# Plasticity and Differentiation of Embryonic Retinal Cells After Terminal Mitosis

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**The relation between terminal mitosis and the events that determine the developmental fate of embryonic precursor cells is not well understood. This relation has now been investigated with [$^3$H]thymidine autoradiography to determine the time of cell birth and with a culture system that allows the testing of the developmental potential of cells isolated from the chick embryo retina. Contrary to the situation in vivo, where neuronal differentiation always precedes photoreceptor differentiation, photoreceptor differentiation occurs prematurely and precedes neuronal differentiation when precursor cells are isolated from the retina at early embryonic stages. Thus, cells born by embryonic day 5 (ED-5) give rise predominantly to photoreceptors when isolated for culture on ED-6 but develop mainly as neurons when isolated on ED-8. This suggests that retinal precursor cells retain after terminal mitosis the capacity to develop either as neurons or as photoreceptors. Moreover, photoreceptor differentiation appears to represent a constitutive or "default" pathway that precursor cells follow in the absence of neuron-inducing signals.**

CENTRAL NERVOUS SYSTEM (CNS) regions such as the retina contain different neuronal cells that are derived from an apparently homogenous population of proliferating neuroepithelial cells. The mechanisms that specify which developmental pathway is followed by each precursor cell are not understood. One hypothesis is that commitment occurs before, or at the time of, the cell's terminal mitosis (Fig. 1A). Alternatively, cells could remain uncommitted through their terminal mitosis, with their developmental pathway being determined by position-dependent signals as they migrate to their ultimate locations (Fig. 1B). Lineage studies in developing rodent (*1*) and amphibian retinas (*2*) suggested that cell specification occurs either at or after terminal mitosis. However, these in vivo studies could neither distinguish between these two alternative scenarios nor directly demonstrate uncommitted postmitotic precursors.

In vitro studies with cells dissociated from 8-day chick embryo retina and grown at low densities have been consistent in the relative proportions of neurons and photoreceptors developing in the cultures (*3*). This finding suggests that some precursor cells are already predetermined by embryonic day 8 (ED-8) to express developmental programs that, as shown by other studies (*4*), are both complex and robust. We have now combined [$^3$H]thymidine autoradiography with in vitro culture to investigate the behavior of cells isolated from retinas at daily intervals between ED-6 and ED-10. Because neuronal differentiation precedes overt photoreceptor differentiation by several days during retinal development in vivo (*5*), a lower frequency of photoreceptors might be expected in cultures derived from younger embryos. However, the opposite result was obtained. Photoreceptors represented approximately 70% of the differentiated cells in cultures derived from ED-6 (stage 29) chick retinas (Fig. 2E). Their relative frequency and absolute number decreased dramatically in cultures from ED-7 retinas and reached a plateau in cultures from ED-8 to ED-10 retinas, in which photoreceptors represented only 20 to 30% of the morphologically differentiated cells. Photoreceptor morphology and opsin-like immunoreactivity in cultures from younger embryos (Fig. 2) resembled those described for cells from 8-day embryos (*3*). The relative frequency and absolute number of nonphotoreceptor neurons showed the opposite trend (Fig. 2, E and F). These changes were not accompanied by detectable changes in overall cell survival. For example, cultures from ED-6 retinas showed 73,200 ± 7,200 cells, as compared to 70,900 ± 5,600 cells in cultures from ED-8 retinas. Moreover, the in vitro microenvironment did not appear to determine the fate of cells isolated at different stages, because cocultures of ED-6 and ED-8 cells showed frequencies of neurons and photoreceptors intermediate between those seen when the same cell suspensions were cultured independently (*6*).

To further characterize the apparent change in cell fate between ED-6 and ED-8 of the cells undergoing terminal mitosis before ED-5 in ovo, we examined the unlabeled cultured cells derived from retinas labeled with [$^3$H]thymidine (Fig. 3). Only those cells that were already postmitotic at the time of the initial thymidine injection would be unlabeled in autoradiograms of the cultures. We could, therefore, test whether the fate of these cells changed as a function of extended exposure to the retinal microenvironment. When retinas thus labeled were dissociated for culture on ED-6, approximately 70% of the unlabeled cells that differentiated after 4 days in vitro could be identified as photoreceptors (Fig. 3, B and C). The ratios of photoreceptors to neurons in two separate experiments were 2.35 and 2.40. However, when cells were isolated on ED-8, photoreceptors represented only 35% of the unlabeled cells that differentiated after 4 days in vitro, with the remaining 65% showing a neuronal phenotype (Fig. 3C). In two separate experiments the ratios were 0.46 and 0.69. Thus, the composition of the populations derived from the cohort of cells undergoing terminal mitosis before ED-5 changes as a function of their exposure to the in vivo microenvironment before they are isolated for culture. Early isolation of precursor cells leads to premature photoreceptor differentiation and to an inversion of the in vivo sequence of events (*5*), in which photoreceptor differentiation is always preceded by extensive neuronal development.

In vivo, the differentiated phenotype expressed by each developing cell correlates

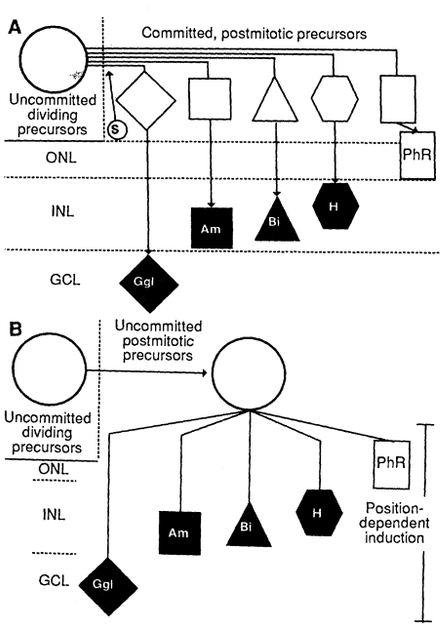


**Fig. 1.** Two hypothetical scenarios for the differentiation of retinal precursor cells, which accommodate cell proliferation, terminal mitosis, and cell migration. Abbreviations: AM, amacrine neurons; Bi, bipolar neurons; GCL, ganglion cell layer; Ggl, ganglion cells; H, horizontal neurons; INL, inner nuclear layer; ONL, outer nuclear layer; PhR, photoreceptor; and S, inductive signals. Müller glial cells are not included in these figures (*8*).

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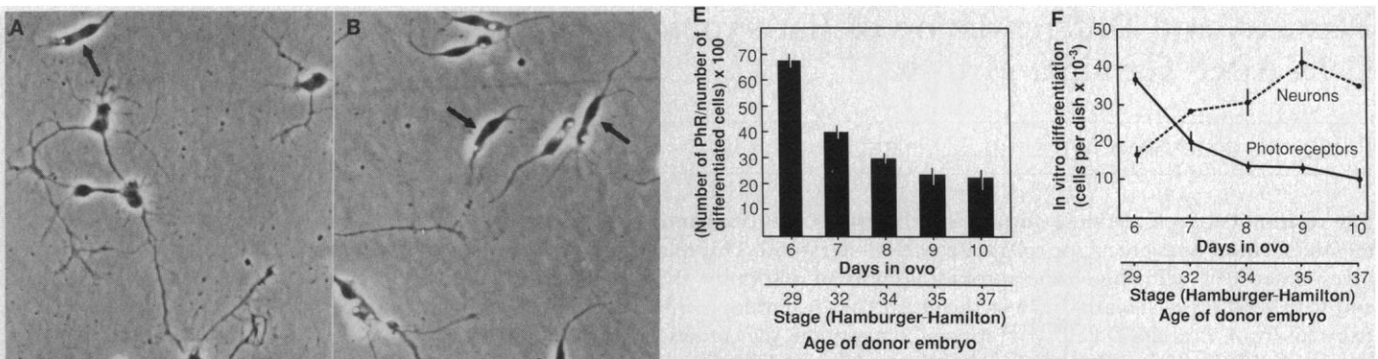


**Fig. 2.** Developmental stage–dependent changes in the frequency of neurons and photoreceptor cells (PhR) differentiating in vitro. Neural retinas were dissected at the indicated times. All of the cell suspensions were seeded at the same density (625 cells per square millimeter), and the cells were cultured as described (*8*) for 4 days before fixation for microscopic analysis or immunocytochemistry. (**A**) Photomicrograph of a culture of 8-day chick embryo retinal cells. Photoreceptors (arrow) were identified by phase-contrast microscopy on the basis of their elongated shape, the presence of a single, short neurite, and an inner segment containing a conspicuous lipid droplet. Their identity has been corroborated by scanning and transmission electron microscopy and by immunocytochemical detection of cell-specific macromolecules (*3*). (**B**) Culture of ED-6 retinal cells. Arrows indicate photoreceptor cells. (**C** and **D**) An ED-6 culture processed for opsin immunocytochemistry (*3*) with a sheep antibody to bovine rhodopsin (*9*). Photoreceptors showed positive immunoreactivity (long arrows), whereas multipolar neurons were negative (short arrows). (C) Phase contrast; and (D) fluorescence. (**E** and **F**) Quantitative analysis. The total number of cells was determined by counting 20 random fields per dish and multiplying their average by the ratio between the area of the dish and the area of the field. The relative frequency of different cell types was established by microscopic analysis of 200 cells, also chosen at random. Process-free, round cells with an undifferentiated morphology were not included in these figures. Results are means of triplicate dishes from two separate experiments, ±SD. (E) Relative frequency of photoreceptors and neurons; and (F) variation in absolute numbers of neurons and photoreceptors.

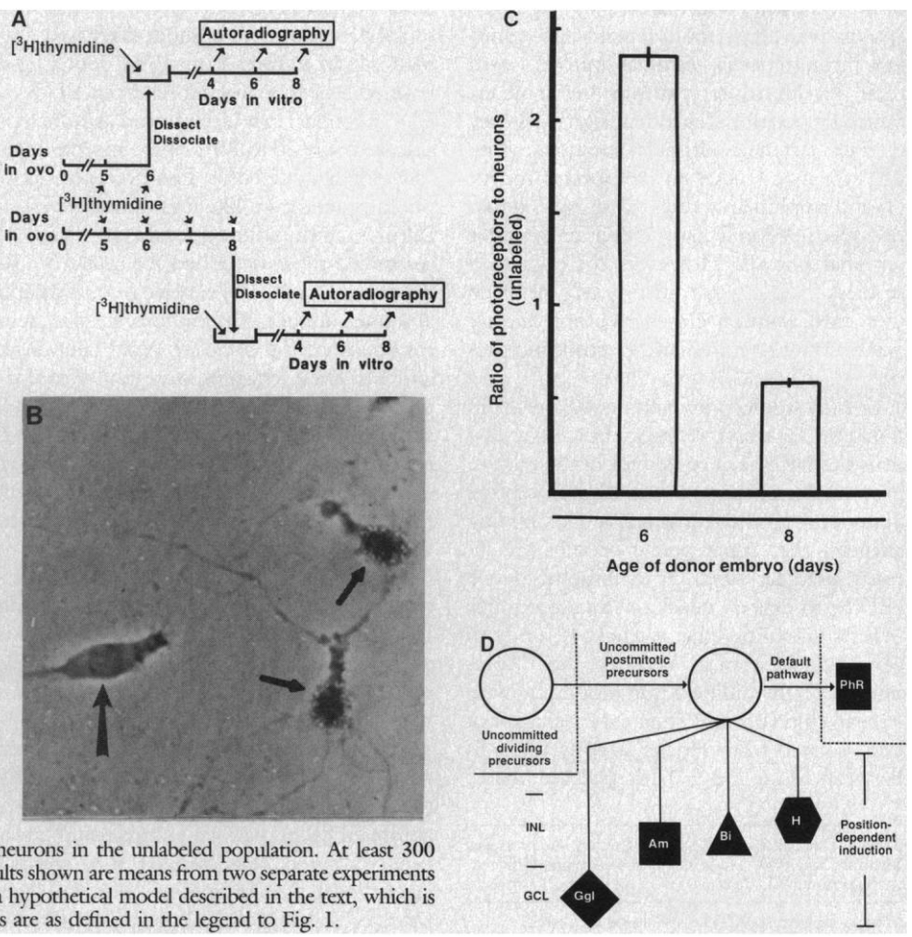


**Fig. 3.** (**A**) Design of an experiment that combined [$^3$H]thymidine autoradiography with cell culture to study the fate of cells undergoing terminal mitosis before ED-5. Similar results were obtained with [$^3$H]thymidine of two specific activities [6.7 Ci/mmole (Du Pont Biotechnology Systems) and 25 Ci/mmole (Amersham)]. The embryos were injected with [$^3$H]thymidine (10 μCi) per embryo on ED-5 through a window in the egg shell. Injections (5 μCi) were repeated daily up to and including the day when the cells were isolated for culture. The cultures were prepared as described above, and [$^3$H]thymidine (0.1 μCi/ml) was added to the medium. After 4 days in vitro the cultures were fixed and prepared for autoradiography with Kodak Nuclear Trak Emulsion NTB2. (**B**) Autoradiogram of a 4-day culture of ED-6 neural retinal cells labeled as described above. Cultures derived from labeled ED-6 and ED-8 embryos were qualitatively and quantitatively similar to cultures from embryos not exposed to [$^3$H]thymidine (*6*). Unlabeled photoreceptor (long arrow) and several labeled cells (short arrows) are shown. (**C**) Ratios of photoreceptors to neurons in the unlabeled population. At least 300 unlabeled cells were scored in each culture dish. The results shown are means from two separate experiments run in triplicate, ±SD. (**D**) Graphic representation of a hypothetical model described in the text, which is consistent with the results reported here. Abbreviations are as defined in the legend to Fig. 1.

with its final position within the retina (*5*). Thus, postmitotic cells that migrate away from the "outer limiting membrane" and toward the inner retina develop as neurons, whereas those that fail to migrate develop as photoreceptors. Cell migration is extensive during the ED-6 to ED-8 period. Therefore, developmental stage–dependent changes in the frequency of differentiated phenotypes observed in vitro are consistent with a model of retinal cell differentiation (Fig. 3D) in which (i) precursor cells remain uncommitted for some time after terminal mitosis, (ii) they differentiate as neurons only after relocating to the inner retina where they are exposed to position-dependent regulatory signals, and (iii) precursor cells that fail to relocate to "neuron-inducing" retinal regions follow a constitutive or "default" pathway and differentiate as photoreceptors. In vivo, this constitutive developmental pathway is followed by those postmitotic cells that remain attached to the outer limiting membrane and have their cell body located in the outer nuclear layer. A similar fate would be followed in vitro by those postmitotic cells that are dissociated from the retina before their migration.

Using retroviral markers to trace cell lineages in the rat retina, Turner and Cepko (*1*) observed that rods, bipolar cells, and amacrine cells have common lineage until late in development, suggesting that determination of cell type may occur at or after the final mitosis of precursor cells. Wetts and Fraser injected fluorescent dextrans into cells of the immature frog optic vesicle and obtained similar results (*2*). Our in vitro observations are consistent with and complementary to the conclusions derived from lineage analysis. However, further studies are needed to determine whether the cells that differentiate as photoreceptors when isolated from the embryo at ED-6 are the same cells as those that differentiate as neurons when isolated at ED-8. This uncertainty is due to the presence, in all of the cultures studied, of some cells that fail to express any differentiated phenotype and remain as process-free, round cells. Our culture technique offers an experimental system for investigating this question and ascertaining whether the fate of individual postmitotic precursor cells changes in response to different microenvironmental conditions.


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8. Cultures of dissociated chick retinal neurons and photoreceptors, free of contamination by connective tissue, glial, vascular endothelial, and pigment epithelial cells, were established as described (*3*). Neural retinas were dissected from White Leghorn chick embryos, briefly trypsinized and dissociated by trituration. The dissociated cells were suspended in medium 199 supplemented with 10% heat-inactivated fetal bovine serum, linoleic acid–albumin (110 μg/ml), and penicillin (100 U/ml) and seeded in polyornithine-coated tissue culture dishes. Glial cells do not develop under these culture conditions (*3*).
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# Interleukin-1 Mitogenic Activity for Fibroblasts and Smooth Muscle Cells Is Due to PDGF-AA


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**Both interleukin-1 (IL-1) and platelet-derived growth factor (PDGF) induce proliferation of cultured fibroblasts and smooth muscle cells. These polypeptide mediators are released by activated macrophages and other cell types in response to injury and are thought to have a role in tissue remodeling and a number of pathologic processes. Analysis of the kinetics of [$^3$H]thymidine incorporation by cultured fibroblasts demonstrated that the response to IL-1 is delayed approximately 8 hours relative to their response to PDGF. IL-1 transiently stimulated expression of the PDGF A-chain gene, with maximum induction after approximately 2 hours. Subsequent synthesis and release of PDGF activity into the medium was detected as early as 4 hours after IL-1 stimulation, and downregulation of the binding site for the PDGF-AA isoform of PDGF followed PDGF-AA secretion. Antibodies to PDGF completely block the mitogenic response to IL-1. Therefore, the mitogenic activity of IL-1 for fibroblasts and smooth muscle cells appears to be indirect and mediated by induction of the PDGF A-chain gene.**


THE INTERLEUKIN-1 MOLECULES (IL-1α and IL-1β) are multipotent inflammatory mediators that are produced by activated macrophages and by epidermal, lymphoid, and vascular cells. They have the capacity to affect mesenchymal destructive and reparative processes during tissue remodeling (*1*). The effects on cultured connective tissue cells include proliferation of fibroblasts (*2*), Balb/3T3 cells (*3, 4*), and smooth muscle cells (*5*), as well as stimulation of collagenase and prostaglandin $E_2$ production (*6*). Confluent fibroblast monolayers express 5,000 to 15,000 IL-1 receptors per cell that bind both IL-1α and IL-1β (*3, 7*). However, in comparison with platelet-derived growth factor (PDGF), IL-1 does not induce early changes in specific cellular proteins in Balb/3T3, and IL-1 produces relatively small changes in DNA synthesis (*4*). In human fibroblasts, IL-1 receptor–mediated ligand internalization is slow (*7*) and the kinetics of IL-1–induced increases in mRNA for c-*fos* and c-*myc* differs from the kinetics of other growth factor–stimulated increases (*8*). PDGF and IL-1 are both secreted by activated macrophages and are potential mediators of fibroblast proliferation (*1, 9*). In this study, we compared the relative mitogenic potencies of the three isoforms of PDGF (PDGF-AA, PDGF-BB, and PDGF-AB) and of IL-1 for human dermal fibroblasts and arterial smooth muscle cells. Our data demonstrate that IL-1–induced proliferation is not due to direct mitogenic stimulation by IL-1 but is mediated by induction and release of PDGF-AA by fibroblasts or smooth muscle cells in an autocrine or paracrine manner, or both.

Initial studies comparing the relative mitogenic activities of IL-1 and PDGF, as measured by [$^3$H]thymidine incorporation at 18 to 20 hours after mitogen addition, demonstrated little or no response to IL-1. Consequently, the kinetics of thymidine incorporation were examined over 48 hours at 8- to 12-hour intervals. The first increase in [$^3$H]thymidine incorporation in response to PDGF-AA, PDGF-BB, and PDGF-AB was seen at 16 hours and was maximal at 24


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