The Journal of Undergraduate Research

Volume 14 *The Journal of Undergraduate Research* Article 7

February 2017

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Cover Page Footnote
This research project was funded by South Dakota State University Department of Chemistry and Biochemistry as well as the Van D. and Barbara B. Fishback Honors College. The authors would like to thank Dr. Hoppe for his guidance and help, George Opoku-Kusi for valuable input on our project, and Dr. Cole-Dai and Dr. Gupta for instructing the course.

Quantitative Analysis of Endocytosis of Macrophage Growth Factor Receptor by Molecular Imaging

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ABSTRACT

Endocytosis is a critical cellular process responsible for nutrient uptake, sampling the environment, and cellular signaling. Endocytosis of growth factor receptors is an important step in the regulation of receptor signaling functions. The macrophage growth factor receptor controls growth and differentiation of macrophages, however, the pathway by which the receptor is internalized is not well understood. The analysis showed that for the first time, knockout of endophilin A2 decreases the internalization of macrophage growth factor receptor in primary bone marrow macrophages. This shows that fast endophilin-mediated endocytosis plays a role in the control of the amount of growth factor receptors internalized in macrophages.

Keywords: endocytosis, endophilin, fast endophilin-mediated endocytosis, FEME, growth factor, CSF-1

INTRODUCTION

Endocytosis is the process by which cargo enters a cell.[1] There are multiple pathways involved in internalizing this cargo, the best researched being clathrin-mediated endocytosis (CME).[2] Recently, a new endocytic pathway that is mediated by endophilin, rather than clathrin, has been discovered.[3] This newly discovered pathway, fast endophilin-

mediated endocytosis (FEME) is unique in that it acts independently of clathrin. FEME has been shown to be involved in internalization of several different G-protein-coupled receptors as well as several receptor tyrosine kinases.[3]

A receptor of note is the colony stimulating factor-1 receptor (CSF-1R), as it is responsible for attachment of the growth factor colony stimulating factor-1 (CSF-1). This growth factor plays a key role in both the differentiation of stem cells into macrophages as well as macrophage survival.[4] Understanding the pathway of internalization of this growth factor has implications for the design of novel therapeutics targeting leukemia and other inflammatory disorders associated with macrophage malfunction.[5] CSF-1R mediates all of the effects of CSF-1, but it is not yet known what endocytic pathway CSF-1R is internalized by.[6]

Although the importance of CSF-1 and its receptor are known, the specific internalization pathway is not known. The authors investigated if CSF-1R is internalized into bone marrow macrophage (BMM) cells by FEME.

The objectives of this study were to develop a quantitative assay to analyze endocytosis of macrophage growth factor receptor by molecular imaging, as well as to apply the quantitative assay to test the role of endophilin A2 protein in internalization of macrophage growth factor receptor. The hypothesis is that FEME is involved in internalization of CSF-1R into BMM cells.

METHODS

The first part to this experiment was to make a quantitative way to measure how much CSF-1 was being internalized into the bone marrow macrophage (BMM) cells. A free program called CellProfiler was used to identify cells and set of instructions for it was created. These instructions were called a pipeline. This pipeline allowed the program to look for specific parts of the cell, so the cell could be located and identified. The cells needed to be dyed with three fluorescent dyes. These dyes were DAPI for the nuclei, Phalloidin for actin, and Dylight 594 for CSF-1.

First, CellProfiler was instructed to identify the nucleus, which allowed an accurate count of how many cells were on the plate and a general location. After the nuclei were found, the

program then took the actin stain, which identifies the cytoskeleton of the cell, and laid it over the nuclei stain. The program then determines what cytoskeleton belongs to what nuclei and maps the cell borders (Figure 1).

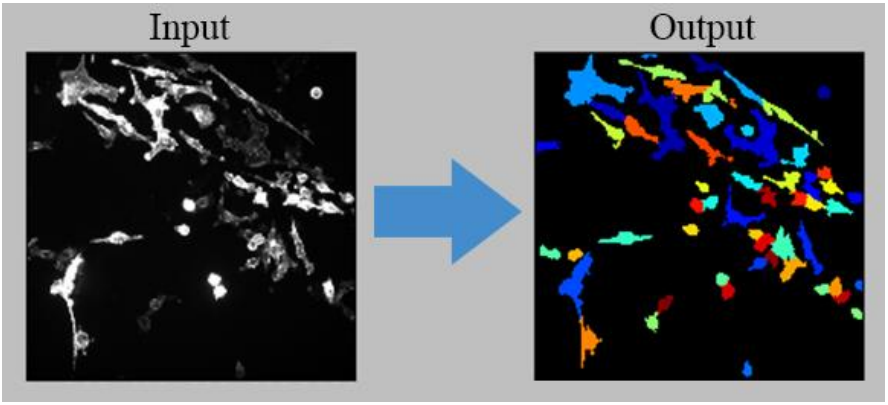

Figure 1: CellProfiler identifying multiple BMM cells. Different colors are used to distinguish between different BMM cells.

These two steps were essential in identifying the location of multiple cells. The program then took the cell outline and measured the CSF-1 fluorescence within the border. CellProfiler then took this raw fluorescence of each cell and graphed it.

Two separate sets of BMM cells from mice were prepared. One set was wild type BMM cells that had a working copy of the gene for endophilin, and the other was BMM cells with the gene for endophilin knocked out using a genome editing tool, CRISPR-Cas9. The two sets were grown in separate wells and starved of CSF-1 for 24 hours. The lines were then given CSF-1 for 5, 10, and 30 minutes. When sufficient time was reached, the cells were fixed to the plate and then dyed so they could be imaged. Both the wild type and the endophilin knockout BMM cells were dyed with the fluorescence stains DAPI (461 nm) for nuclei, phalloidin (514 nm) for actin and Dylight 594 (594 nm) for CSF-1. The dyes were excited with their corresponding wavelengths of light under a high content microscope. Images of both sets of BMM were collected using high content microscopy. The images collected were then analyzed using the previously made pipeline in CellProfiler to calculate the fluorescence per cell. The mean fluorescence per cell was compared between control BMM cells and endophilin knockout BMM cells at intervals of 5, 10, and 30 minutes.

RESULTS AND CONCLUSION

The mean fluorescence intensity for the control BMM cells was 4,816 at 5 minutes, 9,050 at 10 minutes, and 1,596 at 30 minutes, while the mean fluorescence intensity for the endophilin knockout BMM cells was 7,111 at 5 minutes, 10,454 at 10 minutes, and 2,632 at 30 minutes. The endophilin knockout had higher mean fluorescence intensities at all of the time intervals (Figure 2).

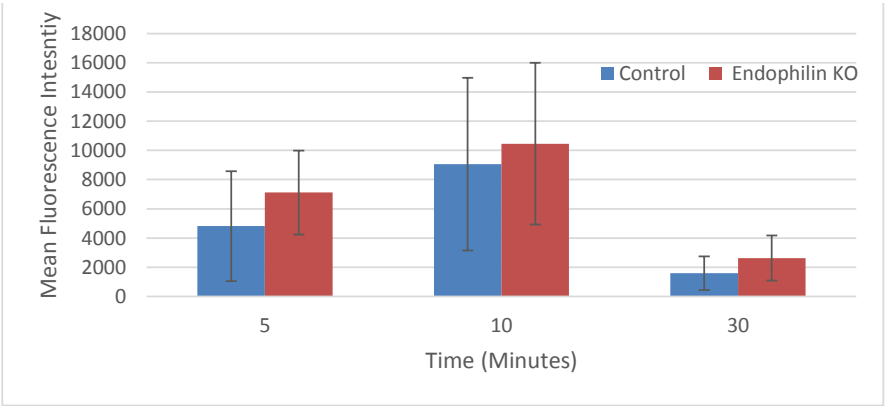

Figure 2: Mean CSF-1R fluorescence intensity of both control and endophilin knockout bone marrow cells at time points 5, 10, and 30 minutes. Bars indicate standard error.

A quantitative assay to analyze endocytosis of macrophage growth factor receptor by molecular imaging was successfully developed. The results suggest that CSF-1 receptors are not internalized into the cell when endophilin is knocked out. The endophilin knock-out cells display higher fluorescence intensity when the receptors remain on the surface of the cell rather than being internalized. The cells remain on the surface because the lack of endophilin prevents the receptors from internalizing into the cell and becoming degraded. In the control cells where FEME is operating, there is less fluorescence due to the decreased concentration of receptors on the surface as they are internalized instead. When the receptors are internalized, they eventually become degraded by lysosomes, indicated by the large drop off in mean fluorescence intensity at 30 minutes. This result suggests that CSF-1 receptors are not internalized into the cell when endophilin is knocked out and, therefore FEME is responsible for internalization of CSF-1 receptor. We conclude that

FEME plays a role in the control of the amount of growth factor receptors internalized in macrophages. The objective of determining if FEME plays a role in the control of the amount of growth factor receptors internalized in macrophages was achieved. Potential applications for this work could be in relation to leukemia cells, or identifying all endocytic pathways present in CSF-1R internalization. Future work to be done includes running statistical tests on the data to verify the significance, replicating the experiment to obtain similar results, and expanding on the results found in this research.

ACKNOWLEDGEMENTS

This research project was funded by South Dakota State University Department of Chemistry and Biochemistry as well as the Van D. and Barbara B. Fishback Honors College. The authors would like to thank Dr. Hoppe for his guidance and help, George Opoku-Kusi for valuable input on our project, and Dr. Cole-Dai and Dr. Gupta for instructing the course.

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