; Hollenberg and Spira, 1973; Hinds and Hinds, 1974). Presence of ectopic cell divisions in the mutant neuroepithelium suggests that the *oko meduzy* neuroepithelial cells may not connect to the ventricular surface in the normal way. To evaluate morphology of individual neuroepithelial cells, we chose to generate mosaic animals. This approach allows one to inspect the morphology of small cell clones and, in parallel, to ask whether any detected mutant phenotype is cell-autonomous. Blastomere transplantations are the method of choice in making zebrafish mosaics (Ho and Kane, 1990). In our experiments, blastomeres labeled with a mix of biotin- and Texas Red-conjugated dextrans were transplanted into unlabelled hosts. A fraction of transplanted blastomeres contributed to eye neuroepithelial cells. Animals containing donor-derived cells in the eye neuroepithelium were collected at 30-36 h.p.f. After fixation, the donor-derived cells were visualized by staining with avidin-HRP complex. Stained embryos were embedded in plastic and sectioned. HRP-labeled
neuroepithelial cells formed small clones surrounded by unlabelled host cells. By analyzing individual clones on consecutive sections, we determined positions of their ventricular termini. The progeny of an ome+/ × ome+ cross were used as both hosts and donors. 25% of host and donor animals were mutant homozygotes in such a cross and displayed the oko meduzy phenotype. Four types of mosaic animals were generated in this experiment: wild-type hosts containing wild-type donor-derived cells, mutant hosts containing mutant donor-derived cells, mutant hosts containing wild-type donor-derived cells and wild-type hosts containing mutant donor-derived cells.

As expected, wild-type clones in wild-type hosts form contact with the ventricular (apical) surface (59 out of 59 clones analyzed; Fig. 5A). In contrast, mutant clones in mutant hosts frequently do not extend to the ventricular surface (8 out of 35 clones analyzed, Fig. 5B). Since host and donor populations contained both wild-type and mutant embryos, this experiment also allowed us to ask whether the loss of ventricular contact was cell-autonomous. Genotypically mutant cells transplanted into wild-type hosts display the wild-type phenotype and always form contact with the ventricular surface (40/40; Fig. 5D). Genotypically wild-type cells transplanted into mutant hosts behave like mutant cells and frequently do not form contact with the ventricular surface (34/79; Fig. 5C). These results show that a substantial fraction of cells in the oko meduzy eye neuroepithelium display abnormal morphology and do not align their termini at the ventricular surface. Furthermore, in mosaic animals this phenotype depends on the genotype of the host but not the donor, indicating cell-nonautonomy. Cell-nonautonomous behavior of the neuroepithelial morphology reveals that cell-cell interactions participate in formation or maintenance of apical integrity in the retinal neuroepithelial sheet.

It is important to note that the basal processes of the neuroepithelial cells are not affected in the oko meduzy animals. In wild-type hosts, 93 out 99 cell clones analyzed (94%) formed contact with the basal surface. Similarly, in mutant hosts, 66 out of 72 clones analyzed (92%) formed contact with the basal surface. It is not surprising that the basal processes are absent in a small fraction of clones because the neuroepithelial cells lose contact with the basal surface during mitosis. Thus the oko meduzy mutations affect the ventricular but not the vitreal surface of the neuroepithelial sheet.

We decided to determine whether the cell-cell interactions identified in the previous experiments also affect the position of centrosomes. To address this question, we generated mosaic animals as described above. After transplantation, embryos were collected at 30-36 h.p.f. and cryosectioned. The donor-derived cells were localized using fluorophore-conjugated avidin (red signal in Fig. 6). Morphology of individual cell clones was further reconstructed with help of confocal microscopy. In addition to morphology, position of centrosomes in the neuroepithelial cells was determined. To locate centrosomes, sections were stained with an anti-gamma-tubulin primary antibody and fluorophore-conjugated secondary antibody (green signal in Fig. 6). As expected in the wild-type environment, the centrosomes of wild-type cells localize centrosomes, sections were stained with an anti-gamma-tubulin primary antibody and fluorophore-conjugated secondary antibody (green signal in Fig. 6). As expected in the wild-type environment, the centrosomes of wild-type cells always localize to the ventricular surface (17 out of 17 clones analyzed; Fig. 6A, ventricular surface indicated with arrowheads). As in the previous experiment, in the mutant environment, mutant cells frequently do not extend to the ventricular surface and their centrosomes are localized ectopically (24 out of 24 clones analyzed contained at least some ectopic centrosomes; Fig. 6B). Mutant clones in the wild-type environment behave in a cell-nonautonomous fashion and, in the majority of cases (20 out of 21 clones analyzed), position all their centrosomes at the ventricular surface (Fig. 6D-F). Wild-type clones in the mutant environment behave mostly like mutant clones and position at least a fraction of their centrosomes away from the ventricular surface (23 out of 26 clones analyzed; Fig. 6C). Again in these experiments, the basal processes of the neuroepithelial cells were unaffected. Cell clones were classified as phenotypically wild-type only if the distance of all their centrosomes from the ventricular surface was smaller than 25% of the total thickness of the neuroepithelial sheet. Clones containing one or more centrosomes that did not fulfill this criterion were

Fig. 5. Morphology of the neuroepithelial cells in mosaic animals. Parasagittal sections through retinae at approximately 36 h.p.f. Mosaic neuroepithelia contain cells originating from two embryos, donor and host. The majority of cells are unlabelled and originate from hosts. The relatively infrequent, HRP-labeled cells are donor-derived. Owing to their dark labeling, shape of the donor-derived cells can be reconstructed on consecutive sections. (A-D) Sections originating from such reconstructions. (A) Wild-type, donor-derived cell clones in the wild-type environment remain in contact with the ventricular (apical) surface. (59 out of 59 clones in 10 eyes from 6 embryos.) (B) In the mutant environment, mutant donor-derived cell clones frequently do not contact the ventricular surface (arrow). (8 out of 35 clones in 4 eyes from 3 embryos.) (C) In the mutant environment, wild-type donor-derived cell clones frequently display the mutant phenotype and do not extend to the ventricular surface (40 out of 40 clones in 7 eyes from 4 embryos.) Some cells are not entirely in the plane of section. They were shown to contact the ventricular surface on adjacent sections. In all panels, arrowheads indicate the choroid fissure, 'le' indicates the lens. Scale bar equals 50 μm. To reduce pigmentation, in some experiments, we used hypopigmented golden animals (D).
Neuronal patterning in the \textit{oko meduzy} retina

classified as phenotypically mutant. All clones were analyzed on multiple optical sections.

This experiment confirmed our previous observations that the \textit{oko meduzy} neuroepithelial cells frequently do not extend to the ventricular surface and that this defect is rescued by interactions with wild-type cells. These cell-cell interactions are sufficient to rescue both the overall morphology of the mutant neuroepithelial cells and the position of their centrosomes. We conclude that due to defective cell-cell interactions in the mutant neuroepithelium, the ventricular termini of the neuroepithelial cells do not align at the ventricular surface but instead terminate in varying positions such that some cells do not form contact with the ventricle. This phenotype does not affect the basal (vitreal) surface of the neuroepithelium where the processes of wild-type and mutant cells behave in the same way.

Which cells rescue the phenotype of genotypically mutant cells in the wild-type environment? The two most obvious possibilities are that the rescue is provided either by the pigmented epithelium (PE) or by the neighboring neuroepithelial cells. The role of the pigmented epithelium in early development of the neural retina has been suggested by tissue reaggregation and cell ablation experiments (Vollmer and Layer, 1986; Raymond and Jackson, 1995). In \textit{oko meduzy}, the pigmented epithelium is defective. The pigmented epithelial cells are no longer recognizable in the central retina by their pigmentation or morphology (Fig. 1B). To test the possibility that the pigmented epithelial cells are responsible for the rescue, we constructed genetically mosaic animals containing wild-type donor-derived pigmented epithelial cells next to mutant host retinal neuroepithelium. The pigmented epithelial cells scored in these experiments were well differentiated as evidenced by their flat morphology and pigmentation granules. Such clones (n=6) did not have any significant effect on the position of centrosomes in the adjacent retinal neuroepithelium (Fig. 6G,H). These results argue that the PE does not play a role in the rescue of the \textit{oko meduzy} phenotype. We cannot, however, entirely exclude the possibility that the wild-type PE cells did not have any effect due to small clone size or movements between the neuroepithelial and the pigmented epithelial sheets. Thus further studies will be required to confirm this conclusion.

DISCUSSION

We have found that during the retinal neurogenesis in the zebrafish mutant \textit{oko meduzy}, the majority of cell types are specified but fail to localize to their normal positions. In the period preceding neurogenesis, the retinal neuroepithelial cells of \textit{oko meduzy} fail to assume the normal morphology and do not align their termini at the ventricular surface. Cell-cell interactions...

\textbf{Fig. 6.} Morphology of the neuroepithelial cells and location of their centrosomes in mosaic animals at approximately 36 h.p.f. Donor-derived cell clones are labeled with Texas Red-conjugated avidin (red). Position of centrosomes is revealed by an anti-gamma-tubulin antibody and a FITC-conjugated secondary antibody (green). Data obtained by confocal sectioning of frozen sections. Each image is a sum of multiple confocal sections. (A) In the wild-type environment, the centrosomes of wild-type cell clones localize to the ventricular (apical) surface. (17 out of 17 clones from 17 embryos.) (B) In the mutant environment, the centrosomes of the \textit{oko meduzy} mutant cell clones are localized ectopically. (24 out of 24 clones from 5 embryos.) (C) In the mutant environment, wild-type cell clones display the mutant phenotype. These cells frequently do not extend to the ventricular surface and at least some of their centrosomes are localized ectopically. (23 out of 26 clones from 16 embryos.) (D-F) In the wild-type environment, mutant cell clones localize their centrosomes in the wild-type ventricular positions. (20 out of 21 clones from 11 embryos.) (G-H) Wild-type pigmented epithelial cells (arrows) in the mutant retina do not have a significant impact on the position of centrosomes in the neighboring retinal neuroepithelial sheet. In all panels, arrowheads indicate the ventricular surface. Arrows indicate the basal termini of reconstructed clones. Scale bar equals 25 \mu m.
interactions participate in development of the proper alignment and morphology of the retinal neuroepithelial cells.

**oko meduzy reveals cell-cell interactions in the developing eye**

The neuroepithelial phenotype of *oko meduzy* is cell-nonautonomous indicating defective cell-cell interactions. In mosaic animals, the phenotype of mutant neuroepithelial clones is rescued by interactions with the surrounding wild-type cells. Which cells participate in this rescue? One of two scenarios is most likely to account for the rescue of the neuroepithelial defect. The first possibility is that *oko meduzy* affects cell-cell interactions within the neuroepithelial sheet. Such interactions are documented in other epithelia and may be mediated via cell junctions (Drubin and Nelson, 1996). The *oko meduzy* defect may, for example, result in mispositioning of cell-cell signaling within the neuroepithelial sheet. A signal that participates in assembly of adherens junctions could be delocalized in the mutant neuroepithelium resulting in displacement of the apical structures such as centrosomes.

Alternatively, *oko meduzy* disrupts cell-cell interactions between the neuroepithelial and the pigmented epithelial sheets. In the vertebrate eye, the ventricular surface of the retinal neuroepithelium is closely apposed by the pigmented epithelium. This possibility was tested by constructing genetically mosaic animals containing wild-type pigmented epithelial cells in otherwise mostly mutant eyes. In these experiments, wild-type PE clones did not have impact on the phenotype of the adjacent mutant neuroepithelial cells. This result can be explained in more than one way. The pigmented epithelium may not play a role in the rescue of the *ome* phenotype. Alternatively, we may not have observed any effect of wild-type PE cells because the pigmented epithelium and the neural retina are moving with respect to each other. Such movements have been suggested to take place in higher vertebrates (Zinn and Marmor, 1979). In our experiments, the wild-type PE clones may thus have moved away from cells that they influenced earlier in development. Further work will be necessary to determine whether *oko meduzy* acts in the pigmented epithelium to contribute to the correct neuroepithelial phenotype.

**Relationship between the neuroepithelial and the neuronal patterning phenotypes in *oko meduzy***

Early in development, the neural retina consists of a single neuroepithelial cell layer. In *oko meduzy*, the neuroepithelial cells are characterized by defective alignment and morphology. Later in development, after the onset of neurogenesis, the *oko meduzy* retinal neurons are severely disorganized. How are these two phenotypes related? One possibility is that the *oko meduzy* locus functions independently in these two aspects of retinal development. Alternatively, the later, neuronal patterning, phenotype could result from the earlier, neuroepithelial, defect.

The possibility that the neuronal patterning phenotype is at least partially caused by an earlier neuroepithelial defect is attractive. In the course of neurogenesis, the neuronal progenitors are born at the ventricular surface and migrate to their proper destinations forming distinct layers (Hinds and Hinds, 1974, 1979). Lineage experiments have left little doubt that the majority of retinal neurons are specified after the last mitotic division (Turner and Cepko, 1987; Holt et al., 1988; Wets and Fraser, 1988). At least in the case of some cell types, specification may occur within minutes after the last mitotic division (Waid and McLoon, 1995). Migration of newly generated neurons must take place in the context of positional cues, which either direct already specified neurons to their proper destinations or, less likely, instruct neuronal progenitors to assume specific fates. The nature of these cues and how they are deposited remains unknown. In one possible scenario, correct development of the retinal neuroepithelial cells and, more specifically, correct elaboration of their apical and basolateral domains may be required for the proper distribution of the positional cues. If this is the case, a neuroepithelial defect, such as in *oko meduzy*, would be expected to result in abnormal patterning of neurons.

**The role of *oko meduzy* in brain neuronal patterning**

The retina and the cortical regions of the vertebrate brain display many similarities. All originate from the pseudostratified epithelium of the neural tube. At early stages of development, in both the retina and brain, the mitotically active cells remain in contact with the ventricular surface of the neuroepithelial sheet, whereas the majority of the postmitotic cells migrate away from the ventricular to the pial surface of the neural tube. In their mature form, the retinal and the cortical neurons are organized into laminae (Cajal, 1893; Rodieck, 1973; Dowling, 1987; Jacobson, 1991). Besides these obvious similarities, the retinal and the cortical neurogeneses display important differences. The inside-out pattern of neurogenesis is not observed in the retina. In vertebrate species studied so far, the earliest born retinal neurons, the ganglion cells, occupy the outermost (by analogy to the cortex, the innermost in the retinal nomenclature) layer of the retina (Alshuler et al., 1991). Another key difference between the cortex and the retina is the role of glia. The Muller glia of the retina become postmitotic and differentiate late in neurogenesis (Young, 1985; Cepko et al., 1996) and thus are unlikely to play a role in the migration of most neuronal types. In the recent years, several genes with important roles in patterning of the cortical neurogeneses have been identified: *reelin, cdk5, mdab1* (D’Arcangelo et al., 1995; Ohshima et al., 1996; Chae et al., 1997; Howell et al., 1997; Sheldon et al., 1997). Although they play prominent roles in brain development, none of them is required in retinal lamination, indicating that different mechanisms control organization of neurons in the retina and brain. The *oko meduzy* phenotype reveals yet another difference between development of these two organs. *oko meduzy* affects the eye but not the brain neuroepithelium, showing that genetic mechanisms that control development of brain and eye neuroepithelia are also different. On the level of genetic circuitry, the *oko meduzy* phenotype may not affect the brain neuroepithelium for one of two reasons: because the *ome* locus does not have a function there or because its function is obscured by another, partly redundant, locus. *oko meduzy* and other patterning mutants of the zebrafish retina (Malicki et al., 1996) may reveal novel aspects of neuronal development in the vertebrate CNS. Further studies of these loci, including thorough molecular analysis, will expose new components of the undoubtedly complex genetic machinery regulating organization of neurons in the vertebrate central nervous system.
We thank Connie Cepko, John Dowling, Eric Weinberg, Geoffrey Doerre, Aletta Schnitzer, Ellen Schmitt, Lila Solnica-Krezel and Eliza Mountcastle-Shah for comments on earlier versions of this manuscript and numerous discussions. We are also grateful to John Dowling for providing us with the zebrasfish opsin probes, Michele Harvanck for assistance in transplantation experiments, Paul Linser for the anti-carbonic anhydrase antibody, and Pavel Draber for the anti-gamma-tubulin antibody. This work was supported by NIH grants RO1 MH56552 and RO1 HD29761 to W. D. and a sponsored research agreement with Bristol-Myers Squibb. J. M. was supported by a fellowship from the Damon Runyon-Walter Winchell Cancer Research Fund.

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