**Investigating the effects of human aldehyde dehydrogenase on vaccinia virus replication in breast cancer stem cells**

**I. Introduction**

According to the CDC, cancer is the second leading cause of the death in the United States. It is responsible for almost one in four deaths and is the second leading cause of death after heart disease (Siegel et al 2014). A total of 1.6 milion cases of cancer are expected to be diagnosed by the end of this year, with an estimated 585 thousand deaths (Siegel et al 2014). Among cancers, prostate and lung cancers are the most common in men, while breast and lung cancers are the most common in women.

Cancer is a group of diseases characterized by the unregulated growth of cells. Since the discovery of stem cells in a number of cancers, much of our attention has been diverted towards understanding the role of stem cells in cancer (Ignatova et al). Stem cells are cells with the capacity to repopulate a tissue and give rise to differentiated progenitor cells. Cancer stem cells are hypothesized to be a population of cells with the capacity to indefinitely renew and drive tumor formation (Reya et al.). Due to their relative quiescence and high expression of drug transporters, tumor stem cells are resistant to traditional mechanisms of therapy such as chemotherapy and radiotherapy (Malik et al) (Deal et al). Additionally, because they represent a small fraction of the tumor mass, surgery often fails to remove the tumor initiating stem cells. As thus, researchers are attempting to identify novel therapeutics to deliver selective toxicity to CSCs in order to prevent cancer recurrence and increase survival for cancer patients.

Oncolytic virotherapy is one way to target CSCs. This utilizes replication-competent viruses to selectively lyse cancer cells and to possibly stimulate anti-cancer immune effects (Smith et al) (Sinkovics et al). Viruses may be engineered for tumor selectivity, and after infiltration of the cell and replication, the cell is lysed and nearby tumor cells are infected, potentially spreading to the heart of the tumor and infecting the resident stem cells. Additionally, the viruses may be immunogenic and stimulate the immune system to further attack the tumor by promoting antigenicity and expression of surface proteins to further aid the body in detecting these cells (Sinkovics et al) (Sze et al). Many classes of viruses have been manipulated to achieve oncolytic capacity, including herpes simplex virus, adenovirus, measles virus, reovirus, and vaccinia virus.

Vaccinia virus (VACV) was instrumental in the battle against smallpox and is currently in clinical trials for metastatic liver cancer (Park et al). The life cycle of the VACV occurs entirely in the cytoplasm. Four types of virions are produced in the life; the intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-assocaited enveloped virus (CEV) and extracellular enveloped virus (EEV) (Smith et al; formation of EEV). After entry and expression of viral genes, IMV particles are assembled and move on microtubules to sites near the microtubule-organizing center where they are subsequently covered by intracellular membrane proteins to form IEV particles. These virions fuse with the plasma membrane to expose CEV on the cell surface. Polymerization of actin propels the virion into surrounding cells, and EEV is released and mediates infiltration in nearby uninfected cells. (Smith et al; Vaccinia Virus Motility)

Because CSCs are phenotypically and functionally distinct from ordinary tumor cells, finding a biomarker to distinguish these undifferentiated cells has been of interest. In particular, aldehyde dehydrogenase has been investigated for such purposes. (Marcato et al). Aldehyde dehydrogenase, as the name suggests, is an oxidoreductase that functions to catalyze the oxidation of aldehydes to their carboxylic acid counterparts. Aldehyde dehydrogenase can function as a maker for normal and cancer stem cells (Ma et al). ALDH expression tends to correlate with poor prognosis in most cancers (Ma et al).

In addition to serving as a marker for CSCs via the ALDEFLUOR assay (Ma et al) (Marcato et al), ALDH is functionally important for the differentiation of CSCs as well as development of drug resistance and cellular protection against stress inducing agents (Ma et al). Retinoid signaling pathways have been indicated in normal and cancer stem cells. Retinol is reversibly converted to retinaldehyde by alcohol dehydrogenase, and retinaldehyde is irresversibly converted to retinoic acid (RA) by ALDH1 (isoform 1). RA then binds to the retinoic acid receptor (RAR). This complex proceeds to regulate gene expression and promote differentiation. In low concentrations of RA, the RAR binds the retinoic acid response element (RARE) and the CCAAT/enhancer binding protein-β to the CCAAT box, which activates transcription of ALDH1, resulting in an increase in RA, and resistance to cytotoxic drugs. The converse is true as well, where a high concentration of RA inhibits transcription of ALDH1, increasing cellular drug sensitivity.

The relationship between CSCs, VACV, and ALDH may not be immediately apparent. However, Wang et al published a study showing that oncolytic vaccinia virus GLV-1h68 strain shows enhanced replication in human breast CSCs in comparison to breast cancer cells. Wang et al classified cells based on ALDH expression using the ALDEFLUOR assay, which separates cells with high ALDH expression from those without it. They found that the VACV strain used in the study replicated more efficiently in ALDEFLUOR-positive cells (cancer stem cells) compared to ALDEFLUOR-negative cells. They found that the viral titer in ALDEFLUOR positive cells was three times higher at an MOI (multiplicity of infection; ratio of virus to cells) of 0.01, and two times higher at an MOI of 10 when compared to ALDEFLUOR negative cells. Additionally, they found that the vaccinia virus GLV-1h68 strain used in the study eliminated tumor xenografts from ALDEFLUOR-positive cells more efficiently than that derived from ALDEFLUOR-negative cells. This was based on a measurement in reduction of tumor size, where ALDEFLUOR-positive cells showed a more tumor size reduction after virus treatments. Additionally, the viral replication was more efficient which resulted in faster tumor regression and earlier detection of flurorescence.

While Wang et al provided convincing results that VACV replicated more efficiently in CSCs, they failed to provide a mechanism as to why this is the case. The interaction between ALDH (or any of its upstream or downstream metabolites or products) and VACV virions or gene products remains unknown. From the results of Wang et al, two conclusions can be made; ALDH or one of its related upstream or downstream products enhances the replication of VACV, and VACV is more lethal to cells with ALDH expression (these can be interpreted as a similar conclusion viewed in different ways). Because there are many steps for Vaccinia virus replication and many possible ways an ALDH positive cell could change the mechanism of replication, it would be pertinent to identify the step in the VACV life cycle that is affected by the presence of ALDH and related compounds. We will quantitatively test this hypothesis as outline in the next section.

**II. Experiment**

The goal of his study is to establish whether infection by vaccinia virus has any effect on the intracellular retinoic acid signaling pathway in stem cells and whether the presence of retinoic acid and related signaling components has an effect on the number of virions produced. Because CSCs saw a greater decrease in mass, I predict that infection by vaccinia interferes with the signaling pathway in that it blocks a critical step or otherwise antagonizes the pathway in order to decrease cellular survival. Additionally, the presence of RA or related metabolites or cellular signaling components increases the number of virions produced at one of the four stages during vaccinia development.

II.A. Biosensors

Due to their low number within a tumor, it will be difficult to measure the concentrations of various metabolites in multiple settings after the infection begins.

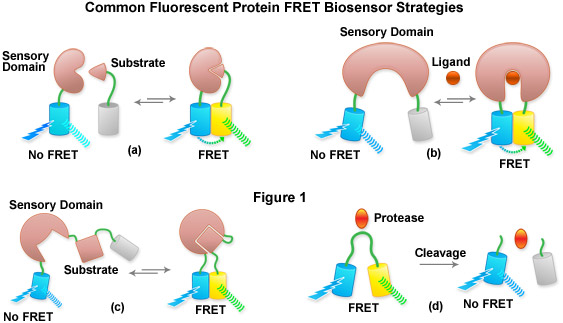


Figure 1: Basic methods and variation behind the FRET biosensors. Fluorescent resonance energy transfer (FRET) is a highly sensitive technique that relies on a protein with two domains and two fluorescent proteins (typically, cyan fluorescent protein [CFP] and yellow fluorescent protein [YFP]). In the absence of metabolites, the domains are separate and so are the fluorescent proteins. As thus, irradiation results in blue fluorescence. When metabolites are present and the domains bind (as in case a), yellow fluorescence is observed instead. The amount of yellow fluorescence can then be measured as an indicator of the amount of metabolite bound. There are multiple ways to utilize FRET relating to how the substrate and sensory domain interact. For our purposes, the most simliar instance would be that visualized in figure (b).

A study conducted by Shimozono et al (2013) utilized genetically encoded probes for retinoic acid (GEPRAs) combined with FRET in order to measure concentrations of free retinoic acid. The retinoic acid receptors were engineered to incorporate cyan emitting and yellow emitting fluorescent proteins as the FRET donor and acceptor. Three GEPRAs were created with different RA affinities, allowing for the quantification of physiological RA concentrations. This was done across a range of developmental states in order to monitor the effects of RA on each stage of development.

While it would be ideal to measure the concentrations of retinol, retinaldehyde, and retionoic acid, biosensors for the former two compounds do not exist (Okumoto et al). As thus, quantifying the levels retinoic acid in order to understand when the presence of the vaccinia virus makes a difference will have to suffice.

II. B. Inserting the biosensor to the stem cell

GEPRAs will be created with different affinities for RA, as done by Shimozono et al. Breast cancer stem cells genetically modified to express GEPRAs will be created in a manner similar to Blurton-Jones et al, who modified neural stem cells to express neprilysin as a model for alzheimer’s disease. Vaccinia virus GLV-1h68 will used to infect the cancer cells at an MOI of 10 and 0.01 (the same concentration and strain used by Wang et al). Levels of RA will be measured using multiple GEPRAs, in a way similar to Shimozono et al. To visualize the change in RA as infection progresses, the levels will be measured in a time dependent fashion. Additionally, viral particles from the infected cells will be released using a freeze-thaw cycle and the titers determined by plaque assays in order to record the number of various virion intermediates in the vaccinia life cycle (the same procedure will be used as Wang et al.)

**III. Discussion**

The expected results are that RA concentrations will be decreased and that virion intermediates will be increased in number (however, I am unable to make predictions as to which of the 4 intermediates will exhibit a change in concentration). It would be appealing but equally unwise and perhaps even reckless to draw a conclusion about the concentrations of upstream and downstream products, as we were unable to directly measure them. Additionally, this experiment will not directly identify *how* virus infection changes RA acid levels or vice versa.

The lack of availability of retinol and retinal bionsensors is crippling to the prospects of this experiment. While it may not have already been investigated, I believe it is possible to synthesize it. Retinol binds to alcohol dehydrogenase (ADH) and retinal binds to Aldehyde dehydrogenase (ALDH). Using a method similar to that employed by Shimozono et al, ADH and ALDH could be tagged with a fluorescent marker as well as their substrate, which would result in fluorescence when they came together (Komatsu et al). However, that would be a resource intensive project on its own, and this study would better be treated as an experiment to allow other, perhaps more enlightening proposals to follow.

An advantage of the biosensors used by Simozono is that they are rather specific for RA, and are not confused by intracellular levels of retinol and retinal. Another advantage of this experiment is that it measures the cellular responses in a time dependent manner, which lends itself for more accurate speculation about molecular mechanisms. Any of the four stages of the virus could interact with the retinol – retinal – RA signaling axis, so being able to reduce the possibilities into a number that can be tested would greatly improve our cause.

A drawback to this experimental design, however, is that it offers no insight into the interaction of other viruses with ALDH and related signaling components or how that affects viral replication efficiency. Additionally, extending the results to other cancers would be risky and unwise, as there is no foundational work showing that viral replication is changed in other cancers. This potentially narrows the scope of this experiment to cells expressing ALDH and those that utilize it in a manner similar to the one described above.

Fortunately, the above drawbacks are minimal and are cited from a cautious point of view. Biosensors are a very potent tool to assess concentrations of specific molecules within the cells, and can be used to further elucidate the mechanisms of interaction between ALDH and related components and viruses. Targeting cancer stem cells may prove to be a powerful new therapeutic option in order to destroy hard to kill cancer, and oncolytic viruses may prove to be the tool to do that.

**Works Cited**

Dean, M., Fojo, T., & Bates, S. (2005). Tumour stem cells and drug resistance. *Nature Reviews. Cancer*, *5*, 275–284. doi:10.1038/nrc1590

Ignatova, T. N., Kukekov, V. G., Laywell, E. D., Suslov, O. N., Vrionis, F. D., & Steindler, D. A. (2002). Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *GLIA*, *39*, 193–206. doi:10.1002/glia.10094

Komatsu, N., Aoki, K., Yamada, M., Yukinaga, H., Fujita, Y., Kamioka, Y., & Matsuda, M. (2011). Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Molecular Biology of the Cell*. doi:10.1091/mbc.E11-01-0072

Ma, I., & Allan, A. L. (2011). The role of human aldehyde dehydrogenase in normal and cancer stem cells. *Stem Cell Reviews*, *7*, 292–306. doi:10.1007/s12015-010-9208-4

Malik, B. (2012). Cancer stem cells and resistance to chemo and radio therapy. *Frontiers in Bioscience*. doi:10.2741/E531

Marcato, P., Dean, C. A., Giacomantonio, C. A., & Lee, P. W. (2011). Aldehyde dehydrogenase: its role as a cancer stem cell marker comes down to the specific isoform. *Cell Cycle*, *10*, 1378–1384. doi:10.4161/cc.10.9.15486

Okumoto, S., Jones, A., & Frommer, W. B. (2012). Quantitative Imaging with Fluorescent Biosensors. *Annual Review of Plant Biology*. doi:10.1146/annurev-arplant-042110-103745

Park, B. H., Hwang, T., Liu, T. C., Sze, D. Y., Kim, J. S., Kwon, H. C., … Kirn, D. H. (2008). Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *The Lancet Oncology*, *9*, 533–542. doi:10.1016/S1470-2045(08)70107-4

Reya, T., Morrison, S. J., Clarke, M. F., & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature*, *414*, 105–111. doi:10.1038/35102167

Shimozono, S., Iimura, T., Kitaguchi, T., Higashijima, S.-I., & Miyawaki, A. (2013). Visualization of an endogenous retinoic acid gradient across embryonic development. *Nature*, *496*, 363–6. doi:10.1038/nature12037

Siegel, R., Ma, J., Zou, Z., & Jemal, A. (2014). Cancer statistics, 2014. *CA: A Cancer Journal for Clinicians*, *64*, 9–29. doi:10.3322/caac.21208

Sinkovics, J., & Horváth, J. (2008). Oncolytic virotherapy. *Orvosi Hetilap*. doi:10.1038/nbt.2287

Smith, T. T., Roth, J. C., Friedman, G. K., & Gillespie, G. Y. (2014). Oncolytic viral therapy: targeting cancer stem cells. *Oncolytic Virotherapy*, *2014*, 21–33. doi:10.2147/OV.S52749

Sze, D. Y., Reid, T. R., & Rose, S. C. (2013). Oncolytic virotherapy. *Journal of Vascular and Interventional Radiology*. doi:10.1016/j.jvir.2013.05.040

Wang, H., Chen, N. G., Minev, B. R., & Szalay, A. A. (2012). Oncolytic vaccinia virus GLV-1h68 strain shows enhanced replication in human breast cancer stem-like cells in comparison to breast cancer cells. *Journal of Translational Medicine*. doi:10.1186/1479-5876-10-167