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

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**Fall 2019 Rotation Proposal Report**

This proposal 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/Optional: Dr. Eric Pearlman

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**Background**

Uncontrolled activation of the highly conserved Hedgehog (Hh) Signaling Pathway, which plays a vital role in mammalian development, causes developmental defects and cancers of the skin such as Basal Cell Carcinoma (BCC). The Hedgehog Signaling Pathway becomes active when a Hedgehog signaling ligand binds to the Patched1 (PTCH1) receptor of a cell, thus activating Smoothened (SMO). SMO then transduces the Hedgehog signal to the cytoplasm where GLI transcription factors are activated and target gene transcription can occur (Varjosalo and Taipale, 2007). GLI2 and GLI3 respond to the initial Hedgehog signal and trigger expression of GLI1 which amplifies the signal response in the cell and potentiates the transcriptional output of Hedgehog Signaling (Hui and Angers, 2011).

Mutations in regulators of the Hh Signaling Pathway such as PTCH1, SMO and GLI transcription factors cause uncontrolled activation of the Hh Pathway and lead to the development of BCC. Although there have been drugs developed to inhibit SMO as a means of modulating the Hedgehog Pathway, it has been found that BCC tumors can acquire resistance to these drugs (Pricl *et al*., 2015). Therefore, it is of interest to investigate recurrent mutations of the GLI1 transcription factor, located downstream of SMO, in cancer cells which can lead to tumorigenesis and drug resistance in BCC (Kasper *et al*., 2006). It has been previously found that multi-site phosphorylation regulates the conversion of GLI proteins into transcriptional activators and kinase activity has been correlated to this activating response in Hedgehog Signaling activity and tumor growth (Niewiadomski *et al*., 2014).

**Rationale and Hypothesis**

The purpose of this project is to further investigate Hedgehog Signaling activation in GLI1 and GLI2 recurrent mutations. As phosphorylation causes regulation of GLI transcription, kinases influence transcriptional activity and Hedgehog Signaling. Therefore, we hypothesize that kinases predicted to be active at new consensus sites due to the presence of recurrent mutations are the source of increased Hedgehog Signaling activity observed in these mutations.

**Specific Aims**

         To test the hypothesis that predicted kinases cause activation of Hedgehog Signaling in recurrent mutations of GLI1 and GLI2 transcription factors, I will begin by identifying serine-threonine kinases which could regulate these mutation sites and promote tumor growth using predictive software. This process will lead me to select kinases predicted to be active at the recurring mutation sites to further evaluate Hedgehog Signaling effects of kinase activity experimentally. After treating wild-type and BCC cell lines with known kinase inhibitors, I will compare mRNA expression of GLI1 among these cell lines as a means of measuring Hedgehog Signaling Activity. Additionally, I will assess BCC cell proliferation over time following drug treatment to determine if kinase inhibition affects Hedgehog Signaling.

**Experimental Approach**

This project will begin with generating a compilation of GLI1 and GLI2 mutations previously identified in the Catalogue of Somatic Mutations in Cancer (COSMIC) database to occur three or more times in cancer cells and screened for activation of the Hedgehog Signaling Pathway in the Atwood Lab. Serine-threonine kinases which could be responsible for regulating these mutation sites and promoting Hedgehog Signaling will be identified using a Group-Based Prediction Software (GPS) and confirmed using the PhosphoNet Kinase Predictor. As kinase consensus sites are within seven to eight amino acids, we will include kinases that are predicted to at phosphosites four positions before or after the recurring mutation site. Using PhosphositePlus and literature searches, I will eliminate kinase candidates that have had previous evidence of mediating phosphorylation and activating Hedgehog Signaling at a mutation site. This process will lead me to select predicted kinases of interest to further evaluate Hedgehog Signaling effects of kinase activity experimentally. After selecting these kinases, I will search the literature to identify known kinase inhibitors tested *in vitro*.

 Known kinase inhibitor drugs, will be diluted for dosing cell lines and a set of Wild Type and Basal Cell Carcinoma cell lines will be treated with these drugs at their IC50s and serially increased on a logarithmic scale to three higher concentrations. A set of WT and BCC cells will be treated with diluent only to provide a cell proliferation control. After incubating the cells in the presence of the inhibitor, the total RNA will be extracted from both diluent and drug treated cells. Extracted RNA will then be quantified using RT-qPCR using primers for GLI1 to quantify Hedgehog Signaling activation, as well as a GAPDH experimental control to confirm successful RT-qPCR. 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. 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.

**References**

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**Experimental Protocols**

**MTT Assay (Adapted from Abcam Protocol)**

1. Seed cells in a 96 well tissue culture plates with 1x106 cells/mL.
2. Add 50 μL of MTT dissolved to 5 mg/mL PBS to each well. Mix gently by tapping the plate down.
3. Incubate at 36°C for 4 hours
4. After incubation add 150 μL of MTT solvent (DMSO) to each well
5. Wrap plate in foil and shake on an orbital shaker for 15 minutes.
6. Read the plate absorbance at an OD of 570 nm within one hour of MTT addition.

**Routine Protocols Used:** Dosing cells with Inhibitor Drug, splitting WT and BCC cells

**Face Sheets Attached:** Zymo Research Direct-zol RNA Mini Prep Kit, BioRad 1-Step Quantitative Reverse Transcription PCR (RT-qPCR) from RNA (iTaq Universal SYBR Green) Kit Literature

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