# Differential Expression Analysis of Fusion Inhibited Mouse C2C12 Cells to Characterize Dysregulation of Key Myogenic Genes

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## Abstract

The regulation of calcium and its signaling pathways play a key role in the expression of several genes involved in myogenesis and muscle differentiation. Prior research has suggested that a lack or excess of calcium can lead to muscular dystrophies associated with the dysregulation of the genes studied. We hypothesized that genes involved in muscular fusion during differentiation would be downregulated or even silenced in calcium-chelated fusion-inhibited cells. However, our research shows the opposite, as some myogenic regulatory genes were upregulated as opposed to the control group. These findings suggest that fusion inhibited nuclei may try to express their own genes to try to continue differentiation even after inhibition, and they may present certain future opportunities for understanding how to tame excess calcium levels in muscular dystrophies such as DMD.

## Introduction

During skeletal muscle development and repair, mononucleated progenitor myoblast cells fuse together to form multinucleated myofibers. Myoblast fusion, a key step in myogenesis, involves the migration, recognition, adhesion, membrane alignment, and union of fusion competent myoblasts (FCMs) and founder cells (FCs)[1]. $Ca^{2+}$ ions are essential factors in regulating myogenesis. On a macroscopic level, $Ca^{2+}$ signaling determines the spatiotemporal location of where new myofibrils will be constructed. However, for the actual activation of myogenesis, $Ca^{2+}$ ions enter muscle cells through calcium-ion channels and activate calcineurin pathways, which in turn trigger the regulation and expression of several transcription factors involved in myogenesis [2]. Calcium chelators such as EGTA reduce the chemical activity of $Ca^{2+}$ ions [3]. Myogenic regulatory factors such as Myod1 and Myog play a vital role in myoblast fusion regulation and inhibition. Myod1 is necessary in muscle regeneration and plays a crucial role in regulating differentiation and myogenesis, while Myog is essential for the development of functional embryonic skeletal fiber muscle differentiation. Mymk is crucial to the membrane remodeling events that drive myoblast fusion [4]. Pax7 is involved in early, undifferentiated cells and regulates Myod1 and Myog, which in turn regulate Mymk. Neb codes for Nebulin, a protein involved in calcium sensitivity [5]. The goal of this project is to determine the effect of calcium chelation on the gene expression, especially of Pax7, Myod1, Myog, and Mymk, and Neb in the C2C12 cell line during multiple stages across myogenesis. This experiment utilized short-read bulk RNA sequencing data to analyze the expression of genes involved in myogenesis and muscle differentiation across a 72 hour timecourse in both a control group and an experimental group treated with 1.5 mM EGTA at 24 hours to inhibit fusion. Fluorescent in-situ hybridization (FISH) was used to visualize gene expression at each time point (0hrs, 6hrs, 24hrs, 48hrs, and 72hrs) in both the control and experimental group. This will be done through the analysis of potentially dysregulated gene expression through RNA-seq data for quantification and FISH for visualization.

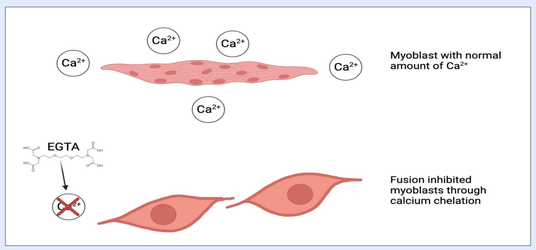


**Figure 1: Comparison of fusion inhibited myoblasts (treated with calcium chelator EGTA) to the normal fusion of myoblasts into myotubes.**

## Methods & Materials

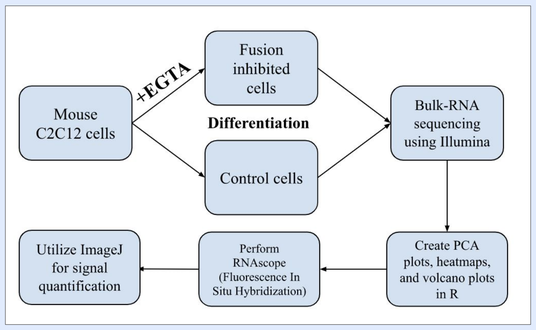


**Figure 2: Methods Flowchart**

## Results

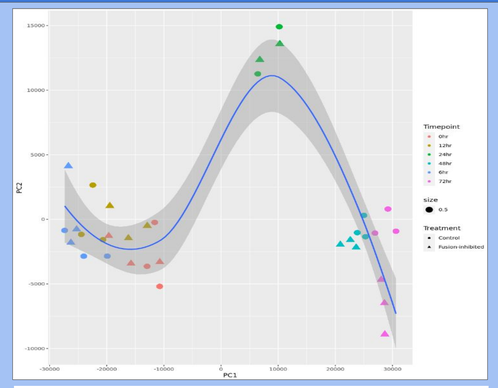


**Figure 3: PCA plot comparing fusion inhibited cells to control cells over 72 hours.** The PCA plot shows that the control and fusion inhibited cells begin to differ significantly in gene expression at 72 hours, after the experimental group was treated with EGTA at 24 hours.

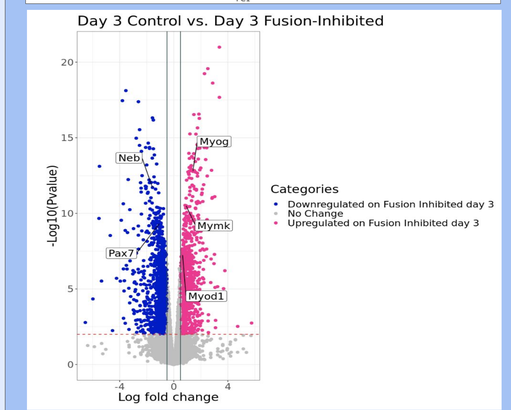


**Figure 4: Volcano plot comparing gene expression of fusion inhibited to control cells after 72 hours.**
**Log fold Change: 0.5**
The volcano plot shows that Pax7 and Neb were downregulated, while Myod1, Myog, and Mymk were slightly upregulated.

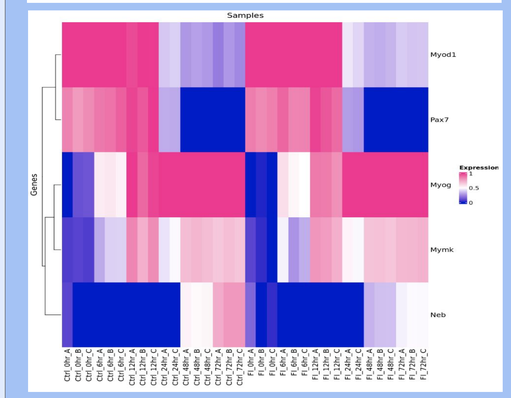


**Figure 5: Heatmap depicting expression of Myod1, Pax7, Myog, Mymk, and Neb in three samples of control (left) and fusion inhibited (right) cells over the course of 72 hours.**
The heatmap shows that the gene expression of the control and fusion inhibited cells remains relatively similar, with the most significant differences appearing at 72 hours.

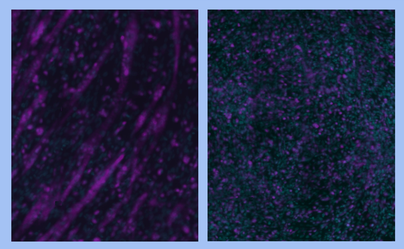

**Figure 6: RNAscope images comparing Neb (magenta) expression with respect to the cell nuclei (cyan) between the 72 hour control (left) and fusion inhibited (right) cells.** The images demonstrate that Neb was slightly downregulated and expressed in the individual cells of the fusion inhibited group.

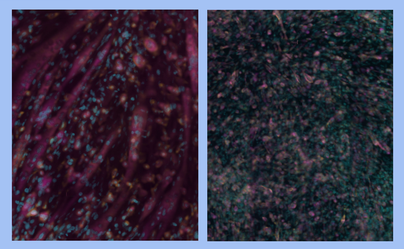

**Figure 7: RNAscope images comparing Myog (yellow) and Mymk (magenta) expression with respect to the cell nuclei (cyan) between the 72 hour control (left) and fusion inhibited (right) cells.** The images demonstrate that Myog and Mymk were slightly upregulated and expressed in the individual cells of the fusion inhibited group.

## Discussion

The expression of myogenic regulatory factors such as Myog and Myod1 are crucial in the development and differentiation of muscle cells, also known as myogenesis. Calcium-signaling plays a major role in the process of myogenesis, allowing for fusion to occur during different stages of differentiation. Thus, regulation of gene expression in calcium-chelated muscle cells analyzed in this experiment play an important role in understanding how calcium deficiency causes the dysregulation of genes involved in muscle differentiation as well as fusion inhibition. However, since the gene expression of the calcium chelated C2C12 experimental group was studied for no more than 72 hours, lack of calcium in cells may have merely delayed myoblast fusion, rather than fully stopping differentiation. Additionally, a potential explanation as to why the genes Myog and Mymk were upregulated is that the fusion inhibition could possibly cause each individual nucleus to express its own genes in an effort to activate the fusion process. In a fused myotube, where the nuclei share a common cytoplasm, not as many genes would need to be expressed since there is nothing obstructing the fusion process. Another potential explanation as to why Neb was downregulated is because Neb codes for a protein, Nebulin, that increases calcium sensitivity in cellular signaling transduction pathways. However, it would make sense that Neb was not activated when calcium chelation was introduced because there would be less calcium which in turn would mean less Neb needed. A reason why Pax7 was downregulated could be because Pax7 is mutually exclusive to Myod1 and Myog. Pax7 is always upregulated in early stages of differentiation and silenced in later stages in favor of Myod1 and Myog. As Myod1 and Myog were both highly expressed in the later time periods in both the control and experimental groups, Pax7 was downregulated during those same time periods. Lastly, Myod1 was very minimally dysregulated which could mean that it isn't as vital to fusion and related to calcium signaling as the other genes being studied.

## Future Directions

- Observe gene expression of the genes of interest at timepoints past 72 hours to observe whether lack of calcium continues to inhibit C2C12 myoblast fusion
- Research if expression of the genes of interest in calcium deficient mouse C2C12 cells is conserved in calcium deficient human myoblast cells
- Use fluorescent in situ hybridization (RNAscope) to analyze the expression of other genes associated with myogenesis
- Explore the possibilities of incorporating EGTA into treatment for diseases induced by an abnormally high intracellular calcium influx due to high calcium levels, such as Duchenne muscular dystrophy[6]

## References

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## Acknowledgements

- **Dr. Ali Mortazavi,** Professor, Developmental and Cell Biology, University of California, Irvine
- **Dr. Kyoko Yokomori**, Professor, Biological Chemistry, University of California, Irvine, School of Medicine
- **Elisabeth Rebboah**, PhD Candidate, Department of Developmental and Cell Biology, University of California, Irvine
- **Jasmine Sakr**, Graduate Student, University of California, Irvine, Department of Pharmaceutical Sciences
- **Stephanie Shirey**, Chemistry Teacher, El Dorado High School