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The golden age of retinal cell culture

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In the late 1950s, the study of retinal cells in vitro was in its infancy. Today, retinal cell and tissue culture is routinely used for studies of cell growth, differentation, cytotoxicity, gene expression, and cell death. This review discusses the major classifications of retinal cell and tissue culture, including primary cell/explant models, retinoblastoma cell lines, and genetically engineered cell lines. These topics are addressed in an historical perspective, coupled with present-day applications for this continually-developing technology.

The culture of retinal cells and tissues is now a widely used tool, with broad applications in the field of Ophthalmology. As with all in vitro systems, both advantages and potential limitations exist. Advantages include highly controllable conditions which allow for measurements on a cell-by-cell basis, isolation from confounding systemic/ocular effects, time course flexibility, and a reduction in the number of animals used for research. Possible limitations include selective loss of specific cell phenotypes/functions, changes in tissue architecture, and the potentially questionable relevance of in vitro findings.

For many applications, the advantages of in vitro studies outweigh the potential limitations. With a balanced approach, in conjunction with animal studies, in vitro studies can provide important basic information about normal retinal functions. Retinal cell and tissue culture exists in three basic forms: primary cultures (reaggregate, explant, and dispersed), retinoblastoma cultures, and genetically engineered cultures.

PRIMARY RETINAL CELL CULTURE

Reaggregate cultures: Any cell that is isolated from a tissue, grown in vitro (prior to subculture) is considered a primary cell culture. The modern era of primary retinal cell culture began in the late 1950's with the work of A. A. Moscona [1,2] in which he elegantly demonstrated the ability of enzymatically dissociated chick neuroretinal cells to reassociate into multicellular, histotypic reaggregates under controlled conditions allowing replicable results [3]. Using this system, Moscona and Linser were able to use reaggregates to study the regulation of retinal enzymes, such as carbonic anhydrase and glutamine synthetase [4-6]. From these early studies, it became clear that cells of the neuroretina were readily isolated, were able to form cell-cell associations, and maintained many of the characteristics of the retina in situ. With this reaggregate system, important intrinsic properties of retinal cells were established. Retinal reaggregates were able to form rosettes, organized cellular structures comprised of a central lumen, surrounded by a circular array of photoreceptors with connecting cilia and external limiting membrane. In the chick reaggregate culture, rosettes formed that were characteristic of the age of origin of the cells [7,8], while the retinal cells therein were able to continue some degree of development with regard to tissue organization, synaptogenesis, photoreceptor differentiation, and enzyme function [3,9-11]. As seen in Figure 1, the cellular organization of retinal reaggregate cultures, feature rosette formation which is visible with light microscopy.

Retinal reaggregate culture has recently undergone a "pellet culture" modification [12], in which slow-speed centrifugation of dissociated retinal cells promotes reaggregation. Reaggregate/pellet cultures are used for studies of differentiation [12], cell-cell interactions [13], and even the disaggregating effects of retroviruses [14]. Importantly, the potential significance of the retinal pigment epithelium (RPE) for the proper cellular stratification of the neuroretina was revealed with this technique, using chimaeric reaggregates of neuroretina and RPE [15,16].

The establishment of the retinal reaggregate cell culture system led to the evolution of other types of primary retinal culture. For the purposes of this discussion, they will be classified as explant cultures and dispersed cell cultures.

Explant cultures: Explant cultures of whole or partial retina were established in an attempt to preserve the tissue architecture characteristic of the retina in situ. With retinal explants, as with other explanted tissues, cells retain many histological and biochemical features and can be maintained for several days, or even weeks. These types of retinal cultures cannot be propogated, exhibit greater degrees of experimental variation among sister cultures, and are difficult to use for some quantitative studies due to variations in geometry and composition [17]. Retinal explant cultures have been ideal for studies of synaptic organization [18], cell-cell interactions [19], outgrowth of cells and their neurites [20-22], and retinal differentiation [23,24]. Retinal explants retain the highest degree of tissue preservation of all retinal cell culture systems and continue to be the method of choice for in vitro studies that require intact cell-cell associations.

Retinal explant cultures have been especially helpful in addressing a fundamental question in the evolution of retinal cell culture, namely, how do cultured retinal cells differ from

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their intact counterparts in vivo? Cultured explants from neonatal rabbit exhibit markers of the GABAergic phenotype for several days in a manner consistent with that seen in vivo [24]. In a comparative study by Germer and colleagues [25], development of an outer plexiform layer, as well as ribbon and conventional synapses of the neonatal rabbit retina in vitro followed a similar pattern to that seen in vivo. The photoreceptor cells in their preparation grew well-developed inner segments and cilia, but not mature outer segments. In a second study of these neonatal rabbit retinal explants [26], glutamine synthetase expression in Müller cells was lower in vitro by an order of magnitude. The Müller cells of the rabbit retinal explant cultures could be stimulated to express additional glutamine synthetase by addition of glucocorticoid hormones or retinal pigment epithelial cell-conditioned medium. Therefore, loss of systemic, circulating factors may account for some of the changes seen when isolated retinal tissue is grown in culture.

Chick retinal explants have been useful in identifying developmental changes characteristic of in vitro conditions. Influences of the substratum (collagen, poly L-lysine, etc.) have been noted with regard to intermediate filament expression in chick embryonic retinal explants [27]. Although neurofilament L was not detected in vitro, the early expression of neurofilament subunits M and H during the maturation process was dependent upon the type of substratum present on the dish (27). In an autoradiographic study of developing chick retina [28], the major differences observed were that the retinal explants grown in culture contained cells that were morphologically more spindle-shaped and the retina, as a whole, was thinner than in vivo. Importantly, cultured chick retinal explant development lagged behind age-matched in vivo retinae by approximately 24 h. These differences can be considered when assessing the use of retinal explants to address experimental problems.

Dispersed cell cultures: Dissociated-dispersed retinal cells, from embryonic, postnatal, and adult animals, lack the cell-cell organization of explant cultures, but can be more useful for some transfection studies, patch-clamp recordings, videomicroscopy, and quantitative analyses where access to individual cells is important or where intercellular communication must be limited.

Since dispersed cell cultures are even further removed from the orderly structure of the intact retina, identification of these dispersed cells is paramount. This problem can be illustrated in the dispersed rat embryonic retinal cell monolayer culture shown in Figure 2. Flat cells and neuronal-like cells are evident, however, morphology alone is not enough to positively identify each of the six major phenotypes of the neuroretina: ganglion, bipolar, photoreceptor, amacrine, horizontal, and Müller cells. With the advent of retina specific antibodies, such as those for rat from the lab of Barnstable [29,30] and those for chicken from Lemmon [31,32], specific retinal cell types could be identified in vitro on the basis of retina-specific marker expression, rather than on less-reliable morphological characteristics. In addition to the development of specific antibodies, retinal cell identification evolved to include tetanus-toxin binding of retinal neurons [33], autoradiographic localization of labelled neurotransmitters [34], and electron microscopic identification of synaptic elements [35]. For particular retinal cell types of interest, highly specific means of identification were introduced. Ganglion cells are identified by pre-labelling in vivo (prior to cell culture) by

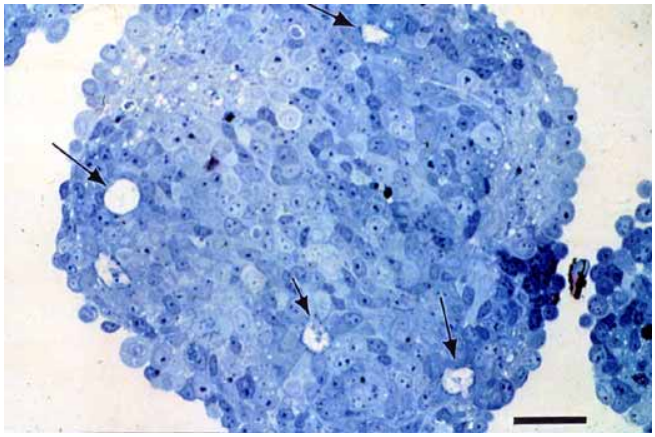

Figure 1. Chick Retinal Reaggregate Culture. Embryonated chicken eggs of C/E genotype from Spafas (Norwich, CN) were allowed to develop in an egg incubator for 7 days. The chick embryo was removed and the neural retinae were dissected free of pigmented epithelium and optic nerve. A single cell suspension was generated by treating the tissue for 10 min at 37 °C with 0.6% trypsin in 1 mM EDTA. The tissue was rinsed with phosphate-buffered saline and seeded into 35 mm suspension-culture dishes at 5 x 10^6cells per dish with Dulbecco's Modified Eagle's Medium with 1 mM L-glutamine, 10% fetal calf serum and 50 µg/ml gentamicin. Dishes were placed on a Junior Orbit Shaker set at 70 rpm in a humidified chamber with 5% CO_2, 95% air at 37 °C. The photo depicts a retinal reaggregate after 7 days in vitro. Rosettes organized around central lumen are visible (arrows). Scale bar is 10 µm.

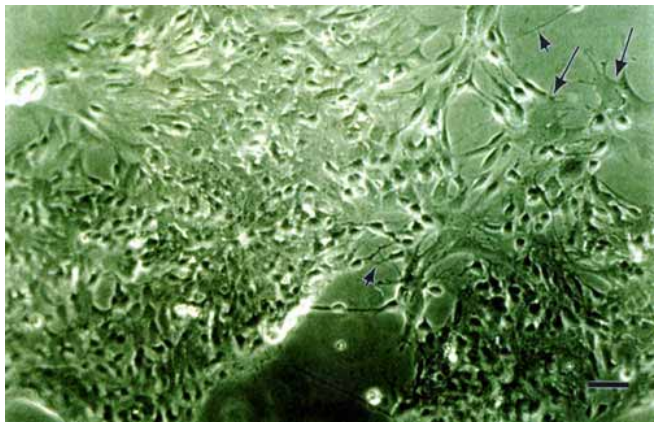

Figure 2. Dissociated E17 rat retina in vitro. Embryonic day 17 rats were enucleated and the neural retina dissected free of pigment epithelium. A single cell suspension was generated by treating the tissue for 10 min at 37 °C with 0.6% trypsin in 1 mM EDTA. The tissue was rinsed with phosphate-buffered saline and plated into 35 mm dishes with Dulbecco's Modified Eagle's Medium containing 1 mM L-glutamine, 10% fetal calf serum and 50 µg/ml gentamicin. Dishes were placed in a humidified chamber with 5% CO_2, 95% air at 37 °C. The E17 culture was photographed after 5 days in vitro. Note the neuritic processes (arrowheads), as well as flat cells (arrows). Scale bar is 10 µm.

intraxonal retrograde transport of horseradish peroxidase [36]. Horizontal cells have been identified based on specific glutamate-modulated K^+ current conductance, in conjunction with immunoreactivity to parvalbumin [37]. For amacrine cells, autoradiographic evidence of acetylcholine [38,39], GABA immunocytochemistry [40] and autoradiographic demonstration of ^{3}H-muscimol uptake [40] have proven useful for identification purposes.

Retinal cell identification procedures were also adapted to enrich cultures for specific retinal cell populations. As with neural cell culture, enrichment procedures involved retrograde labelling, gradient separation, both positive and negative selection procedures (chemical and immunological), and mechanical means of separation. Retrograde labelling [41] coupled with metrizamide gradient separation [42] has been used to select for ganglion cell types. Another ganglion cell enrichment procedure has relied upon the Thy-1 marker and other ganglion-specific antibodies for immunological panning [43]. For positive selection of different cell types, retina-specific cell surface markers used for fluorescence-activated cell sorting [44] can separate mixed retinal preparations into nearly purified cultures. Selective destruction, such as anti-opsin/complement treatment of cell cultures, can help exclude photoreceptors from a final cell population [45]. Photoreceptor enrichment has also been possible through the chemical destruction of undesired retinal neurons by the photoreceptor-sparing toxins kainic acid and β-bungarotoxin [46] and by the non-ionic detergent Triton X-100 [47,48]. Mechanical selection through the adept use of a vibratome has been used as a physical means of photoreceptor purification [49]. Sequential papain and DNAse enzymatic digestion coupled with density gradient centrifugation has allowed for pure Müller cell cultures [50]. Enzymatic dissociation in combination with velocity sedimentation has been reported for isolation of purified horizontal cells [51]. For more general applications, rat retinal sublayers can be separated by mild enzymatic treatment with trypsin followed by physical separation of retinal tissue into the outer nuclear layer, inner nuclear layer, inner plexiform layer, and ganglion cell/nerve fiber layer [52].

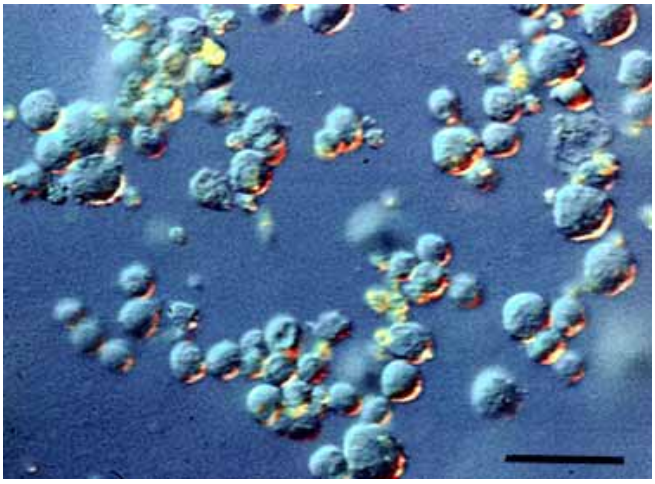

Figure 3. Y79 cell suspension culture. Y79 retinoblastoma cells were obtained from the American Type Culture Collection (ATCC# HTB-18) and maintained in RPMI-1640 medium (Sigma, St. Louis, MO) with 15% fetal calf serum (heat-inactivated; Hyclone, Logan, UT), 0.02% L-glutamine and 50 μg/ml gentamicin. Cells were grown in suspension flasks in a 37 °C humidified chamber with 5% CO_2, 95% air. Note cells in "grape-like" clusters. Scale bar is 10 μm.

The origin of retinal tissue used for monolayer and explant cell culture has included a diverse assortment of creatures, including human [53-59], rabbit [24,25], rat [30], mouse [60], pig [61] chicken [46], drosophila [62], moth [63], zebrafish [64], carp, [65], goldfish [66,67], frog [68,69], turtle [70], gecko [71] and even camel [72,73]. As a rule, embryonic/postnatal tissue has been easier to propogate than adult tissue. Survival, as well as the differentiation of many primary retinal cell cultures, has been enhanced with the addition of specific factors to the culture medium, such as basic fibroblast growth factor [74], acidic fibroblast growth factor [75], ciliary neurotrophic factor [76,77], neurotrophin-3 [78], glial-derived neurotrophic factor [79] and retinoids [80,81]. RPE conditioned medium has been shown to possess potent survival and maturation factors for neural retinal cells in vitro [82,83], including pigment epithelium-derived factor (PEDF) [84,85].

Retinal Cell Differentiation in Primary Culture: Primary retinal cell culture models have been ideal for retinal developmental studies. Isolated from confounding ocular/systemic influences, retinal cells and explants have been used to address basic questions as to the intrinsic vs. extrinsic nature of retinal cell differentiation. Retinal progenitor cell fate was shown to be affected by cell-cell interactions [86] and was dependent upon retinal progenitor cell "competence" to respond to environmental cues [87]. In the absence of extrinsic signals, murine retinal precursor cells grown in clonal density cultures (as single cells) could generate both neurons and glia [88]. In the chicken, the majority of similarly isolated retinal progenitor cells tended to develop into photoreceptors in the absence of intraretinal influences, a "photoreceptor default" pathway [89]. Furthermore, retinal cell fate could be determined after the time of terminal mitosis [90]. All of these developmental findings relied upon coordination between in vivo and in vitro experimentation to identify some of the powerful differentiation influences of the retina.

With the advent of improved purification procedures, such as a recently modified vibratome-mediated purification of photoreceptors [91], primary retinal cell culture methods continue to be refined and re-defined. More detailed technical advice beyond the scope of this overview is available [92], and continues to evolve. Primary retinal cell and tissue culture remains the most widely-used form of retinal cell culture.

RETINOBLASTOMA CELLS

The major drawbacks of primary retinal cell cultures are their limited supply and finite lifespan. Investigators have addressed this problem by establishing proliferative retinal cell cultures of virtually infinite lifespan from retinoblastoma tumors. These retinoblastoma cell lines are not only immortal, but can also be stored frozen in liquid Nitrogen for later use, unlike primary cell cultures. The first retinoblastoma cell line described was the Y79 cell line, derived from the intraocular tumor of a 2.5 year old human [93]. Shortly thereafter, the WERI-Rb1

[94], WERI-Rb24, and WERI-Rb27 [95] cell lines were also established from human intraocular tumors. Other human retinoblastoma cell lines include FMC-Rb1 [96], Rb 355-7 [97], SO-Rb50 [98], and HXO-Rb44 [99]. Through analysis of genetic polymorphisms, it was determined that Y79, Rb 355-7, and WERI-Rb27 are genetically related [100]. These human retinoblastoma tumor cell cultures have several characteristics in common, such as growth in suspension as "grape-like" clusters (Figure 3), immunoreactivity to glial and/or neuronal markers [101-103], and the ability to form tumors in vivo [94,99,104]. Retinoblastoma cells were found to exhibit alterations in the expression of the retinoblastoma susceptibility gene, *Rb* [105]. Alterations in *Rb* expression sparked significant interest in the use of retinoblastoma cells as tools to gain insight into mutational mechanisms of *Rb* inactivation.

In the flurry of *Rb* research in the late 1980s and early 1990s, retinoblastoma cells were used to elucidate the potential role of *Rb* as a tumor suppressor. Although replacement of a wild-type *Rb* gene into *Rb*-defective or deficient cells decreased growth rate, colony-formation in soft agar, and tumorigenicity [106], *Rb* reconstitution alone was not enough to completely prevent retinoblastoma cells from forming tumors in vivo [107,108]. The *Rb* gene was shown to be an important component in the coordination of normal retinal growth and differentiation [109] though it did not appear to be a strong tumor suppressor. Elements of the *Rb* gene family were differentially expressed during mammalian embryogenesis [110] and mice deficient in the *Rb* gene were non-viable, with defects in neurogenesis and hematopoiesis [111]. Significant strides were made in the study of *Rb* gene expression with the aid of retinoblastoma cell culture systems.

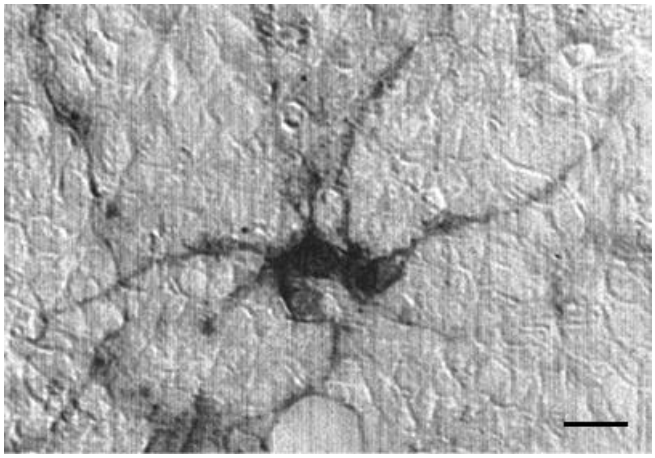

Figure 4. R28 retinal cells immunoreactive to Thy 1.1. The photograph depicts R28 cells immunoreactive for Thy 1.1, a retinal ganglion cell marker. Scale bar is 5 µm. R28 cells were grown in DMEM with 10% calf serum, 1 mM L-glutamine, and 50 µg/ml gentamicin. For Thy 1.1 staining, cells were fixed for 10 min at room temperature in 2% paraformaldehyde and permeabilized in 0.25% Triton X-100 for 5 min. After a rinse in phosphate-buffered saline (PBS), cells were incubated for 1 h with 10 µg/ml anti-Thy 1.1 antibody (Chemicon, Temecula, CA). After rinsing 3 times for 5 min in PBS, cells were incubated with biotinylated anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA) for 60 min. Cells were equilibrated in Tris-buffered saline (50 mM Tris-HCl, pH 7.6, 0.9% NaCl) and incubated for 20 min with horseradish peroxidase-conjugated avidin (Elite kit, Vector Laboratories). The cells were rinsed in 0.05 M Tris and developed with a diaminobenzidine kit (Pierce, Rockford, IL) and the brown/black reaction product visualized by light microscopy. Negative controls consisted of incubations in control serum without primary antibody, and did not generate reaction product.

The undifferentiated nature of the Y79 retinoblastoma cells also made them very useful in bioassays to test putative retinal differentiating agents. Morphological observations could be made, once these cells were attached to suitable matrices [112,113]. Differentiating agents, such as dibutyryl cAMP, retinoids, and sodium butyrate [114-118] were found to affect the morphology, growth rate, and biochemistry of Y79 cells in vitro. Our own studies showed that the growth inhibitor succinylated concanavalin A (SCA) could reversibly decrease the expression of the N-MYC protein in Y79 cells and induce expression of the photoreceptor marker interphotoreceptor retinoid-binding protein [119]. Profound morphological effects were seen in adherent Y79 cells upon treatment with PEDF, including the formation of large, organized rosettes and significant neurite outgrowth [84,85]. However, even this differentiating treatment was not enough to block the tumor-forming abilities of this cell line [120]. Interestingly, sodium butyrate, a less neuritogenic compound than PEDF, inhibited tumor-formation in vivo [121], presumably due to its growth-inhibiting effects.

Recently, Y79 cells have been utilized for studies on the efficacy of anti-cancer drugs [122-125], radiation [126], and photodynamic therapies [127]. Under some of these treatment conditions, Y79 cells undergo apoptotic cell death through a p53-dependent mechanism [128]. These findings suggest a potential role for the manipulation of pro-apoptotic genes in the treatment of retinoblastoma. In addition to the human retinoblastoma cell lines, mouse [129] and rat [130] cell lines exist that continue to be useful in studies of retinal cell differentiation, tumor biology, and anti-cancer therapeutics.

GENETICALLY ENGINEERED RETINAL CULTURES

In rare instances, continuously-growing cell lines arise spontaneously from primary cultures [131-134]. More commonly, cell-cycle-related genes that induce cell proliferation, such as SV40 large T antigen, E1A/E1B gene of adenovirus, or src are introduced by investigators to promote an immortalized, growth stimulated culture. In genetically engineered retinal cultures, some resulting cell lines will lack the tumor-forming potential of the aforementioned retinoblastoma cell lines, and possibly retain more differentiated features. Like the retinoblastoma cell lines, the genetically engineered immortalized retinal cell cultures can withstand long-term freezer storage.

In the late 1980s, human retinoblasts were transformed with SV40 virus, adenovirus, and *ras* genes for the purpose of studying oncogene activity, efficiency of transformation, and tumorigenicity [135]. In these studies, retinal cell lines were used to demonstrate that stable, malignant transformation of primary human cells required the cooperation of more than one oncogene [136]. As with retinoblastoma cells, these ge-

netically engineered cells, called Ad 12 HER 10, responded to dibutyryl cAMP in a differentiation process characterized by cell rounding and neuritic-like process formation [137].

KGLDMSM, another human retinal cell line, developed by Dutt and colleagues [138], was induced to progress through the cell cycle by expression of SV40 Large T Antigen. KGLDMSM cells were shown to express neuron-specific enolase, neurofilament, glycine receptor, synaptophysin and secretogranin [139]. These cells also responded to cAMP and the phorbol ester TPA by undergoing morphological changes characterized by extension of neuritic processes and c-fos expression [139]. A retinal Müller cell line, of rat origin, was also established by the introduction of SV40 large T antigen [140]. The Müller cell line, termed rMC-1, was shown to express GFAP, a marker for reactive gliosis and cellular retinaldehyde binding protein (CRALBP), a marker for Müller cells in the adult retina. Similarly, a rat retinal Müller cell line established by introduction of the human papilloma virus E6/E7 gene [141] retained expression of Müller cell markers, including S-100, carbonic anhydrase C, CRALBP, and GFAP, but not glutamine synthetase.

In the early 1970s, Pessac, Calothy, and colleagues developed a retinal cell culture system to study the effects of oncogenes, such as *v-src*, on the proliferative and differentiating capacity of chicken and quail neuroretinal cells [142,143]. These Rous sarcoma virus transformed avian retinal cells were still able to retain retinal characteristics, such as choline acetyl transferase, glutamic acid decarboxylase, tetanus-toxin binding [144], and A2B5 immunoreactivity [145]. Retrovirally or oncogenically transformed avian retinal cells have been used recently for the identification of new retina-specific genes, such as the melanocyte specific gene QNR-71 [146], and the characterization of new avian cell lines expressing mature retinal markers [147].

Rous sarcoma virus-transformed chick neuroretinal cells do not always remain growth-stimulated. In the case of LA29NR, a chick neuroretinal cell line infected with Rous sarcoma virus, cells were proliferative for 15 passages, but then succumbed to a senescent process [148]. The senescence was associated with a loss of transformed phenotype and eventually cell death. Nevertheless, even 15 passages represented a prolonged lifespan for these cells and allowed for an additional 4 months of experimentation, not possible with primary cultures.

Retinal cells induced to progress through the cell cycle often maintain features of retinal precursor cells, rather than a mature retinal phenotype. Such retinal precursor features are characteristic of the cell lines developed in our own laboratory, namely the E1A-NR.3 and the clonally-derived R28 cell cultures [149]. These cells were established from postnatal day 6 Sprague-Dawley rat retinae and were forced to remain active in the cell cycle by a replication-incompetent retrovirus containing the 12S portion of the adenovirus E1A gene. The 12S portion of the E1A gene was used to promote cell growth, while avoiding tumor-forming potential seen in SV40 large T antigen-expressing cells [150,151]. E1A-NR.3 and R28 cells exhibit contact-inhibited growth [149] and were transplanted intraocularly into rats with no tumor-formation seen in 42 eyes [152]. The E1A-NR.3 and R28 retinal cells have been useful for retinal transplantation experiments [152], and as targets for cytotoxicity and neuroprotection studies [153-155]. These cells have been shown to express markers of photoreceptor [149,156], Müller [157], and ganglion cell phenotypes (Figure 4).

Genetically engineered retinal cell lines continue to be used for studies requiring large numbers of cells, and can be cloned by limiting dilution if homogeneity is desired. Additionally, these cell lines can be custom-designed from isolated retinal cell types and offer a great deal of flexibility in experimental design.

Table 1. A Comparison of Retinal Culture Systems: Characteristics of Retinal Cell Cultures

	Primary Cultures	Retinoblastoma Cultures	Engineered Cell Lines
Examples	Explants, Reaggregates Monolayers	Y79, WERI-Rb1, FMC-Rb1, RB355-7	KGLDMSM, R28 rMC-1
Lifespan	Finite (2-4 weeks)	Indefinite	Indefinite
Freezer storage	No	Yes	Yes
Phenotypic Marker Expression	Excellent	Immature	Variable
Growth Characteristics	Post-mitotic or slow-growing	Highly proliferative	Mitotic
Tumor-forming ability	None	High	Range: none to high

Summary of the major differences between the basic types of retinal cultures discussed in the text.

SUMMARY

Retinal cell culture has proven to be a powerful tool in Ophthalmic research. While no cell culture described can replace the intact eye, these cultures do provide controllable experimental systems for the examination of fundamental retinal processes. The major characteristics of retinal culture systems are summarized in Table 1. The relevancy of retinal cell culture experiments remains a subject of debate. This debate will continue as we seek to improve upon retinal cell culture systems and create cell lines that more closely approximate the retina in situ.

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All animal experimentation was accomplished in accordance with the ARVO statement on the use of animals in Ophthalmic research.

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