

Defining retinal progenitor cell competence in *Xenopus laevis* by clonal analysis

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Extrinsic cues and intrinsic competence act in concert for cell fate determination in the developing vertebrate retina. However, what controls competence and how precise is the control are largely unknown. We studied the regulation of competence by examining the order in which individual retinal progenitor cells (RPCs) generate daughters. Experiments were performed in *Xenopus laevis*, whose full complement of retinal cells is formed in 2 days. We lineage-labeled RPCs at the optic vesicle stage. Subsequently we administered a cell cycle marker, 5-bromodeoxyuridine (BrdU) at early, middle or late periods of retinogenesis. Under these conditions, and in this animal, BrdU is not cleared by the time of analysis, allowing cumulative labeling. All retinal cell types were generated throughout nearly the entire retinogenesis period. When we examined the order that individual RPCs generated daughters, we discovered a regular and consistent sequence according to phenotype: RGC, Ho, CPr, RPr, Am, BP, MG. The precision of the order between the clones supports a model in which RPCs proceed through stepwise changes in competence to make each cell type, and do so unidirectionally. Because every cell type can be generated simultaneously within the same retinal environment, the change in RPC competence is likely to be autonomous.

KEY WORDS: *Xenopus*, Eye, Retinogenesis, Cell fate determination, Cellular competence, 5-Bromodeoxyuridine

INTRODUCTION

The question regarding the mechanism(s) for achieving cell type diversity in the nervous system is a major one in biology. For many reasons, the vertebrate retina has become a favorite model of central nervous system (CNS) development. All vertebrate retinas have the same six basic cell types: photoreceptors [both rods (RPr) and cones (CPr)], horizontal cells (Ho), bipolar cells (BP), Müller glia (MG), amacrine cells (Am) and retinal ganglion cells (RGCs), arranged in the same basic cytoarchitecture. Within this 'basic' plan, vertebrate retinas vary widely in properties such as cell morphology, neurotransmitter, size of cell populations and distribution across the retinal surface. Thus, there is both diversity and simplicity.

Landmark studies of retinal cell lineage were published nearly simultaneously for the frog and rodent (Wetts and Fraser, 1988; Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990). These demonstrated that retinal progenitor cells (RPCs) produced clones that vary greatly in size and could be composed of any combination of cell types. The dominant hypothesis accounting for these data was that RPCs produced uncommitted offspring that were acted upon by signals in the environment and instructed in what to mature as. Gradual change in environmental signals during development was invoked to account for the temporal periods of genesis for each retinal cell type (Harman and Beazley, 1989; La Vail et al., 1991; Prada et al., 1991; Rapaport et al., 2004). Specifically, RGCs are always the first cell type to be born, followed by a group including Ho, Am and CPr, and culminating with a group including RPr, BP and MG.

However, evidence that retinal cell fate is not defined by the environment began rapidly accumulating. Heterochronic cell mixing experiments (in vitro or in vivo) showed that raising young RPCs in

an older environment (or vice versa) did not change cell fate acquisition or the schedule of expression of cell-specific markers (Watanabe and Raff, 1990; Belliveau and Cepko, 1999; Belliveau et al., 2000; Rapaport et al., 2001). Clonal density cell culture demonstrated that RPCs from embryonic day (E) 16-17 rat retinas grown in serum free medium behave similarly to those in retinal explants (Cayouette et al., 2003). Eventually the 'induction by environmental cues' model of retinal cell fate determination evolved to include the property of competence, defining the ability of a cell to respond to inductive cues, and an intrinsic cell property.

Numerous molecules have been shown to be involved in retinal cell fate signaling (Harris, 1997; Cepko, 1999). However, the nature of competence and the mechanism of its control is more difficult to study and largely unknown. Competence appears to function as a permissive property intrinsic to RPCs that allows them to respond to instructive signals for cell type production (Cepko et al., 1996). In such a way Notch-Delta has been shown to keep RPCs in an undifferentiated, proliferative state (Austin et al., 1995; Dorsky et al., 1995; Dorsky et al., 1997), whereas agents that antagonize Notch, such as Numb (Wakamatsu et al., 1999; French et al., 2002), may be part of the mechanism instilling competence.

The order of retinal cell production opens a window into the regulation and limits of cellular competence. Studies in a number of vertebrates (La Vail et al., 1991; Rapaport et al., 2004) show that the birth of cells of each phenotype during retinogenesis is staggered, demonstrating a temporal, though overlapping sequence. Several models of cellular competence are consistent with this 'order with overlap'. For instance, the control of competence may be imprecise, even stochastic, allowing the genesis of any cell type at any time but exhibiting a developmentally changing bias towards or away from specific types. For example, at any point in time cell types A, B, C, D and E could be generated, but early on the probability of A might be high and D low, and vice versa late in development. We call this the 'random model' of competence. In another scheme, RPCs may pass through 'competence periods' to make one or more cell types. Again, for example, early in development an RPC may be competent to make cell types A and B, then subsequently move on to make C, D and E. We call this the 'phasic model'. In this model,

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one would expect shifting of cell genesis order within a phase (A-B or B-A, or C-D-E or E-C-D, etc.), but not between phases (never A-C-B, C-B-E, etc.). Finally, the most extreme form of competence control is where RPCs pass sequentially through periods to make each cell type separately. In our example, a single RPC would produce cell type A, then B, C, etc., although each RPC does not necessarily make all cell types. We call this the ‘stepwise model’. A strict stepwise model would fit data that show a sequence of genesis with the ordinal position of each phenotype invariant. Both the random and phasic models require RPCs to possess multiple competence states simultaneously, whereas the stepwise model suggests that an RPC adopts a single competence state at a given time to allow the production of a specific cell type.

Besides ordered cell genesis, these models make different predictions concerning the stage of cell production of individual RPCs at a given developmental stage. If the development of the population is synchronous, most RPCs would be expected to undergo symmetric mitotic divisions at early stages, then all transition around the same time to asymmetric mitoses as daughters leave the cell cycle, and eventually end with terminal divisions at a late embryonic stage. Both the random and phasic models predict synchrony between the developmental staging of RPCs, but the stepwise model is compatible with asynchrony.

To determine the synchrony of cell production, and whether it is sequential, we studied cell genesis in the frog, *Xenopus laevis*, whose retinogenesis completes in 48 hours. Prior attempts to establish ordered retinal cell birth in this species (Holt et al., 1988; Stiemke and Hollyfield, 1995) were unsuccessful. Given these two facts, *Xenopus* presents a rigorous test of ordered retinogenesis, a challenge that we sought to address by combining lineage and cell birthday markers allowing us to look at the level of resolution of single RPCs. We demonstrate that the population of *Xenopus* RPCs is heterogeneous, and they generate all cell types at almost all periods of retinogenesis. We also show an ordered and regular sequence of cell production: RGC, Ho, CPr, RPr, Am, BP, MG. Although not all cell types are present in each clone, in each one the cells display these ordinal relations. These findings support the model in which RPCs follow an intrinsic program with stepwise changes in competence to produce one cell type at a time in a conserved order.

MATERIALS AND METHODS

Embryos

Embryos from *Xenopus laevis* were obtained by in vitro fertilization. They were maintained in 10% Holtfreter’s solution at 16–21°C and staged by external morphology according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). The jelly coat and vitelline membrane of embryos were removed manually at stages 17–19. All animals were held and treated experimentally according to University of California and NIH guidelines.

Labeling clones

A 0.7 kb fragment of *gfp3* was excised from *pCS2-nls-gfp3* (provided by S. Evans) using *Xho1* and *Xba1*. This fragment was subcloned into vector, *pCS2-ITR* [provided by S. Evans (Fu et al., 1998)] to create *pCS2-gfp3-ITR*. This expression vector containing the ITR sequences of AAV has been demonstrated to allow even segregation of plasmids among daughters. GFP was detected throughout the cytoplasm, including all cell processes. We transfected *pCS2-gfp3-ITR* DNA mixed, 1:3 by weight, DNA with the lipofectant, DOTAP (Roche). Approximately 65 μ l of this mix was injected with a micropipette connected to a Picospritzer pneumatic system (General Valve Corporation, Fairfield, NJ, USA). Stage 20–21 embryos were injected (Fig. 4A), and two distant sites per vesicle were targeted, with two injections performed per site. The lipofection technique has been shown to produce lengthy, stable expression (Holt et al., 1990), well beyond the 55 hours of

this study, so dilution of the lineage tag is unlikely to be a problem. This was confirmed by the presence of lineage-labeled profiles in large clones that were BrdU⁺ (62.5%) from a late stage of BrdU injection. In such cases these would be expected to have undergone the highest number of cell divisions and to have most likely diluted the lineage tracer.

Labeling mitotic cells

At early, middle or late stages of retinal development (Fig. 4A), *gfp*-transfected embryos were injected with three 15 nl pulses of BrdU (5 mg/ml). We targeted the abdomen, which early in development is primarily composed of yolk. BrdU only slowly becomes cleared from here providing cumulative labeling. This was verified by ensuring that the ciliary marginal zone, which proliferates throughout the life of the tadpole/frog, was entirely labeled with BrdU, indicating no dilution. At analysis (stage 41), BrdU⁺ and BrdU[−] cells represent those that were post-mitotic and mitotic, respectively, at the time of injection.

Histology and immunohistochemistry

At stage 41, tadpoles were anesthetized and then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. They were then cryoprotected in 30% sucrose for at least an hour before 10 μ m coronal sections were cut through the eyes. Sections were screened for GFP fluorescence using a Nikon SMZ1000 fluorescence stereoscope. If at least one section demonstrated GFP⁺ profile(s) all sections from that case were immunolabeled. Sections from all other retinas were excluded from further consideration.

To immunostain incorporated BrdU, we denatured DNA with heat. Slides were immersed in 250 ml of 10 mM sodium citrate buffer (pH 6.0) and heated in a microwave oven (700 W) for 3 minutes. They remained in the hot buffer for 5 minutes, and were microwaved again. Slides then cooled for 30 minutes. The sections were immunostained with antibodies to GFP (rabbit polyclonal, Molecular Probes, 1:1500), BrdU (mouse monoclonal IgG1, Becton Dickinson, 1:15) and rhodopsin [4D2, mouse monoclonal IgG2b, 1:100, gift of R. Molday (Molday and Mackenzie, 1983)]. Secondary antibodies were, respectively, goat anti-rabbit Alexa-594 (Molecular Probes, 1:500, red), goat anti-mouse FITC (IgG1, Southern Biotechnology, 1:150, green), and goat anti-mouse Alexa-350 (IgG2b, Molecular Probes, 1:150, blue).

Data collection and analysis

Sections were imaged with a Nikon E800 epifluorescence microscope; images were captured with an Apogee KX85 CCD camera and analyzed using ImagePro software (Media Cybernetics). All data were collected from the mature, central retina. Approximately 50% of transfected eyes contained GFP-labeled cells that were clustered. A cluster was often confined to one section and seldom spanned more than two to three. To be considered a clone, a GFP⁺ cell cluster must be isolated by at least seven cell diameters from any other GFP⁺ cell(s). Retinal phenotypes were identified by morphology (Fig. 1). RPr were distinguished by anti-rhodopsin (4D2) immunoreactivity. Each section was first examined for GFP⁺ cells, to identify clones. Once a clone was defined, the phenotype of each cell was determined. Finally, the BrdU-labeling status of each constituent was determined. The occasional complex clone containing overlapping cells was examined with a BioRad MRC 1024 confocal microscope and the information used to aid in cell classification.

RESULTS

Identifying the progeny of *gfp*-transfected retinal progenitor cells

The DNA denaturation step needed for BrdU immunohistochemistry extinguished the native fluorescence of GFP, necessitating the use of a GFP antibody for detection. We found this enhanced the imaging of the GFP signal by giving stronger, more uniform fluorescence (Fig. 1). As others have shown (Strettoi and Masland, 1995; Jeon et al., 1998), we were able to reliably identify cell types based on the laminar and sub-laminar location of their cell bodies and the disposition of processes.

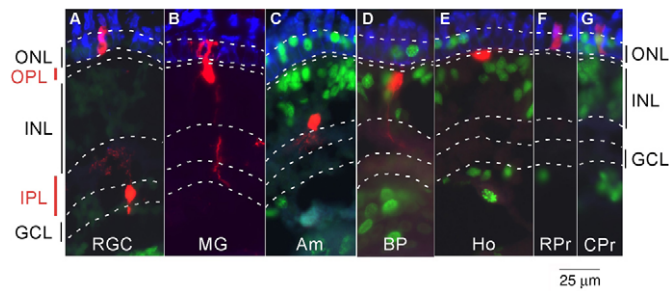


Fig. 1. GFP⁺ retinal sections of stage 41 *Xenopus* tadpoles.

Immunolabeled for GFP (red), BrdU (green) and rhodopsin (blue). Sclera is up, vitread is down. Each cell type has a readily recognized morphology. Am, amacrine cell; BP, bipolar cell; CPr, cone cell; GCL, ganglion cell layer; Ho, horizontal cell; INL, inner nuclear layer; IPL, inner plexiform layer; MG, Müller glia; ONL, outer nuclear layer; OPL, outer plexiform layer; RPr, rod cell; RGC, retinal ganglion cell.

Examples of the seven major retinal cell types are shown in Fig. 1. The inner nuclear layer (INL) contains four phenotypes: Am, Ho, BP and MG (Fig. 1B-E). The somas of Am (Fig. 1C) and Ho (Fig. 1E) are on opposite sides of the INL, with processes that extend laterally into the inner plexiform (IPL) and outer plexiform layers (OPL), respectively. BP (Fig. 1D) and MG (Fig. 1B) both extend radial processes, but can be reliably distinguished because the former terminate in the plexiform layers whereas the latter extend beyond, to the outer and inner limiting membranes. RGCs (Fig. 1A) are easily identified by their cell bodies in the ganglion cell layer (GCL), their processes in the IPL and their single axons extending into the nerve fiber layer. The one distinction that could not be based on morphology is between RPr and CPr (Fig. 1F,G). For this, we used an antibody, 4D2 (kindly provided by R. Molday), that labels RPr only. As the somas of photoreceptors are confined to the

outer nuclear layer (ONL), RPr and CPr are readily distinguished by 4D2 immunoreactivity (RPr) (Fig. 1F), or its absence (CPr) (Fig. 1G). Experiments on dissociated retinal cells from stage 41 tadpoles confirm no cross-reactivity of this antibody with a cone-specific marker (Chang and Harris, 1998) and give an expected 1:1 ratio of RPr to CPr (data not shown).

GFP-labeled cell clusters are clones

GFP⁺ profiles in the mature retina were usually found in aggregates (Fig. 2), suggestive of a lineage relationship. These clusters occurred at low density (Fig. 2A-C) and, if more than one was present in an eye, they were well separated. Individual cells within a cluster were radially aligned (Fig. 2A,B), although some tangential dispersion was seen (Fig. 2C). A criterion of six soma diameters distance was adopted for a profile to be included as a member of a cluster (Wetts and Fraser, 1988), although there was seldom any doubt.

The *gfp*-DOTAP injection dosage was titrated, seeking that which gave a high number of single clusters, and few with more than one cluster, per eye. At this dose 30% of injected embryos had no discernible GFP fluorescence in either eye. We eventually analyzed 111 tadpoles that demonstrated GFP fluorescence in at least one eye. Fig. 3A shows the frequency of cell clusters per eye. Of the 222 optic vesicles transfected, 134 (60%) eyes had 162 GFP-labeled cell clusters. Of the retinas with labeled cells, the majority contained only one cluster (Fig. 3A). Both the infrequent occurrence of eyes with labeled cells and the low number of clusters per eye indicated that the dose used was on the cusp between allowing or not allowing a transfection ‘hit’ and, when hit, a single occurrence was the predominant outcome.

To further demonstrate that the labeled cell clusters were clones, we compared the size distribution that we obtained with that using a more traditional technique of lineage tracing, single cell injection (Holt et al., 1988). The distribution of clone sizes (Fig. 3B) was very similar across techniques. Each demonstrated a median cluster size

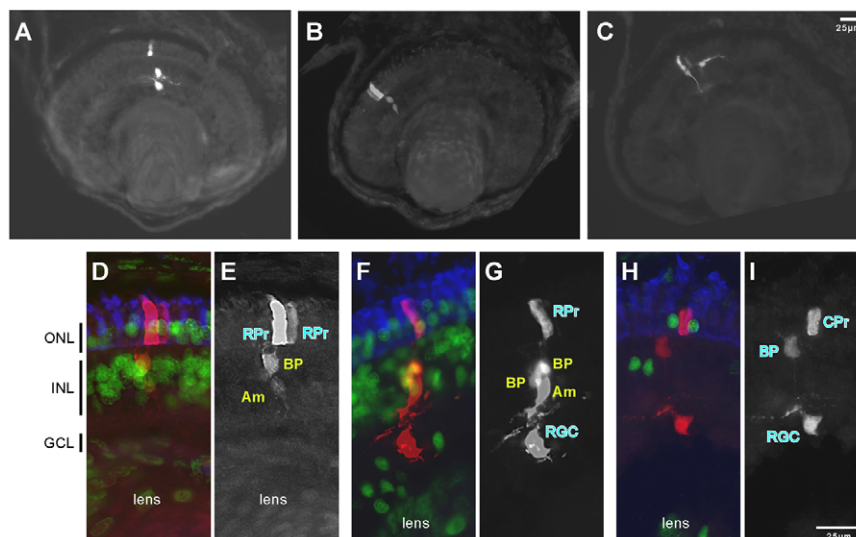


Fig. 2. Retinal sections of stage 41 *Xenopus* containing clusters of GFP-labeled cells. Sections were immunolabeled as in Fig. 1. (A,B,C,E,G,I) Monochrome images showing GFP labeling only. (A) BrdU injected at stage 30. This cluster has at least one member in each of the retinal layers, and they are tightly radially aligned. (B,C) BrdU injected at stages 33/34 (B) and 30 (C). Clusters contain radially aligned cells in the ONL and INL, except C, where one cell exhibits some tangential dispersion. (D-I) Pairs of images from the same clone; E,G,I show only the GFP label allowing identification of cell phenotype. The BrdU labeling of each cell is indicated by the color of its identifier: yellow, BrdU⁺; blue, BrdU⁻. (D,E) BrdU injected at stages 33/34. In Clone #146, the RPr were post-mitotic at the time of BrdU administration, but the BP and Am were not yet post-mitotic. (F,G) BrdU injected at stage 31. In Clone #90, an RGC and an RPr are BrdU⁻ whereas two BP and an Am are BrdU⁺. (H,I) BrdU injected at stages 33-36. In Clone #153, all members are BrdU⁻.

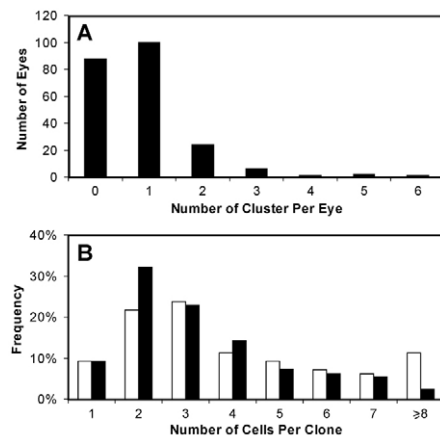


Fig. 3. Features of GFP-labeled clusters suggest that members are progeny from individually labeled retinal progenitor cells. (A) Bar chart of the number of GFP-labeled clusters per eye. Almost as many eyes did not have GFP-labeled cells as had them. The proportion with more than one cluster decreased dramatically. (B) Bar chart of our data (black) obtained using DNA lipofection on the size of GFP lineage-labeled cell clusters compared with data (white) produced by intracellular injection of HRP (Holt et al., 1988). All cell cluster parameters for the two studies are similar, indicating that both successfully label cell clones.

of three cells, and a positive skew (Fig. 3B). Based on many lines of evidence, we conclude that the GFP-labeled cell clusters in this study can justifiably be regarded as clones.

The developmental staging of RPCs is heterogeneous throughout retinogenesis

To assess progenitor developmental stage relative to embryonic stages, we administered BrdU at various time points after lineage labeling and examined the proliferative history of RPCs. We define retinogenesis trimesters (Fig. 4A) as early, when more than 66% of the cell population is BrdU⁺, middle, when between 66 and 33% of cells are BrdU⁺, and late, when less than 33% of cells are BrdU⁺. Fig. 4A gives the timing of lineage and BrdU labeling in the context of hours post-fertilization (top), traditional developmental stages (Nieuwkoop and Faber, 1967) (middle) and the mitotic index [bottom, modified from Holt et al. (Holt et al., 1988)].

The proportion of BrdU⁺/GFP⁺ cells showed a gradual decrease from early (62%) through middle (41%) to late periods of retinogenesis (31%) (Table 1; Fig. 4B) ('all cell types' histogram). However, while a large proportion [45% (22/49)] of clones contained exclusively BrdU⁺ constituents early in retinogenesis (Table 2), 31% (15/49) were composed exclusively of BrdU⁻ profiles, and 24% (12/49) had mixed BrdU⁺/BrdU⁻ labeling. These data indicate that at this early period about half of RPCs had yet to begin to produce post-mitotic daughters, whereas one-third had

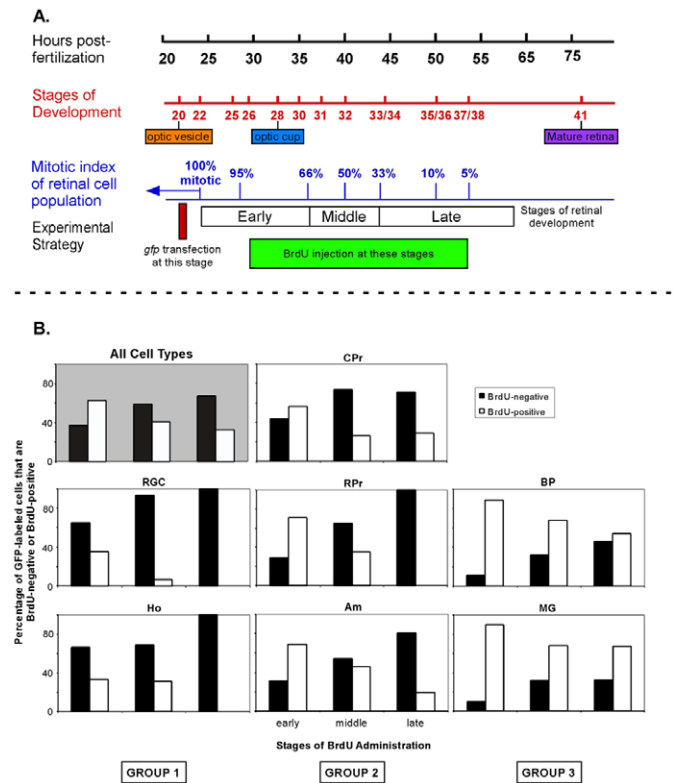


Fig. 4. Mitotic landmarks of retinal development in *Xenopus laevis* and the pattern of genesis of the seven major cell types at the population level. (A) The timing of *gfp* transfection and BrdU injection according to three different scales: (1) hours post-fertilization at 22–24°C; (2) embryonic stage according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967); and (3) mitotic index of retinal cell population [modified from Holt et al. (Holt et al., 1988)]. (B) The frequency of BrdU⁺ and BrdU⁻ cells of each phenotype for three stages of retinogenesis. Every cell type was generated at early and middle periods, and all but RGC, Ho and RPr were produced at the late period.

already reached the end of their proliferative life. Similarly, during the middle period, 19% of clones had yet to produce a post-mitotic daughter, whereas 49% had generated all their progeny. Even at the late period, we found that 7% of clones were composed of all BrdU⁺ profiles, even though 55% were mitotically arrested (BrdU⁻). These data indicate that RPCs at early, middle and late developmental stages coexist at all stages of retinogenesis.

Most cell types are born at all stages of development

It is possible that the clones containing all BrdU⁻ cells from early stage injections produce only cell types born early, whereas those containing all BrdU⁺ cells from middle period injections produce

Table 1. The mitotic index of GFP-labeled retinal cells from the early, middle or late retinogenesis period is consistent with how each period is defined

BrdU administration		BrdU ⁺ GFP-labeled cells		BrdU ⁻ GFP-labeled cells		Total <i>n</i> cells
Stage		<i>n</i>	%	<i>n</i>	%	
Early	26-30	89	62	54	38	143
Middle	31-32	91	41	131	59	222
Late	33-38	55	31	121	69	176
Totals		235		306		541

only late-generated phenotypes. Fig. 4B shows the percentage of each cell type that are BrdU⁺ or BrdU⁻ at early, middle and late periods of retinogenesis. We found that some individuals of all cell types were post-mitotic (BrdU⁻) at any stage of development. Further, but for RGC, Ho and RPr, of which genesis had ceased by the late period, some individuals of any other phenotype were BrdU⁺ at all stages. These data showed that at early and middle periods of development, all cell types were being generated, and even at the late period, all but three types were still being produced. Further, we observed that the timing of peak genesis tended to segregate cell types into three groups: Group 1, RGC and Ho, tend to be early; Group 2, CPr, RPr and Am, are predominately middle stage; and Group 3, BP and MG, tend to be generated late (Fig. 4B). These groupings suggest that cell production in *Xenopus* occurs in three highly overlapping phases. Such phases have been observed in other vertebrate retinas (La Vail et al., 1991; Rapaport et al., 2004). To determine whether the significant overlap in the timing of retinal cell birth of different cell types in *Xenopus* is genuine or reflects heterogeneity of the developmental schedule of each RPC that may have masked a sequence, we analyzed the birth order within individual RPCs.

Clonal analysis reveals that retinal cell genesis follows a common sequence

Fig. 5 shows a graphical display of 61 RPC clones. Each clone is represented by a row, with the boxes representing each cell type. If a box has a circle in it, it means that the clone has at least one cell of that phenotype, and if the circle is white it means that all of them are BrdU⁺. A black circle means all clone members of that phenotype are BrdU⁻. Finally, a half-black half-white circle indicates more than one cell of that phenotype in the clone, some BrdU⁻, some BrdU⁺. The number of BrdU⁻ and BrdU⁺ cells, if greater than one, is indicated to the left and right of the circle, respectively.

Five of the clones shown contain exclusively BrdU⁺ cells (top), and five BrdU⁻ cells (bottom). These were randomly selected from the larger cohort with these labeling patterns. (For a complete list of clones, BrdU injection times and other details, see Table S1 in the supplementary material.) Between them are all 51 heterogeneous clones, those that contain BrdU⁺ and BrdU⁻ cells. These clones provide information on the order of genesis of retinal cell types. Take, for example, clone #90 (asterisk), which is illustrated in Fig. 2G. In this five-cell clone an RGC and an RPr are BrdU⁻, while an Am and two BP are BrdU⁺, indicating that the RGC and RPr were post-mitotic, whereas the RPC, at the time of BrdU administration (stage 31), had yet to produce the Am and BP. Similarly, in clone #146 (asterisk; shown in Fig. 2E), the two RPr, both BrdU⁻, were born before the Am and the BP, both of which are BrdU⁺. Each clone provides a ‘snapshot’ of the ordinal relationships between two or more cell types. As we consider more and more clones, the full picture of the cell birth relationships in the frog retina becomes clear, and tells an unambiguous story, that a sequence of genesis is apparent at the clonal level of resolution. If a clone has one or more

BrdU⁺ RGCs, then all other clone members are BrdU⁺ (10 of 10 clones) (see Table S1 in the supplementary material). Similarly, if one or more MG is BrdU⁻, then all other clone members are as well (12 of 13 clones, see Table S1 in the supplementary material; exception, clone #148). These data indicate that RGCs are the first cells in the frog retina to be generated, and MG the last. The columns, which represent each cell type (Fig. 5) are arranged, and produce a pattern whereby white circles transition to black circles, sweeping from the superior right corner to the inferior left. If the cell birth relations between individuals within a clone were random, then no matter how we arranged the rows or columns we would not be able to achieve a smooth transition from BrdU⁺ to BrdU⁻ cells. The pattern allows us to assign ordinal relationships between cell types. For example, 17 heterogeneous clones containing at least one BrdU⁺ Am had BrdU⁻ RGC (6), Ho (5), CPr (2) and RPr (5), but all the BP (6) and MG (3) were BrdU⁺. This indicates that Am are generated between these two groups. Performed for all heterogeneous clones, this analysis allows us to recognize the ordinal birth relationships between each phenotype. Specifically RGC is always the first cell type to become post-mitotic, followed by Ho, then CPr, then RPr, then Am, then BP, and finally MG.

Among the 51 clones with heterogeneous BrdU labeling, there were only four with ordinal relations that did not fit this sequence (arrows, Fig. 5). In clone #94, at the time of BrdU administration, the Ho was not yet born (BrdU⁺), but the CPr had been generated (BrdU⁻). Clones #59 and #44 contained more than one occurrence of two ordinally adjacent cell types, some BrdU⁺ and some BrdU⁻. In these cases at least one Am was post-mitotic when at least one RPr was still mitotic. Finally, in clone #148, three out of five MG were generated (BrdU⁻) before the single BP (BrdU⁺). In all four of these clones, all other cell types displayed BrdU labeling consistent with the overall sequence of genesis. It is possible that these few outliers indicate some variability in the order of genesis. However, such variation would seem to be very limited as it is always between adjacent phenotypes in the cell birth sequence. Although they need to be explored further, the rarity of ‘out-of-order’ events makes them difficult to study. Their occurrence should not be the cause for rejecting the robust finding that the great majority of observations indicate that cell genesis order in the frog retina is regular, rigid and unidirectional.

The rigidity of cell birth sequence is further demonstrated in a quantitative analysis. We made pairwise comparisons between two cell types under the condition: when one was 100% BrdU⁻ (reference cell type) and the other 100% BrdU⁺ (comparison cell type). The results are presented in a matrix (Fig. 6). The diagonal black-filled rectangles give the number of clones with all individuals of that (reference) phenotype BrdU⁻. The other rectangles in the row give the quantity of the reference clones in which all members of that type (comparison) are BrdU⁺. For example, there are 49 clones that contain exclusively BrdU⁻ RGCs. Of these, five contain all BrdU⁺ Ho, five all BrdU⁺ CPr, and so on. This shows that all six non-RGC cell types are born after RGCs, and this is the only cell type for

Table 2. The developmental schedule of GFP-labeled RPCs is highly heterogeneous

BrdU administration	Clones containing BrdU ⁺ cells only		Clones containing BrdU ⁻ cells only		Clones containing BrdU ⁺ and BrdU ⁻ cells		Total <i>n</i> clones	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%		
Stage								
Early	26-30	22	45	15	31	12	24	49
Middle	31-32	13	19	33	49	22	32	68
Late	33-38	3	7	25	55	17	38	45
Totals		38		73		51		162

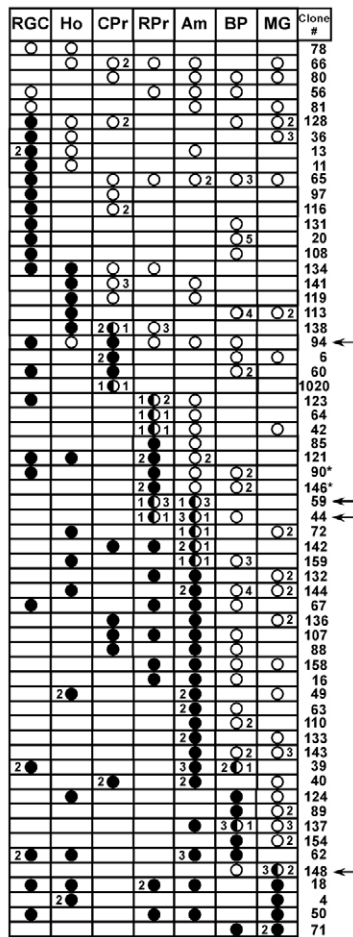


Fig. 5. Representation of 51 clones containing heterogeneous BrdU labeling, plus five each of randomly selected clones with all BrdU⁺ and all BrdU⁻ profiles. When the retinal phenotypes (columns) are ordered as shown, we see a smooth transition from white (BrdU⁺) to black (BrdU⁻) that sweeps from the top right to the bottom left. Empty box, no cells present; white circle, all cells BrdU⁺; black circle, all cells BrdU⁻; half-black half-white circle, some cells BrdU⁻, some BrdU⁺. Arrows indicate the clones that do not fit the overall sequence. Asterisks indicate the clones shown in Fig. 2D-G.

which this can be said. In another example, there are 41 clones containing exclusively BrdU⁻ RPr. In these there are no RGC, Ho or CPr that are BrdU⁺, indicating that these cell types were born before or at the same time as RPr. However, these clones do contain Am, BP and MG that are BrdU⁺. These cell types must be born after RPr. For some pairwise comparisons the numbers are small: specifically, those between CPr and RPr, and CPr and Am, are limited to only one example each. However, the fact that these comparisons fit so well in the overall context based on many more pairwise comparisons gives us confidence in them. The ‘Totals’ in the right and left columns indicate for each reference phenotype the number of comparisons that fit and do not fit, respectively, our cell production sequence. Of 89 comparisons, we find only a single incongruent case (clone #94). χ^2 tests comparing these data to what would be expected if there were no order to cell genesis were significant at $P < 0.01$ or less for all rows, except the BP-MG comparison, which failed to reach this significance level, probably because of the small number of these pairs. However, when the reversed matrix was considered (reference cell type all BrdU⁺, and comparison cell type

	Total	RGC	Ho	CPr	RPr	Am	BP	MG	Total
RGC	0	49	5	5	2	6	9	3	30
Ho	0	0	26	3	2	3	3	5	16
CPr	1	0	1	36	1	1	5	3	10
RPr	0	0	0	0	41	4	6	2	12
Am	0	0	0	0	0	44	9	8	17
BP	0	0	0	0	0	0	23	3	3
MG	0	0	0	0	0	0	0	12	3

Fig. 6. A matrix of pairwise comparisons of clones where one phenotype (reference) is 100% BrdU⁻, but the others (comparisons) are 100% BrdU⁺. This analysis reveals that the order of retinal cell birth is highly regular. Black boxes indicate the number of clones of the reference cell phenotype, i.e. the number of clones that exclusively contain BrdU⁻ cells of this phenotype. White boxes indicate the number of clones of the comparison cell phenotype, i.e. the number of comparison clones that exclusively contain BrdU⁺ cells for a given reference phenotype. For example, of the 26 clones for horizontal cells (all Ho BrdU⁻), there are three cone clones (all CPr BrdU⁺).

all BrdU⁻; data not shown), the sequential relationship between BP-MG was highly significant ($P < 0.001$). χ^2 tests on paired cell types with ordinal relations (i.e. RGC-Ho, Ho-CPr, etc.), that further account for clone size, were all significantly different from random ordering at the $P < 0.01$ level. Both graphical and quantitative analyses demonstrate that retinal cell genesis in the frog follows a regular and consistent order within clones.

DISCUSSION

Retinal progenitor cells are heterogeneous

Variation in a number of clone parameters demonstrates that RPCs are heterogeneous. Among these are clone size, clone composition and progress through development relative to the developmental stage of the embryo. Concerning clone size, the majority in the frog retina contained two to three cells, but four had eight or more (Fig. 3B). Our clone size histogram matches well that reported for this species using intracellular injection to label lineage (Holt et al., 1988) (Fig. 3B). Many factors influence clone size, including the length of time that RPCs remain proliferative, cell cycle timing and mode of cell division. Given that the proliferative life of a *Xenopus* RPC is maximally 48 hours, and cell cycle time is about 12 hours (Rapaport, 2006), 1-16 cells per clone would be expected.

As in previous studies (Wetts and Fraser, 1988; Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990), we had a number of one-cell clones, which raises the question as to how they are produced. Do they reflect a technical limitation? Their ubiquity suggests otherwise. The only way to produce a one-cell clone involves apoptosis, and the simplest mechanism is either for an RPC to die after it produces a single post-mitotic daughter, or for one of a pair of post-mitotic sisters to die. Cell death is a feature of the developing retina, including RPCs (Voyvodic et al., 1995). Despite its occurrence, apoptosis is unlikely to affect the conclusions of this study. For it to produce apparent cell birth order, or to vary the sequence, it must be targeted to specific phenotypes at specific times, and no evidence for such ‘directed’ cell death has ever been seen in the retinas.

We see, as others have, many two-cell clones. These are significant because they could represent daughters of a symmetric, terminal mitosis that, if RPCs pass sequentially through single competence states, might be expected to be composed of two cells of the same phenotype. However, this was clearly not the case (see Fig. S1 and Table S1 in the supplementary material). Rather, by far the most common pairing in two-cell clones is one cell along on the

sequence of cell genesis. Cell death may contribute to formation of heterogeneous two-cell clones. However, these data are also consistent with the finding that not all retinal cells are determined and/or committed at the time they leave the cell cycle (Belecky-Adams et al., 1996). It follows that competence extends into post-mitotic stages – so long as the post-mitotic cell is undetermined, the competence clock continues to run. Thus a heterogeneous two-cell clone could arise by a terminal division in which one daughter is determined at cell division (perhaps by asymmetric segregation of cellular components) and the other sometime later while in a subsequent competence state. The fact that two-cell clones are very commonly composed of two phenotypes that are immediate neighbors in the sequence of genesis (see Fig. S1 in the supplementary material) supports this idea.

Confirming the findings of others (Wetts and Fraser, 1988; Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990), we find that the output of RPCs is highly variable. Clones containing a variety of combinations of two or more phenotypes are observed (Fig. 5; see Tables S1 and S2 in the supplementary material) supporting the idea that, as a population, RPCs are not restricted or limited in their ability to produce any cell type(s) at the onset of retinogenesis. However, cell type composition within clones is not altogether random (Alexiades and Cepko, 1997). Similarly, the cell type associations within our clones demonstrate some preferential pairings (see Table S2 in the supplementary material). For example, clones that contain RPr will also tend to include Am, and clones containing RGCs have a lower probability of including MG. We are not certain of the cause of high- and low-probability associations, but we suggest that the explanation may include factors such as the proximity of cells in the sequence of genesis, the proportion in the retinal population, and exposure to environmental cues that may allow or prohibit the production of cell type(s).

A significant and surprising heterogeneity of RPCs is seen in their developmental staging. RPCs at the same embryonic age can be on very different developmental schedules, even at extremes of their proliferative capacity (Table 2). Thus, a clone of all BrdU⁺ constituents can neighbor one with all BrdU⁻ members. This heterogeneity probably accounts for the failure of earlier studies to detect sequential cell genesis in the frog (Holt et al., 1988; Stiemke and Hollyfield, 1995). These studies analyzed the population of retinal cells, which does not provide the resolution to reveal a sequence against the ‘noise’ of the variability in developmental timing we herein observe. Further, heterogeneity of RPC timing suggests that the environment in which they grow does not determine their schedule. Rather, it supports the hypothesis that the temporal shifting of cellular competence is governed by a program intrinsic to each RPC. Finally, given the heterogeneity of RPCs on many parameters, it is perhaps not surprising that they also exhibit significant heterogeneity in gene expression (Trimarchi et al., 2008). The challenge will be to relate gene expression differences to different properties of RPCs and the clones they produce.

In a retinal clone certain phenotypes are always generated before others

Clones made up of a mixture of BrdU⁻ and BrdU⁺ cells capture a ‘snapshot’ of the genesis relationship(s) between these phenotypes. These snapshots indicate that cells in a clone are not generated in a stochastic order. Were this the case, a two-cell clone of heterogeneous BrdU labeling would just as likely have one cell BrdU⁺ as the other. However, we observed that the potential identity of BrdU⁺ profile(s) of such a pair was limited by the identity of the BrdU⁻ profile. For example, six clones that had at least one BrdU⁻ Am had BrdU⁻, but

not BrdU⁺ RPr. However, 11 clones with at least one BrdU⁻ Am (some the same clones) had BrdU⁺, but not BrdU⁻ BP. In these cases, Am are generated after RPr, but before BP. No case exhibiting different ordinal relationships between these three cell types was seen.

The sequence of retinal cell genesis can be derived from the ordinal relationships within clones

Whereas clones give a ‘snapshot’ of the ordinal relationships of genesis between cell types, the totality of these pictures provides a coherent ‘movie’ of the entire retinal population. Specifically, our data establish that retinal cell genesis follows a defined sequence: RGC→Ho→CPr→RPr→Am→BP→MG. This sequence is rigid in that there is almost no variation in any of these ordinal relationships. Further, although cell genesis is dynamic and temporally shifting, it shifts only in one direction. Once an RPC generates a phenotype different from those previously produced it never goes back to generate the earlier cell types again. This finding validates an earlier suggestion that competence shifts unidirectionally (Cepko et al., 1996). These data do not accord with a model whereby RPCs possess multiple competence states simultaneously to form some subset of cell types. Were this model to operate one would expect consistent ordinal relationships between some cell types, but not between others. For example, if the phases of retinal cell birth reported in a number of vertebrates (Harman and Beazley, 1989; La Vail et al., 1991; Rapaport et al., 2004) represent competency periods for subsets of cell types, we would have expected to see variable ordinal relations within, but invariant relations between, phases. We always observe rigid ordinal relationships and this accords only with the stepwise model for shifting competence states of RPCs, a cell type at a time.

The order of cell genesis in *Xenopus* retina apparent at the clonal level is very similar to that of other vertebrates [wallaby (Harman and Beazley, 1989); monkey (La Vail et al., 1991); rat (Rapaport et al., 2004)]. The only difference is that RPr are generated in close step with CPr in *Xenopus*, whereas in the wallaby, rat and non-foveal region of monkey retina, they are generated late, after Am. We collected only two heterogeneous clones (94 and 138) that directly compared RPr and CPr (Fig. 5). This low number probably reflects the fact that these cell types are generated in close step so that there is a very narrow window for BrdU administration to reveal the birth order between these two cell types. When we administered BrdU at stage 22, and identified unlabeled (post-mitotic) cells in the ONL at stage 41, we found unlabeled CPr in a 4:1 ratio with unlabeled RPr (unpublished observation). This is consistent with Chang and Harris’s (Chang and Harris, 1998) report that, despite significant overlap in the genesis periods of RPr and CPr in *Xenopus*, the first CPr were born before the first RPr. In the cone-dominated chick retina (60% CPr, 40% RPr), CPr and RPr were generated before cells found in the INL (Belecky-Adams et al., 1996). The addition of an amphibian to the database of retinal cell genesis sequences adds weight to the observation that they are highly similar in all vertebrates, possibly conserved during evolution. However, the relatively minor interspecies differences in ordinal position, particularly for CPr and RPr, may reflect adaptation of cell production to lifestyle demands of the species. The more RPr to be made, the later in development the peak of RPr genesis occurs. Even though we do not think the demand on cell production in terms of numbers affects the rigidity of their production, demonstration of a rigid cell genesis sequence in other vertebrates will substantiate the hypothesis that mechanism(s) for regulating cellular competence in retinal development is conserved.

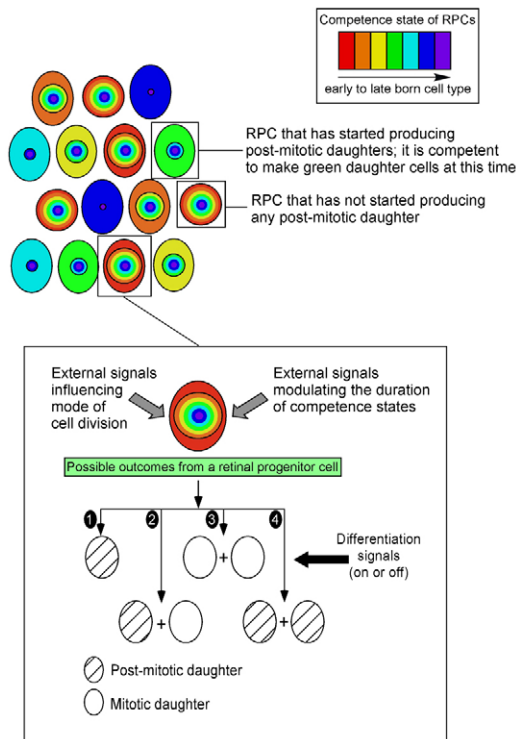


Fig. 7. Model for retinal cell type specification and production.

Our model for retinal cell type specification and production accounts for the heterogeneity of RPC development and invokes the stepwise change of cellular competence for the primary control of the orderly production of cell types found in the vertebrate retina.

Mechanisms for RPC competence and a model

How is competence regulated during neurogenesis? In *Drosophila* embryonic and larval neurogenesis, temporal regulation of transcription factors suppresses or promotes cellular competence (Isshiki et al., 2001; Zhu et al., 2006). Four transcription factors are expressed in rigid sequence (Hunchback→Krüppel→Pdm→Castor) as embryonic neuroblasts produce daughters that can either commit to a specific neuronal lineage or continue to divide. Indeed, a single division can give both outcomes – a commitment with the daughter maintaining expression of the transcription factor, becoming determined and differentiating, or a progression with the other daughter remaining in the cell cycle, shifting expression to the next transcription factor and establishing a unique lineage of different cell types. In development of post-embryonic *Drosophila* brain, competence of neuroblasts appears to be governed by the concentration of another transcription factor, Chinmo. High levels lead to the production of neuron types that are born early, whereas low to medium levels lead to late-born types. As these transcription factors can regulate cell fate determination temporally without specifying particular fate(s) they are thought to be ‘competency genes’. Despite their identification in *Drosophila*, the regulation of their activity is still poorly understood. Nevertheless, the rigid sequential formation of cell types that is produced by regulating competency is very similar to what we see in the frog retina. The molecular mechanism(s) for competence regulation in invertebrate neurogenesis may be conserved in the vertebrate CNS. An ortholog of the gene *hunchback*, a player in the competence cascade in *Drosophila*

neurogenesis, called *ikaros* (*IKAROS* family zinc finger 1 – Mouse Genome Informatics), has been shown to be necessary and sufficient to produce early-born cell types (RGC, Ho and Am) in the developing mouse retina (Elliott et al., 2008).

We do not yet understand the mechanism for maintaining and changing retinal cell competence. However, we know that it does not involve counting cell divisions because embryos in which cell division was inhibited during retinogenesis form at least some cell types in the retina (Harris and Hartenstein, 1991). This observation supports the presence of a clock or program for shifting competence in post-mitotic neurons before determination and differentiation. We speculate that cellular activities such as protein and/or RNA accumulation or degradation may be a mechanism for shifting competence. This is seen in rat optic nerve oligodendrocytes, in which an intrinsic clock counts time (not cell divisions) to differentiation (Gao et al., 1997), and the concentration of the cyclin-dependent kinase p27 (Kip1) is likely to be part of the counting mechanism (Raff, 2007).

We propose a model in which retinal cell competence regulates cell fate determination. Fig. 7 depicts a pool of heterogeneous RPCs, each at a unique developmental stage that cannot be predicted by its neighbors, but partly defines its competence state. The shape of the RPC indicates their developmental stage: circular, yet to make post-mitotic daughters; oval, producing post-mitotic daughters. The temporal switching of cellular competence is intrinsic to RPCs and their undetermined post-mitotic daughters, and exerts control over the fate of these cells. This does not imply that external environmental cues are uninvolved. They may act as permissive signals that allow or prohibit the production of a cell type and may control the number of each type produced. For example, extrinsic signals may affect the mode of cell division (Mu et al., 2005; Poggi et al., 2005). The presence or absence of differentiating factors or inhibitors can regulate the timing of commitment and/or differentiation (Cepko et al., 1996; Zhang and Yang, 2001; Kay et al., 2005). Finally, the duration of a competence state can be modulated during retinal development (Kim et al., 2005). In our model (Fig. 7), intrinsic cellular competence acts as an instructive signal, whereas external environmental signals act as permissive signals for retinal cell type specification and production. An RPC may be multipotent over its proliferative life, but is competent to make only a single cell type at a given time.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/10/1707/DC1>

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