**(TITLE)**

**Role of Kinase Driven Phosphorylation on Hedgehog Signaling Activation**

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**Fall 2019 Rotation Report**

This report was written by the student, has a distinct and reachable goal, and represents a reasonable amount of work for one rotation project earning 4 units of academic credit. The student will learn new techniques and approaches consistent with the goals of the rotation program.

Rotation Supervisor’s Name: Dr. Scott Atwood

 Signed\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  Date \_\_\_\_\_\_\_\_\_\_

Faculty Advisor’s Name: Dr. Eric Pearlman

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**Introduction**

Basal Cell Carcinoma (BCC) is the most prevalent skin cancer worldwide (Lucena *et a*l., 2019). Evidence supports that this cancer depends on uncontrolled activation of the Hedgehog (Hh) Signaling Pathway. The Hedgehog Signaling Pathway becomes active when a Hedgehog Signaling ligand binds to the Patched1 (PTCH1) receptor on the primary cilia of a cell, thus activating the signal transducer Smoothened (SMO). SMO then moves to the cilium where it binds and inhibits Suppressor of Fused (SUFU), resulting in transcription factor activation of Glioma-Associated Oncogene (GLI) (Varjosalo and Taipale, 2007). Mutations that occur in regulators of the Hh Signaling Pathway such as PTCH1, SMO and GLI cause uncontrolled activation of the Hh pathway leading to the development of cancers such as BCC (Atwood *et al*., 2012). Small molecule drugs that inhibit SMO have been used to modulate the Hedgehog Signaling pathway and treat BCC. It has been found that BCC tumors can acquire resistance to these SMO inhibitor drugs, which motivates investigation of recurrent mutations of the GLI1 transcription factor, located downstream of SMO (Pricl *et al*., 2015, Kasper *et al*., 2006). It has been previously found that multi-site phosphorylation regulates the conversion of GLI proteins into transcriptional activators and kinases activate the response in Hedgehog Signaling activity and tumor growth (Niewiadomski *et al*., 2014).

Mitogen-Activated Protein Kinase 2 (MAP2K1) is active at re-current GLI mutations sites determined experimentally to activate Hh Signaling. Mutations in MAP2K1 are correlated with melanoma and inhibiting this kinase reduces metastasis in pancreatic cancer (Homisko *et al*., 2019, Gu *et a*l., 2018). Additionally, MAP2K1 stabilizes GLI and activates Hedgehog Signaling (Liu *et al*., 2014). Cobimetinib is effective as a MAP2K1 inhibitor used to treat melanoma with an IC50 of 4.2 nM (Rowdo *et al*., 2016). Pyruvate Dehydrogenase Kinase (PDHK) regulates glycolysis which is more highly activated in cancer cells than normal cells (Golias *et al*., 2018). A PDHK inhibitor, Dichloroacetic acid (IC50, 81.03 mM), upregulates apoptosis of ovarian cancer cells (Zhou *et al*., 2018). The prediction of PDHK activity on re-current GLI mutation sites, makes it of interest in assessing its inhibition in Basal Cell Carcinoma. NIMA Related Kinase (NEK1) affects Hh signaling through its role in ciliogenesis and is correlated to cancer (Melo-Hanchuk *et al*., 2017, Evangelista *et al*., 2008). Zinc05007751 is a novel selective NEK1 inhibitor with an IC50 of 3.4 μM. Evidence suggests that inhibition of NEK1 would reduce GLI activation and Hedgehog Signaling. The purpose of this project is to investigate Hedgehog Signaling activation in GLI recurrent mutations. As MAP2K1, PDHK and NEK1 kinases are predicted to be active at recurrent mutation sites in GLI, their inhibition is predicted to influence transcriptional activity and Hedgehog Signaling.

**Hypothesis**  
 We hypothesize that MAP2K1, PDHK and NEK1 kinases predicted to be active at recurrent GLI mutation sites are the source of increased Hedgehog Signaling activity observed in these mutations.

**Materials and Methods**

**Kinase Prediction**

A compilation of GLI1 and GLI2 mutations that occurs three or more times in cancer cells in the Catalogue of Somatic Mutations in Cancer (COSMIC) database have been generated and previously screened for activation of the Hedgehog Signaling Pathway in the Atwood Lab. Using Group-Based Prediction Software (GPS) and PhosphoNet Kinase Predictor, kinases which are predicted to be active at phosphosites four positions before or after a recurring mutation side are compiled. Kinase candidates having previous evidence of mediating phosphorylation and activating Hedgehog Signaling at a mutation site were eliminated from the compilation. Previous insight into their activity in Hedgehog Signaling or cancer allowed for the selection of MAP2K1, PDHK and NEK1 to evaluate Hedgehog Signaling effects due to kinase activity experimentally.

**Cell Dosing, RNA Extraction, RT-qPCR**

Cobimetinib, Dichloroacetic Acid, and Zinc 05007751 are selected as inhibitors for MAP2K1, PDHK and NEK1 due to their specificity and IC50 range. 3T3 (Wild Type) cells are serum starved and serum starved with Hedgehog conditioned media and treated with these inhibitors at their IC50s and serially increased on a logarithmic scale to three higher concentrations. Basal Cell Carcinoma Cells are serum starved and treated under the same conditions of inhibitors as Wild Type cells. Additionally, these cells are all treated with diluent only (DMSO) as a control. After 24 hours of incubation, RNA is extracted from both diluent and drug treated cells using the Zymo Research Direct-Zol RNA Miniprep Plus kit. Extracted RNA is amplified using RT-qPCR with primers specific for the GLI1 gene as a means of quantifying Hedgehog Signaling activation, as well as a GAPDH experimental control to quantify GLI expression.

**Proliferation Assay**

To confirm kinase inhibitors do not affect cell proliferation, an MTT Assay is conducted on diluent (DMSO) and drug treated 3T3 and BCC cells. Cells are seeded at 2,000 cells/well and grown to 50% confluency. Cell proliferation is quantified by measuring the absorbance at 570 nm for 3T3 cells treated with drugs and diluent only at 0, 2 and 4 days and for BCC cells at 0, 2, 4 and 6 days. Percent viability is determined by comparing the absorbance value for the drug treated and diluent only sample to its respective measurement at day 0.

To confirm that this result contributes to cell proliferation, I will conduct an MTT Assay on diluent and drug treated WT and BCC cells. This assay utilizes NADPH dependent cellular oxidoreductase enzymes to degrade a yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT) to formazan which is purple in color to measure cell viability. Using a plate reader to measure absorbance, we would expect that if the kinase inhibitor drug is affecting the Hedgehog Signaling Pathway, BCC cell proliferation will be reduced over time as compared to diluent treated BCC cells. An observation of a dose dependent reduction of GLI1 mRNA when treated with kinase inhibitor and a reduction of cell proliferation between diluent and drug treated BCC cells will provide evidence that the predicted kinases could be responsible for activating the Hedgehog Signaling Pathway

Discussion

qPCR Prediction

If the presence of selected kinases are responsible for the activation of Hedgehog Signaling, decreased mRNA levels for GLI1 in BCC cell lines are expected in a dose response manner in the presence of kinase inhibitor as compared to diluent treated BCC cells.

MTT prediction

Using a plate reader to measure absorbance, we would expect that if the kinase inhibitor drug is affecting the Hedgehog Signaling Pathway, BCC cell proliferation will be reduced over time as compared to diluent treated BCC cells. An observation of a dose dependent reduction of GLI1 mRNA when treated with kinase inhibitor and a reduction of cell proliferation between diluent and drug treated BCC cells will provide evidence that the predicted kinases could be responsible for activating the Hedgehog Signaling Pathway