Molecular Vision 2014; 20:301-306 <http://www.molvis.org/molvis/v20/301>
Received 10 December 2013 | Accepted 11 March 2014 | Published 14 March 2014
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# Review: R28 retinal precursor cells: The first 20 years

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**The R28 retinal precursor cell line was established 20 years ago, originating from a postnatal day 6 rat retinal culture immortalized with the *12S E1A* (NP-040507) gene of the adenovirus in a replication-incompetent viral vector. Since that time, R28 cells have been characterized and used for a variety of in vitro and in vivo studies of retinal cell behavior, including differentiation, neuroprotection, cytotoxicity, and light stimulation, as well as retinal gene expression and neuronal function. While no cell culture is equivalent to the intact eye, R28 cells continue to provide an important experimental system for the study of many retinal processes.**

Cell lines have been developed and studied since the 1950s as a means of examining cellular behavior under controlled environmental conditions. Until the early 1990s, most continuous retinal cell cultures, such as Y79 [1], WERI-RB24, and WERI-RB27 [2], were derived from retinoblastoma tumors while other retinal cell lines were immortalized with the oncogenic SV40 large T antigen [3,4]. However, there was a need for a non-tumorigenic, immortalized retinal cell line with controlled growth characteristics suitable for experimental retinal transplantation and in vitro studies of retinal cell activity. This was the impetus for the creation of the R28 retinal cell line as a research tool.

## DISCUSSION

*Establishment of 12S E1A*-immortalized cells: The first *12S E1A*-expressing retinal cell line was designated E1A-NR.3 [5]. The E1A-NR.3 cells were created by incubating postnatal day 6 rat retinal tissue explants with $10^5$ colony-forming units/ml of the psi2 E1A virus, a replication-incompetent retrovirus containing the *12S E1A* gene of the adenovirus [6]. The *12S E1A* gene was chosen for its ability to stimulate the growth of rodent cells without tumorigenesis [6]. The *12S E1A*-expressing cells were selected following two-week incubation with the antibiotic geneticin (G418) as the vector containing the *12S E1A* gene also expressed neomycin/geneticin resistance. The presence of E1A in the surviving cells was determined by western blot and immunocytochemistry. Immunoreactivity to the photoreceptor-specific markers S-antigen and interphotoreceptor retinoid binding protein (IRBP) was confirmed. The E1A-NR.3 cells were anchorage-dependent as they did not grow in soft agar [5]. As additional evidence for growth control, these cells did not form tumors in an ocular transplantation study of 42 animals [7]. These results suggested that the E1A-NR.3 retinal cell line was distinct from typical retinal tumor cell lines and worth further examination.

Once the E1A-NR.3 cells were established, it was clear that there was some degree of heterogeneity in the cultures as seen by the mixed cellular morphologies as well as the fact that no marker of interest was expressed in 100% of the cells [5]. To attempt to create a more homogeneous cell line, the E1A-NR.3 cells underwent three rounds of limiting dilution (one cell per well in 96 well plates) to ensure that the final culture was derived from a single cell. During the last round of limiting dilution, the 28th well of the dish contained a particularly healthy-looking culture (based on the growth rate, percentage of live cells, and morphology), which was expanded for further analysis. This culture was named “R28,” with the “R” designating “Rochester” (the university of origin) and “28” indicating the 28th well of the 96-well dish from which the cells were expanded. Three rounds of limiting dilution required approximately 40 passages before R28 cells could be scaled up for storage in liquid nitrogen. This meant that most of the experiments that validated the phenotype of R28 cells occurred during cell passage numbers in the 40s. Interestingly, although R28 cells were derived from a single E1A-NR.3 cell through limiting dilution, they remain heterogeneous to this day, suggestive of a precursor type cell. Table 1 summarizes the strengths and limitations of R28 cells in terms of their immortalization, rat origin, and heterogeneity.

*Characterization of R28 cells:* The characterization of cell lines is very important, especially in light of the recent findings on the RGC-5 cell line, once thought to be a rat retinal ganglion cell line [8] but now reported to be the unrelated SV40-transformed mouse photoreceptor cell line 661W [9]. The existence of cross-contaminated and uncharacterized cell lines can lead to questionable results and faulty scientific

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Table 1. Advantages and Limitations of R28 cells.

| Characteristics | Advantages | Limitations |
|---|---|---|
| Immortalized | Unlimited lifespan<br>Can be grown in large numbers<br>Can survive long-term storage in liquid nitrogen<br>Not tumorigenic in vivo | Immortalization affects gene expression and morphology<br>Not the same as primary retinal cells<br>Expresses geneticin resistance, so stable transfection requires another selection marker |
| Rat retinal origin | Mammalian retinal cells<br>Compatible with rat models | Not the same as human retinal cells<br>Some antibodies of interest may not be available in a rat-specific format |
| Heterogeneous | May more closely simulate a retinal explant or in vivo retina with multiple cell types<br>Diversity of cell types can respond to a variety of stimuli<br>Considered a retinal precursor cell line with differentiation potential | The degree of heterogeneity is unknown and may depend on culture conditions.<br>Not a homogeneous cell line as desired for some applications |

conclusions. To avoid these pitfalls, strict cell culture protocols have been followed and validating experiments published regarding the identity of R28 cells. Following the establishment of R28 cells, strict cell culture protocols in the laboratory of origin were established as follows: a) a separate bottle of medium was used for each cell line, b) only one cell line was used at a time in the laminar flow hood, c) the hood was cleaned with ethanol between the use of different cell lines, and d) hand washing/glove changing occurred between the use of cell lines.

Several published studies support the origin and identity of R28 cells. A microarray analysis of R28 cells at passage 48 indicated that 4,131 genes of rat origin were expressed as “present” [10], while other studies have demonstrated the successful use of rat-specific PCR probes [e.g., 11]. These studies support the assertion that R28 cells are of rat origin. Furthermore, microarray analysis has indicated the presence of a female-specific form of cytochrome C (Cyp2c12), coupled with the absence of a male-specific form of cytochrome p450, suggesting a female origin for these cells [10].

Functional studies indicate that R28 cells possess retinal neurotransmitter receptors that can respond to neurotransmitter stimulation [12,13]. Subpopulations of R28 cells respond to dopamine, serotonin, glycine, and acetylcholine stimulation in patch-clamp analyses [12]. Subpopulations of R28 cells are also immunoreactive to GluR1, 2, and 3, N-methyl-D-aspartate (NMDA), and γ-aminobutyric acid-a (GABAa) receptors [13]. R28 cells lack voltage-gated channels, but some cells generate inward currents in the presence of non-NMDA, NMDA, GABAa, and GABAb receptor agonists [13]. Due to the heterogeneous and precursor-like nature of R28 cells, not every cell responds to every neurotransmitter agonist, but further study is warranted. It is unlikely that additional enrichment through cell sorting or further limiting dilution will result in a homogeneous culture, which leaves these cells more comparable to the heterogenous population of the intact retina. More likely is the possibility that specific culture conditions (as yet unidentified) will promote a more specific and mature phenotype in R28 retinal precursor cells.

*Caveats:* Despite efforts to maintain the identity and integrity of the R28 cell line, it is always possible that once cells leave the laboratory of origin, they may be grown under less than stringent conditions. Previous studies of R28 cells were conducted within the standards and available technology of the time, without detailed cell line identity statements in the methods sections of publications. Despite these caveats, it is important to note that R28 cells have always reacted to rat-specific probes and have expressed markers consistent with their retinal cell origin [10,14,15]. There is a strong glial component to the R28 retinal precursor cell line, as evidenced by the expression of glial fibrillary acidic protein (GFAP), S-100 (50%), and vimentin (nearly 100%), with co-expression in some cells of both vimentin and IRBP [15], an indication of the uncommitted nature of these precursor-like cells. Additional characterization of R28 cells is welcome in light of new technologies that were not available at the time of the earlier studies. Additional studies could include a more detailed assessment of heterogeneity and the development of conditions that promote specific retinal phenotypes, such as glial cells or retinal ganglion cells.

*R28 cells used for studies of in vitro toxicity and neuroprotection:* The ability to grow large numbers of R28 or E1A-NR.3

cells with consistent growth characteristics makes them especially useful for studies involving in vitro toxicity. The first toxicity studies involved the E1A-NR.3 cell line and the apoptotic effects of anti-recoverin [16] and anti-enolase [17] antibodies as a model for cancer-associated retinopathy. Soon after, R28 cells were used for a variety of in vitro toxicity experiments, including the effects of triamcinolone [18,19], avastin [20], indocyanine green [21], benzo(e)pyrine [22], oxysterol [23], nicotine [24], and trypan blue [25], as well as the human immunodeficiency virus (HIV) Tat protein [26].

The natural link between toxicity and neuroprotection led to studies using R28 cells to examine the neuroprotective effects of insulin-like growth factor 1 (IGF-1) [27], L-deprenyl [28], latanoprost [29], the gap junction inhibitor PQ1 [30], erythropoietin [31], unoprostone [32], tetracycline [33], stromal cell-derived factor-1 [34], and insulin [35-37]. Often, these in vitro studies were performed before in vivo validation. In the case of latanoprost [29], the study showed neuroprotection both in vitro and in vivo in the same series of experiments. Studies of in vitro toxicity and neuroprotection remain the most common experimental paradigms for R28 cell studies.

*Studies of gene expression and cell signaling pathways:* The microarray data on R28 cells [10] give some guidance about which rat-specific genes may be expressed by the cell line and have been helpful in the experimental design of specific studies of gene expression and function. These genes and pathways include synapsin and synaptophysin I [38], pigment epithelium-derived factor mechanisms [39,40], and insulin signaling [41], as well as protein kinase C (PKC) and extracellular-signal-regulated kinase (ERK) signaling [42].

*Studies of retinal function:* In vitro studies of R28 cells have led to significant findings related to retinal function and specific disease states. For example, studies on autoantibodies to small heat shock proteins in glaucoma [43], serum from glaucoma patients [44], and R28 cells grown under increased atmospheric pressure [45] (methodology comment) have been used to simulate the damaging metabolic effects of glaucoma on retinal cells. As an in vitro model of retinal differentiation, R28 cells undergo increased neurite outgrowth in response to pigment epithelial-derived factor [46] and mouse acetylcholinesterase [47]. Other studies have examined the visible light responses of R28 cells in vitro [21,48,49]. This is not to conclude that R28 cells are light sensitive through visual pigments, but rather that they respond to light-damaging effects. In the case of blue light (411 nm), R28 cells responded to illumination by increased production of mitochondrial superoxide radicals and caspase-3 cleavage products [49]. These responses were not seen at 471 nm. Taken together, these findings suggest that blue light-mediated apoptosis in R28 cells is wavelength-dependent and occurs by direct mitochondrial damage and increased oxidative stress [49].

*Current status:* Standard culture conditions for R28 cells have evolved over the years. The most current maintenance medium consists of Dulbecco's Modified Eagle's Medium (DMEM, D5523, Sigma Chemical Co., St. Louis, MO) with 10% calf serum (Thermo Fisher Scientific, Waltham, MA), 0.37% sodium bicarbonate, 0.058% l-glutamine, and 100 ug/ml gentamicin. Another culture medium has been used to promote a more neuronal phenotype [36] as follows: R28 cells are plated at low density on 60-mm dishes coated with laminin on glass coverslips and grown in DMEM, 10% newborn bovine serum, and 250 M pCPT-cyclic AMP, a cell-permeable cAMP analog (Sigma). These culture conditions reduce GFAP (glial marker) expression and growth rate, and promote neurite outgrowth [36]. Neurite outgrowth is best seen at low cell density. Ideally, one could imagine culture conditions that lead to 100% phenotypic outcomes. So far, this has not been the case. Other culture conditions that promote specific retinal phenotypes have not yet been identified but would be very interesting for future studies. A comparison between R28 cells and other neuronal cell lines would also be informative but would be challenging at this time due to the lack of published comprehensive and validated neuronal gene expression studies pertaining to other cell lines.

R28 cells have been useful in a variety of experimental systems in the field of retinal cell biology, ranging from toxicity/neuroprotection to gene expression, retinal function, and cell signaling pathways. In summary, R28 cells possess useful characteristics, such as contact inhibited, anchorage-dependent growth, the expression of rat and retinal-specific genes, and functional processes that have allowed them to contribute to several interesting projects over the past 20 years. With proper care and attention to cell identity and integrity, ocular cell lines such as R28 will continue to play an important role in the study of retinal gene expression and behavior.

Note Added in Proof: R28 cells have undergone verification by IDEXX-RADIL to be of rat origin without contamination by other mammalian cell lines. A baseline genetic profile of these cells is available upon request.

## ACKNOWLEDGMENTS

The author thanks the former laboratory members and collaborators who have made this work possible. Grant support for the original R28 cell line was through NIH grant R29EY10676 and the Lucille Markey Charitable Trust to the University of Rochester. Current grant support through

Cornell Center on the Microenvironment & Metastasis through Award Number U54CA143876 from the National Cancer Institute, R21CA127061 and NYSTEM C026412. GMS has a commercial interest in the sale of R28 cells through KeraFAST, Inc. (soon to be Symbiotic Scientific).

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 14 March 2014. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.