Childhood Eye Cancer Trust (CHECT) Research Grant Final Report (end of grant report)

Please complete all sections of this report and return to petra.maxwell@chect.org.uk.

Project title:	**A retinal organoid platform for retinoblastoma drug development**		
Project reference:	23/01/Seigel2		
Total award:	**£50,000**		
Details of any additional funding:	N/A		
Lead investigator:	Gail M. Seigel, Ph.D.	Administering institution:	University at Buffalo
Start date of award:	July 1st, 2023	End date of award:	June 30th, 2026

1. **Final Report**
   Please structure your report as follows:

   a. Summary of findings/results/outcomes of this project (with reference to the aims and objectives stated in your original application).
   b. If your aims and objectives changed during the course of the project, please explain why and in what way.
   c. Any problems or challenges impacting on the findings / results / outcomes of this project.
   d. How will these findings or outcomes impact patients or the public, and in what timescale? Do you foresee any obstacles / barriers to patients benefitting from the research findings?

A. Summary of Results

In the original application, our overall goal was to 1) Establish a cell toxicity profile for ADCs using monocultures and cocultures of hiPSC-derived retinal organoids and RB cells and 2) Assess cell death and DNA damage in ADC-treated co-cultures of hiPSC-derived retinal organoids and RB cells. We were able to generate co-cultures of RB cells and organoids, distinguish RB cells from organoids with a fluorescent tracer, treat co-cultures with ADCs, and develop a new way to detect ADC payload presence within RB cells. We were able to measure cell death with ADC treatment, although it was confounded by a loss of organoid viability between the time of their initiation at the University of Colorado and their propagation at the University at Buffalo. Since retinal organoids took about 120 days to generate, we used this time to address additional subgoals of our project, which were to identify new targets for RB therapy and to improve upon drug testing models for RB.

We began working on a new ADC with GD2 as the target molecule. Work continues, although it is proprietary and cannot be published yet. We also identified new target genes for RB, including ALYREF and YBX1. We found that YBX1 is expressed in RB cell lines and tumors, but not retinal organoids. We identified an anticancer compound with strong effects on RB and YBX1, namely luteolin, Luteolin can 1) decrease expression of YBX1, 2) inhibit growth of RB cells, 3) induce

death of RB cells. These results suggest a role for YBX1 in RB cell growth regulation and its potential as both a target and a biomarker for tumor growth inhibition by anti-cancer therapies such as luteolin. In addition, YBX1 may be a useful marker for RB cells in co-culture with retinal organoids.

This work was enhanced by the addition of 4 new students in the UB lab in the Spring-Summer of 2025, namely Sean Yares, Rydwan Hossain, Daniel Dedrick, and Noor Fatima. Rydwan and Noor remain in the lab as undergraduates, while Daniel will be returning to our lab for his Master's degree in August 2026. Sean graduated in May 2026. Thanks to results from our CHECT project, we were able to obtain a small grant for the students from the University at Buffalo Undergraduate Research Program. They have presented posters on RB-on-a-chip as described under "Presentations".

Finally, we developed the first of its kind RB-on-a-chip, a millifluidic system for RB drug testing. RB-on-a-chip will vastly improve most in vitro studies of retinoblastoma by ourselves and others going forward. Studies of RB tumor cells and organoids, until this point, have involved cells growing in a static cell culture dish. We have improved on this by growing RB cells in a chamber with a circulatory system to mimic the tumor environment in the body. This RB-on-a-chip system will allow us to study retinoblastoma cell growth, invasion, and response to therapeutic drugs in a dynamic 3D environment. This RB-on-a-chip study was presented as a "hot topic" at the Association for Research in Vision and Ophthalmology in Denver, CO on May 3rd, 2026 (my birthday!) and as a public webinar in June 2026. We have found that drug treatment, specifically luteolin, has more potent effects in a circulatory environment versus a static environment. Our future plans include 1) exploring these chip results to determine the reason for enhanced circulatory effects on drug potency against RB and 2) to test ADCs in this new system.

B. Modified Aims and Objectives

With 120 days preparation of retinal organoids and their reduced viability in transit, we took the time to address important subaims of the project, namely identifying new targets and developing a more physiologically relevant 3-D drug screening model (RB-on-a-chip). These subaims did not disappoint.

C. Problems/Challenges

The biggest challenge was that the 3-D organoids appeared to lose some viability between their initiation at the University of Colorado and their propagation at the University at Buffalo. This complicated the analysis of viability and expression of cell death markers in organoids. We then pivoted to another 3-D drug testing model, namely the RB-on-a-Chip, as well as other subgoals that provided us with robust results, presentations and publications.

D. Impact on Patients: Timescale and Potential Barriers

Development of new RB drug testing models will benefit patients by providing a way to more precisely measure drug and dosing effects in a dynamic circulatory environment. In turn, this will get us closer to targeted RB therapies with more accurate dosing, with the goal of killing tumor cells and sparing the normal surrounding tissue, thereby preserving vision and maintaining a good quality of life for people with RB. This work is under review for publication at the Digital Journal of Ophthalmology. Once it is published, other scientists will be more aware of the new process. In addition, the presentation of this work at the ARVO international conference (attendance ~11,000) and other public venues and webinars is also getting the information out to scientists who could use this method for their own projects. I remain personally accessible and available to help anyone who wishes to learn how to run RB-on-a-chip experiments.

2. **Plain English summary (please refer to appendix 1).**
   Please provide a brief plain English summary of your final report above, including any findings or outcomes, and their potential impact on patients or the public. CHECT (and funding partners) will publish this summary in the public domain to demonstrate how we support research, therefore please do not include any confidential or commercially sensitive information.

Retinoblastoma is an aggressive eye tumor that affects young children. Although current treatments can save lives, they can cause serious side effects including vision loss. To improve safety, we have developed a more realistic way to test new drugs. Previously, drugs have been tested on flat cells in a dish, which is a lot different than a real human eye. Our project improves this by growing cells using a new device called "RB-on-a-chip." This chip mimics the natural blood flow of the eye and lets us test new drugs in a more realistic way. The chip will let us find the best drugs and doses to prevent harmful side effects. We have also found new ways to kill RB tumor cells while sparing healthy eye tissue. Our results move us closer to safer treatments that can save lives, preserve sight, and improve quality of life for children with RB.

3. **Publications**
   Please list all published or accepted papers and abstracts from the work of this grant (journal style) (attach copies where available)

Publications

1. Seigel GM, Onwumere O, Sauane M, Lopez S, Shang E, Habiba H, Redenti S, Grossniklaus HE, Gharbaran R. 2024. ALYREF/THOC4 expression and cell growth modulation in retinoblastoma. Pathol Res Pract. Jun 14;260:155392. doi: 10.1016/j.prp.2024.155392. Epub ahead of print. PMID: 38880039.

2. **Seigel, GM**, Dedrick D, Fatima N, Hossain R, Redenti S and Gharbaran R. 2026. RB-on-a-chip: A Dynamic Drug Testing Platform for Retinoblastoma, submitted to the Digital Journal of Ophthalmology.

3. **Seigel, GM**, Furey, K, Hannah, W, Dinh, T, Habiba, H, Fatima N, Yares S, Hossain R, Onyekwere, O, Redenti, S, Grossniklaus H, and Gharbaran R. 2026 YBX1 Expression and Cell Growth Modulation in Retinoblastoma, submitted to Current Eye Research.

4. Seigel, GM, Sauane M, Onwumere O, Lopez S, Kastrat E, Kim A, Vemalapally L, Shang E, Grossniklaus H, Redenti S, Gharbaran R. 2026. Piperlongumine inhibits methylation of N5-methylcytosine (m5c) via suppression of NSUN2/ALYREF oncogenic signaling cascade in human retinoblastoma. submitted to Molecular and Cellular Neuroscience.

5. Shi C, Huang K, Onwumere O, Gharbaran R, Bensalel J, Gallego-Delgado J, Li S, Flores-Bellver M, Benito-Martin A, **Furey F, Seigel GM**, Guzik DesMarais V, Heissel S, Lewis ER, Young M, Jakobs TC, Redenti S. 2026. Retinoblastoma Extracellular Vesicle LY6E Cargo Facilitates Metastatic Niche Formation in Optic Nerve Head Astrocytes. Submitted to Extracellular Biology.

Abstracts

1. **Seigel GM**, Dedrick D, Fatima N, Hossain R, Yares S, Redenti S and Gharbaran R. 2026. RB-on-a-chip: A Dynamic Drug Testing Platform for Retinoblastoma in Vitro. Platform Presentation at the Association for Research in Vision and Ophthalmology in Denver, CO, chosen as a "hot topic" and press release.

2. Gharbaran R, Sauane M, Onwunmere O, Lopez S, Kasrat E, Mandal P, Shang E, Grossniklaus H, Redenti S, **Seigel GM**. 2026. MYCN modulation of polycomb repressive complex 2 (PRC2) oncogenic activities in pediatric retinoblastoma. Poster presentation at the Association for Research in Vision and Ophthalmology in Denver, CO.

3. Hossain R, Fatima N, Dedrick D, Gharbaran R, Redenti S, **Seigel GM**. 2026. Retinoblastoma-on-a-chip for anticancer drug testing. Experiential Learning Showcase and Celebration poster presentation, University at Buffalo, Buffalo, NY, April 20, 2026.

4. Seigel, GM, Furey, K, Wolf, H, Dinh, T, Habiba, H, Onyekwere, O, Redenti, S, Grossniklaus H, and Gharbaran R. YBX1 Expression and Cell Growth Modulation in Retinoblastoma. 2025. Platform Presentation at the Association for Research in Vision & Ophthalmology.

5. Gharbaran R, Furey, K, Wolf, H, Dinh, T, Habiba, H, Onyekwere, O, Redenti, S, Grossniklaus H, and **Seigel, GM**. A novel small-molecule MYC:MAX antagonist that potently induces anti-tumor effects inhibits N5-methylcytosine (m5c) oncogenic cascade in human retinoblastoma. 2025. Poster Presentation at the Association for Research in Vision & Ophthalmology.

6. **Seigel, GM**, Redenti, S, Suane M, Grossniklaus H, Onwumere O, Gharbaran, R. 2024. ALYREF Expression and Cell Growth Modulation in Retinoblastoma. Poster Presentation at the Association for Research in Vision & Ophthalmology.

7. Gharbaran R, Suane M, Onwunmere O, Lopez S, Kasrat E, Mandal P, Shang E, Grossniklaus H, Redenti S, **Seigel GM**. 2024. Piperlongumine targeting of the NSUN2/ALYREF oncoepitranscriptomic signaling cascade in retinoblastoma. Poster Presentation at the Association for Research in Vision & Ophthalmology.

4. Dissemination of results

Please list where and by whom any results/findings have been disseminated (e.g. conferences, workshops, public engagement events)

We had one UB graduate student successfully defend her Master's thesis entitled “Targeted Drug Therapy of Retinoblastoma” by Kiarra Furey, M.S. May 2024, Dr. Gail M. Seigel (mentor). Ms. Furey is now pursuing her Ph.D. studies at City University of New York (CUNY).

We had an undergraduate student presentation at the University of Colorado:
Lee, XA, Ha M, Howell A, Shah D, Seigel GM, Vergara, MN. Stem Cell-Derived Retinal Organoids for Screening Retinoblastoma Therapeutics. Presented at the University of Colorado in 2024.

There were six poster and platform presentations at the Association for Research in Vision and Ophthalmology conferences in 2024, 2025 and 2026 presented by Gail Seigel and/or Raj Gharbaran. See abstract list above.

A live remote webinar presented by Gail Seigel, sponsored by React4Life (Genoa, Italy) on June 18th, 2026. This webinar is still available on demand at:

https://www.react4life.com/retinoblastoma-on-a-chip-and-therapeutic-strategies/

University at Buffalo Undergraduate Research and Creative Activities (URCA) poster presentations in 2024 (Kiarra Furey) and two in 2026 [April and July 2026] (Noor Fatima, Rydwan Hossain, and Daniel Dedrick), all on campus in Buffalo, NY, presented by our undergraduate students.

5. Intellectual property (IP)
Please list any IP arising from the research, and whether it is wholly owned by the researcher.

N/A

6. Collaborations
Please list any collaborations which have arisen during or as a result of this research.

On this project, we have been collaborating with the following laboratories:

Dr. M. Natalia Vergara, University of Colorado
Dr. Stephen Redenti, City University of New York
Dr. Rajendra Gharbaran, Bronx Community College
Dr. Dhaval Shah, University at Buffalo

7. Future research and funding
Please provide details of any further research/ideas planned and where potential funding will be sourced from as a result of this project

We are planning to continue our work with RB-on-a-chip to 1) better understand drug responses in a circulatory environment and 2) expand our chip studies to Antibody-Drug Conjugates for personalized medicine approaches. We have already obtained some internal funding from the University at Buffalo for the undergraduate students working on this project. We plan to apply to both governmental sources and private foundations for our future work, including CHECT when the next opportunity arises.

8. Any further comments

I want to take this opportunity to say how thankful I am for the strong support from CHECT. Their enduring support over the years has been a key to our success, empowering our team to make important discoveries and disseminate this information to benefit the RB field. We remain profoundly grateful for this partnership.

Report completed by:	Gail M. Seigel, Ph.D.	Date of report:	Aug 1, 2026

Appendix 1: Writing a plain English summary in your Childhood Eye Cancer Trust (CHECT) final report [i]

A plain English summary is a clear explanation of your research that should be accessible by an interested audience.

Your final report will be reviewed by experts on the CHECT Scientific Advisory Committee but also by lay members of the SAC and CHECT Board members who are not scientific experts. It will also be accessible through the CHECT website.

A good quality plain English summary providing an easy to read overview of your whole study will help:

- those carrying out the review (reviewers and Board and panel members) to have a better understanding of your research
- inform others about your research such as those affected by retinoblastoma (Rb), members of the public, health professionals, policy makers and the media
- research funders to publicise the research that they fund.

The summary is important. If it is felt that your plain English summary is not clear and of a good quality then you may be required to amend your summary prior to the final closure of the project.

What to include in your plain English summary

Your plain English summary should be 300 words or less. When writing the summary consider including the following information:

- Aim(s) of the research
- Findings or outcomes
- Potential impact on Rb individuals and families

How to write a plain English summary

The people who will read your summary will be an interested audience, but are not necessarily specialists. Therefore write your summary with this audience in mind, for example at the same level as an article in a newspaper.

There are a few simple rules for writing in plain English. In summary these are:

- Avoid wherever possible using jargon, abbreviations and technical terms. If you have to use them provide a clear explanation
- Avoid complicated English or uncommon words
- Use active not passive phrases: for example say ‘we will do it’ rather than ‘it will be done by us’
- Keep sentences short
- Think about the order and structure
- Break up the text. For example use bullet lists
- Ask patients / carers / colleagues to read a draft to find out if anything is unclear.

The plain English summary is not the same as a scientific abstract. Please do not cut and paste this or other sections of your final report to create the plain English summary. Further guidance on plain English summaries for research is available at:

- **www.amrc.org.uk/blog/how-to-write-a-lay-summary-of-a-research-project**

- **www.cancerresearchuk.org/funding-for-researchers/patient-involvement-toolkit-for-researchers/planning-your-patient-involvement/writing-for-a-lay-audience**

[i] Based on the NIHR guidance available at https://www.nihr.ac.uk/about-us/CCF/PPI/Plain_English_summaries_in_National_Institute_for_Health_Research_funded_research.pdf

RB-on-a-chip: A Dynamic Drug Testing Platform for Retinoblastoma

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Keywords: Retinoblastoma, Drug Development, Microfluidics, Chip Technology

Document Summary: We present proof of concept for the first of its kind RB-on-a-chip platform optimized for drug testing with a fluid velocity in the range of retinal capillary flow. RB-on-a-chip represents an improved *in vitro* method with greater physiological relevance for the testing of new therapies for retinoblastoma.

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Abstract
Purpose: Retinoblastoma (RB) is the most common intraocular tumor of childhood. Until now, in vitro studies of RB in static cell cultures have had limited relevance to tumors in vivo. To address this limitation, we have developed a millifluidic platform to mimic physiological flow, shear stress, nutrient delivery, and drug activity in a controlled environment.

Methods: RB cell lines were set up in MIVO millifluidic chambers (React4Life, Genoa, Italy). The settings on the pump were 4.6 rpm with 2.0 mm diameter tubing, a flow rate of 1 mm/minute and average velocity below the insert at 0.35 mm/sec, comparable to retinal capillary flow. For survival studies, 50,000 cells were placed in a 24-well insert (0.4μ pore size) in RPMI 10% FBS, grown at 37 degrees/5% CO2 incubator for up to 2 weeks and tested for cell viability by trypan blue exclusion. Cells were counted every day and compared with control static suspension cell cultures. For cytotoxicity studies, cells were treated with luteolin (a cytotoxic flavonoid with known efficacy against RB) or control DMSO in the circulation and compared with static cultures.

Results: RB cell cultures were maintained in MIVO for up to 2 weeks, the integrity limit of the MIVO tubing. Luteolin cytotoxicity studies suggest that the drug is more potent in dynamic versus static culture at 24 hours of treatment.

Conclusions: Here, we present proof of concept of the first of its kind RB-on-a-chip miilifluidic system designed to recapitulate physiological flow, shear stress, nutrient delivery, and drug activity in a controlled environment. RB-on-a-chip will allow for high throughput testing of new drugs and therapeutics with decreased cost, ethical advantages over animal studies and reproducible conditions that more closely approximate the circulating in vivo tumor environment.

Introduction

Retinoblastoma is the most common pediatric intraocular malignancy in children, comprising 3% of all pediatric cancers [1]. It is an aggressive form of tumor with profound effects on patients. A prominent challenge faced in the treatment of retinoblastoma is that the preclinical assays do not fully encapsulate human tissue complexity. Intra-arterial chemotherapy (IAC), although a solid modality for RB treatment, still has implementation limitations and side effects such as ocular toxicity which could cause vision impairment despite tumor control, and it is also reported that IACs efficacy is decreased in cases of extensive vascular presence [2]. Chemotherapy resistance mechanisms (e.g. Topotecan resistance) are also on the rise [3]. Current RB treatments are optimal with preserving life but not vision in many cases or overcoming drug resistance, requiring more precise tumor specific treatment. There is an urgent medical need to develop targeted anti-cancer therapies for RB that can minimize off-target side effects and preserve visual function.

Until now, the in-vitro studies pertaining to RB have revolved around static cell cultures which have limited bearing on tumors in vivo, in terms of nutrient gradients and the tumor microenvironment. As such, these paradigms may not accurately predict drug response in humans which leads to a limited ability to test targeted therapies and drugs that may work in vitro but not clinically [4].

By creating a millifluidic system to replicate physiological flow, shear stress, nutrient delivery, and drug activity in a controlled environment, we believe we can enhance the in vitro study of RB. This method could save time and money while producing robust, repeatable outcomes. To investigate RB cell growth and response to therapeutic drugs in a dynamic three-dimensional setting we created the first ever RB-on-a-chip millifluidic system to investigate RB cell proliferation and drug response in a dynamic three-dimensional setting.

Aligning with the emphasis on targeted therapy, this idea recreates realistic tissue architecture and microenvironmental conditions that are absent in traditional static cultures [5]. Microfluidic organ-on-a-chip systems can enable dynamic investigation of drug resistance by integrating controlled gradients [6]. RB-on-a-chip serves as a translational model that bridges the gap between laboratory research and clinical application while facilitating the development of more precise and less vision-compromising treatments. We present here the first RB-on-a-chip results that show proof of concept for future cytotoxicity studies.

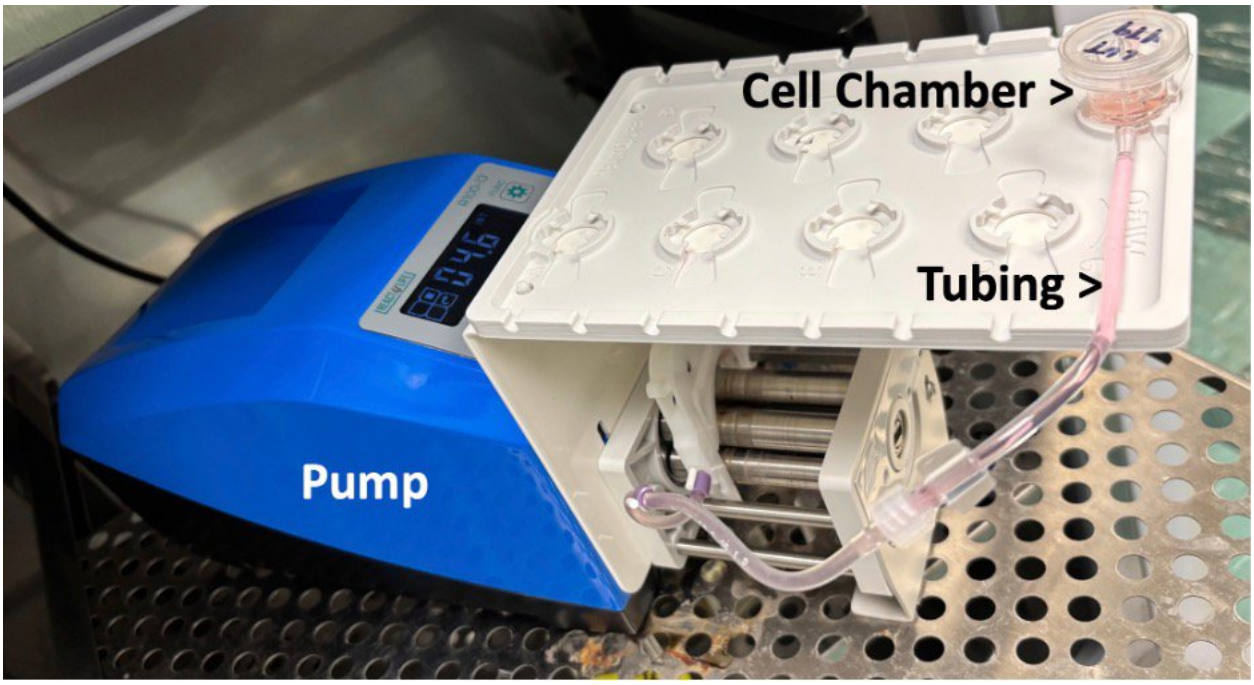
Methods

Cell Maintenance and Chip Set-Up: Multiple retinoblastoma cell lines (Y79, RB143, and WERI-RB27) were grown and maintained in separate static cultures of RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) in an incubator kept at 37ºC with 5% CO2. We used MIVO chambers (React4Life, Genoa, Italy) already proven for cytotoxicity testing in other tumors [7]. When setting up the MIVO chambers, 50,000 cells were placed into a 24 well insert (Avantor, Radnor, PA) with a pore size of 0.4 microns with the growth medium used to grow the cells. We connected the pump tubing to complete the loop and injected drug or carrier into the circulatory system to treat the cells. Once the chips were set up, we secured them to a tray and placed a tension device on the tubing to secure them to the pump (React4Life, Genoa, Italy) allowing proper movement of fluid. Once the tension device was secured to the pump, the tension was set to 3 clicks. The settings on the microfluidic pump were 4.6 rpm using 2.0 mm diameter tubing, with a flow rate of 1 mm/minute and average velocity below the 24 well insert at 0.35 mm/sec, comparable to retinal capillary flow (0.14 to 1.85 mm/sec) [7].

Drug Treatment and Cell Survival: 50,000 cells were placed in a 24-well size insert (0.4 micron pore size) in RPMI 10% FBS and grown at 37º/5% CO2 incubator. Cells were either untreated, treated with 0μM or 100μM luteolin (Selleck Chemicals, Houston, TX) (a cytotoxic flavonoid with known efficacy against RB [9]), or control DMSO (ATCC, Manassas, VA) carrier in the closed-loop circulation for 24 hrs and compared with static cultures. Static cultures included both 60mm dishes as well as chips set up with the pump turned off. Cell counts were then taken from both the fluidic and static conditions (control and treated), and tested for viability using trypan blue. After 24 hours we carefully drew up 10uL of medium out of each chip and dish (Luteolin and DMSO for both) and combined them in a 1:1 ratio with 0.4% trypan blue (GIBCO). The mixture was then placed on a hemocytometer to be viewed under the microscope at 10x magnification. While viewing under the microscope, we then counted the cells both dead and alive to find the % viability to compare to the original viability. Cells were also counted with a CellDrop FLi automated cell counter (DeNovix, Wilmington, DE) for trypan blue viability. Graphpad Prism was used to generate statistics (average, standard deviation, p value).

Results

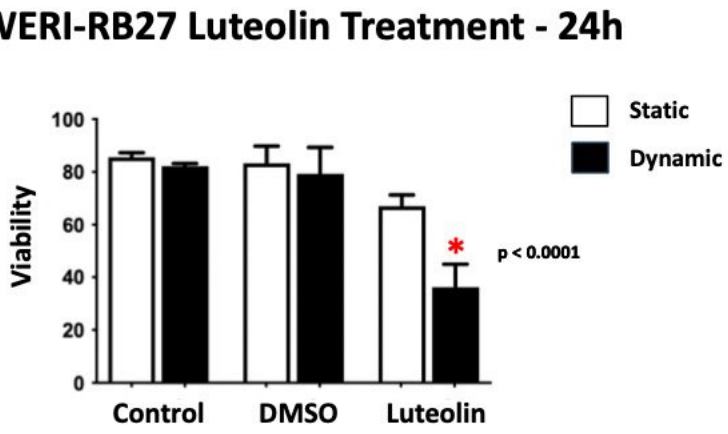
RB-on-a-chip in action We set up RB-on-a-chip as a cytotoxicity platform as shown in Figure 1. Medium with drug was introduced via a port into the circulatory system. RB cells were placed into a 24 well insert within the MIVO chamber. A pump circulated the medium with/without drug through the circulatory tube with a unidirectional pulsatile flow as the apparatus sat within a 5% CO2 chamber at 37º. Cells were removed from the 24 well insert for counting and could be returned to the incubator for continued treatment if needed. RB cells could be maintained in this system with greater than 90% viability for up to 2 weeks (the integrity limit of the circulatory tubing).



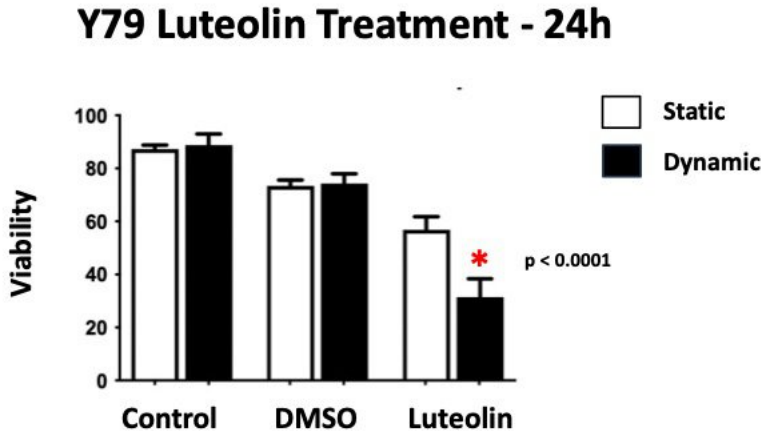
Drug Treatment Luteolin (3′,4′,5,7-tetrahydroxyflavone) is an anti-cancer flavonoid derived from many types of plants including fruits, vegetables, and medicinal herbs [for review, see 10]. Since we have already examined concentration-dependent drug effects of luteolin on RB cells [9], it seemed reasonable to use this known entity to test the RB-on-a-chip system as a potential cytotoxicity platform. We compared the effects of 100μM luteolin in the RB-on-a-chip platform versus static conditions at 24 hours, as shown in Figure 2. For all three RB cell lines (WERI-RB27 [Panel A], Y79 [Panel B] and RB143 [Panel C]), there was a significant difference ($p<0.0001$) between cell survival in the dynamic conditions versus static conditions. In contrast, cell viability in the untreated conditions or with DMSO (luteolin carrier) were not significantly different between the chip and static conditions.

Figure 2: Luteolin Shows More Potency in Dynamic versus Static Conditions
In panels A, B and C, 100 μM luteolin showed enhanced potency after 24 hours under dynamic conditions versus static conditions ($p > 0.0001$). There was no significant difference between control (untreated or DMSO) between static and dynamic conditions.

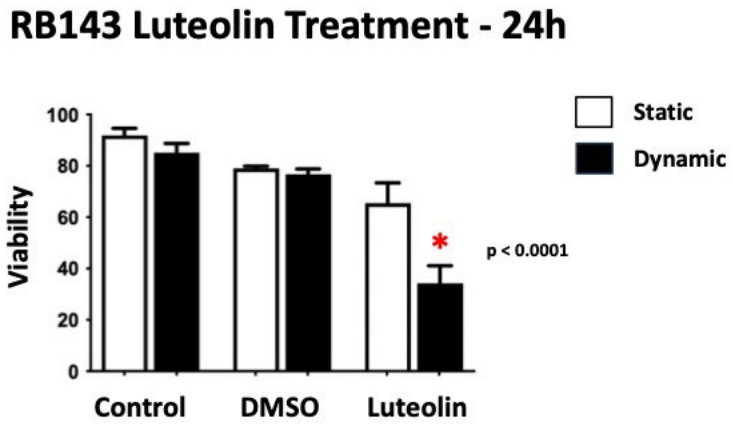
A



B

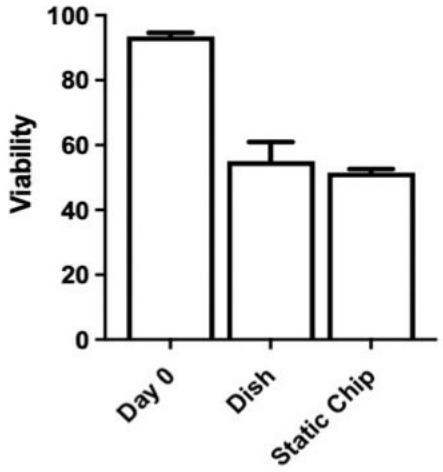


C



We then tested static chip (with the pump turned off) versus static dish as shown in Figure 3. We saw no significant differences in the cytotoxic effects of luteolin between the two conditions, suggesting that it is not the physical configuration of the chip versus dish, but rather the circulation of the drug that seems to have the most influence on luteolin cytotoxicity.

Figure 3: Static Chip Versus Static Dish. For luteolin treatment, both static chip (with the pump turned off) and static cell culture dish showed similar luteolin effects, with no statistically significant difference between them. Day 0 indicates untreated control cells that remained greater than 90% viable throughout the experiment.



Discussion

The majority of cancer drugs fail in clinical trials, primarily because traditional pre-clinical models (2D cell cultures and mice) poorly mimic human physiology. We present here proof of concept of the first reported RB-on-a-chip model with physiological relevant application to therapeutic drug testing in vitro. The optimized pulsatile circulatory flow under the insert (0.35mm/sec) is within the range of human retinal capillary flow (0.14 to 1.85 mm/sec) [8], supporting physiological relevance. Both systemic and intra-arterial administration of chemotherapy for RB involve drug traveling within the blood vessels, supporting the concept that circulatory movement of drugs is relevant in both treatment paradigms and could be more precisely configured in future studies to match these and other modalities. RB-on-a-chip represents a new platform that will enable precise measurement of drug cytotoxicity in a dynamic environment. As per the FDA Modernization Act 2.0 in 2022, data from organ-on-a-chip platforms can now be accepted as evidence in drug approval processes, which may reduce the need for animal testing.

Interestingly, we found that luteolin displayed more potent killing effects in the millifluidic chip environment versus static conditions. This enhanced toxicity phenomenon in dynamic conditions has been seen with other drugs, including doxorubicin and cisplatin [11]. One possible explanation for this difference is that the continuous flow of drugs through the circulatory tubing leads to improved drug bioavailability and cellular

exposure in the chamber [12]. Another possibility is that physiological forces and fluid shear stress in the circulating system enhance drug potency [13]. In addition, enhanced cell-cell interactions [14] and bystander effects [15] could lead to increased cell death in the dynamic environment. Differences in cytotoxicity between dynamic and static drug treatments show the importance of developing drug testing platforms with optimized physiological relevance. RB-on-a-chip shows strong promise as a valuable tool for future in vitro studies of retinoblastoma.

Acknowledgements: This research was funded in part by The Childhood Eye Cancer Trust (Dr. Judith Kingston Research Fund) and the Undergraduate Research and Creative Activities Program at the University at Buffalo (GMS), Professional Staff Congress-City University of New York Research Award (Award # 68676-00 56) (RG). The authors declare no conflicts of interest. No generative AI was used in preparation of this manuscript.

References

1. Alkatan, H. M, Al Marek, F, Elkhamary, S. (2019). Demographics of Pediatric Orbital Lesions: A Tertiary Eye Center Experience in Saudi Arabia. *Journal of epidemiology and global health*, *9*(1), 3-10. https://doi.org/10.2991/jegh.k.181224.001
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ABSTRACT

TITLE: RB-on-a-Chip: A Dynamic Platform to Study Retinoblastoma In Vitro

ABSTRACT BODY:

Purpose: Retinoblastoma (RB) is the most common intraocular tumor of childhood. To date, in vitro studies of RB have involved static cell cultures with limited relevance to tumors in vivo. We hypothesized that we could improve the in vitro study of RB by developing a microfluidic platform to recapitulate physiological flow, shear stress, nutrient delivery, and drug activity in a controlled environment. Here we present the first of its kind RB-on-a-chip microfluidic system as a means to study RB cell growth and response to therapeutic drugs in a dynamic 3D environment.

Methods: RB cell lines including Y79, RB143, and WERI-RB27 were set up in MIVO microfluidic chambers (React4Life, Genoa, Italy). The settings on the microfluidic pump were 4.6 rpm with 2.0 mm diameter tubing, a flow rate of 1 mm/minute and average velocity below the insert at 0.35 mm/sec. For survival studies, 50,000 cells were placed in a 24-well insert (0.4µ pore size) in RPMI 10% FBS, grown at 37 degrees/5% CO2 incubator for up to 2 weeks and tested for cell viability by trypan blue exclusion. Cells were counted every day and compared with control static suspension cell cultures. For cytotoxicity studies, cells were treated with 0µM to 100µM luteolin (a cytotoxic flavonoid with known efficacy against RB) or control DMSO in the circulation for up to 72 hrs and compared with static cultures.

Results: RB cell cultures were maintained in MIVO for up to 2 weeks, the integrity limit of the MIVO tubing. All three untreated RB cell lines survived in MIVO chambers ranging from 91% to 97% viability, comparable to static cultures. In drug testing studies, by 48 hrs of luteolin treatment, RB cells in MIVO were already 6 to 8-fold lower in viability compared with RB cells in static culture ($p<0.05$). By 72 hrs, at least 97% of luteolin-treated RB cells in MIVO were dead, compared with 75% in the dish ($p<0.05$), suggestive of more potent luteolin effects as a circulating drug. Additional drug testing and invasion experiments are now underway.

Conclusions: RB-on-a-chip can be used for in vitro studies of RB cell growth and response to therapeutics with enhanced recapitulation of the native tumor microenvironment. This RB platform will allow for high throughput tests of new drugs and therapeutics with decreased cost,

ethical advantages over animal studies and reproducible conditions that more closely approximate the circulating in vivo tumor environment.

Layman Abstract (optional): Retinoblastoma (RB) is the most common intraocular tumor of childhood. Most studies of RB involve cells growing in a dish. We have improved on this by growing RB cells in a chamber with a circulatory system to mimic the tumor environment in the body. We have developed the first of its kind RB-on-a-chip system to study retinoblastoma cell growth, invasion, and response to therapeutic drugs in a dynamic 3D environment.

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ALYREF/THOC4 Expression and Cell Growth Modulation in Retinoblastoma

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Abstract

In this study, we tested the hypothesis that ALYREF/THOC4, a poor prognostic factor in different cancer types, has potential as a drug target and prognostic biomarker for retinoblastoma (RB). Immunostaining (IHC), Western blot, and RT-qPCR analyses detected overexpression of ALYREF in the RB cell lines Y79, RB143, WERI-RB1, and RB116. IHC analysis on RB tumor array showed that 11/14 of RB tumors were ALYREF+ to varying degrees, with eight tumors at maximum 3+ intensity. The IHC analysis also detected ALYREF+ cells in normal retina, mainly in the inner nuclear and ganglion cell layer, while some tumor-bearing human eyes were ALYREF+ in the optic nerve suggesting a role in optic invasion/tumor invasion. The expression of ALYREF within the tumor itself, in the optic nerve, as well as in adjacent "normal" retina, suggest that this pattern of expression may lead to ALYREF being a potentially useful prognostic indicator for RB, as it is for other tumors. siRNA knockdown of ALYREF resulted in a 40% decrease in cell growth

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Declaration of competing interest

The authors declare no potential conflicts of interest

CRediT authorship contribution statement

Rajendra Gharbaran, Gail M. Seigel, Onyekwere Onwumere, Moira Sauane, Sual Lopez, Enyuan Shang, Habiba Habiba, Stephen Redenti: Data curation, Formal analysis, Manuscript review, Editing, Methodology. **Rajendra Gharbaran, Gail M. Seigel:** Writing – original draft, Figure, Data table generation. **Rajendra Gharbaran, Gail M. Seigel, Moira Sauane, Hans Grossnikaus, Stephen Redenti:** Funding acquisition. **Rajendra Gharbaran, Gail M. Seigel:** Scientific design, Conceptualization, Investigation, Project administration. All authors approved the manuscript.

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in both WERI-RB1 and Y79 cells ($p<0.05$) and this was associated with decreased expression of mRNAs for the cell proliferation markers Ki67 and PCNA ($p<0.005$). These results suggest a role for ALYREF in RB cell growth regulation and its potential as both a target and a biomarker for tumor growth inhibition by anti-cancer therapies.

Keywords

retinoblastoma; WERI-RB1; ALYREF/THOC4; Y79; RB116; metastasis; proliferation; retinoblastoma

Introduction

Retinoblastoma (RB) is the most common intraocular pediatric cancer, affecting approximately 300 children each year in the US [1]. In high-income countries, RB has a five-year survival rate of over 90%, mainly due to early detection and advanced treatment options [2]. However, the global incidence/survival of this malignancy remains poor, with one case per 15,000–20,000 live births [3] and an estimated mortality rate of 30 to 50% [4]. Therefore, there is a need to gain greater insights into the molecular mechanisms of RB pathology, essential for developing effective therapeutic strategies.

To date, the etiology of RB is not completely understood. However, it is presumed that the primary RB tumor cells descended from proliferating precursors with biallelic genetic defects in the RB1 tumor suppressor gene, most frequently before age 5 [5]. While biallelic inactivation of RB1 on chromosome 13 [6] remains the most significant event in RB oncogenesis, the pathology of the proportion of RB cases that are RB+/RB+ may be driven in part, by other factors such as amplification of MYCN [7], MDM2/4 and E2F3 [8], among others.

ALYREF (THOC4) functions as a molecular chaperone with important roles in RNA metabolism, RNA nuclear export, RNA stability, and regulation of gene transcription [9–12]. Emerging studies showed deregulated ALYREF in human cancers is associated with poor prognosis and plays important roles in cell proliferation and migration [13]. Currently, there is no data on the putative role(s) of ALYREF in RB.

Therefore, the aim of the present study was to gain insights into the putative roles of ALYREF in RB.

Methods

Cell lines and cell cultures.

The human-derived RB cell lines WERI-RB1 and Y79, and normal human retinal pigmented cells ARPE-19, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). WERI-RB27 cells were generously provided by Dr. John Ludlow. RB116 cells were generously provided by Dr. Bruce Ksander from Harvard University. WERI-RB1, WERI-RB27, and Y79 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Gaithersburg,

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MD, USA), 1% L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) and penicillin/streptomycin (Thermo Fisher Scientific). RB116 cells were cultured in similar media but with 1% HEPES (Gibco). ARPE19 was maintained similarly but in a 1:1 DMEM/FAM medium containing 20% heat-inactivated serum. U251 cells were kindly provided by Dr. Enyuan Shang of Columbia University. The culture condition for U251 cells was similar to that of WERI-RB1 and Y79 but in DMEM. All cells were maintained in a humid environment of 5% CO_2 at 37°C.

Immunohistochemistry

Paraffin sections of human RB tumors (5 microns thick) were generously provided by Dr. Hans Grossniklaus, Emory University. A human RB tissue array with 14 RB cases and adjacent ocular tissues (BC35111a, USBiomax, now TissueArray.com, Derwood, MD) was also used for analysis. Negative controls received isotype control antibody instead of the primary ALYREF antibody. Tumor sections were baked for one hour at 60°C to promote adherence. For immunostaining, RB tumor sections were deparaffinized for 8 minutes in xylene, followed by three minutes of incubation each in graded alcohols (100%, 95%, 70%), followed by water. Paraffin sections were boiled in antigen retrieval buffer (sodium citrate, pH 6.0 with 0.05% Tween-20) for 25 minutes. Cytospins and deparaffinized tissues were stained using the CRF Anti-Polyvalent HRP Polymer kit (ScyTek, Inc. Logan, Utah). Sections and cells were incubated in PBS with 0.05% Tween-20 for five minutes at room temperature. Tumor sections received primary ALYREF rabbit monoclonal antibody (clone D3R4R, Cell Signaling Technology, Danvers, MA, USA, cat. #12655) (1:100 dilution in PBS-Tween) incubated for 1 hour at room temperature. HRP-Polymer and diaminobenzidine (DAB) were used according to the CRF kit to develop a brown reaction product. After DAB staining and water rinse, paraffin sections were counterstained with hematoxylin for 1 minute, rinsed in water, followed by dehydration through a series of graded alcohols (70%, 95%, 100%) back to xylene. Paraffin slides were mounted in EcoMount (Biocare Medical, Pacheco, CA). Cytospins were mounted with KPL aqueous mounting medium (KPL, Gaithersburg, MD). Digital images were captured with a SONY ICX 285AL SPOT camera (Diagnostic Instruments, Sterling Heights, MI).

Cellular immunofluorescence

Cellular immunofluorescence was carried out as described [14, 15] with some modifications. Cells adhered to poly L-lysine (Sigma-Aldrich, MO, USA cat. #: 27964-99-4) coated number 1 coverslips microwell (MatTek Corporation, Ashland, MA, USA) overnight at 37°C in 5 % CO_2, were fixed for 15 min with 4 % paraformaldehyde (Electron Microscopy Sciences 15710, Hatfield; PA, USA). Fixed cells were permeabilized with 0.25 % PBS-TX100 (Triton X-100 purchased from Sigma–Aldrich, MO, US, cat. #: T9284) for 10 min then blocked for 30 min in 1 % BSA prepared in 0.1 % PBS-Tween20, followed by incubation in the ALYREF rabbit monoclonal antibody, diluted at 1:400 in blocking buffer, overnight at 4°C. Next, the cells were rinsed three times with 1 × PBS, then incubated with Alexa fluor 488-conjugated secondary antibody diluted at 1:1000 in PBS containing Alexa 594-conjugated Phallodin, for 1 h in a dark chamber, followed by rinses as described. Stained cells were mounted in ProLong Gold anti-fade reagent containing DAPI (Life Technologies, Carlsbad; CA, USA, cat. #: P36931) and images were captured with a

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CoolSNAP HQ2 CCD camera (Cool SNAP EZ, Photometrics, Tucson; AZ, USA) coupled to a Nikon Ti Eclipse inverted microscope (Melville, NY, USA).

RNA isolation, cDNA, and RT-qPCR

Total RNA was isolated by Trizol (Life Technologies, Carlsbad; CA, USA, cat. # 155960218) according to the manufacturer's instructions. cDNA was prepared by ProtoScript® II First Strand cDNA Synthesis Kit (New England BioLabs, Rowley; MA, USA, cat. #: E6300S) according to manufacturer's instructions, from 1 μg of RNA per 20 μl reaction. For qPCR, each reaction consisted of cDNA that is equivalent 2.5 ng of mRNA, 10 mM primers, and 10 μl 2X SYBR GreenER™ qPCR SuperMix (Life Technologies, cat. #: 11761-100). Reactions were carried out in a MicroAmp Fast Optical 96-Well Reaction Plate on a BioRad Icycler Thermal Cycler. The reaction was performed using the standard mode (initial denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute). Each qPCR reaction was done in triplicate. The amount of target mRNA was normalized to the expression levels of the housekeeping gene GAPDH. The PCR primers and their respective sequences are listed in Table 1. The ΔΔCt method was used to calculate the fold-change relative to controls.

Protein analysis by Western blot

Western Immunoblot analysis was accomplished as described by Persaud et al (2018) [16] with modifications. Briefly, total protein was extracted from cells using 50 μL Pierce IP lysis Buffer (Thermo Scientific Fisher) and 100X protease inhibitor. Protein lysates were centrifuged at 12,000 RPM at 4°C for fifteen minutes. Supernatants were mixed with 6X sample buffer and heated at 75° C for 15 minutes prior to electrophoresis (Cell Signaling Technology, Inc.). Following normalization, 5 μg of each protein sample was added to 12% gels, loaded into a Mini Gel Tank (Invitrogen), with 1X running buffer, and electrophoresed at 100 volts for 45 minutes. The gels were transferred to nitrocellulose membranes and incubated in Odyssey blocking solution for 45 minutes followed by overnight incubation at 4°C with primary antibody against ALYREF. The membrane was then rinsed 3 times with 1X TBS-Tween for 5 minutes each followed by incubation with secondary antibody for 45 minutes at 4°C. The membrane was washed three times with 1X TBS-T for 5 minutes each, and the resulting protein bands were analyzed using the Odyssey Clx imaging system.

siRNA Transfection

siRNA transfection was carried out as described by Gharbaran et al (2021) [15] and Gharbaran and Aisemberg (2013) [17] with modifications. 5×10^4 cells/ml cultured in poly L-lysine-coated 6-well plate overnight, were treated with 25 nM of either siRNA to ALYREF (Dharmacon, CO, USA, cat #: L-012078-00-0010--ON-TARGETplus SMARTpool) or off-target control (Dharmacon, CO, USA, cat #: D-001810-10-05-ON-TARGETplus Non-targeting Pool) for 48 h. In brief, siRNA was complexed with Lipofectamine RNAiMax (Invitrogen, cat. #: 13778075; lot #: 2018673) in Opti-MEM reduced serum media (Gibco, cat. #: 31985070; lot #: 2003883) in 1X siRNA buffer (Dharmacon, cat #: B-002000-UB-100), before applying to cultured cells. The sequence of siRNAs included in the ON-TARGETplus SMARTpool from Dharmacon, consists of the following sequences: UCU CAG ACG CCG AUA UUC A (J-012078-05), GUU AAA CAG

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ACC AGC AAA U (J-012078-06), GGA ACU CUU UGC UGA AUU U (J-012078-07), CAA AAC AAC UUC CGA CAA (J-012078-08 and those of the off-target control, ON-TARGETplus Non-targeting Pool (cat. # D-001810-10-05) are UGG UUU ACA UGU CGA CUA A, UGG UUU ACA UGU UGU UGA, UGG UUU ACA UGU UUU UCU GA, UGG UUU ACA UGU UUU CCU A.

WST-1 cell proliferation assay

Cell viability assay was performed as described by Gharbaran et al [18]. In brief, 100 μl of cell suspension containing 1×10^5 cells/ml were seeded in a 96-well plate, incubated overnight then treated with siRNA to ALYREF (ALYsi) or off-target control sequences for controls (OTCsi) for 48 h. Cells were then incubated with WST-1 and absorbance was read at 450 nm on a Synergy H1 Hybrid microplate reader (BioTek Instruments, Winooski, VT, USA). Cell growth (as a percentage) was computed as a ratio of the absorbance (A450) of treated (either ALYsi or OTCsi) cells to the absorbance of the untreated control.

Statistical analyses.

Data analyses were performed using SAS 9.1.3 (SAS Institute Inc., Cary; NC, USA), StatView 5 (SAS Institute Inc.), and JMP 16.2.0 (SAS Institute Inc.). RT-qPCR fold change and cell growth data were presented as the mean standard of the mean (SEM), derived from triplicates. Paired *Student t-test* and analysis of variance (ANOVA) were used to determine differences between the means as defined by $p<0.05$. Each experiment was repeated at least three times.

Results

Detection of ALYREF in RB cell lines

The present study reports on the detection of the ALYREF/THOC4, a transcription factor and RNA nuclear exporter protein. To determine whether ALYREF is overexpressed in RB, we first compared expression between RB cell lines WERI-RB1, WERI-RB27, RB116, and Y79 to ARPE-19 cells (normal retinal epithelial cells). RT-qPCR conducted on Trizol-extracted mRNA showed significantly higher levels of endogenous ALYREF transcripts in RB116, WERI-RB1, and Y79 cells compared to either ARPE-19 or glioblastoma U-251 cells (Figure 1). We also carried out Western blot studies on whole cell lysates to access the translational levels of ALYREF in the RB cell lines. The results showed a protein band of approximately 27 kDa which corresponded to the expected molecular weight of ALYREF (Figure 1B). This band showed a higher intensity for RB cells compared to either ARPE-19 or U-251 cells. In these experiments, β-actin was used for internal control. The relative levels of ALYREF mRNA for each cell line appeared comparable to the relative protein levels detected by Western blot. Intriguingly, the Y79 cells which display greater metastatic potential [19], appear to express higher levels of ALYREF at the protein and mRNA levels, even marginally greater than the WERI-RB1 cells. We also conducted immunocytochemistry for the detection of ALYREF in RB cell lines. Each cell line showed immunoreactivity for ALYREF (Figure 1C–F) which was absent in negative controls.

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Primary human RB tumors are immunoreactive to ALYREF

For clinical relevance, we next examined primary human RB tumors for ALYREF immunoreactivity using a tumor tissue array with 14 cases of RB. (Fig. 2). The tumor array slide was immunostained as per Methods and evaluated for staining intensity as shown in Figure 2, Table 2, where individual results for each tumor sample are shown alongside clinical information. We found that 11/14 RB tumors were ALYREF positive and 8/11 of these were +++. The staining intensity did not appear to correlate with tumor staging. Examples of the 0 to +++ immunoreactivity scale are shown below the Table 2. No immunoreactivity was seen with cornea, sclera, or choroid in tumor-bearing eyes. However, we did observe ALYREF immunoreactivity in advancing RB in the optic nerve (Figure 3, Panel A).

ALYREF immunoreactivity in "normal" RB adjacent retina and non-RB retinal tissues

We followed up on one of the findings in Table 2, specifically ALYREF immunoreactivity seen in RB adjacent "normal" retina. Figure 3, Panel B shows immunoreactivity in "normal" RB adjacent retina particularly in the inner nuclear layer (INL) and ganglion cell layer (GCL), with some in the outer nuclear layer (ONL). In addition, we saw ALYREF immunoreactivity in these same layers of retinal tissues from normal eyes (Figure 3, Panel B).

Inhibition of cell growth by genomic depletion of ALYREF

To gain insights into the putative role(s) of ALYREF in RB pathology, we next studied RB cells for changes in growth in response to genomic depletion of ALYREF. To accomplish this, we treated WERI-RB1 and Y79 cells with 100 nM siRNA to either ALYREF (ALYsi) or off-target control (OTCsi) for 48 h and studied the cells for changes in cell growth using WST-1 cell proliferation assay. As shown in Figure 4, siRNA-depletion of ALYREF was associated with decreased cell growth, compared to OTCsi-treated controls. In each cell line, RT-qPCR on the treated cells revealed a decrease in mRNA fold changes for the cell proliferation markers Ki67 and PCNA (Figure 4). The ALYsi-treated WERI-RB1 and Y79 cells showed a 74.31% and 76.49% decrease in ALYFEF expression, respectively (data not shown). These results suggest that ALYREF may be associated with the growth of RB.

Discussion

ALYREF activities have been reported for a number of cancer types, where it may play essential roles in processes such as proliferation [20] and metastasis [21] which drive development and progression, and prognostication of malignant phenotype [22]. Here, we report novel insights involving ALYREF and RB.

Our IHC analyses showed strong staining for ALYREF in some primary RB tumors, with less immunoreactivity in other tumors. This observation is similar to other tumor biomarkers, such as HER2 [23] where some breast cancer tumors are more immunoreactive than others, and even some regions within the same tumor may differ in immunoreactivity. In the case of HER2 and breast cancer, clinical decisions about whether to use HER2-specific anti-cancer therapies are made based on the degree of HER2 immunoreactivity within a tumor [24], so this is not a new challenge in tumor management. Strong

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ALYREF immunoreactivity was also detected in regions that corresponded to the optic nerve, indicating a potential role in disease metastasis for RB, as optic nerve invasion is characteristic of disease progression [25]. In the metastatic stage of head and neck squamous cell carcinoma, ALYREF along with the methylase NSUN5, are overexpressed [26]. It is suspected the metastatic role for ALYREF involves RNA shuttling and modifications [27] processes which are yet to be explored in RB. In addition to our IHC results, we also showed the presence of ALYREF in RB cells using Western blot, RT-qPCR, and cellular immunofluorescence, important for downstream in vitro drug testing platforms.

Our *in vitro* cell growth assay also showed that knockdown of ALYREF in RB cells resulted in decreased cell growth and this was associated with transcriptional inhibition of the cell proliferation markers Ki67 and PCNA. Similar findings were reported for other cancer types. Knockdown of ALYREF resulted in decreased cell proliferation and this was associated with decreased Ki67 and PCNA in bladder cancer and hepatocellular carcinoma [28, 29]. In RB, the knockdown of ALYREF decreased tumor cell proliferation and inhibited tumor formation ability in the RB orthotopic xenografts [30]. Despite this, given the roles of ALYREF in mRNA modifications and metabolism, future studies to determine the role of this transcription factor in RB pathology, especially RB with disease metastasis, are warranted.

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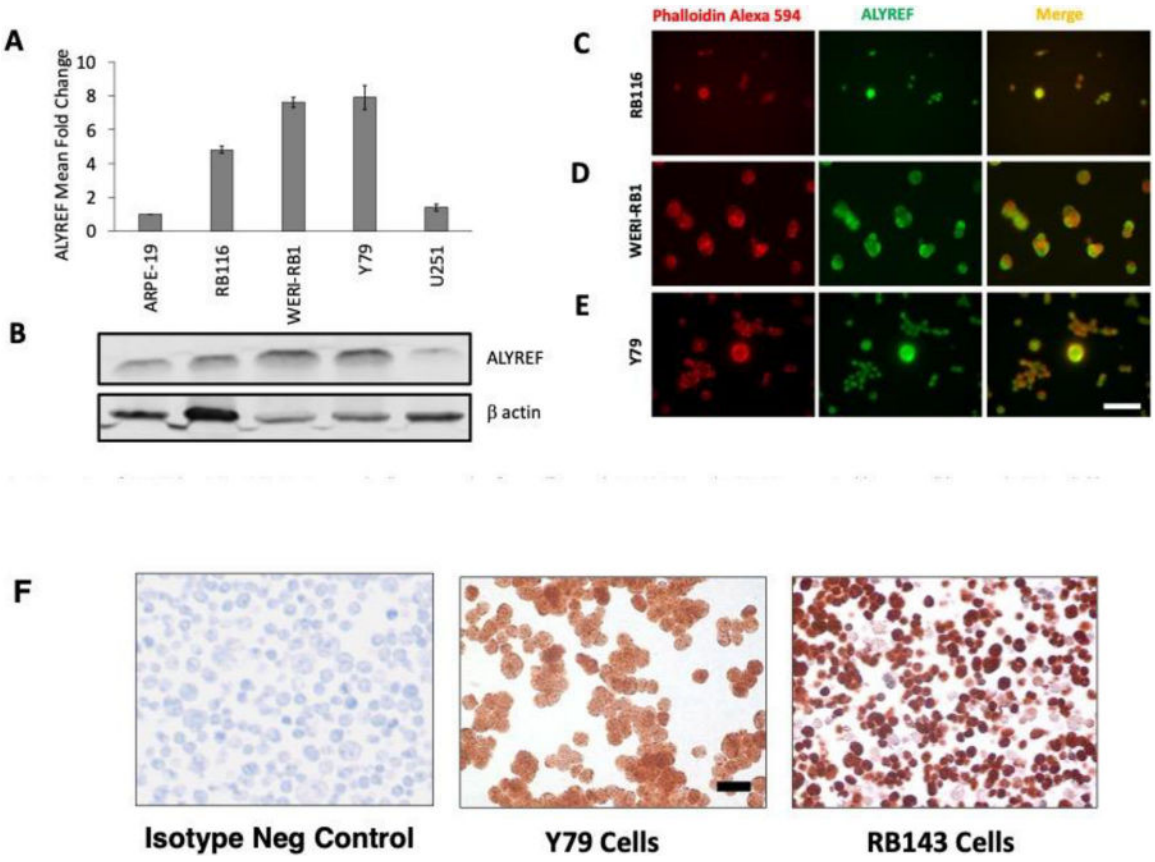


Figure 1. Detection of ALYREF in RB Cell Lines.

Panel A: ARPE-19 pigmented cells were used as ”normal” control. RB116, Y79 and WERI-RB1 are retinoblastoma cell lines, and U251 is a glioblastoma cell line. RNA levels were normalized to GAPDH then the fold changes were determined using the ΔΔCt method. B. Detection of ALYREF by Western blot in the same cell lines as in A. C-E. In this and subsequent figures, *, denotes $p<0.05$, **, $p<0.005$. ARPE-19 pigmented cells were used as ”normal” control. Panel F: ALYREF immunoreactivity in Y79 cells and RB143 cells. Scale Bars = 10 microns.

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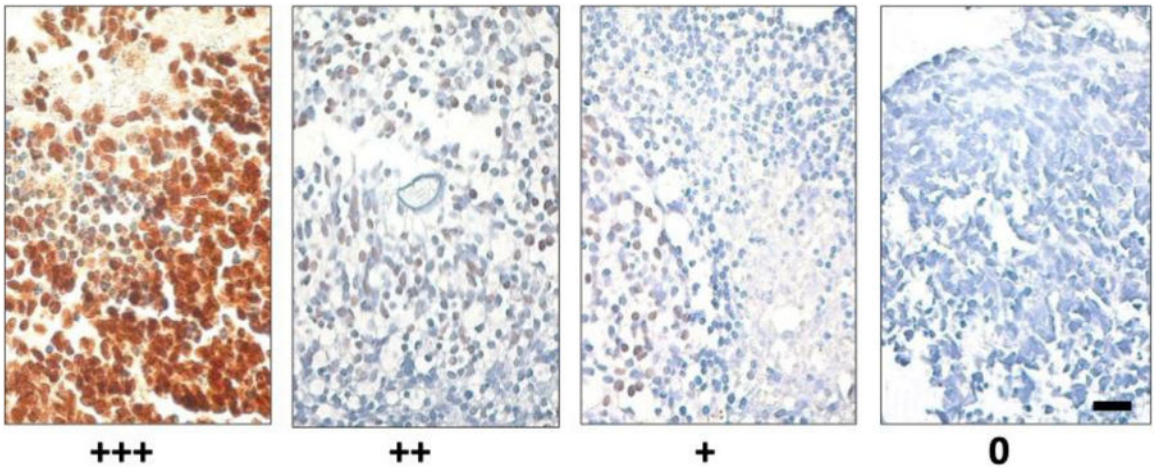


Figure 2. ALYREF Immunoreactivity in RB Tumors.

ALYREF immunohistochemistry was performed, with individual results for each tumor sample, alongside clinical information as available. 11/14 RB tumors were ALYREF + and 8/11 of these are +++. Examples of the 0 to +++ scale are shown below the table.

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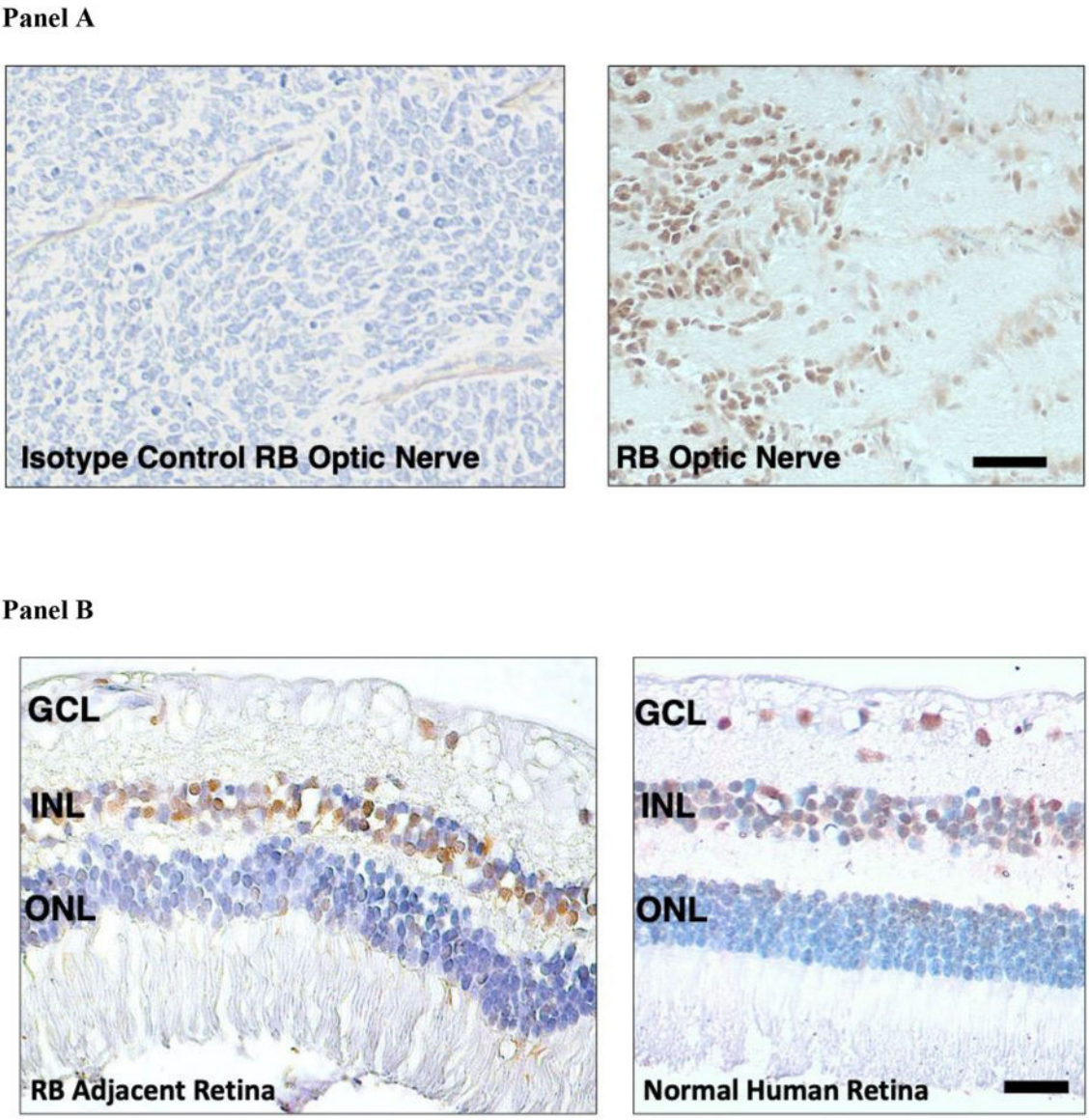


Figure 3. ALYREF Immunoreactivity in RB Optic Nerve, Adjacent Human Retina and Normal Human Retina.

Panel A: ALYREF immunoreactivity is shown in the human optic nerve of an eye with advancing RB. An isotype negative control image is shown for comparison. Scale bar = 20 microns.

Panel B: For RB adjacent retina and normal retina, immunoreactivity is predominantly in the inner nuclear layer (INL) and ganglion cell layer (GCL). Scale bar = 20 microns.

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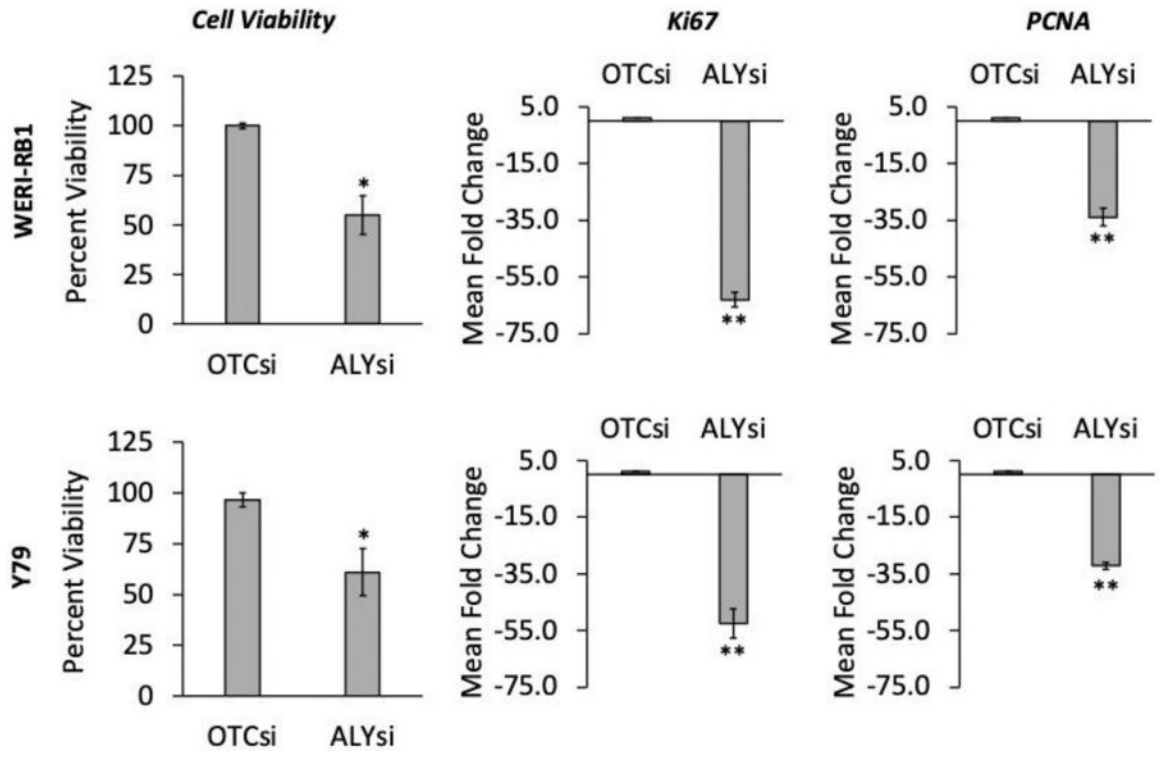


Figure 4. Knockdown of ALYRFEF decreases cell growth.

Depletion of ALYREF transcript with siRNA (ALYsi) compared to off-target control siRNA (OTCsi), resulted in decreased cell growth in WERI-RB1 and Y79 cells. In both cell lines, ALYsi was associated with decreased mRNA fold changes for the cell proliferation markers Ki67 and PCNA.

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Table 1.

List of PCR primers

Gene symbol	Forward Sequence	Reverse sequence
GAPDH	CATGGCCTCCAAGGGAGTA	AGGGGTCTACATGGCAAC
ALYREF	GCAGGCCAA AACAACTTCCC	AGTTCCTGAATATCGGCGTCT
PCNA	GGCTCCATCCTCAAGAAGGT	AGTCCATGCTCTGCAGGTTT
Ki67	AATCCAACTCAAGTAAACGGGG	TTGGCTTGCTTCCATCCTCA

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Table 2.

Summary of ALYREF immunoreactivity

Sample	TNM	Stage	ALYREF
A 1, 2 RB	T3cN0M0	IIC	+
A 3, 4 RB	T4bN0M0	IIIB	+++
A 5, 6 RB	T2N0M0	IB	+++
A 7, 8 RB	T2N0M0	IB	0
B 1, 2 RB	T3cN0M0	IIC	++
B 3, 4 RB	T2N0M0	IB	+++
B 5, 6 RB	T3aN0M0	IIA	+++
B 7, 8 RB	T2N1M0	IV	0
C 1, 2 RB	T3N0N0	II	+++
C 3, 4 RB	T2N0M0	IB	++
C 5, 6 RB	T2N0M0	IB	+++
C 7, 8 RB	T3bN0M0	IIB	+++
D 1, 2 RB	T3aN0M0	IIA	0
D 3, 4 RB	TIN0M0	IA	+++
D 5, 6	“Normal” Adj retina	NA	+
D 7, 8	“Normal” Adj retina	NA	+
E 1, 2	“Normal” Adj cornea	NA	0
E 3, 4	“Normal” Adj retina	NA	+
E 5, 6	“Normal” Adj sclera/choroid	NA	0
E 7, 8	“Normal” Adj sclera/choroid	NA	0
Adrenal tumor	control	-	0