Identifying Pathways that Regulate Flagellar Assembly

Keywords: *Chlamydomonas reinhardtii*, flagella assembly, kinase, length regulation

Introduction: Recent discoveries concerning cilia assembly suggest complex signaling pathways play a prominent role in cilia length regulation and function.[1] Far less is known about the about the kinases that regulate these pathways. The proposed research will attempt to uncover the signaling pathways responsible for growth and regulation by establishing which kinases and mechanisms are responsible for the regulation of cilia length.

Background and Rationale: Cilia and flagella are found on almost every cell in the human body and consist of microtubules that extend from the cell surface. Cilia are typically divided into two types, primary and motile, which both sense extracellular signals. Primary cilia, found on the majority of cells in the human body are immobile. Motile cilia, found on the majority of epithelial cell surfaces, create wave-like patterns to propagate fluid. As flagella and motile cilia have identical structures, the words cilia and flagella are used interchangeably.

The process of assembling cilia, ciliogenesis, is highly regulated as the centrioles that nucleate cilia are also required for cell division. The mechanisms regulating ciliogenesis, including initiation, assembly and resorption, are poorly understood. Learning more about these mechanisms will facilitate the study and treatment of diseases involving ciliary dysfunction.

Flagella of the unicellular green alga *Chlamydomonas reihardtii* are essentially identical to cilia of vertebrate cells and provide an excellent model to study ciliogenesis. Chemical studies in *Chlamydomonas* have demonstrated length-regulating roles for G-protein coupled receptors[2]. Similarly, flagella assemble in a length-dependent manner, with rapid early assembly and very slow assembly as they approach their steady-state length. The rate of disassembly is length independent and the length at which assembly and disassembly are in equilibrium is known as the balance point[3]. To identify kinase pathways that affect flagellar assembly, we performed a small-molecule screen using a kinase inhibitor library. Preliminary data show inhibiting Protein Kinase A and G causes an increased rate of flagellar assembly during regeneration over of 2 hours as compared to wild type. Also, inhibiting Protein Kinase C causes a slower rate of assembly during regeneration at 1 hour as compared to wild type **(Figs. 1,2)**. *We hypothesize these inhibitors target regulators that control the switch from rapid to slow assembly rates. Reversing this switch has the potential to rescue defects caused by slow or impaired assembly.*

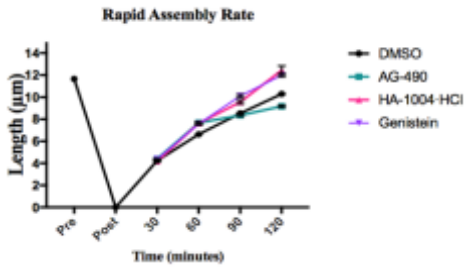

Figure 1. Preliminary kinase inhibitors with increased rate of assembly during regeneration.

Aim 1: Validate Targets and Phenotypes with Novel Inhibitors and Activators: To confirm phenotypes and identify the kinases responsible for the observed phenotypes, we will use different inhibitors of the same targets. In contrast to the kinase inhibitors, the activation of these kinases should show us the opposite effects, confirming the targets and phenotypes previously identified (**Figs. 1,2**). Following pH shock to induce flagellar loss and regrowth, we will treat *Chlamydomonas* cells (CC125) with inhibitors and activators of protein kinase A, G, C and tyrosine kinases (**Table 1**). Flagella will be imaged using

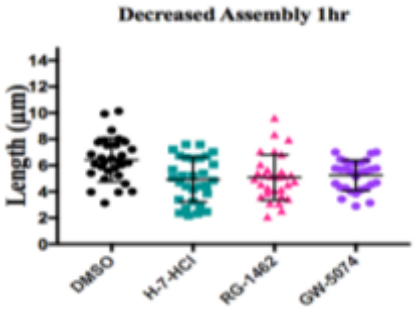

Figure 2. Preliminary kinase inhibitors with decreased rate of assembly during regeneration.

automated phase contrast microscopy and flagellar length measurements will be performed using ImageJ software. With these experiments, we expect to confirm the data seen in **Figures 1 and 2** while helping us to further confirm these kinases as regulators of the switch from rapid to slow assembly.

Table 1. Inhibitors and Activators of proposed targets to be used in Aim 1

PKA	H89 dihydrochloride	Inhibitor
PKA	PKA inhibitor fragment (6-22) amide	Inhibitor
PKC	Calphostin	Inhibitor
PKC	K-252C	Inhibitor
PKG	RP 8 Br PET cGMPS	Inhibitor
PKG	KT5823	Inhibitor
PKA	Taxol	Activator
PKA	8-PIP-cAMP	Activator
PKC	Oleadic Acid	Activator
PKC	Bryostatin 1	Activator
PKG	8-Bromoguanosine 3',5'-cyclic monophosphate sodium salt	Activator
PKG	Guanosine 3'-5'-cyclic monophosphate	Activator

Aim 2: Identify Regulatory Pathways for Flagellar Assembly: As many of the kinases identified in the preliminary screen have both cytoplasmic and nuclear downstream targets, we will identify which subset of targets are responsible for the flagellar assembly phenotype. To discriminate between the targets, we will use inhibitors from the preliminary screen simultaneously with cyclohexamide, a protein synthesis inhibitor that will prevent gene expression in downstream transcription factors (**Table 2**). Next, we will perform an epistasis experiment by inhibiting or activating a preliminary target as well as a potential downstream targets to see if they are in the same pathway. The process of pH shock and flagellar length measurement will be followed according to the steps described in Aim 1. These experiments will narrow down the pathway components regulating the switch from rapid to slower assembly rates.

Aim 3: Determine the Role of Identified Kinases in Trafficking of Flagellar Cargo: We will use total internal reflection fluorescence (TIRF) microscopy to determine the role of kinases in trafficking of flagellar cargo by assessing the preliminary targets' effect on transportation of tagged proteins in regenerating flagella**.** We will treat the cells with the kinase inhibitors during flagellar regeneration and use TIRF imaging to compare the amount of cargo traveling from the base to the tip of the regenerating flagella by quantifying fluorescence intensity of tagged cargo.[4,5] Results from this visualization and quantification of tagged cargo will identify the mechanism with which identified pathways regulate flagellar assembly.

Table 2. Cytoplasmic and Nuclear compounds to be used for experiments in Aim 2.

Cytoplasmic		Nuclear	
Target	Drug Name	Target	Drug Name
RhoA Kinase	HA-1077 2HCl	NFkB	Luteolin
RhoA Kinase	Y-27632 2HCl	NFkB	Cardamonin
Raf	GW 5074	CREB	666-15
Raf	Erastin	CREB	C-646
14.3.3	R18	APC	TAVE hydrochloride

<u>Intellectual Merit/Broader Impacts</u>: My familiarity with the culture conditions and flagellar phenotyping of the model organism *Chlamydomonas reinhardtii* will facilitate the proposed experiments. I will gain the necessary skills to perform TIRF microscopy through future mentoring from Dr. Avasthi. The initial microscopy work in the outlined project, allows Rockhust University undergraduate students participating in research at the University of Kansas Medical Center to be trained on microscopy. The findings from these experiments impact the science community through the identification of fundamental principles of ciliary regulation. Society is influenced by these findings as they will provide the foundation for the treatment of diseases of ciliary dysfunction. Results will be shared in relevant conferences, preprints and publications. Also, the proposed experiments and results, will be shared with undergraduate students at Rockhurst University with the intention of using relevant basic science research to engage future students. This will capture their attention and spark their interest for research opportunities at the University of Kansas Medical Center. Support from the NSF through the Graduate Fellowship Research Program will promote my success as a future scientist by facilitating research during my graduate career, but will also benefit society by providing a more approachable path to science careers for women.

[1] Nachury,Maxence V.(2014) *Philosophical Transactions of the Royal Society B: Biological Sciences* 369.1650
[2] Avasthi, Prachee et al. (2012). *ACS Chemical Biology* 7.5 [3] Marshall, Wallace F. et al. 2005.*Molecular Biology*

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