Final Progress Report

Research projects funded by the Childhood Eye Cancer Trust

All information provided in this report will be kept confidential to the Childhood Eye Cancer Trust, unless stated

Project reference: (to be completed by CHECT)

Name: Gail M. Seigel, Ph.D.

Date: May 21, 2019

Project title: Targeted Antibody-Drug Conjugates for Retinoblastoma Therapy

Date awarded & value: 9/15/17 25,000 £

Amount claimed to date:

Expected end date: 9/15/18

Aims / Objectives of project:

The objectives of this project were: (i) *in vitro* evaluation of the efficacy of the ADCs, and (ii) evaluation of the dose-exposure (i.e. pharmacokinetics - PK) relationship, exposure-efficacy (i.e. pharmacodynamics - PD) relationship, and toxicity of the ADCs (via histological evaluation) in an orthotopic mouse model of RB. The results from the proposed experiments will provide invaluable information about the 'druggability' of the novel ADCs. The results will allow us to establish preliminary PK-PD relationships for the ADCs, and determine the safety profile of the ADCs, which are necessary for submitting an IND (Investigational New Drug) application to regulatory agencies.

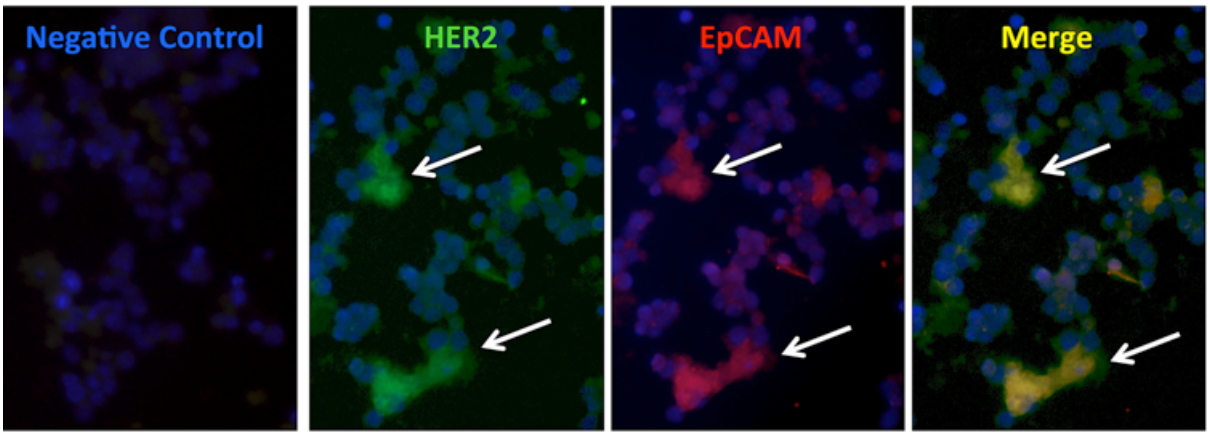
Summary of progress for CHECT scientific advisory committee including targets and outcomes that have been achieved or missed, and why:

Since this may be our final report, we will combine information from previous reports with more recent updates to provide a comprehensive summary:

Initial CHECT Report (October 2018):
We made progress on several fronts, including co-localization studies of HER2 and EpCAM in both RB cell lines and tumors, as well as the assembly of a real time *in vivo* mouse retinal imaging instrument that will allow us to visualize intraocular injections and subsequent tumor growth of fluorescent RB cells in mice.

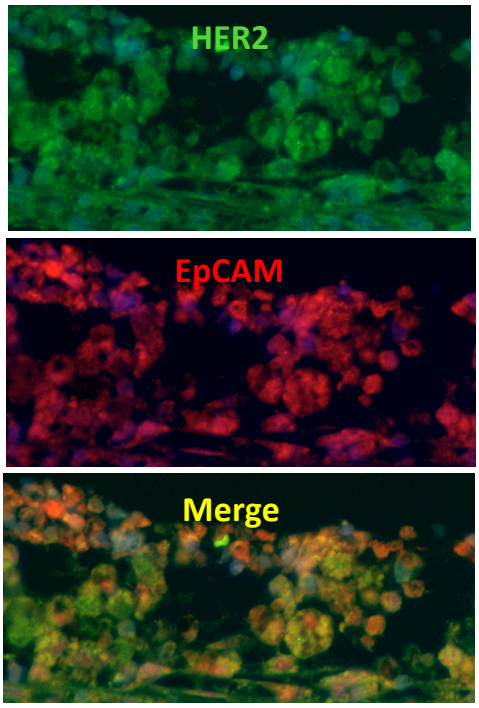
Real Time In Vivo Mouse Retinal Imaging System
Our co-PI Dr. Dhaval Shah assembled a real time *in vivo* mouse retinal imaging system (RIS) in his laboratory that will enable us to visualize intraocular injections, as well as tumor progression in living mice. The Retinal Imaging System (RIS), shown below, will be used to both perform intraocular injections and image ocular fluorescence *in vivo*. RIS has a 3D micromanipulator and pipette holder, which allows for precise manipulation of microneedles used for intraocular injections. The system will be used for intravitreal injection of GFP-labelled RB cells and quantitative monitoring of RB tumor progression or regression in mouse eyes.

Co-localization studies of HER2 and EpCAM in Y79 human RB cells
As part of our goal to examine HER2 and EpCAM as potential drug targets in retinoblastoma, we carried out co-localization immunofluorescence studies of Y79 human retinoblastoma cells, as well as human tumor specimens from Emory University. In the figure below, HER2 (green) and EpCAM (red) show areas of co-localization in Y79 cells.



Co-localization studies of HER2 and EpCAM in human RB tumors

We continued our HER2/EpCAM co-localization study with human RB tumors obtained from an ocular tumor repository at Emory University, curated by Dr. Hans Grossniklaus. As seen in the accompanying photomicrographs, HER2 (green) and EpCAM (red) show areas of co-localization in the merged image of the bottom panel (orange/yellow). The presence of HER2 and EpCAM within the same tumor, often in close proximity, provides important information for the optimization of anti-HER2 and anti-EpCAM targeted drugs, including the possibility of bivalent targeting.

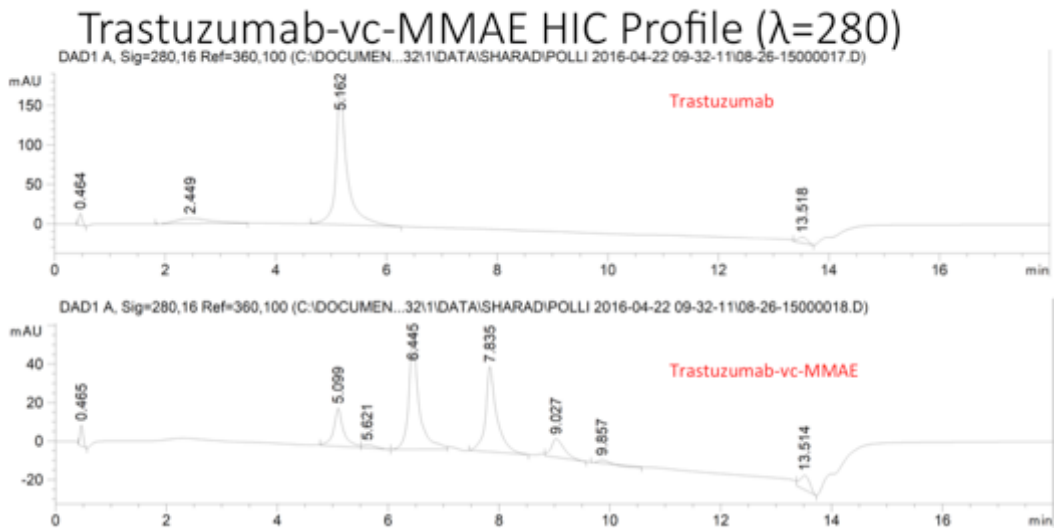


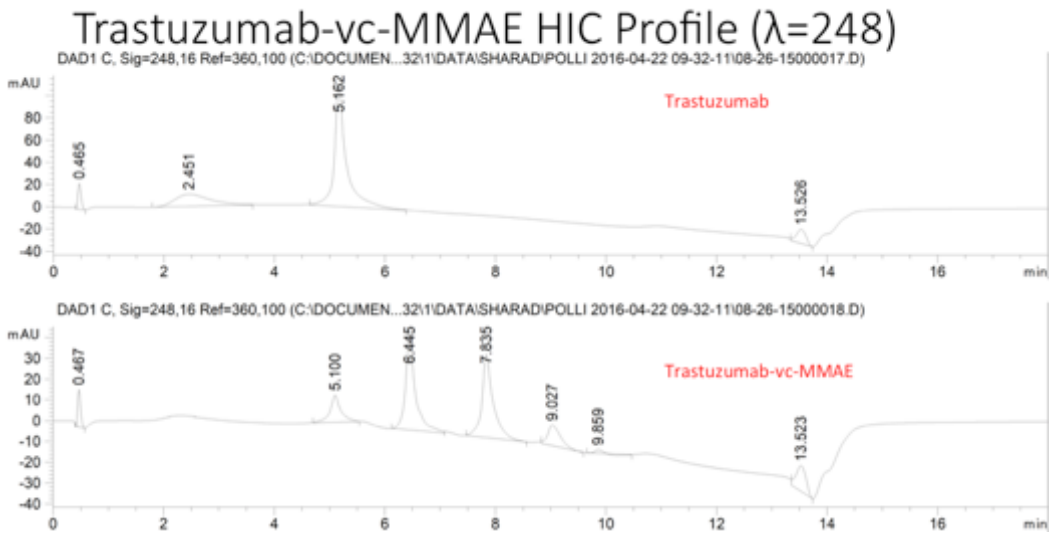
In addition to the results shown here, we have also obtained a new, more potent payload for our antibody drug conjugates. MC-Val-Ala-PBD will be utilized (in addition to MMAE) for our drug conjugates and optimized on human RB cells prior to use *in vivo*.

Second CHECT Progress Report (April 2018):

We have synthesized and analyzed new ADCs, demonstrated cytotoxicity, and begun use of our real-time in vivo mouse retinal imaging system. We have successfully transfected Y79 and WERI-RB27 human retinoblastoma cells with fluorescent GFP, as well as LacZ, giving us two easy ways to detect the presence of these cells in the living mouse eye. Results from these experiments are shown here:

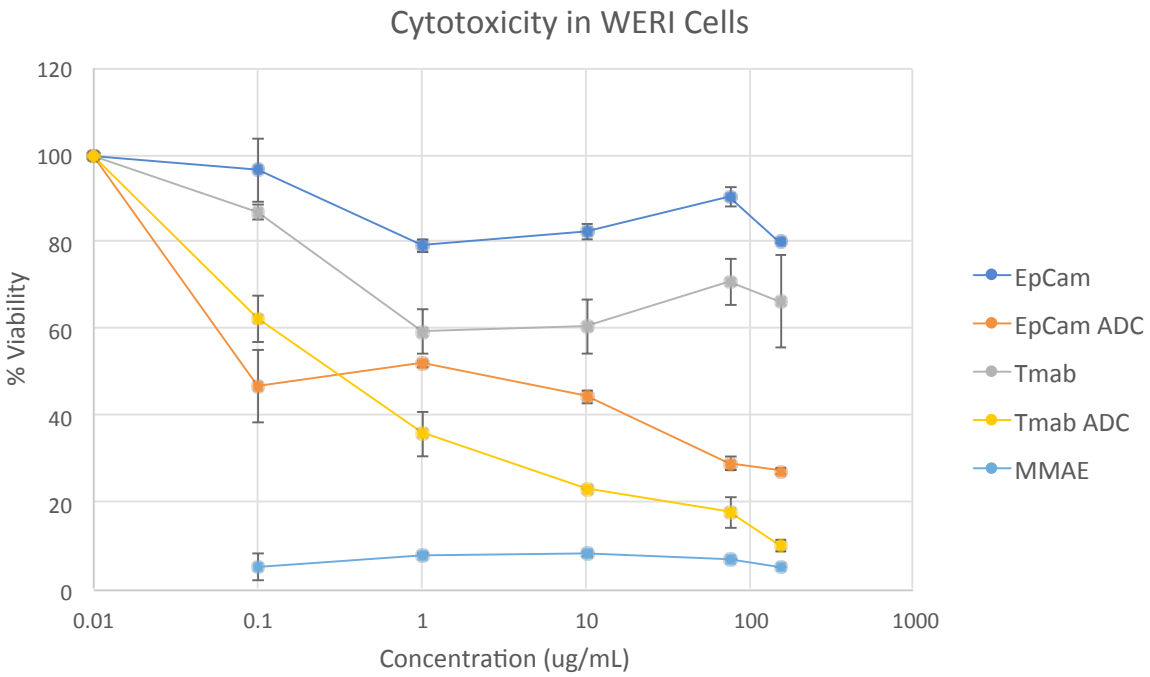
ADC analysis by hydrophobic interaction chromatography
We now have both Trastuzumab and EpCAM ADCs on hand. These plots represent spectra from hydrophobic interaction chromatography for trastuzumab and the trastuzumab-vc-MMAE conjugate at 280nm and 248nm wavelengths (corresponding to the absoroption maxima of both antibody and MMAE respectively). Profiles show conjugation from the appearance of four additional peaks indicating conjugation of 2, 4, 6, and 8 molecules of MMAE. These MMAE variations will be used *in vitro* and *in vivo* as experimental anti-tumor therapy.



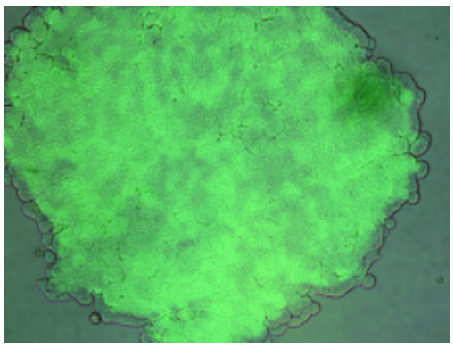


ADC cytotoxicity in WERi-RB27 cells

WERI-RB27 cells were incubated for 96 hours with EpCAM-ADC, Trastuzumab-ADC, as well as unconjugated EpCAM, Trastuzumab and MMAE at concentrations of 0.1, 1.0, 10, 75 and 150 ug/ml. Cell viability was measured by MTT assay. As seen in the graph below, both ADCs exhibited cytotoxicity *in vitro*, while 150 ug/ml of Trastuzumab-MMAE showed the greatest killing effect. This is equivalent to a dose of approximately 0.75 ug/ml in a mouse eye.



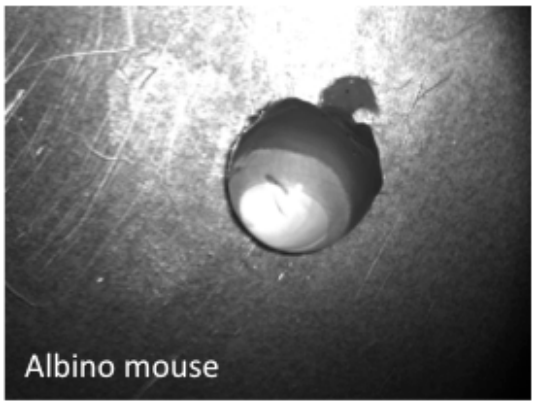
Establishment of GFP+/LacZ+ retinoblastoma cell lines

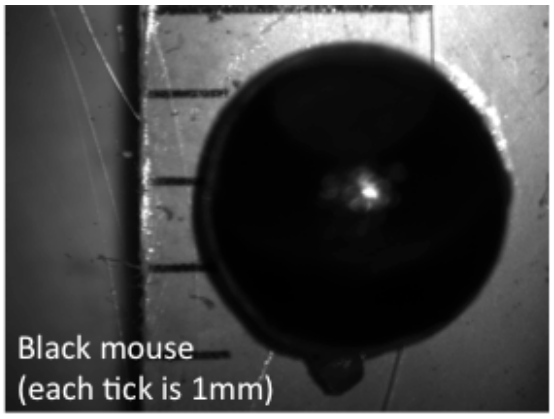


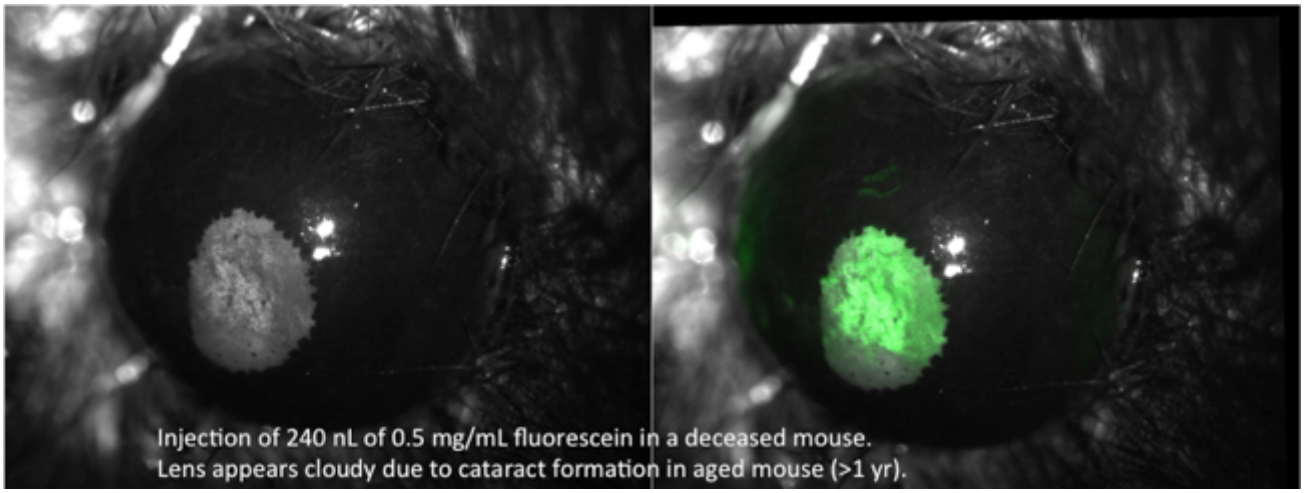
In order to facilitate tumor size measurements in vivo, as well as cytotoxicity experiments in vitro, we successfully transfected Y79 and WERI-RB27 human retinoblastoma cells with a plasmid that confers neomycin resistance, along with GFP+ and LacZ+ expression. To maintain this phenotype, the cells are grown in 250ug/ml of G418 added to the cell culture medium. An image of green fluorescent GFP+ Y79 cells is shown here. These cells will be used for intravitreal injection and quantitative monitoring of RB tumor progression or regression in mouse eyes.

Pilot images with the real-time in vivo retinal imaging system

The images below were taken to calibrate the new real-time *in vivo* retinal imaging system using mouse eyes. The albino mouse provides a clearer view of the intraocular space and fluorescein is visible once injected into the mouse eye. This system will allow us to visualize intraocular tumor progression of GFP+ retinoblastoma cells over time. Once the eyes are harvested, they can be separated into retina, posterior cup, lens/vitreous humor, cornea/ciliary body for further analysis.







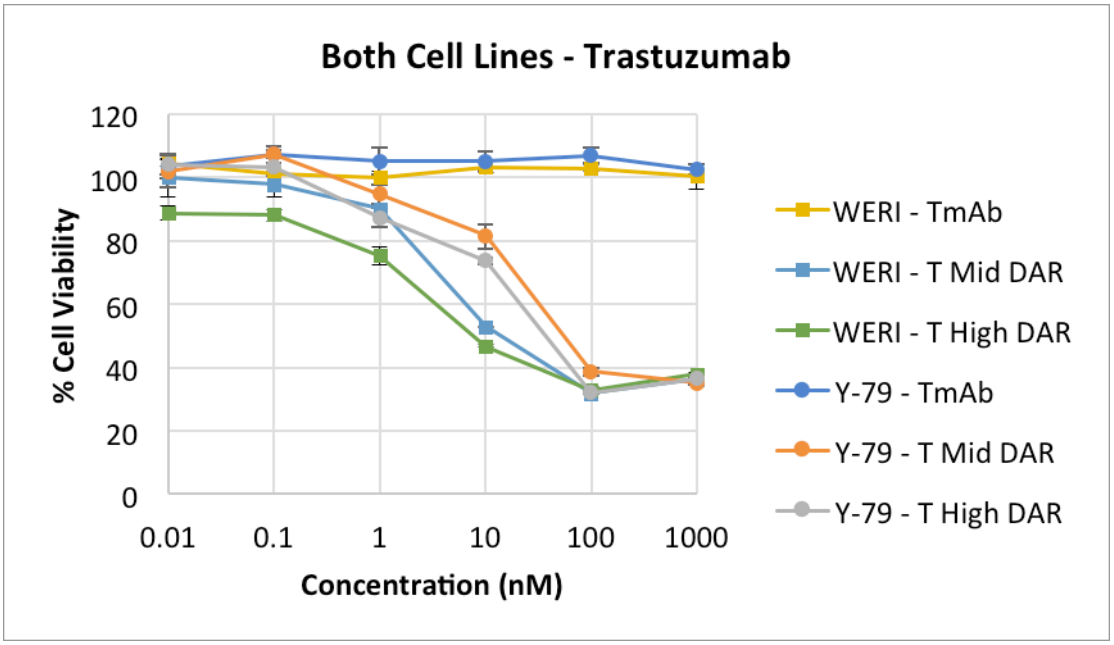
In summary, we demonstrated in our second progress report that both Trastuzumab and EpCAM ADCs show cytotoxicity for human retinoblastoma cells. The availability of GFP+ LacZ+ retinoblastoma cell lines will allow us to visualize tumor progression in the eye of a living mouse with our new real-time in vivo ocular imaging system that is now functional.

Third progress report (November 2018)

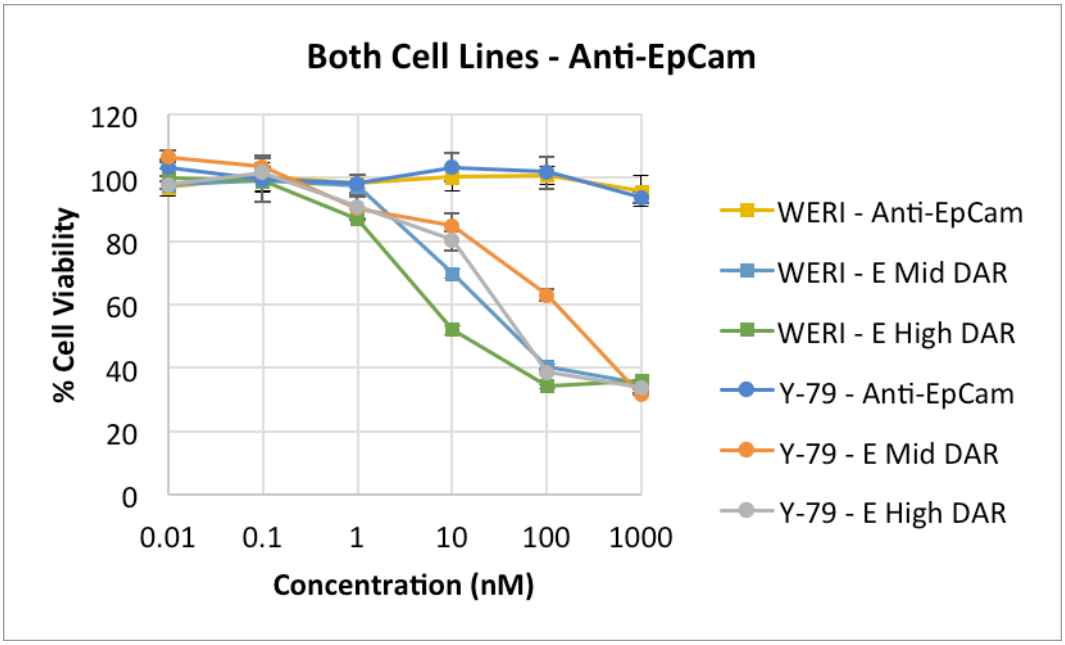
1. We have tested new antibody-drug conjugates with a more cytotoxic payload—pyrrolobenzodiazepine (PDB)
2. We have verified Her2 RNA expression in retinoblastoma tissues by two methods of *in situ* hybridization.

New and more potent antibody-drug conjugates for HER2 and EpCAM:
The following cytotoxicity graphs show the potency of the new antibody-drug conjugates (ADCs) developed against the HER2 and EpCAM targets with a new and improved cytotoxic molecule pyrrolobenzodiazepine (PDB). The trastuzumab (HER2) ADCs showed better efficacy than the anti-EpCam ADC, and the effect was greater in WERI-Rb27 cells as compared with Y79 cells. The maximum ADC concentration (1000 nM) led to ~35% cell viability regardless of antibody and DAR (drug-antibody ratio). The IC50s of these ADCs are approximately 2-5 nM for both formats in the WERI cells, and around 10-20 nM for both formats in the Y79 cells.

Trastuzumab (HER2) data:



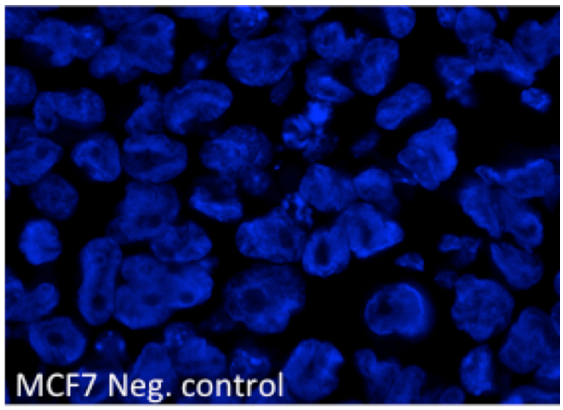
anti-EpCAM ADC data:

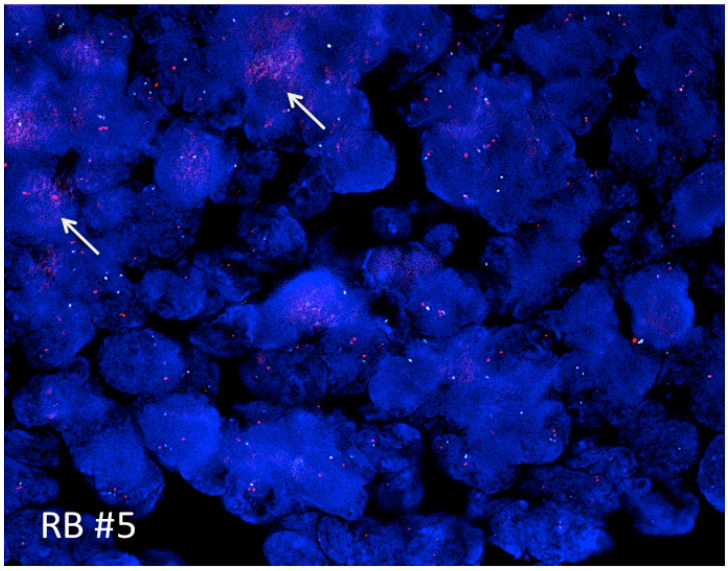


Her2 expression analysis by in situ hybridization
Previously, we had shown HER2 expression in retinoblastoma by immunohistochemistry, flow cytometry, western immunoblot, and PCR analysis. Nevertheless, questions remained about the detection of Her2 in retinoblastoma by *in situ* hybridization as a way of determining Her2 copy number and amplification for diagnostic purposes, as well as another means of verifying Her2 expression in RB. We addressed this issue in two ways: 1) Fluorescence *in situ* hybridization (FISH) performed by an independent molecular genetics core and 2) Colorimetric *in situ* hybridization (CISH) of Her2 using RNAScope in our own lab.

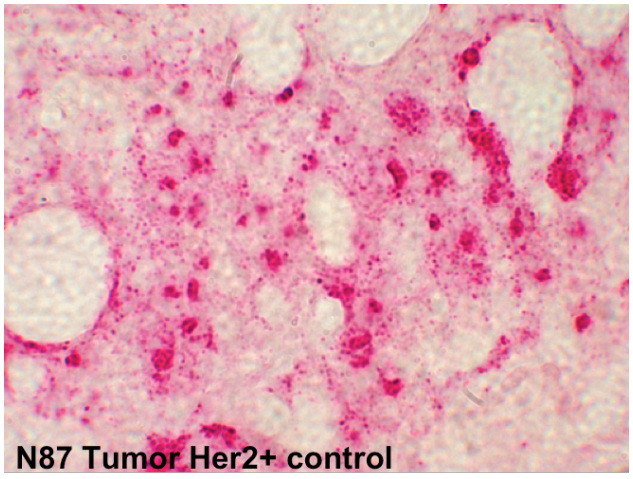
Results: We examined 24 RB tumors, along with normal adjacent tissues and appropriate controls for Her2 expression by both FISH and CISH. A total of 20/24 RB tumors expressed some Her2 by in situ hybridization. Of those 20 that were positive for Her2, three were considered to have low levels of expression. Interestingly, we also saw Her2 signal in six separate cases of "normal" adjacent retinal tissue, with potential implications for RB tumor progression. Examples of FISH and CISH results are shown below:

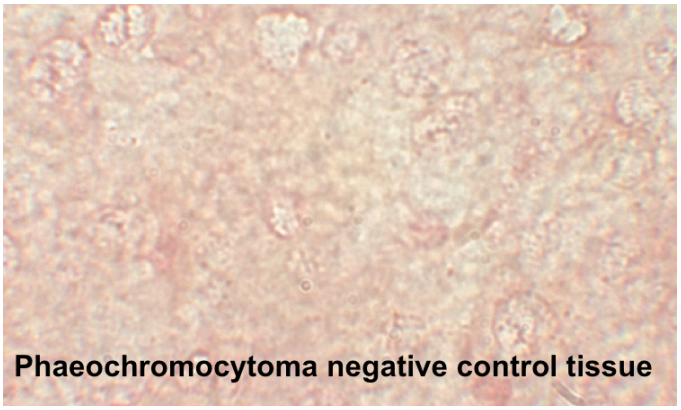
Fluorescence in situ hybridization of Her2 in retinoblastoma
In the first pair of images, fluorescence in situ hybridization is shown for an RB tumor, along with a negative control (MCF7 breast cancer cells). Eight individual RB tumors, along with a tissue array of 17 RB cases were analyzed by the Molecular Cytogenetics Core of Albert Einstein School of Medicine. In the accompanying images, the red dots correspond with a positive signal for Her2. Two representative images (MCF7 negative control and RB Tumor #5) are shown here:

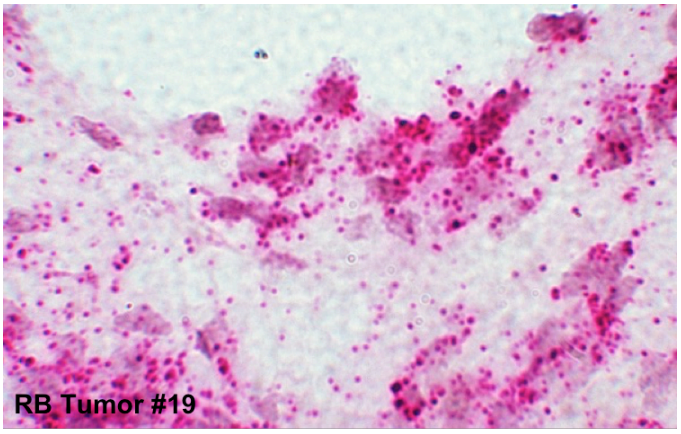




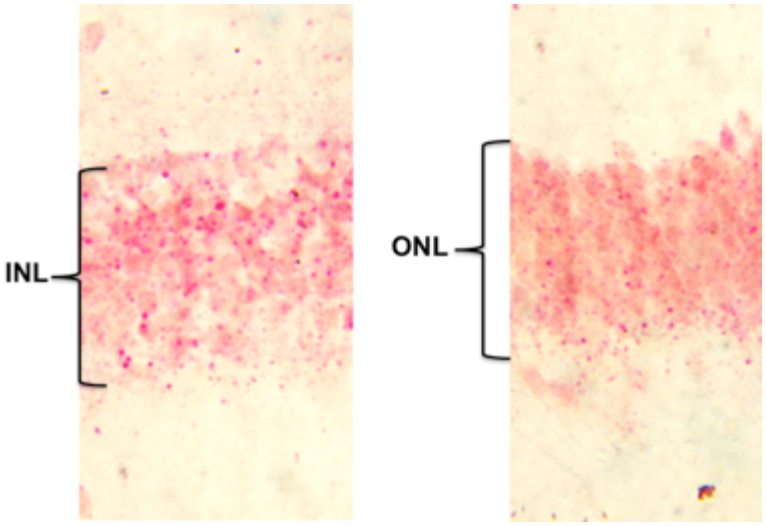
Colorimetric in situ hybridization of Her2 in retinoblastoma by RNAScope analysis Colorimetric in situ hybridization (RNAScope, Advanced Cell Diagnostics, Newark, CA) detects Her2 signal in RB, as shown by red specks. Representative images are shown below:





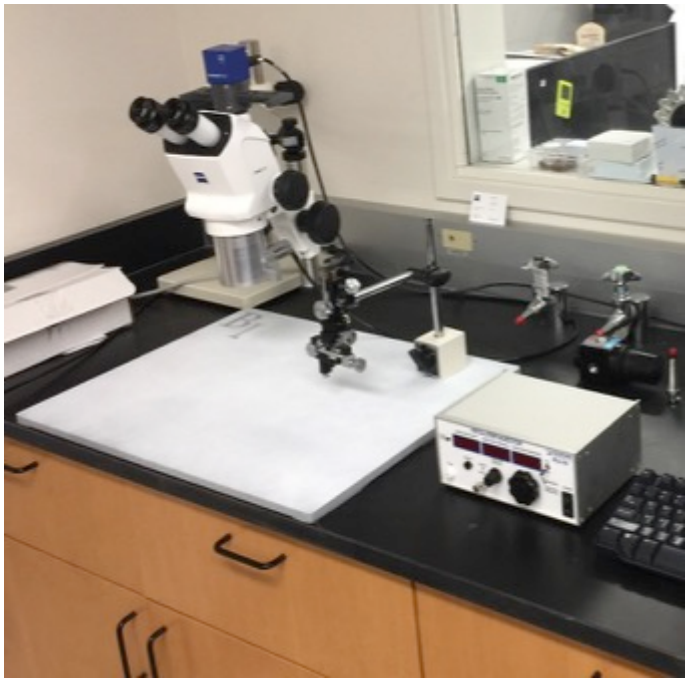


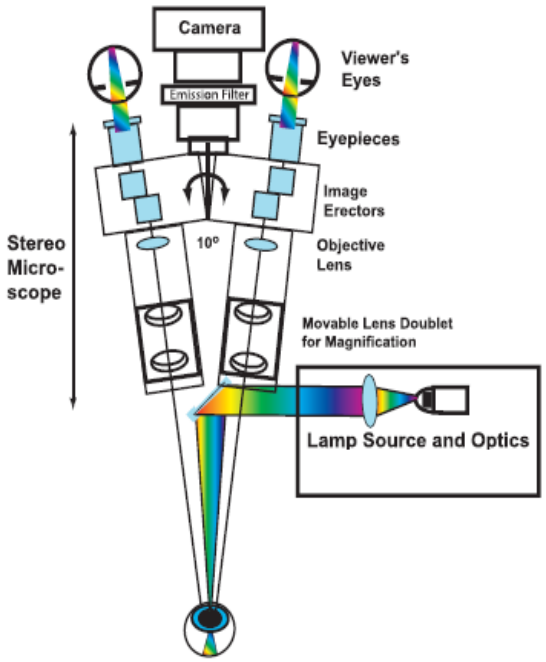
Examples of “normal” adjacent retina with Her2 signals, predominantly in the inner nuclear (INL) and outer nuclear (ONL) layers are shown below:



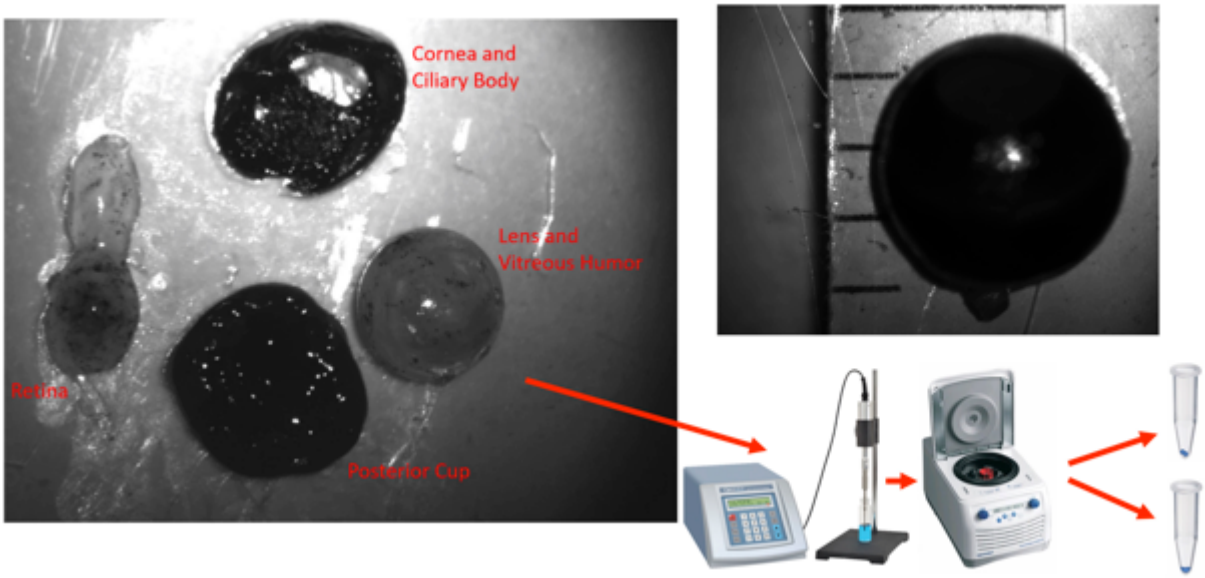
Latest updates (May 2019):

The real time in vivo imaging system is in use, now with the capability of isolating ocular tissues and quantifying drugs and metabolites in the eye from each ocular region. Diagrams are shown below that demonstrate the set-up, tumor imaging, dissecting and analysis capabilities:





Eye Dissection Capability



Details of any associated publications / presentations of results at meetings etc. All to include acknowledgement "This research was funded / part-funded by The Childhood Eye Cancer Trust."
We presented our work at the annual Association for Research in Vision and Ophthalmology conference in Vancouver, Canada in May 2019 in a "hot topic" poster session. The abstract was entitled, "Her2 RNA expression in retinoblastoma and adjacent retina detected by *in situ* hybridization". Our results have implications for Her2 drug targeting in retinoblastoma and may also provide important information about HER2 expression and RB tumor progression.

Our work has been accepted for peer-reviewed publication [Seigel, Shah, Mendoza, Mendoz, Szalai, Grossniklaus, Song and Shan, In situ analysis of Her2 DNA and RNA in retinoblastoma and adjacent retina, Oncoscience in press, 2019]. We thanked CHECT for their support in the acknowledgements section.

We have leveraged our CHECT-supported work to obtain additional funding from the Developmental Studies Hybridoma Bank for a project entitled, "New Antibody Tools for Shared Biomarkers of Breast Cancer and Retinoblastoma" [Gail M. Seigel, PI]. The new award will start within the next few weeks, with the goal of identifying new drug targets for retinoblastoma.

Other comments / potential problems / barriers to progress etc.
Due to the complexity of the pound to dollar exchange, the majority of supply funds were administratively unavailable for project purchases until mid-August 2018. Therefore, most of the supplies were purchased in the final 30 days of this project. As such, experiments will continue well beyond the end date of the award, particularly the *in vivo* tumor studies that depended on the development of the new and more powerful ADC. We are happy to provide further updates on publications that will appear beyond the date of this final report. We thank CHECT for all of their support.