Optogenetic Control of RhoA Signaling in HeLa and 3T3 Cells

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Introduction

The cytoskeleton controls cell motility and maintains cell shape. The RhoA GTPase signaling pathway regulates the cytoskeleton, making it a valuable target for studying cell dynamics, including mechanotransduction via Yes-associated protein (YAP) transcriptional co-activator. Optogenetic tools allow us to precisely control cellular signaling pathways using light-sensitive proteins that change conformation upon exposure to specific wavelengths of light, allowing for control of cell signaling pathways without the use of drugs or widespread genetic perturbations. This study uses opto-RhoA to activate RhoA signaling and increase cytoskeletal tension. The opto-RhoA tool consists of BcLOV4, a blue light-sensitive protein from the fungus, *Botrytis cinerea*, fused to the RhoA GTPase. BcLOV4 is cytosolic in the dark, preventing RhoA activation. Upon blue light exposure, BcLOV4 undergoes a conformational change allowing it to bind the plasma membrane, bringing RhoA with it and allowing it to be activated.

Previous studies established the use of opto-RhoA in HEK cells, which are easy to work with but not highly relevant for cell migration studies due to their lower cytoskeletal activity, and HUVEC cells, which are primary migrating cells but less genetically manipulable. This study aims to establish optogenetic control of RhoA signaling in HeLa and 3T3 cells, which have more extensive cytoskeletons than HEK cells but are still easy to transfect, making them suitable for high-throughput analysis. This approach will enhance the study of cellular processes including cell migration.

Materials & Methods

Culturing cells: HeLa and NIH-3T3 cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin and incubated at 37°C with 5% CO_2.
Transfection: Cells were seeded at ~40% confluency in 35 mm glass bottom dishes and transfected at ~75% confluency using Lipofectamine™ 3000 following manufacturer's instructions with either BcLOV4-mCherry or opto-RhoA (RhoA GTPase-BcLOV4-mCherry) in pcDNA3.1 backbones. Cells were imaged 24-48 hours post-transfection.
Fluorescence microscopy: Cells were imaged with a Leica DMI6000B fluorescence inverted microscope, using a sCMOS camera (pco.edge), an LED illuminator (Lumencor Spectra-X), and a 63× oil immersion objective. mCherry fluorescence was imaged every 15 or 30 seconds, with 1.5 seconds of blue light delivered every 30 seconds for a total time course of 10 minutes. Cell area was measured with ImageJ software at 0 minutes (T0) and 10 minutes (T10), and the percentage change in cell area was calculated. Statistical differences were assessed using a Mann-Whitney U test. N = 15-45 cells per condition.

Results & Discussion

In this study, we introduced plasmids expressing the BcLOV4-mCherry (control) or opto-RhoA-mCherry to both HeLa and 3T3 cells and visualized them using fluorescence microscopy. In both cell types, BcLOV4 localized to the membrane in response to blue light. We then measured the responses of the cells to pulsatile blue light stimulation (Figure 1A). While some HeLa cells showed subtle yet observable modifications in the cell shape, with some cells decreasing in area, this change was not statistically significant compared to BcLOV4 (control) cells (Figure 1B). In contrast, the 3T3 cells exhibited more dramatic decreases in cell area, emphasizing the cell line-specific effects of RhoA activation (Figure 1C).

The different responses between HeLa and 3T3 cells may be attributed to their distinct cellular properties. HeLa cells, derived from human cervical carcinoma, have different cytoskeletal dynamics compared to 3T3 cells, which are fibroblasts derived from embryonic tissue, making them inherently more responsive to changes in the cytoskeleton. In addition, the differential responses observed in these cell lines could also highlight potential challenges with plasmid constructs, such as differences in transfection efficiency, expression levels, and plasmid stability.

Conclusion

Here, we successfully implemented optogenetic tools to control RhoA signaling in 3T3 cells, enabling precise manipulation of signaling in fibroblasts, including detailed mechanistic studies of RhoA signaling pathways, high-throughput screening for potential therapeutic targets, and comparative analysis with other cell types. Next, we will use Opto-RhoGEF in 3T3 cells. Opto-RhoGEF can provide more nuanced control of the RhoA pathway by activating endogenous RhoA in response to light. Experiments with Opto-RhoGEF and opto-RhoA could also reveal differences in downstream signaling effects compared to direct RhoA activation, offering insights into the regulation and dynamics of RhoA signaling.

Overall, optogenetic control of RhoA signaling in 3T3 cells allows for precise manipulation and study of cellular processes, such as cytoskeletal dynamics and cell morphology, with high spatial and temporal resolution. This technique enables a deeper understanding of cellular behavior which is crucial for the development of novel therapeutic approaches.

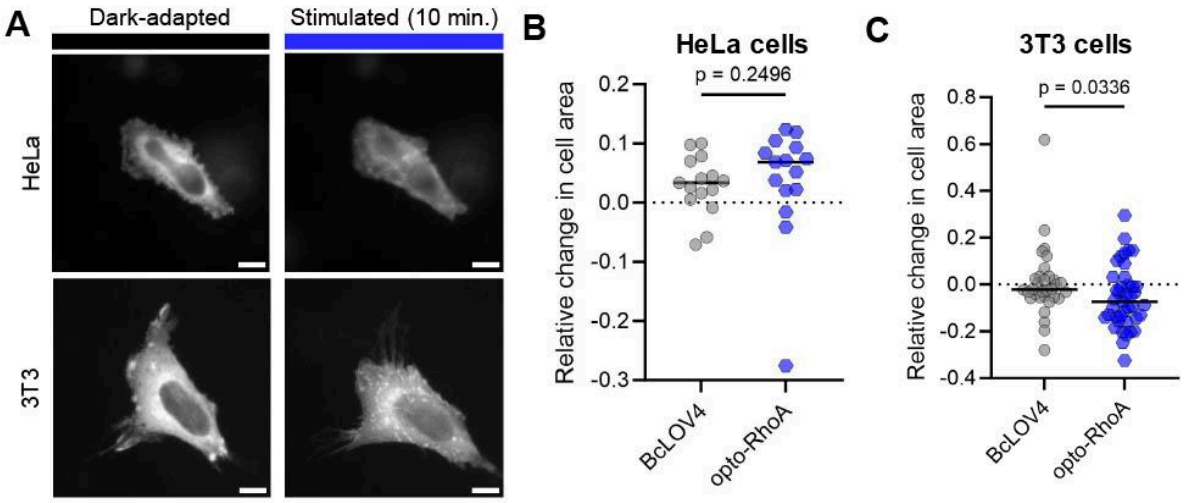

Figure 1. Optogenetic RhoA activation in 3T3 and HeLa cells. A. Opto-RhoA visualized in the mCherry channel in both HeLa (top) and 3T3 (bottom) cells under dark-adapted and blue light-stimulated conditions. Scale = 10 μm. B-C. The relative change in cell area over a 10 minute stimulation time course was quantified HeLa (B) and 3T3 (C) cells expressing either BcLOV4 or opto-RhoA constructs. Statistical difference was quantified by a Mann-Whitney U test.

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