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Retinal engineering: Engrafted neural cell lines locate in appropriate layers

(central nervous system/transplantation/cell migration and differentiation/cell recognition/pattern formation)

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ABSTRACT **A major question in central nervous system development, including the neuroretina, is whether migrating cells express cues to find their way and settle at specific locations. We have transplanted quail neuroretinal cell lines QNR/D, a putative amacrine or ganglion cell, and QNR/K2, a putative Müller cell into chicken embryo eyes. Implanted QNR/D cells migrate only to the retinal ganglion and amacrine cell layers and project neurites in the plane of retina; in contrast, QNR/K2 cells migrate through the ganglion and amacrine layers, locate in the inner nuclear layer, and project processes across the retina. These data show that QNR/D and QNR/K2 cell lines represent distinct neural cell types, suggesting that migrating neural cells express distinct address cues. Furthermore, our results raise the possibility that immortalized cell lines can be used for replacement of specific cell types and for the transport of genes to given locations in neuroretina.**

The development of the central nervous system (CNS) is a complex process made up of distinct, but overlapping, stages of cell proliferation, migration, and differentiation. An important question is whether migrating precursor or determined neural cells express cues to find their way and settle at a specific location. The neuroretina is a convenient paradigm to approach this question, because it is an evagination of the CNS, readily accessible in the avian embryo. Previously, we derived from embryonic day (E) 7 quail neuroretina cultures transformed by Rous sarcoma virus (1) permanent cell lines that display distinct phenotypes (2, 3), suggesting that they may represent different progenitors or committed cell types. In particular, clone QNR/D, a putative candidate for amacrine or ganglion cells, binds ganglion cell and amacrine cell-specific mAb 94C2 (2) and expresses preproenkephalin mRNA (4). Enkephalin is restricted to a subset of ganglion cells (5) and to a subset of amacrine cells (6) in avian retina. In contrast, QNR/K2 cells bind mAbs 96B11 (7) and 105E2 (D.T., unpublished data), which are Müller cell-specific in chicken and quail retinal sections (D.T. and B.P., data not shown). Here we engraft these cells into embryonic chicken retina and find that the distinct cell lines migrate to their appropriate locations and differentiate according to their phenotypes predicted from *in vitro* molecular markers. These data suggest that QNR/D cells and QNR/K2 cells express distinct address codes that direct them to locate in the appropriate stratum, indicating that these committed cells know who they are and therefore where to localize.

MATERIALS AND METHODS

Cultures of QNR/D and QNR/K2 cells were dissociated in 0.075% crystalline trypsin (Cooper Biomedical) in Dulbecco's phosphate-buffered saline (PBS), washed with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum ($DMEM_{10}$), and labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ['DiI'/$DiIC_{18}$(3)] (8) as follows. QNR/D (2×10^6) and QNR/K2 (2×10^6) cells were incubated in parallel for 15 min at 21°C in 1 ml of $DMEM_{10}$ containing 2% of a freshly prepared stock solution of 5 mg/ml DiI in absolute ethanol. Cells were resuspended every 5 min during the incubation by agitation. They then were washed with 15 ml $DMEM_{10}$ three times and resuspended in 1 ml $DMEM_{10}$. DiI-labeled cells (5×10^4) in 25 μl $DMEM_{10}$ were injected intraocularly into the vitreous of E9 chicken embryos as described (9) for introducing mouse hybridoma cells into embryonic chicken eyes. The vitreal chamber provides the only space in the eye with a capacity to accept a volume of injected cells. Injected embryos were incubated at 38°C for 3 and 6 days to E12 and E15, the eyes removed, the cornea, lens, sclera, and pigmented epithelium were dissected away, and the neural retina and vitreous were fixed in 4% paraformaldehyde in PBS at 4°C. The paraformaldehyde then was exchanged with PBS and the retinas removed from the vitreal bodies, cut into flower-shaped flat whole mounts, examined by Zeiss fluorescence microscopy under rhodamine optics, and photographed. Laser confocal images of implanted cells were generated with a Zeiss Axiovert microscope. Regions of the retina containing DiI-labeled QNR cell implants were selected, equilibrated in 20% sucrose, frozen, and cut into 16 μm thick cross-sections with a cryostat. Some retinal sections were stained with 0.5 ng/ml 4′,6-diamidino-2-phenylindole

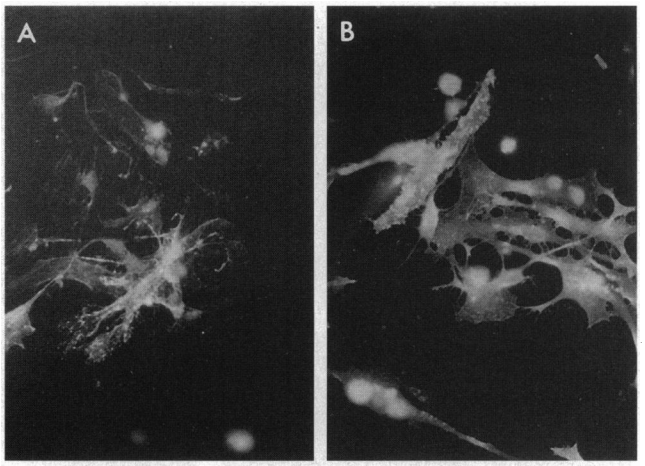

FIG. 1. Immunofluorescence images of Rous sarcoma virus transformed quail neuroretina clonal cell lines QNR/D (*A*) and QNR/K2 (*B*) stained *in vitro* with mAbs 94C2 and 96B11, respectively, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse $F(ab')_2$.

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Abbreviations: QNR/D, quail neuroretina clone D; QNR/K2, quail neuroretina clone K2; CNS, central nervous system; E, embryonic day.

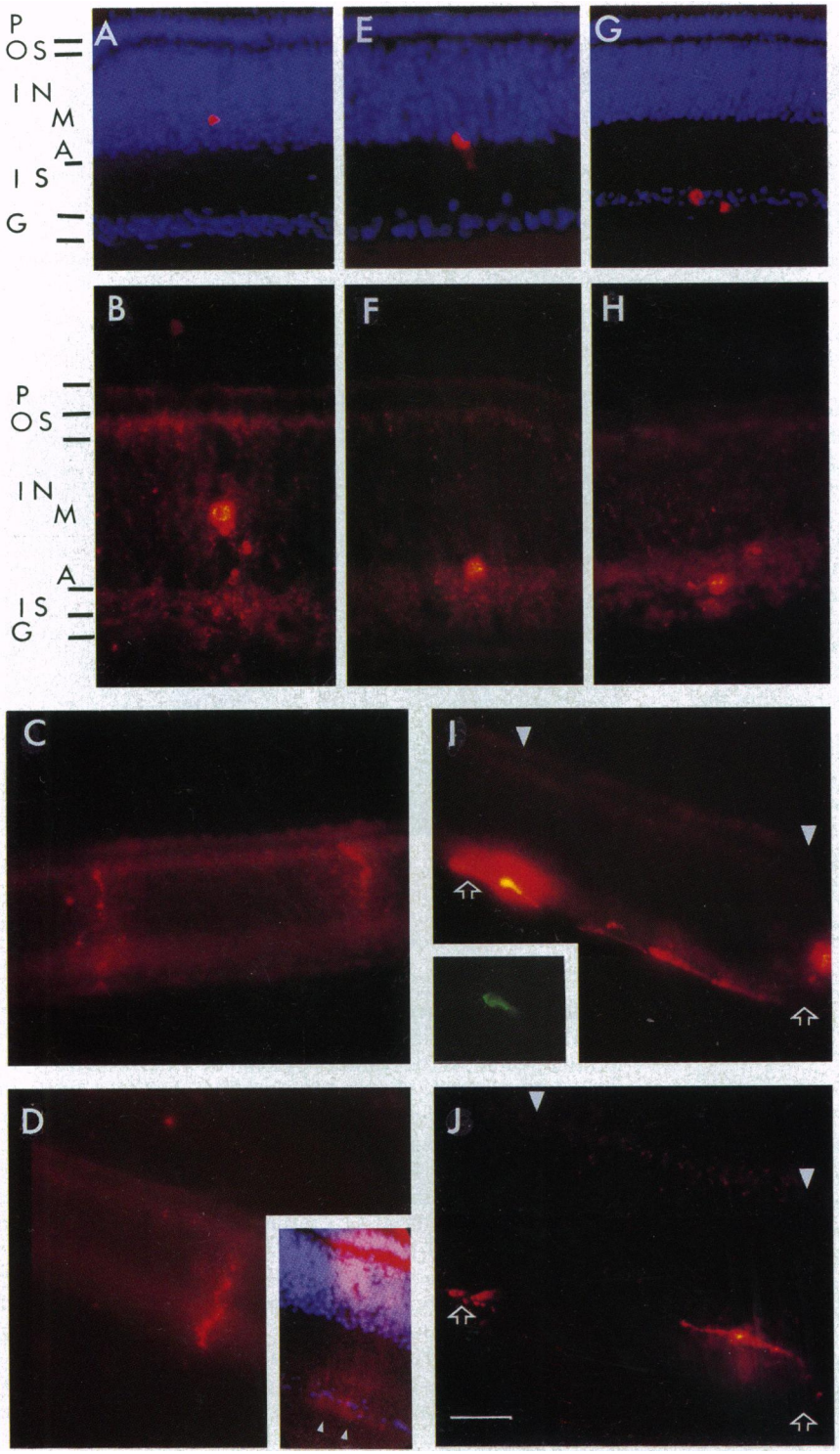

FIG. 2. Implantation of DiI-labeled QNR/K2 and QNR/D to specific cell strata in the developing embryonic chicken retina after inoculation at E9. QNR/K2 cells migrated to the middle of the inner nuclear layer by E12 (*A* and *B*) and projected processes across the retina by E15 (*C* and *D*). (*D Inset*) DiI-labeled QNR/K2 cells 11 days after implantation extended from the vitreal margin (arrowheads) to the outer margin of retina. (*E–H*) QNR/D cells migrated to the amacrine and ganglion cell layers by El2 and (*I* and *J*) extended neurites along the plane of retina by El5. P, photoreceptor layer; OS, outer synaptic layer; IN, inner nuclear layer (containing horizontal and bipolar cell layers in the outer portion of the IN); M, Müller cell layer in middle of IN; A, amacrine cell layer at inner portion of IN); IS, inner synaptic layer; G, ganglion cell layer. (*A*, *E*, *D Inset*, and *G*) Retinas stained with DAPI exhibit blue cell nuclei with red DiI-labeled implanted QNR cells. (*I Inset*) FITC-conjugated goat anti-mouse $F(ab')_2$ binding to chicken anti-quail antibody on implanted DiI-labeled QNR/D cell. Solid arrowheads mark the photoreceptor margin of retina cross-sections; Open arrows mark ganglion cell margin of retina. (*A*, *E*, and *G*, bar = 60 μm; *B*, *F*, and *H*, bar = 40 μm; *C*, *D*, *I*, and *J*, bar = 75 μm.)

(DAPI) in PBS for 15 min at room temperature and washed three times with PBS. The retina sections were examined as above and photographed.

Retinas with implanted DiI-labeled QNR/K2 cells were fixed with 4% paraformaldehyde and prepared for electron microscopy by standard methodology with osmium tetroxide and epon after photoconversion of the DiI to a diaminobenzidine product (10).

RESULTS AND DISCUSSION

QNR/D and QNR/K2 cells, which express the ganglion and amacrine cell marker 94C2 and the Müller cell marker 96B11, respectively, *in vitro* (Fig. 1), were labeled with the fluorescent dye DiI (7) and transplanted to embryonic chicken retinas by intraocular injection (9) into the vitreous. Injections were made in E9 chickens—a stage of active histogenesis in retina when central retina is differentiating to form the early inner synaptic layer and ganglion cells are maturing, but much of the retina toward the periphery remains an undifferentiated stratified epithelium. In cross-sections of the eye made 3 days after engraftment, QNR/D and QNR/K2 cells were seen in the vitreous, although the majority of QNR cells were present in the neuroretina. That DiI-labeled QNR/D and QNR/K2 cells in chimeric retinas were indeed quail cells was clearly shown by their labeling

Table 1. Number of QNR cells engrafted in specific retinal strata

Cell line	Chicken age	Amacrine/ ganglion*	Müller†	Other‡
QNR/D	E12	125	1	12
	E15	296	0	5
QNR/K2	E12	4	135	10
	E15	0	250	2

DiI-labeled QNR cells were injected intraocularly into chicken embryos at E9 and retinas were taken at E12 and E15. Individual DiI-labeled engrafted cells were scored on retinal cross-sections from 45 injected eyes.

*Ganglion cell layer and the inner portion of the inner nuclear layer containing amacrine soma.

†Middle portion of the inner nuclear layer containing the nuclei of Müller and bipolar cells.

‡Other layers that contained QNR/D cells included the photoreceptor, horizontal, and bipolar cell layers and those containing QNR/K2 cells included the photoreceptor and ganglion cell layers.

with the chicken antiserum from Lance-Jones and Lagenaur (11) (Fig. 2). The striking finding was that the vast majority of QNR/D and QNR/K2 cells were localized in their respective cell strata in the retina (Table 1). Indeed, QNR/D cells were found only in the ganglion and amacrine cell layers and QNR/K2 cells were found in the middle of the inner nuclear layer where Müller cell nuclei reside (Fig. 2). A small fraction of QNR/D cells were found in the photoreceptor, horizontal, and bipolar cell layers, whereas some QNR/K2 cells were present in photoreceptor and ganglion cell layers. At this stage (3 days after injection), most quail cells were spherical or had only short processes. When E15 retina sections were observed 6 days after injection, the engrafted cells had remained in their distinct stratum and at that time 98% of QNR/D and QNR/K2 cells were at their proper location, i.e., ganglion and amacrine cell layers and inner nuclear layer, respectively. Furthermore, QNR cells had elaborated complex processes (Figs. 2–4). In retinal cross-

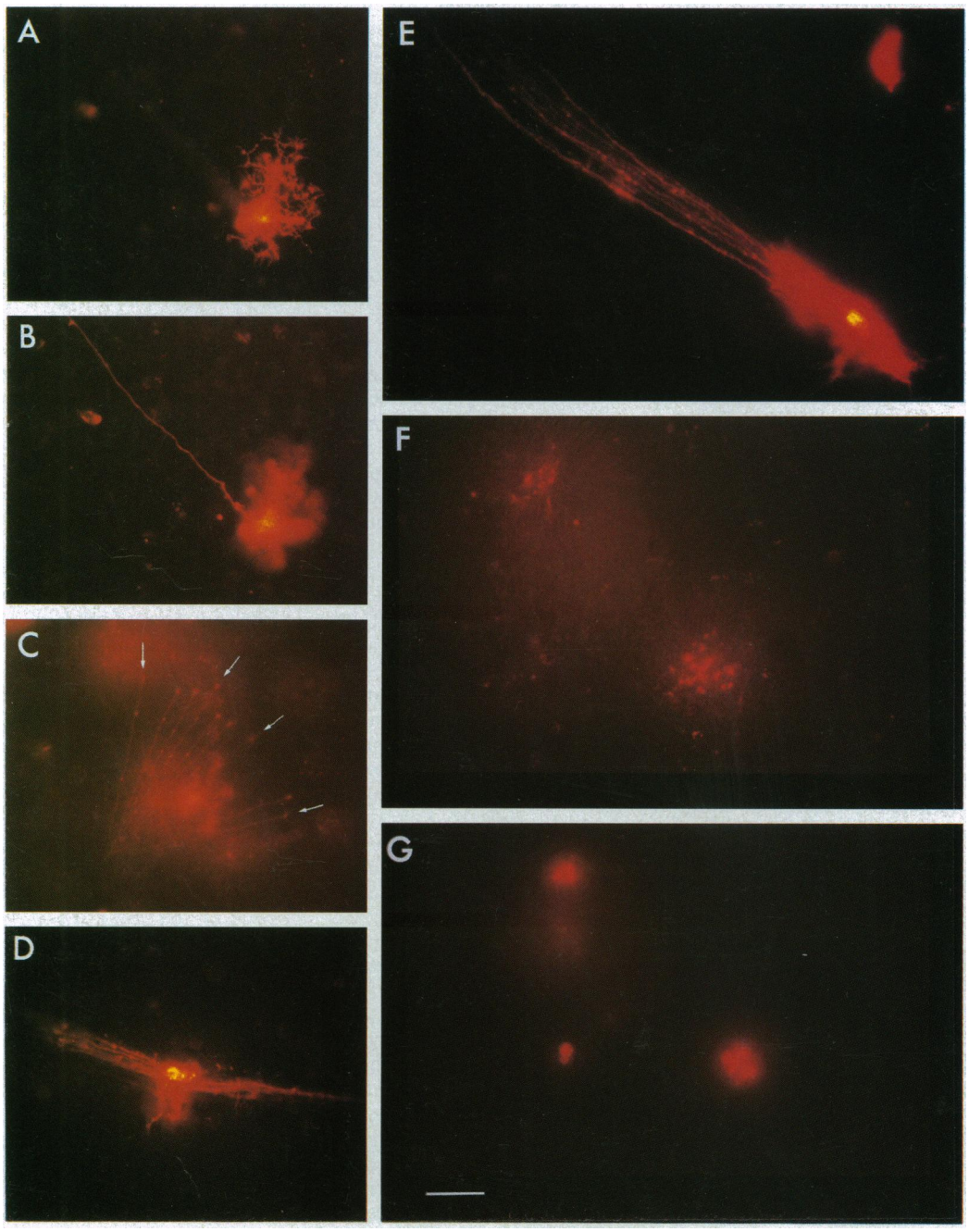

FIG. 3. *En face* images of engrafted DiI-labeled quail neuroretina cells, QNR/D and QNR/K2, in whole mounts of E15 embryonic chicken retinas 6 days postinjection. (*A* and *B*) Two focal planes of an engrafted QNR/D cell showing dendrite-like (*A*) and axon-like (*B*) processes. (*C*) QNR/D cell projecting an array of processes each ending in a growth cone (selected growth cones indicated by arrows). (*D* and *E*) QNR/D cells projecting processes along the plane of retina. (*F* and *G*) Two focal planes of three engrafted QNR/K2 cells: (*F*) multiple Müller cell-like endfeet of the three cells at the vitreal margin of the retina and (*G*) cell bodies of the three cells at a deeper focal plane in the retina. (Bar = 50 μm.)

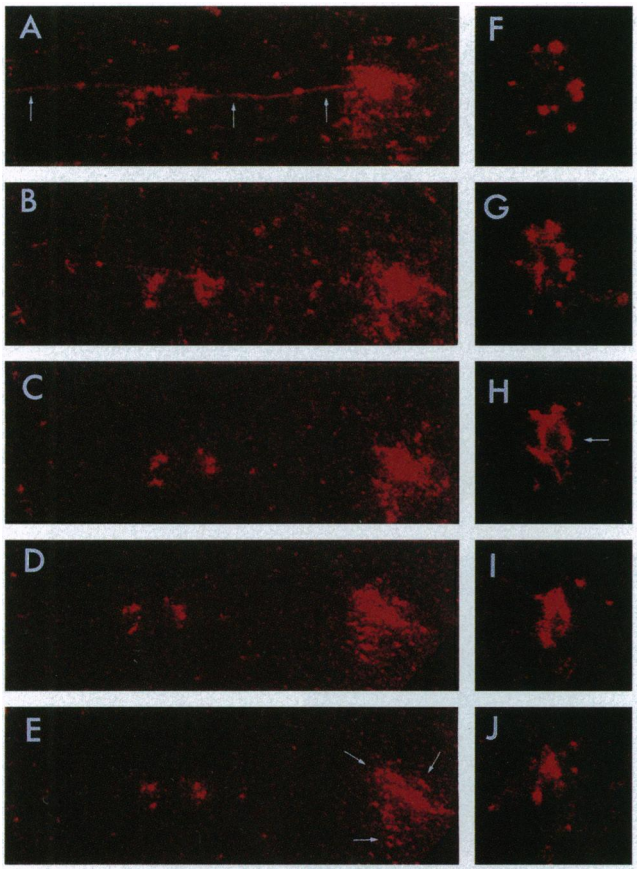

FIG. 4. A Z-series of laser confocal microscope images of a DiI-labeled QNR/D cell (*A–E*) and a DiI-labeled QNR/K2 cell (*F–J*) in an El5 retina 6 days after implantation. The QNR/D cell extends a long axon-like process in the ganglion cell axon layer (*A*, arrows) and projects multiple dendrite-like processes into the inner synaptic layer (*E*, arrows). The QNR/K2 cell extended multiple processes with Müller cell bulbous endfeet-like morphology at the vitreal margin (*F*), perinuclear cytoplasm in the inner nuclear layer (*H*), and multiple processes terminating at the outer margin of retina (*J*).

sections, QNR/D cells, in concordance with their location, extended intricate neurites along the plane of retina in the amacrine and ganglion cell strata, reminiscent of dendritic trees and axons, whereas QNR/K2 cells projected Müller-like processes across the retina, perpendicular to QNR/D cells. *En face* observation of retinal whole mounts revealed that QNR/D cells expressed elaborate neuritic arborizations and axon-like processes (Fig. 3) similar to amacrine and ganglion cell morphologies first described by Ramon y Cajal (12) and QNR/K2 cells expressed a cluster of multiple filamentous processes that extended from the cell soma to the vitreal margin of retina in one direction (Fig. 3) and to the photoreceptor layer in the opposite direction, like Müller cells (12). Laser confocal Z-series images of implanted QNR/D and QNR/K2 cells (Fig. 4) in retinal whole-mounts show an example of a QNR/D cell with a long axon-like process in the ganglion cell axon layer and dendrite-like processes projecting into the inner synaptic layer and a QNR/K2 cell transversing the retina with multiple bulbous endfeet at the vitreal margin (Fig. 4*F*), the perikaryium in the inner nuclear layer (Fig. 4*H*), and multiple filamentous projections ending at the outer margin of retina (Fig. 4*J*). By 11 days postimplantation, QNR/K2 cells had extended processes fully from the inner limiting membrane at the vitreal margin of retina to the outer limiting membrane in the photoreceptor layer (Figs. 2*D Inset* and 4). In E21 embryos, 12 days after injection, QNR/D and QNR/K2 cells were present in their same respective strata as in earlier ages and retained their differentiated morphology. Cells that remain in the vitreous of E12, E15, and E21 embryos failed to extend processes. Although DiI-labeled QNR cells continued dividing normally *in vitro*, there was no indication of QNR cell division or QNR tumor formation up to 12 days (E21) after migration into the retina.

We cannot ascertain from their integration in the amacrine and ganglion cell layers or from their cellular morphology whether QNR/D cells are progenitors of amacrine cells or ganglion cells or both because some amacrine cells are found in the ganglion cell layer of normal chicken retina, the so-called displaced amacrine cells that make up 30–35% of the cells in the ganglion cell layer (13). Likewise, displaced ganglion cells appear in the amacrine cell stratum. In addition, the shapes of amacrine and ganglion cell dendritic trees can be virtually indistinguishable when viewed *en face*. Also, both ganglion cells and subsets of amacrine cells (12, 14) extend axons and axon-like processes along the plane of retina. Although we cannot designate whether QNR/D cells have the potential to become amacrine or ganglion cells or both, they constitutively do have the capacity to differentiate into an array of distinct morphologies representative of numerous subclasses of amacrine and ganglion cells.

Analogously, QNR/K2 cells, on reaching the region of the inner nuclear layer composed of bipolar and Müller cell nuclei, extend Müller-like processes across the retina (Figs. 2–5). The QNR/K2 cells resemble Müller cells after implantation in that they extend multiple processes that span the entire retina from the outer limiting membrane at the base of the outer segments of photoreceptor cells at the pigmented epithelial surface of the neural retina to the inner limiting membrane at the vitreal surface (15). At the vitreal margin, the cluster of filamentous processes that emanate from the QNR/K2 cell body terminate in bulbous endings like Müller cell endfeet that envelop ganglion cell bodies and axon bundles and form the inner limiting membrane (Figs. 4 and 5). These *in vivo* morphologic results as well as expression of Müller cell markers *in vitro* suggest that QNR/K2 cells represent a Müller cell precursor that behaves as a Müller cell after implantation in retina.

Signals that convey instructions for cell migration and differentiation also persist in retinas of older embryos. Indeed, QNR/D and QNR/K2 cells injected into El5 eyes and observed at E21 in cross-sections were found in the ganglion and amacrine layers and the inner nuclear layer, respectively, and exhibited morphologies typically seen in embryos implanted at E9 (Fig. 3). However, at El5, penetration of the chicken embryo retina by the QNR cells was restricted to peripheral retina because, in central retina, DiI-labeled cells were found only between the vitreal body and the neural retina, indicating a diminished capacity of quail cells to migrate into central retina probably as a consequence of the fusion of Müller cell endfeet forming an impenetrable retinal inner limiting membrane. Nevertheless, where the cells were able to penetrate at the periphery they migrated correctly.

These data show that cell lines that may represent distinct precursors or committed cell types from the neural retina can migrate into the host neural retina to their appropriate sites, where they integrate and differentiate accordingly, suggesting they know where to go and where to stop. The data also imply that the chicken retina itself expresses molecular markers or signals that identify the diverse cellular strata and that these address signals are interpreted by the migrating quail cells. The differences in migration between the two cell lines is not merely a difference in penetration capacity. Indeed a very small fraction of cells from both the QNR/D and QNR/K2 lines were found in "ectopic" strata. QNR/D

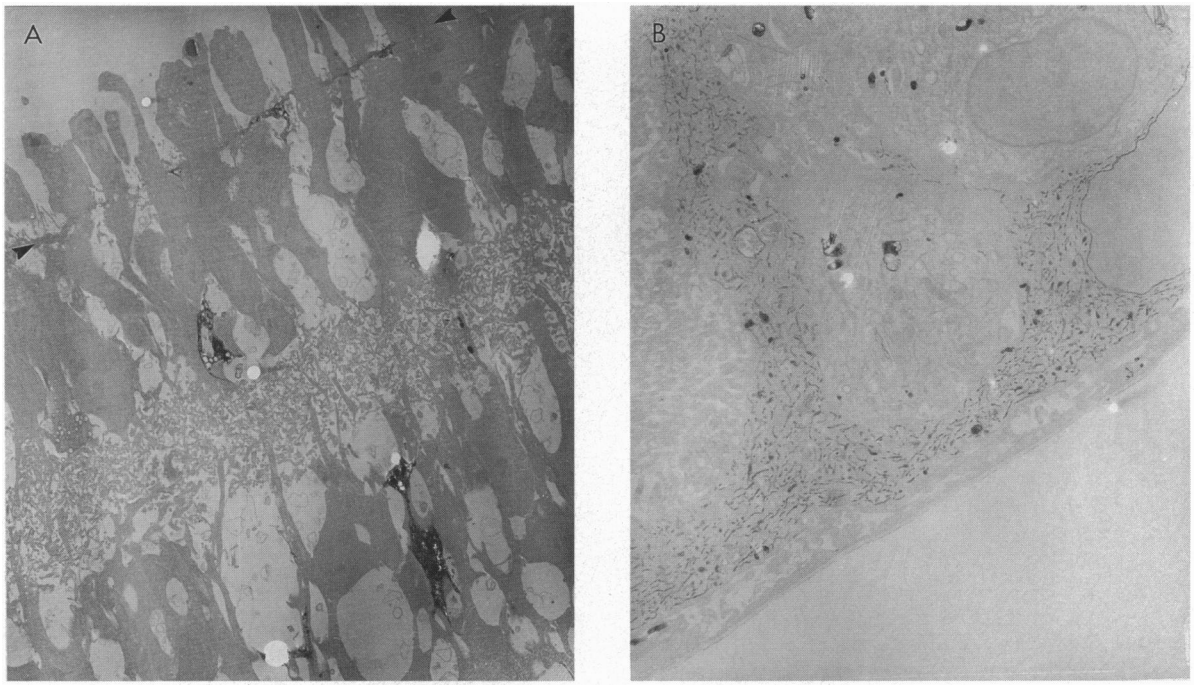

FIG. 5. Electron micrograph of implanted DiI-labeled QNR/K2 cells in retina after photoconversion of the DiI label to a diaminobenzidine product (7). QNR/K2 cells exhibited Müller cell morphology forming the outer limiting membrane (arrows), interdigitating the photoreceptor cells and inner nuclear layer cells (*A*) to enveloping ganglion cell bodies and axon bundles with endfeet at the inner limiting membrane of the retinal vitreal margin (*B*). (*A*, ×2000; B, ×4500.)

cells were present in deeper layers of retina as were QNR/K2 cells, which were also seen in the ganglion cell layer. Also, cells reached their proper layer despite migrating from the vitreal margin into the retina in the opposite direction of normal cell migration from the proliferative zone at the pigmented epithelial margin of retina.

In addition, these results indicate that molecular markers expressed *in vitro* might help identify immortal cell lines as belonging to particular cell classes because we could predict the ultimate migration, localization to specific strata, and differentiated phenotype of these cell lines from their molecular makeup. This leads one to investigate whether during normal development, differentiating neural precursors indeed acquire cues that direct their localization to a specific layer in retina and then arrest their migration. This question may be investigated by asking if QNR cell clones express distinct address codes that may be qualitatively or quantitatively different and which can read and interpret positional information of the host retina.

Immortalized neural cell lines have been used previously to approach the complexities of cell type determination. In these studies, multipotent human retinoblastoma Y79 cells were shown to differentiate into neurons or glial cells *in vitro* in response to culture conditions (16). Multipotent neural cell lines derived from hippocampus and cerebellum were engrafted into the developing CNS and gave rise to apparently mature neural cells. Their conditional differentiated morphology was contingent on the implant site. Hippocampal HiB5 cells (17) integrated into regions of the hippocampus and cerebellum where cells had recently undergone proliferation at the time of implant and HiB5 cells manifested morphologies characteristic of either neurons or glial cells endogenous at the implant site: dentate granule neurons in the dentate gyrus and cerebellar granule neurons and Bergmann glia. Cerebellar C27–3 cells (18) developed morphologies of granule cell neurons, astrocytes, and oligodendrocytes when implanted into the cerebellum. To our knowledge, these studies showed for the first time that neural cell lines can be successfully implanted and differentiate into distinct cell types with morphologies indistinguishable from that of the indogenous host cells. In contrast to these multipotent or totipotent cell lines, QNR cell clones have a restricted pathway of differentiation. QNR/D and QNR/K2 cell clones evoke the possibility of engineering a panel of diverse cell lines that are developmentally restricted and determined to migrate to a particular region of the CNS and differentiate into the appropriate cell class or subclass for that region. These immortalized surrogate cell lines might be genetically manipulated to then correctly augment damaged or reconstitute missing cell types in the CNS.

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1. Crisanti-Combes, P., Lorinet, A.-M., Girard, A., Pessac, B., Wassef, M. & Calothy, G. (1982) *Cell Differen.* **11,** 45–54.
2. Pessac, B., Girard, A., Romey, G., Crisanti, P., Lorinet, A.-M. & Calothy, G. (1983) *Nature (London)* **302,** 616–618.
3. Pessac, B., Girard, A., Romey, G., Crisanti, P., Wassef, M., Privat, A. & Calothy, G. (1983) *Brain Res.* **275,** 53–59.
4. Joshi, J. & Trisler, D. (1990) *J. Cell. Biochem.* **14F,** 68 (abstr.).
5. Britto, L. R. G. & Hamassaki-Britto, D. E. (1992) *Visual Neurosci.* **9,** 389–398.
6. Brecha, N., Karten, H. J. & Laverack, C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3010–3014.
7. Trisler, D. (1987) *Curr. Top. Dev. Biol.* **21,** 277–308.
8. Honig, M. G. & Hume, R. I. (1986) *J. Cell Biol.* **103,** 171–187.
9. Trisler, D., Bekenstein, J. & Daniels, M. P. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4194–4198.
10. Sandell, J. H. & Masland, R. H. (1988) *J. Histochem. Cytochem.* **36,** 555–559.
11. Lance-Jones, C. C. & Lagenaur, C. F. (1987) *J. Histochem. Cytochem.* **35,** 771–780.
12. Ramon y Cajal, S. (1972) *The Structure of the Retina*, translators Thorpe, S. A. & Glickstein, M. (Thomas, Springfield, IL).

13. Binggeli, R. L. & Paule, W. J. (1969) *J. Comp. Neurol.* **137,** 1–18.
14. Catsicas, S., Catsicas, M. & Clarke, P. G. H. (1987) *Nature (London)* **326,** 186–187.
15. Dowling, J. E. (1987) *The Retina: An Approachable Part of the Brain* (The Belknap Press of Harvard Univ. Press, Cambridge, MA), pp. 15–16.
16. Kyritsis, A. P., Tsokos, M., Triche, T. J. & Chader, G. J. (1984) *Nature (London)* **307,** 471–473.
17. Renfranz, P. J., Cunningham, M. G. & McKay, R. D. G. (1991) *Cell* **66,** 713–729.
18. Snyder, E. Y., Deitcher, D. L., Walsh, C., Arnold-Aldea, S., Hartwieg, E. A. & Cepko, C. L. (1992) *Cell* **68,** 33–51.