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Original Article

Lateral and radial growth uncoupled in reaggregated retinospheroids of embryonic avian retina

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ABSTRACT According to an earlier presented model (Layer and Willbold, *Int. Rev. Cytol. 146:* 1-47, 1993), growth of the retina can be conceived of as an areal increase of an epithelial tissue sheet ("lateralization") plus a concomitant establishment of the layered retina ("radialization"). To provide further support for this model, here we have reaggregated dissociated retinal plus pigmented cells from chick or quail embryos and observed their development into histotypic three-dimensional spheres in rotation culture. These so-called stratospheroids consist of a continuous fully laminated retinal part with a coiled-up pigmented epithelial core. Using BrdU-labeling, we show that radial growth, i.e. the sequential production of cell types in spheroids, is comparable to normal vitreal-scleral retinogenesis. The region next to the pigmented epithelial core represents a «lateral growth zone» (equivalent to an ora serrata *in vivo*), where mitotic cell numbers are highest, even when in the laminated part proliferation has already ceased. Gradients of lateral differentiation emanate from this growth zone into the retinal tissue, as revealed by immunostaining of the photoreceptor protein opsin and the cell recognition molecule F11. Moreover, we found that stratospheroids derived from older embryos consist only of a hollow monolayered neuroepithelium which develops in the absence of any radial growth. This indicates that cell production is sustained longer in lateral than in radial direction. These differently staged stratospheroids will be excellent models to characterize genes involved in the regulation of lateral and radial growth processes.

KEY WORDS: *retina, F11-antigen, BrdU, proliferation, regeneration*

Introduction

The mature retina can be conceived of as a sheet of tissue composed of many radially stacked cell columns, which are considered as the structural building units of a retina (Turner and Cepko, 1987; Holt *et al.*, 1988; Wetts *et al.*, 1989; Turner *et al.*, 1990). Cells contained within a column are clonally related, implicating radial migration of cells (Williams and Goldowitz, 1992; Reichenbach *et al.*, 1994; Willbold *et al.*, 1995) although lateral migration of cells has been also observed (Fekete *et al.*, 1994; Reese *et al.*, 1995). Even if we knew how columns are specified, this would not tell us how the retina increases its areal size in time and space from a monolayered neuroepithelium, thereby always being neatly placed between the vitreous body and the pigmented epithelium within the growing eye cup. For the developing neocortex, Rakic has presented the "radial unit hypothesis", according to which a first period of symmetric cell divisions will determine the areal size, thereby providing the founder cells for the ontogenetic columns (Rakic, 1988, 1995).

How is areal versus radial growth and the overall number of neural and glial cells controlled and regulated in the retina? The mechanisms regulating retinal growth in different vertebrate species are not understood. In fishes, amphibians and reptiles the eye cup and its constituents continue to grow throughout life, while in birds and mammals it approaches its final size during embryogenesis. In general, after invagination of the early eye anlage, the pigmented epithelium and the retinal neuroepithelium form a double-sheeted structure. Both epithelia, which make intimate back-to-back contact, have the same origin and remain fully continuous with each other (see Fig. 1A). As development proceeds, the retina will increase its radial width to a final thickness of about 200 μm, while the pigmented epithelium remains a monolayered sheet.

To study these mechanisms, a reaggregation culture model producing so-called stratospheroids is most helpful. This experimental system exploits the regenerative capacity of the embryonic

Abbreviations used in this paper: BrdU, 5-bromo-deoxyuridine; div, days *in vitro*; E, embryonic day; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RPE, retinal pigmented epithelium.

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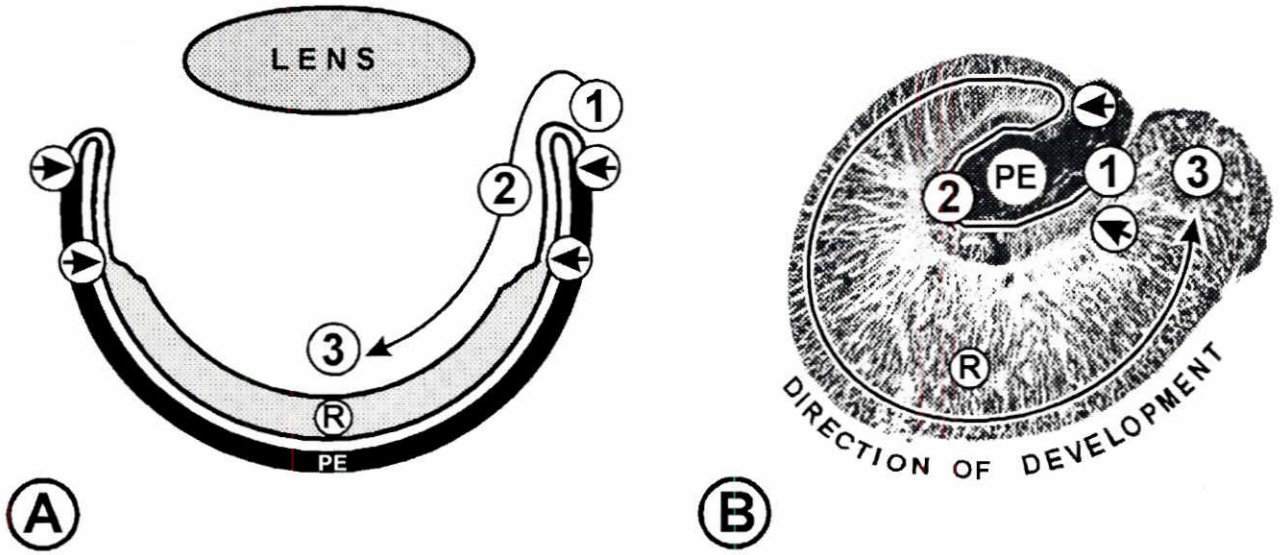

Fig. 1. Structure of an eye cup compared with stratospheroids. *Schematic drawing of an eye cup* **(A)** *and a computer-digitized image of an eosin/hematoxylin-stained paraffin section of a chick stratospheroid (starting from E6) after 14 days in culture* **(B)**, *to correlate the different tissue parts in vivo and in vitro. The pigmented epithelium (PE; dark center of stratospheroid) is condensed to form a cluster from which the neural retina emerges (R) as a monolayered neuroepithelium (its origin is marked by a «1»; its course is followed by an arrow). This monolayered neuroepithelium (region «2»; its beginning and ending are marked by encircled arrows) back-loops, becomes thicker and multilayered to form the differentiated retina («R»; «3»). Three-dimensionally, this continuous structure is coiled up like a snail's shell. Therefore, the developmentally youngest part of the retinal regenerate ensheathes the pigmented core, while developmentally older parts may approach the pigmented core (without contacting it) at the opposite side. (Encircled 1: transition zone between PE and monolayered neuroepithelium; encircled 2: monolayered neuroepithelium; encircled 3: differentiated retina; encircled arrows: beginning and ending of neuroepithelium; compare also Fig. 7).*

chicken retina under rotation culture conditions (Layer and Willbold, 1993, 1994). Stratospheroids are three-dimensional cellular spheres which closely resemble embryonic retinas, since they develop all cellular and plexiform layers with their cell types arranged in the correct architecture (Fig. 2). Unlike explant cultures, they are produced from dissociated cells and cell clusters derived from the ciliary margin, including neuroepithelial and pigmented epithelial cells. The neuroblasts are capable of re-entering an intense phase of cell proliferation; more than 80% of the spheroids' cells are generated in culture (Vollmer and Layer, 1986b, 1987; Willbold and Layer, 1992b).

Based on a long series of experiments using this system, we presented earlier a model that divides the entire development of the retina into three basic steps (Layer and Willbold, 1993, 1994). According to this model, the growth of the retina can be conceived of as an areal increase of the epithelial tissue sheet (which we call "lateralization") plus a simultaneous establishment of the layered retina (which we call "radialization"), followed by processes of "lamination". The noted gradients of retinal differentiation – scleral-vitreal and central-peripheral – reflect these two growth dimensions (Layer and Kotz, 1983; Liu *et al.*, 1983; Prada *et al.*, 1991). Accordingly, the rates of thymidine uptake and of self-renewal of stem cells first diminish near the center of the retina, while it is still pronounced towards the periphery (Dütting *et al.*, 1983).

Up to now, the available evidence from different studies supports our model, however, it is far from sufficient to fully validate it. With this study on stratospheroids, we try to present a set of data that focuses on all three parts of the model. In particular, we define radial growth patterns in spheroids by BrdU-immunocytochemistry. This part is much more extensive than what has been described in an earlier thymidine-uptake study (Vollmer and Layer, 1986b), now allowing us to describe the formation of individual cell layers. Then, lateral growth of the spheroids is investigated both by BrdU-uptake and expression of opsin and F11 antibody reactivity, revealing a distinct gradient of lateral growth in spheroids. Finally, the sustained proliferative activity in the neighborhood of the RPE, plus the disappearance of radial development in spheroids derived from older tissue are major steps to complement the model.

Results

Stratospheroids are in vitro regenerates of the embryonic retina

Stratospheroids originate from cells which are located *in vivo* in the eye margin. They are composed of pigmented and non-pigmented compartments (Figs. 1B, 2). The pigmented cells represent a prominent epithelium which is densely packed and coiled-up. Typically, it fills parts of the spheroid center (to replace the missing vitreous body), protruding to the surface at one pole. The retinal part of the spheroid emerges from the pigmented epithelium as a monolayered neuroepithelium which then becomes thicker and multilayered (Figs. 1B, 2). Thus, the whole stratospheroid is a continuous structure consisting of a pigmented and a retinal part. Since it is coiled-up like a snail's shell, the transition from RPE to the retinal part may be cut once or twice, depending on the particular sectioning direction (compare Figs. 1B, 2). The differentiation of layers and cell types in stratospheroids has been described elsewhere (Layer and Willbold, 1993, 1994).

Radial growth of stratospheroids: a vitreal-scleral gradient of proliferation

When placed in rotation culture, retinal stem cells derived from the eye margin re-enter an extended phase of cell proliferation (Fig. 3). During the first day in culture, only few BrdU-positive cells are found throughout the early stratospheroid, implicating that proliferation is still low. After two days in culture, most of the cells forming the pseudostratified neuroepithelium proliferate, reaching its maximal proliferation rate after five days in culture. At this stage, the cells at the outer rim of the spheroid, which later will become photoreceptors, are already postmitotic like most of the cells towards the center of the spheroid including varying numbers of prospective ganglion cells. After seven days in culture, the three nuclear and two plexiform layers can be clearly distinguished. The remaining BrdU-positive cells are located exclusively in the INL, particularly in its outer two thirds. After nine and twelve days in culture, proliferation slowly ceases and shifts towards the outer part of the INL. The last mitotic cells are found in a single row in the outer third of the INL.

Lateral growth of stratospheroids: a central-peripheral gradient of photoreceptor and fiber differentiation

In stratospheroids, the equivalent of a central-peripheral gradient of cellular and fiber differentiation can be easily demonstrated (Fig. 4). Differentiation of photoreceptor cells after eight days in culture is visualized by their expression of the photoreceptor-specific marker rhodopsin. In that part of the spheroid most distant to the pigmented core, opsin expression is clearly detectable, whereas approaching the pigmented epithelium the staining progressively diminishes (Fig. 4A). This demonstrates that the youngest part of the retinal tissue is located next to the pigmented epithelium. Staining of a stratospheroid after fourteen days in culture with the fiber-specific antibody F11 shows that OPL formation proceeds in a similar "central-peripheral gradient", which is indicated by the increasing thickness of the layer from the pigmented core towards the retinal part. Again, its formation has barely started in the neighborhood of the pigmented cells, as indicated by pearl-like patches, while its more mature parts are found further away (Fig. 4B). Thus, the developmentally youngest part of the stratospheroid is located next to the pigmented cluster, while its oldest part is more distant (note that, depending on the direction of sectioning, the younger retinal part can approach the pigmented epithelium again from the opposite side, since it coils up like a snail's shell).

Lateral growth is sustained in the vicinity of pigment epithelial cells: the ora serrata equivalent

Proliferation in the radial dimension has mostly ceased by nine days in culture (Fig. 3) and the vast majority of cells has already become postmitotic (Figs. 3, 5). However, a large number of BrdU-positive cells is still found in the vicinity of the pigmented core (Fig. 5; the actual numbers will depend on the spheroid's orientation during sectioning). Thus, proliferation remains highest near the pigmented epithelium, directly correlating with the "central-peripheral" differentiation gradient as shown above. Noticeably, proliferation near the pigmented epithelium is detectable throughout the culture period of about fourteen days, whereas *in vivo* cell proliferation stops around embryonic days E9-E10. Pigmented and retinal regions merge in this transition zone of the spheroid, which hence represents the point at which the mitotic neuroepithelium differentiates into a laminated retinal tissue. Therefore, this zone represents the *in vitro* equivalent of the ora serrata of a normal retina.

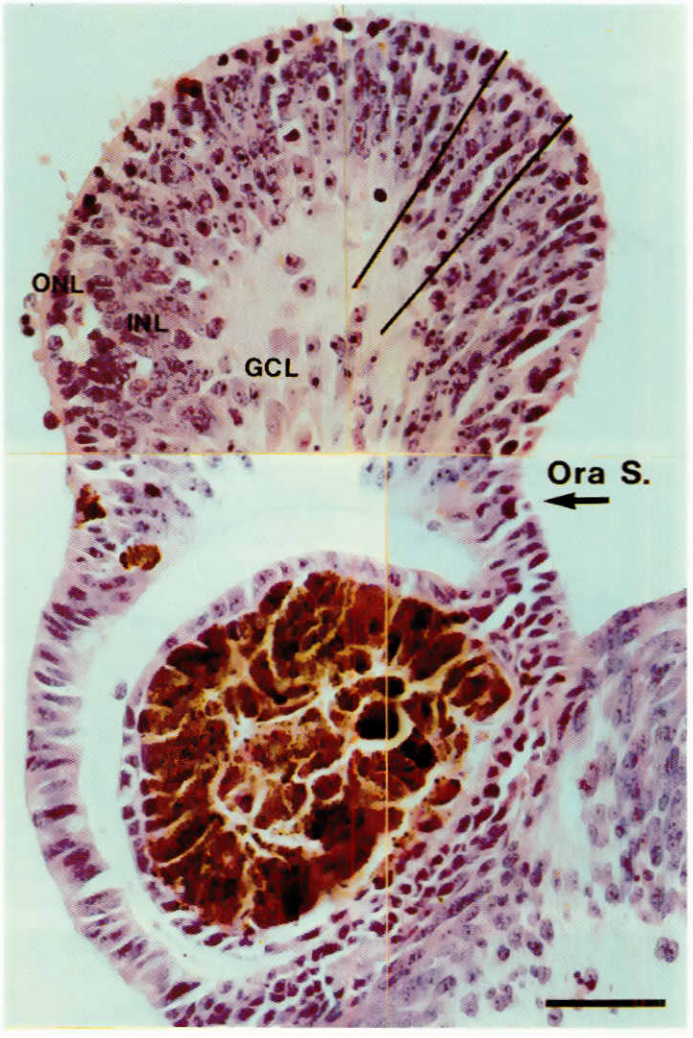

Fig. 2. Lateral growth zone in stratospheroids. *Histological structure of a stratospheroid which has developed from dissociated chick (E6) and quail (E5.5) cells after 14 days in culture, stained with eosin-hematoxylin. Here the transition zone (corresponding to the ora serrata in vivo) between the monolayered neuroepithelium emerging from the pigmented epithelium and the laminated neural retina can be easily recognized on both sides of the spheroid. The outer nuclear layer (ONL), the inner nuclear layer (INL) and some dispersed ganglion cells (GCL) can be clearly distinguished. The black lines delineate the borders between adjacent radial cell columns. Bar, 20 μm.*

Radial but not lateral growth is arrested in stratospheroids from older tissue

Using older tissue for production of stratospheroids provides direct evidence that lateral stem cell production can be maintained in the complete absence of any radial growth under our *in vitro* conditions. With increasing age of the embryonic tissue, the percentage of normally multilaminated stratospheroids decreases. Instead, another type of monolayered spheroid is detected, depending on the age of the original tissue and the preparation. In Figure 6, such

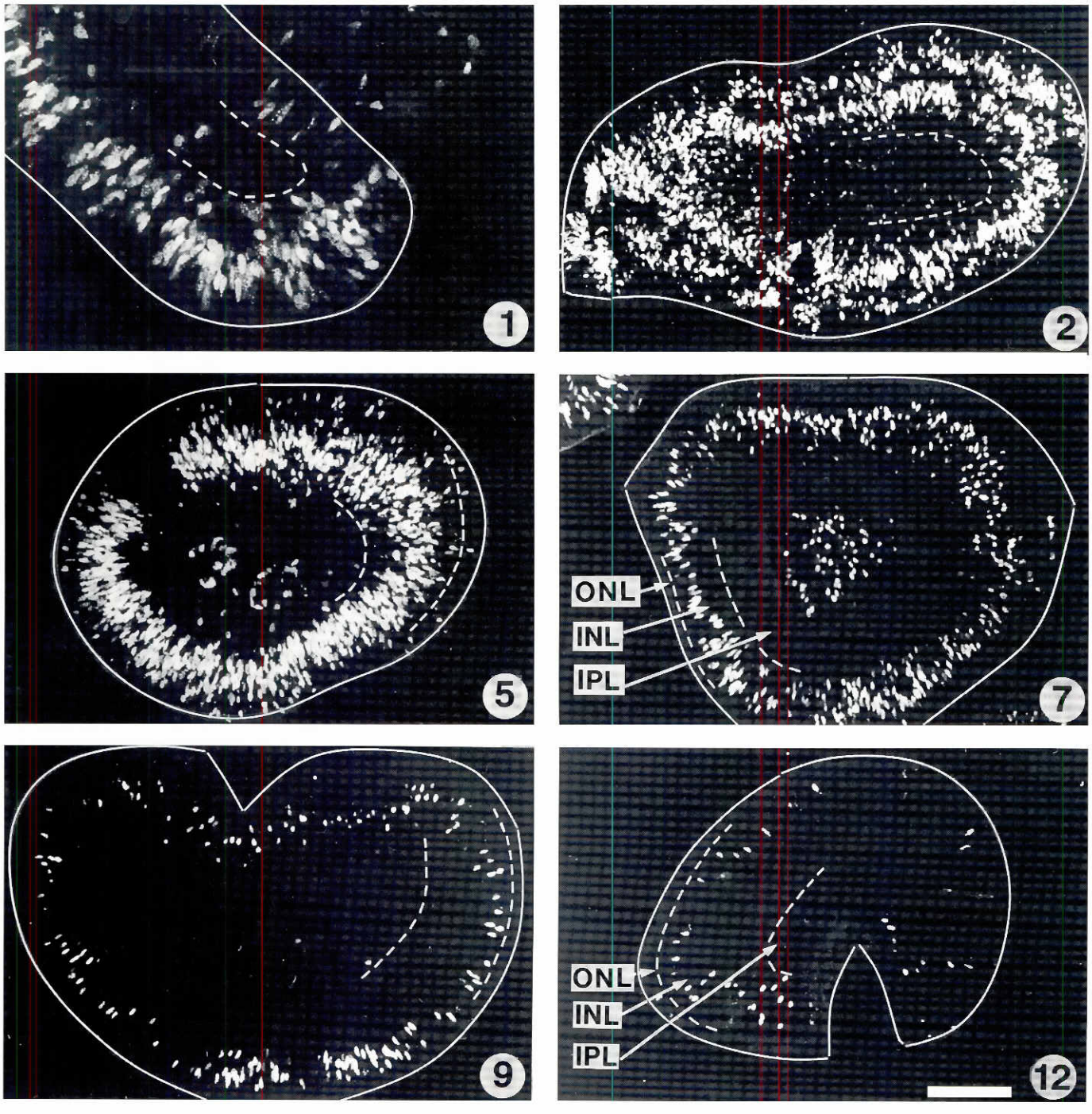

Fig. 3. Radial proliferation in stratospheroids. *Radial cell growth of chick stratospheroids (starting from E6) as demonstrated by BrdU-uptake after 1, 2, 5, 7, 9 and 12 days in vitro (div). During 1 div, only few BrdU-positive cells are found. After 2 div the cells seem to split up into an inner and an outer compartment (see Discussion). Up to 5 div, proliferation increases and covers the entire width of the growing neuroepithelium. At 7 div, most of the cells in the ONL, the GCL and the inner part of the INL are postmitotic (BrdU-positive cells in the center of this spheroid belong to a non-organized cell mass). After 9 and 12 div, proliferation shifts towards the outer part of the INL. For better orientation, the outer borders of the spheroids (full lines) and both borders of the INL (dashed lines) are outlined. Bar, 50 μm, (1), 100 μm (2-12).*

monolayered spheroids derived from embryonic day seven quail tissue are shown. Here we have used quail cells, since the cellular structure can be more easily resolved than in chicken stratospheroids. In spheroids derived from older stages (later than embryonic day E6 in quail and embryonic day E7 in chick), it can be clearly seen that the retinal area of the stratospheroid consists of only a monolayered epithelium forming a more or less hollow sphere. The neuroepithelium is still able to lateralize and form an epithelium; however, it is no longer able to radialize and form cell columns.

Discussion

Stratospheroids are valid models to study retinogenesis

Stratospheroids represent spheroidal structures of retinal tissue that are regenerated in rotation culture. Starting from only a few cells, stratospheroids are constructed by processes comparable to normal development, that can often be better followed and/or manipulated than under conditions of normal retinogenesis (Willbold and Layer, 1992a; Layer and Willbold, 1994; Willbold *et al.*, 1995). For the chicken retina, a series of studies have established that the first cells leaving the cell cycle around embryonic days E4-E6 are ganglion cells, followed by cones, then amacrine, bipolar and horizontal cells. This vitreal-scleral pattern is superimposed by differentiation of layers and particular cells along a central-peripheral gradient (Layer and Kotz, 1983; Liu *et al.*, 1983; Spence and Robson, 1989; Prada *et al.*, 1991; Dütting and Thanos, 1995).

In earlier thymidine-uptake studies, we have compared the localization of mitotic cells in spheroids with the expression of cholinesterases (Vollmer and Layer, 1986b, 1987); however, there we did not relate the patterns with the formation of particular layers or cell types. By applying the BrdU labeling technique, we have now concentrated on the establishment of radial patterns within the spheroids. We know that most cells of spheroids are generated *in vitro* (Willbold and Layer, 1992b), and that the histological course of production of individual cell layers is also very similar to normal retinogenesis (the respective *in vivo* data are not shown).

Production of stem cells leads to lateral growth of the neuroepithelium: a model for lateralization and cell column formation in retinogenesis

After cell proliferation has come to an end in most parts of the spheroid, we could still trace a prominent number of mitotic cells in the direct vicinity of the pigmented core. This shows that a) this zone is the youngest part of the retinal tissue, thus again underlining its ora serrata-like nature; and b) it must be from this part that the stem cells of the developing retina are derived. This notion is strongly supported by the gradients of opsin and F11 differentiation which also originate from this zone.

These observations lead directly to the major question of this study about the regulation of lateral and radial growth in a retinal tissue. With his "radial unit hypothesis" to explain neocortical development, Rakic has distinguished between radial and transversal growth. He stresses that the extension of the tissue must be brought about mainly by symmetrical divisions, while the radial growth requires asymmetric cell production. For both processes, different (groups of) genes may be responsible. Thus, the phylogeny of differently sized neocortices may require merely minor mutations of transversally relevant genes (Kornack and Rakic, 1995; Rakic, 1995).

To explain growth of the eye, we have introduced a similar scheme. Accordingly, the entire growth could be conceived of as three spatially and temporally overlapping processes which we call *lateralization*, *clonal radialization* and *lamination* (Fig. 7). First, self-renewal of stem cells leads to a lateral expansion of the primitive neuroepithelium *(lateralization)*, thus supplying the retina with the progenitors of the cell columns. The individual cells of the columns

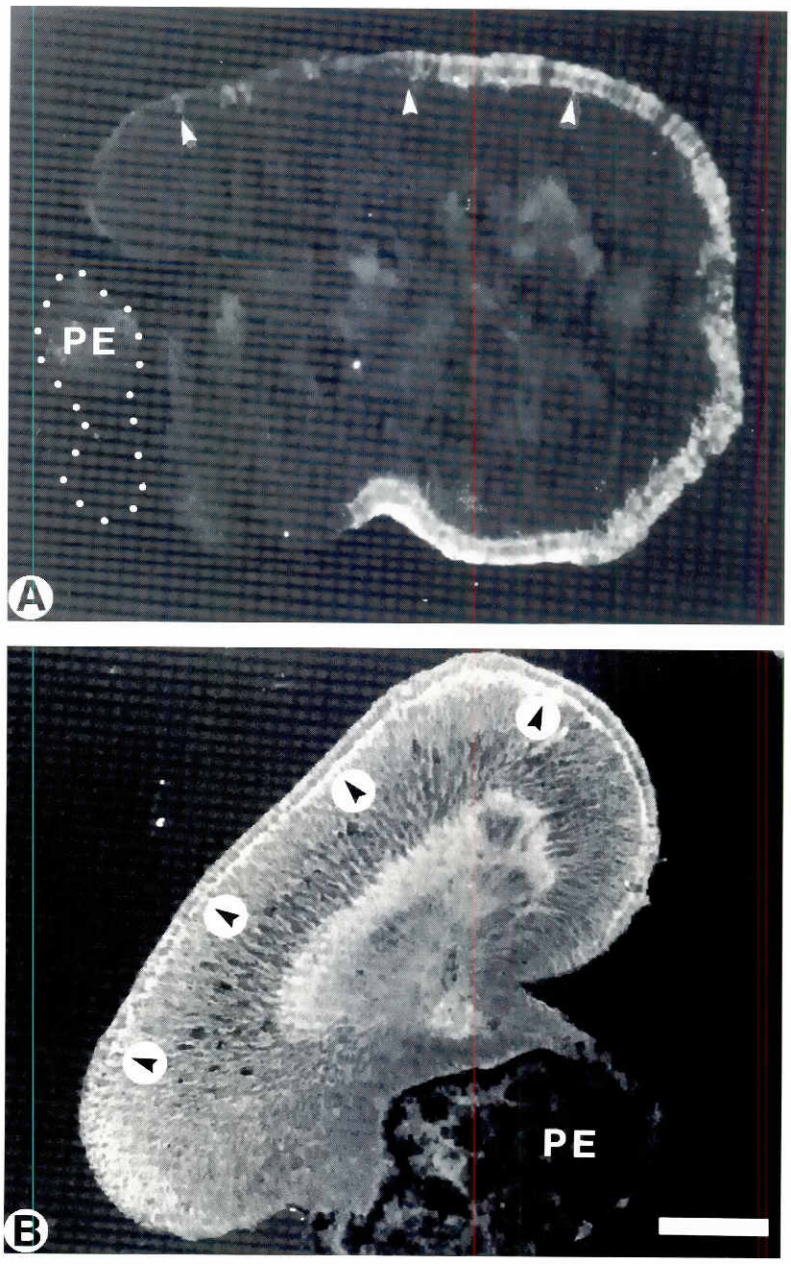

Fig. 4. Lateral differentiation in stratospheroids. *Lateral gradient of photoreceptor* **(A)** *and fiber differentiation* **(B). (A)** *Chick stratospheroid (starting from E6) after 8 div, stained with the opsin-specific antibody rho4D2. The pigmented cells (PE) are located at the left side of the spheroid (white dots). In the vicinity of the pigmented cells, the prospective photoreceptors show no opsin expression. More distally, single photoreceptors are faintly stained (arrowheads). In the more mature parts (towards the right), more and more photoreceptors become opsin-positive.* **(B)** *Chick stratospheroid (starting from E6) after 14 div, stained with the fiber-specific antibody F11, which allows visualization of OPL development (arrowheads). OPL formation starts with the appearance of pearl-like patches (left arrowhead) which later fuse to form a continuous fiber layer (right arrowheads). PE, pigmented epithelium. Bar, 100 μm.*

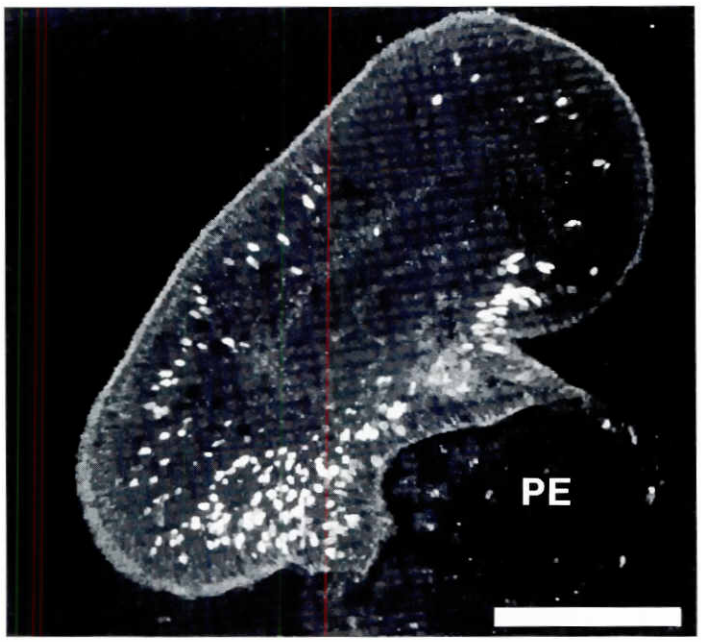

Fig. 5. Cell proliferation is sustained in the vicinity of the pigmented epithelium (PE). *Lateral cell growth is sustained in the vicinity of the PE of a chick stratospheroid (starting from E6) after 14 div. At this time, only a few BrdU-positive cells are still found in the laminated part of the spheroid (outer part of INL, compare Fig. 3), whereas close to the PE many cells show mitotic activity. Bar, 200 μm.*

are later generated by a radial proliferation process *(clonal radialization)*. Concomitantly, the cell bodies segregate first into two neuroblastic layers (Willbold and Layer, 1992a), and then into three nuclear layers (*lamination*; note that the processes of *clonal radialization* and *lamination* overlap in space and time, however, for the sake of clarity they appear sequentially in our scheme; the sequence is now slightly modified compared with our earlier model). Only then the cells begin to differentiate according to their spatial location to establish their final synaptic connections (Gierer and Müller, 1995). In particular, lateral growth by stem cell divisions is suggested to represent a first important period of retinal development to supply the retina with a sufficient number of stem cells.

Such a scheme is fully consistent with the observation that *in vivo* self-renewal of stem cells decreases first in the center of the retina (Dütting *et al.*, 1983; Hernandez-Sanchez *et al.*, 1994). Thus, we suggest that the "lateral growth zone" first occupies most of the neuroepithelium in the very early eye anlage, and later retracts circumferentially towards the rim of the eye cup. In fish, amphibians and reptiles the remaining "lateral growth zone" is active throughout life to fit the eye's volume to the increasing size of the whole organism. In fish, some stem cells may also remain in the ONL of the functional retina (Maier and Wolburg, 1979; Raymond *et al.*, 1988; Hitchcock and Cirenza, 1994). However, *in vivo* it is impossible to uncouple the two processes of lateral and radial growth clearly. Here we have succeeded to uncouple these processes. In an earlier study we found that laminated stratospheroids arose only from chicken tissue younger than embryonic day E9 (Vollmer and Layer, 1986a). We have shown here in quail, where development proceeds more rapidly, that already from embryonic day E7 onwards, the neuroepithelial (non-pigmented) part of the deriving spheroid now consists of an increasing number of monolayered spheroids. This demonstrates that radial growth is inhibited under these circumstances. If this does not merely reflect an *in vitro* effect (which can never be entirely ruled out), this experiment indicated that lateral growth is possible, even when radial growth has been shut down. These different types of spheroids should allow us to isolate the genes responsible for the regulation of lateral and radial retinal growth. Such genes should be most decisive for the capacity of retinal regeneration.

It is difficult to decide on the precise nature of the founder cells from which the lateral extension emanates. Since the initial introduction of stratospheroids as developmental model systems, it was clear that the pigmented epithelium of the eye margin is needed to re-establish a proliferative period and to develop a correctly laminated retinal spheroid, indicating influences of the pigmented epithelium on cells located in its close vicinity (Vollmer *et al.*, 1984). By culturing retinal explants in the absence or

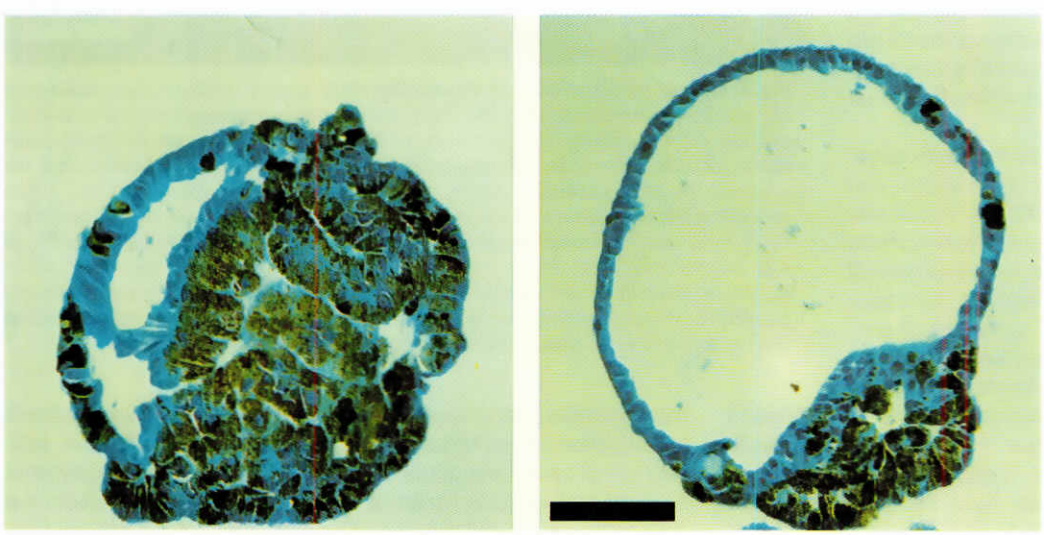

Fig. 6. Uncoupling of lateral and radial growth. *Radial growth is absent in 14-day-old stratospheroids, which are derived from E7 quail tissue. Spheroids derived from E7 or older tissues increasingly consist of a monolayered epithelium; this indicates that lateral growth is still possible while radial growth is fully inhibited. Paraffin sections stained with methyl-green-pyronine. Bar, 100 μm.*

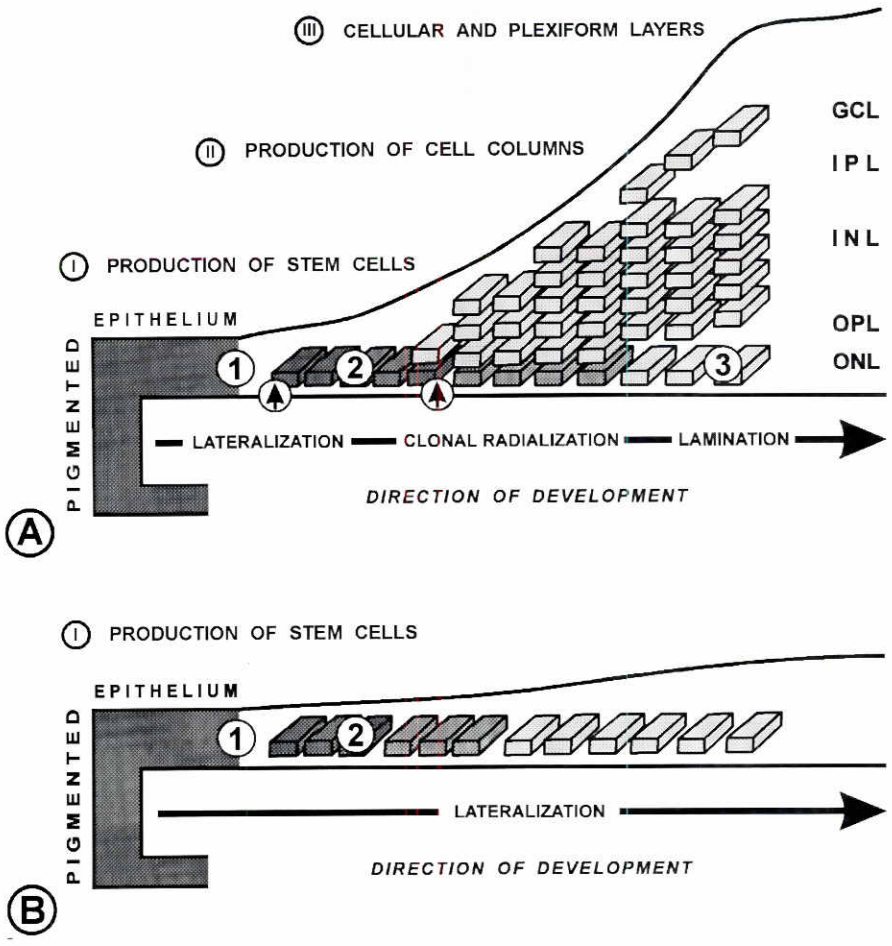

Fig. 7. Basic mechanisms of stratospheroid formation. *Hypothetical scheme showing steps of lateralization, clonal radialization and lamination during stratospheroid formation.* **(A)** *In vitro retinogenesis closely depends upon the pigmented epithelium. Lateral proliferation and self-renewal of multipotent precursor cells (dark color) leads to the formation of a monolayered neuroepithelium (= lateralization). Later, radial proliferation gives rise to cell columns (= clonal radialization). Concomitantly, the cell bodies segregate to form the three nuclear cell layers (= lamination). Comparable mechanisms may also be present during retinogenesis in vivo.* **(B)** *Lateralization and radialization processes uncoupled at older stages (compare Fig. 6). Here, prospective stem cells are still produced by lateralization (symmetric divisions?), however, proliferation in the radial direction (asymmetric divisions?) does not occur. (Encircled 1: transition zone between PE and neuroepithelium; encircled 2: monolayered neuroepithelium; encircled 3: differentiated retina; encircled arrows: beginning and ending of neuroepithelium; compare also Fig. 1).*

presence of pigmented tissue slices, a strong stimulation of cell proliferation by the pigmented epithelium was directly proven (Liu *et al.*, 1988). It could also be shown that the pigmented epithelium produces a set of specific growth factors (Jaynes and Turner, 1995; Sheedlo *et al.*, 1995). Moreover, it was recently demonstrated that ablation of the RPE during early ocular development using lethal transgenic constructs led to severe abnormalities in retinal histogenesis (Raymond and Jackson, 1995). We assume that the founder cells for lateral growth are neuroepithelial non-pigmented cells, whereby the pigmented epithelium may be closely involved in the determination of these cells. Since the pigmented epithelium and the retina are continuous and interconnected by the «lateral growth zone», both a tangential gradient based on lateral cell-cell contacts, as well as a radial gradient based on the close back-to-back contacts seem possible to explain the decreasing competence of the stem cells for lateral and radial growth and the switch from proliferation to differentiation.

Using the stratospheroid system we plan to further characterize the neuroepithelial cells and to seek for the mechanisms regulating radial and lateral growth.

Materials and Methods

Cell preparation and rotary culture

Whole eyes from appropriate staged chicken (normally from embryonic day 6; White Leghorn, purchased from a local hatchery) or quail embryos (purchased from a local hatchery) were isolated, washed with Hanks' solution and dissected free from connective tissue. The lens and the vitreous body were removed. The eyes were cut with a pair of eye scissors circumferentially along the retinal side of the ora serrata to separate the tissue into i) a peripheral part composed of ciliary pigment epithelium and ciliary neuroepithelium, and ii) a central part which contains the functional part of the retina. For the production of stratospheroids only the peripheral part is used. The tissues were collected separately in F12-medium on ice. For dissociation for the rotary culture system, the tissues were first incubated in 1 mg/ml collagenase and 0.3 mg/ml hyaluronidase (Boehringer, Mannheim, Germany) in F12-medium for 7 min at 37°C, washed once in F12-medium and treated with 1 mg/ml trypsin (Cooper Biomedical/Technicon, Bad Vilbel, Germany) for 10 min at room temperature. The tissues were rinsed in F12-medium and mildly dissociated with a fire-polished Pasteur pipette in the presence of 0.05 mg/ml DNase (Worthington/Technicon). After a 3-fold wash in F12-medium, the cells were resuspended in aggregation medium (10% fetal calf serum (GIBCO BRL, Berlin, Germany), 2% chicken serum (GIBCO BRL), 1% L-glutamine (SERVA, Heidelberg, Germany), 0.1% penicillin/streptomycin (SERVA), 0.02 mg/ml gentamycin (SERVA) in DMEM-medium. Suspensions (2 ml) of cells (8 eyes per dish) were aggregated in 3.5 cm plastic dishes (Greiner, Nürtingen, Germany) by rotation on a gyratory shaker (self-made; 60 rpm, 37°C, 97% air/3% CO_2). Medium was changed every two days.

Preparation of paraffin sections

After the appropriate times in culture, the retinospheroids were collected, washed in phosphate buffered saline (PBS), fixed in 4% PBS-formalin (commercial formalin) for 3 h at 4°C, embedded in paraffin and

cut in 2-3 μm sections according to standard procedures. After deparaffination, the sections were stained by an eosin-hematoxylin procedure (10 min blue staining, 0.1% eosin for 5 min) as described by Hutson and Donahoe (1984) or by a methyl-green-pyronine procedure (10 min) as described in Romeis (1989).

Preparation of frozen sections

After 10-14 days in culture, the retinospheroids were collected, washed in phosphate buffered saline (PBS) and fixed in 4% PBS-formalin (commercial formalin) for 3 h at 4°C. After a 3-fold wash in PBS they were soaked in 25% sucrose-PBS overnight. Frozen sections were cut at 10-16 μm thickness on a cryostat (Reichert-Jung, Nußloch, and Microm, Walldorf, Germany).

Immunohistochemistry

Sections were preincubated in PBS/3% bovine serum albumin (BSA) at room temperature for 10 min followed by incubation with the primary antibodies (mouse-α-opsin antibody rho4D2 at a dilution of 1:10.000 [Hicks and Molday, 1986; Hicks and Barnstable, 1987]; rabbit-α-F11 antibody at a concentration of 25 μg/ml [F11 is a neurite-specific fasciculation antigen, which is found especially in fiber-rich regions; Rathjen *et al.*, 1987]; mouse-α-BrdU antibody [Bio-Science Products AG, Emmenbrücke, Switzerland] at a 1:10 dilution in PBS). All antibodies were applied for 60 min at room temperature. The sections were washed 3 times (5 min each) in PBS and 5 min in PBS/rabbit serum, followed by a 60 min incubation with a biotin-conjugated sheep-α-mouse (Amersham Buchler, Braunschweig, Germany) secondary antibody (or a fluorescein isothiocyanate conjugated goat-α-rabbit secondary antibody; Dianova, Hamburg, Germany) at a 1:100 dilution in PBS. After 3 short washes in PBS, the biotin-conjugated secondary antibody was detected with a 30 min incubation with streptavidin-conjugated Texas Red (Amersham Buchler). After 3 washes in PBS (5 min each) the sections were mounted in glycerine-gelatine (Merck, Darmstadt, Germany). Controls were always performed by incubating sections without the primary antibody. We found no non-specific binding of secondary antibodies in these control sections (data not shown).

BrdU-labeling

BrdU (5-bromo-deoxyuridine, SERVA) at a final concentration of $5x10^{-5}$ M was added to the culture medium, protected from light, and further incubated for 16 h. The specimens were then washed in PBS and frozen sections were prepared. For staining, the sections were washed for 10 min in PBS and then treated for 10 min with 2N HCl at 37°C, followed by 2 washes (5 min each) with 0.1 M sodium borate (pH 8.5), two 5 min washes in PBS, 10 min in PBS plus 5% rabbit serum. Then the BrdU monoclonal antibody was applied according to the described procedure.

Microscopy and photography

Stained sections were observed using a Zeiss Axiophot microscope with Nomarski and fluorescence optics. To avoid any complications with doubled-labeled fluorescein isothiocyanate and Texas Red sections, we used a blocking filter which cuts off any light below 560 nm to clearly separate the signals. For documentation, we used Agfapan 25 and Kodak Tmax 400 films.

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