

Natural Sciences

Gene Expression During C2C12 Differentiation

Zoe Storaasli¹, Yan Zhao², Marlin Touma MD, PhD²

¹Vanderbilt University, Nashville, TN, USA

²Touma Neonatal Congenital Heart Laboratory, UCLA David Geffen School of Medicine, Los Angeles, CA, USA

Abstract

Congenital heart disease (CHD) is one of the most common types of birth defect and is very poorly understood. To comprehend this disease better, the normal genetic expression associated with myocyte differentiation must be mapped out. This gives a deeper understanding of how cardiomyocytes (cardiac muscle cells) divide and specialize, which can contribute to understanding the mechanisms of CHD better. The workflow of this specific project began with culturing C2C12 mouse muscle myoblast cells and harvesting them at six different time points: 50% confluency, 100% confluency, 24-hours postdifferentiation, 48-hour postdifferentiation, 72-hour postdifferentiation, and 96-hour postdifferentiation. The expression levels of MyoD1, Myogenin, MyoM2, MyoZ2, GATA4, Tbx5, NKX2.5, Ki67, Tcap, and RBFOX1 were then measured and compared with qPCR. It was expected that those gene markers associated with muscle differentiation and structure would increase in expression levels over time while those markers associated with cell proliferation would decrease in expression levels. The qPCR showed the expected induction of myogenic differentiation marker genes, with the following deviation: MyoD1 expression did not increase during differentiation, and cardiogenesis markers did not follow a clear expression pattern during differentiation. Future studies on human-induced pluripotent stem cells differentiation to cardiomyocytes are needed to understand the patterns of gene expression in cardiomyocytes compared to skeletal myocytes. The outcomes of these studies may contribute to a deeper understanding of heart development and abnormalities in patients with CHD, and may improve diagnosis and treatment methods.

INTRODUCTION

Congenital heart disease (CHD) affects approximately 1 in 40,000 births in the United States and is considered to be one of the most common types of birth defect (Mai, 2012). CHD is a broad term for all congenital heart defects, which are generally defined as a structural abnormality of the heart and/or major vessel that exists at birth. The incident of death from CHD on live births is 81:100,000.

Despite the relatively large prevalence and mortality of congenital heart defects, only about 15% can be attributed to a known cause (van der Bom, 2011). The mechanisms of CHD are poorly understood, but some genetic associations have been identified. These include chromosomal aneuploidy and mutations of the FBN1, TBX5, JAG1, NKX2.5, and GATA4 genes, among others (Richards, 2010). Pathogenic variation accounts for approximately 40% of human CHD cases (Yasuhara & Garg, 2021). The molecular mechanisms underlying CHD could encompass mutations in elements of the cardiac gene network, changes in hemodynamics, disruptions in the regulatory pathways of cardiac genes, dysfunction of micro-RNAs, impacts of epigenetics, occurrences of adult congenital

heart diseases, and various other factors (Huang et al., 2010). One must navigate the intricacies of genetic inheritance and the phenotypic variability associated with CHDs to grasp their nature better.

A substantial amount of recent work has been done to gain a better understanding of the connections between the genome and the mechanisms of congenital heart defect. One of the approaches for this is mapping out the expression of different markers involved in the process of heart development during normal differentiation. On a broader level, understanding normal gene expression during myogenesis may help to identify abnormalities that occur during CHD. The specific markers chosen in this study fall into different categories: myogenesis (MyoD1, Myogenin, MyoM2, and MyoZ2); cardiogenesis (GATA4, Tbx5, and NKX2.5); cell proliferation (Ki67); later muscle structure, including sarcoma assembly, T-tubule function, and apoptosis (Tcap); and cardiac hypertrophy/heart failure (RBFOX1). Mutations in GATA4, along with other members of the GATA family, have already been identified in some patients with CHD (Rajagopal et al., 2007).

Comparisons of myogenic and cardiogenic gene marker expression during C2C12 myogenic differentiation can provide insight into the normal expression patterns during differentiation, which has yet to be carefully examined under the lens of CHD. As new genes of interest are established, gene expression can be compared between instances of myogenesis, cardiogenesis, and CHD in order to identify novel abnormalities present in cases of CHD. Additionally, Ki67, Tcap, and RBFOX1 provide a larger scope perspective of various expression patterns by including processes beyond those in individual cell differentiation. C2C12 cells, derived from a mouse skeletal muscle cell line, were selected due to their established and prevalent choice as a model for studying myogenesis (Science-Direct, n.d.). They can be easily induced to undergo steps in differentiation from a muscle precursor to myoblast, myocyte, and finally, multinucleated myotube via serum starvation. This allows for neat quantification of transcriptional factors involved in the differentiation process.

RESEARCH QUESTION

How does the expression of gene markers involved in myogenesis, cardio genesis, cell proliferation, and myotube structure change during C2C12 myogenic differentiation?

HYPOTHESIS

During myoblast differentiation, gene markers associated with muscle differentiation and structure (MyoD1, Myogenin, MyoM2, MyoZ2, and Tcap) will increase in level of expression, gene markers associated with cell proliferation (Ki67) will decrease in level of expression, and gene markers associated with cardiogenesis (GATA4, Tbx5, and NKX2.5) will remain constant in level of expression.

METHODS

C2C12 cells, a mouse myoblast cell line, were plated at an approximate confluency of 0.2 million cells per plate with 10% Fetal Bovine Serum (FBS) growth media. Once cells reached 50% confluency, they were passaged at a 1:6 ratio. Then, as the plates reached 50% confluency again, the first time point of cells was harvested. The second time point was harvested at 100% confluency before spontaneous differentiation began. At this point, the remaining plates had their media changed to 2% horse serum differentiation media to induce differentiation via serum starvation. The third, fourth, fifth, and sixth time points were harvested 24, 48, 72, and 96 hours, respectively, after the addition of differentiation media.

RNA was extracted from each of the six samples, and a reverse transcription was performed to create cDNA. A real-time qPCR with two technical replicates was performed with the sample cDNA and primers for MyoD1, Myogenin, MyoM2, MyoZ2, GATA4, Tbx5, NKX2.5, Ki67, Tcap, and RBFOX1. The internal control for the qPCRs was S26. An outline of this process is shown in Figure 1. No statistical tests were performed due to small sample size.

Results

Images of the cells at each of the six harvesting time points can be seen in Figure 2. Table 1 summarizes the results from all 10 qPCRs, and Figure 3 summarizes this data graphically. Tcap expression had no change until 48

hours after the addition of differentiation media, where it increased dramatically by about 100,000 fold (Figure 4). It then decreased by approximately 30% after every subsequent 24 hours in differentiation media. Final expression levels were still approximately 48,000 fold higher than initial expression levels. Ki67 expression decreased to about 0.25 times the starting expression amount in a linear fashion (slope of -0.143), as seen in Figure 5. The largest decrease was from 1 at 50% confluency, to 0.47 at 100% confluency.

The next section of data corresponds to those genes involved in myogenesis. Notably, MyoD1 expression remained constant during differentiation, as seen in Figure 6. Expression levels were consistent over the time course of the experiment, ranging from 0.77 to 1.72 fold initial expression levels. Myogenin expression increased overall during differentiation and showed a spike in expression to 508 times the initial amount at the 24-hour timepoint, as seen in Figure 6. After this dramatic jump, expression remained relatively constant at around 280 times initial expression levels. MyoM2 expression increased somewhat linearly (slope of 38.9) to about 180 times the initial expression level, as seen in Figure 6. Expression levels increased at a consistent rate, except for a relatively high jump from 12 fold at 100% confluency, to 83 fold at 24 hours in differentiation media. MyoZ2 expression increased drastically and linearly (slope of 3884) to about 18,000 times the initial expression level, as seen in Figure 6. A small deviation from this consistent increase is a large jump from 256 fold to 5850 fold, similar to that of MyoM2, between the 100% confluency to 24 hours in differentiation media.

Moving on to those genes specific to cardiac muscle, RBFOX1 expression increased during differentiation. It reached the highest expression levels 96 hours after the addition of differentiation media, at 38.3 times the initial expression level (Figure 7). It did not increase before during differentiation, while in growth media. GATA4 expression decreased 24 hours after the addition of differentiation media and maintained lower expression levels throughout differentiation, as seen in Figure 8. Similar to RBFOX1, there was no change in expression before during differentiation, while in growth media. Tbx5 expression levels did not show a clear pattern during differentiation and stayed within a relatively small range of 0.79 to 3.35 fold initial expression, as seen in Figure 8. NKX2.5 expression increased by a small amount during differentiation to about 2.3 times the initial expression level, as seen in Figure 8. This slight increase occurs across the entire time course, including before and during differentiation.

DISCUSSION

Three of the four studied myogenesis genes supported the hypotheses that gene markers associated with muscle differentiation and structure will increase in expression and that gene markers associated with cell proliferation will decrease in expression during differentiation. These were Myogenin, MyoM2, and MyoZ2 (Figure 6). Tcap expression, associated with later muscle structure, supported this hypothesis as well (Figure 4). The one exception was MyoD1, which did not express as expected because

its expression remained constant as opposed to increasing during differentiation (Figure 6). Because MyoD1 is a transcriptional factor associated with myogenesis, its expression was expected to increase during the differentiation process. A potential explanation for this deviation may be later involvement of MyoD1 in myogenesis than was studied in this time course (from 50% confluency to 96 hours after differentiation).

The hypothesis that those genes involved in cardiogenesis would remain constant in expression level during differentiation was not supported. Two of the cardiogenesis markers, *Tbx5* and *NKX2.5*, had irregular melt curves with multiple peaks, in addition to high cycle numbers. This may be due to unspecific primers, which could be addressed by increasing primer length. Because of this, the data from the *Tbx5* and *NKX2.5* qPCRs are unreliable. The *GATA4* expression level decreased during differentiation, which varied from the expectation that it would remain constant (Figure 8). This could be due to crosstalk between *GATA-4*, *TAL1*, and *Sirt1* during regulation of myogenic differentiation (Wang et al., 2012).

The expression level of *RBFOX1* increased during differentiation (Figure 7). Because *RBFOX1* is involved in cardiac hypertrophy/heart failure, there were no expectations for the role of *RBFOX1* in normal myogenesis. Previous studies have connected increased *RBFOX1* expression to reduced cardiac hypertrophy manifestation (Gao, 2016). The newfound increase in *RBFOX1* expression during differentiation gives insight into the potential relationship between *RBFOX1* expression and CHD. Expression levels of *RBFOX1* should be further studied to establish a potential relationship between cardiac hypertrophy, CHD, and abnormal heart cell differentiation.

All of these results are limited by the fact that there was only one biological replicate due to time constraints. Despite this reduced statistical significance, the exploratory nature of this project is useful for guiding subsequent research, especially considering the limited the archive of information relating to variance in myogenic and cardiogenic gene expression in individuals with CHD. In the future, this entire experiment should be repeated with more biological replicates. Further study should be completed on *MyoD1*, *Tbx5*, *NKX2.5*, *GATA4*, and *RBFOX1* using human-induced pluripotent stem cells differentiated to cardiomyocytes to compare patterns of gene expression in cardiomyocytes to those in skeletal myocytes. Once normal myogenic and cardiogenic gene expressions are established during differentiation, they can be compared against gene expression in individuals with CHD. Discrepancies between the two may point to unidentified mechanisms of CHD.

ACKNOWLEDGMENTS

Thank you to Marlin Touma, Yan Zhao, and Emil Bogenman, in addition to the entire LA-HIP2.0 program funded by the Saban Research Institute at Children's Hospital Los Angeles.

CONFLICTS OF INTEREST

We have no conflicts of interest to disclose.

REFERENCES

- Gao, C., Ren, S., Lee, J. H., Qiu, J., Chapski, D. J., Rau, C. D., Zhou, Y., Abdellatif, M., Nakano, A., Vondriska, T. M., Xiao, X., Fu, X. D., Chen, J. N., & Wang, Y. (2016). *RBFOX1*-mediated RNA splicing regulates cardiac hypertrophy and heart failure. *The Journal of Clinical Investigation*, 126(1), 195–206. <https://doi.org/10.1172/JCI84015>
- Huang, J., Liu, Y., Sun, P., Lv, X., Du, M., & Fan, X. (2010). Molecular mechanisms of congenital heart disease. *Cardiovascular Pathology: The Official Journal of the Society for Cardiovascular Pathology*, 19(5), 183–193. <https://doi.org/10.1016/j.carpath.2009.06.008>
- Kang, X., Zhao, Y., Van Arsdell, G., Nelson, S. F., & Touma, M. (2020). *Ppp1r1b*-lncRNA inhibits PRC2 at myogenic regulatory genes to promote cardiac and skeletal muscle development in mouse and human. *RNA*, 26(4), 481–491. <https://doi.org/10.1261/rna.073692.119>
- Mai, C.T., Riehle-Colarusso, T., O'Halloran, A., Cragan, J.D., Olney, R.S., Lin, A., Feldkamp, M., Botto, L.D., Rickard, R., Anderka, M., Ethen, M., Stanton, C., Ehrhardt, J., & Canfield, M. (2012). Selected birth defects data from population-based birth defects surveillance programs in the United States, 2005–2009: Featuring critical congenital heart defects targeted for pulse oximetry screening. *Birth Defects Research Part A: Clinical and Molecular Teratology*, 94(12), 970–983. <https://doi.org/10.1002/bdra.23098>
- Rajagopal, S. K., Ma, Q., Obler, D., Shen, J., Manichaikul, A., Tomita-Mitchell, A., Boardman, K., Briggs, C., Garg, V., Srivastava, D., Goldmuntz, E., Broman, K. W., Benson, D. W., Smoot, L. B., & Pu, W. T. (2007). Spectrum of heart disease associated with murine and human *GATA4* mutation. *Journal of Molecular and Cellular Cardiology*, 43(6), 677–685. <https://doi.org/10.1016/j.jmcc.2007.06.004>
- Richards, A. A., & Garg, V. (2010). Genetics of congenital heart disease. *Current Cardiology Reviews*, 6(2), 91–97. <https://doi.org/10.2174/157340310791162703>
- ScienceDirect. (n.d.). C2C12 cell line—An overview. Retrieved February 9, 2024, from <https://www.sciencedirect.com/topics/immunology-and-microbiology/c2c12-cell-line>
- Touma, M., Reemtsen, B., Halnon, N., Alejos, J., Finn, J. P., Nelson, S. F., & Wang, Y. (2017). A path to implement precision child health cardiovascular medicine. *Frontiers in Cardiovascular Medicine*, 4, Article 36. <https://doi.org/10.3389/fcvm.2017.00036>
- van der Bom, T., Zomer, A. C., Zwinderman, A. H., Meijboom, F. J., Bouma, B. J., & Mulder, B. J. (2011). The changing epidemiology of congenital heart disease. *Nature reviews Cardiology*, 8(1), 50–60. <https://doi.org/10.1038/nrcardio.2010.166>
- Wang, L., Jia, Y., Rogers, H., Wu, Y. P., Huang, S., & Noguchi, C. T. (2012). *GATA-binding protein 4 (GATA-4)* and *T-cell acute leukemia 1 (TAL1)* regulate myogenic differentiation and erythropoietin response via cross-talk with *Sirtuin1 (Sirt1)*.

The Journal of Biological Chemistry, 287(36), 30157–30169. <https://doi.org/10.1074/jbc.M112.376640>
Yasuhara, J., & Garg, V. (2021). Genetics of congenital heart disease: A narrative review of recent advances and clinical implications. Translational Pediatrics, 10(9), 2366–2386. <https://doi.org/10.21037/tp-21-297>

Table 1: Summary of gene expression at different time points from qPCR results.

		Relative Gene Expression					
Gene Category	Gene	50% (control)	100%	24 hr	48 hr	72 hr	96 hr
Later Muscle Structure	Tcap	1	1.7	1.07	1.04E+05	6.92E+04	4.82E+04
Cell Proliferation	Ki67	1	0.47	0.3	0.37	0.05	0.24
Myogenesis	MyoD1	1	1.7	1.72	0.95	1.56	0.77
Myogenesis	Myogenin	1	79.5	508.29	270.16	327.16	253.5
Myogenesis	MyoM2	1	12.02	83.29	150.37	143.42	180.77
Myogenesis	MyoZ2	1	256.58	5850.06	10218.91	13922.6	18116.74
Cardiac Hypertrophy	RBFox1	1	0.77	17.31	18.17	8.33	38.3
Cardiogenesis	GATA4	1	0.99	0.47	0.44	0.26	0.3
Cardiogenesis	TbX5	1	3.35	3.04	0.79	2.26	1.27
Cardiogenesis	NKX2.5	1	1.43	1.29	1.27	1.64	2.31

Note. Red color indicates unreliable melt curve in qPCR.

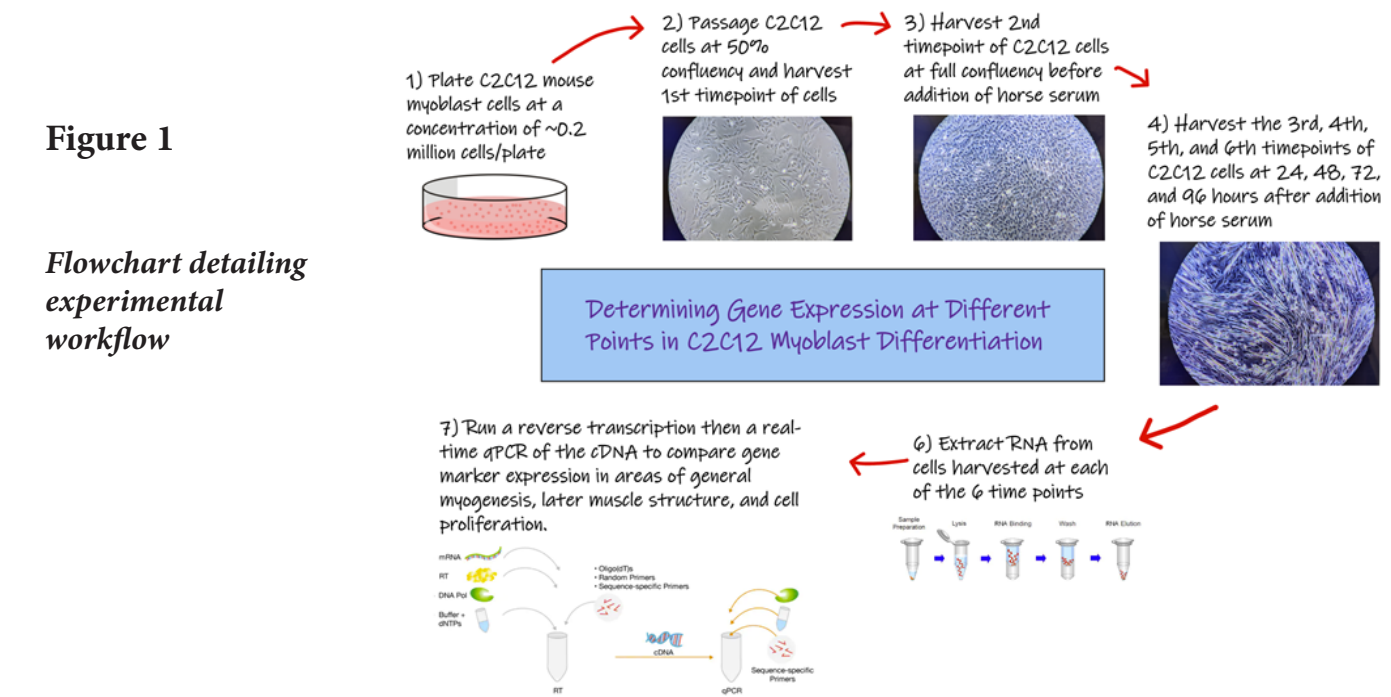


Figure 2

Images of cells at each of the six harvest time points

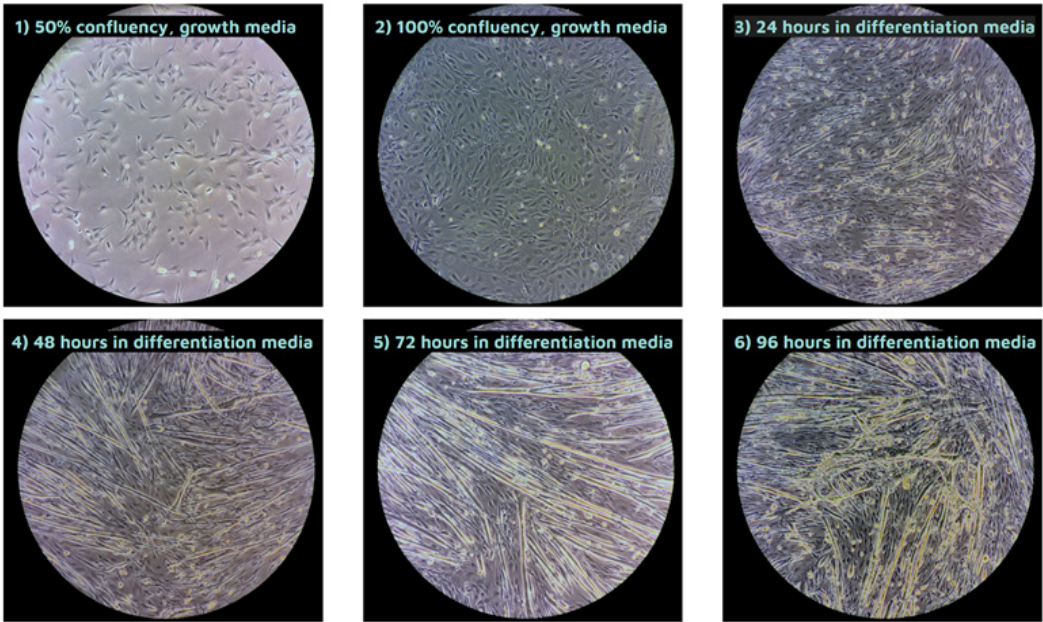
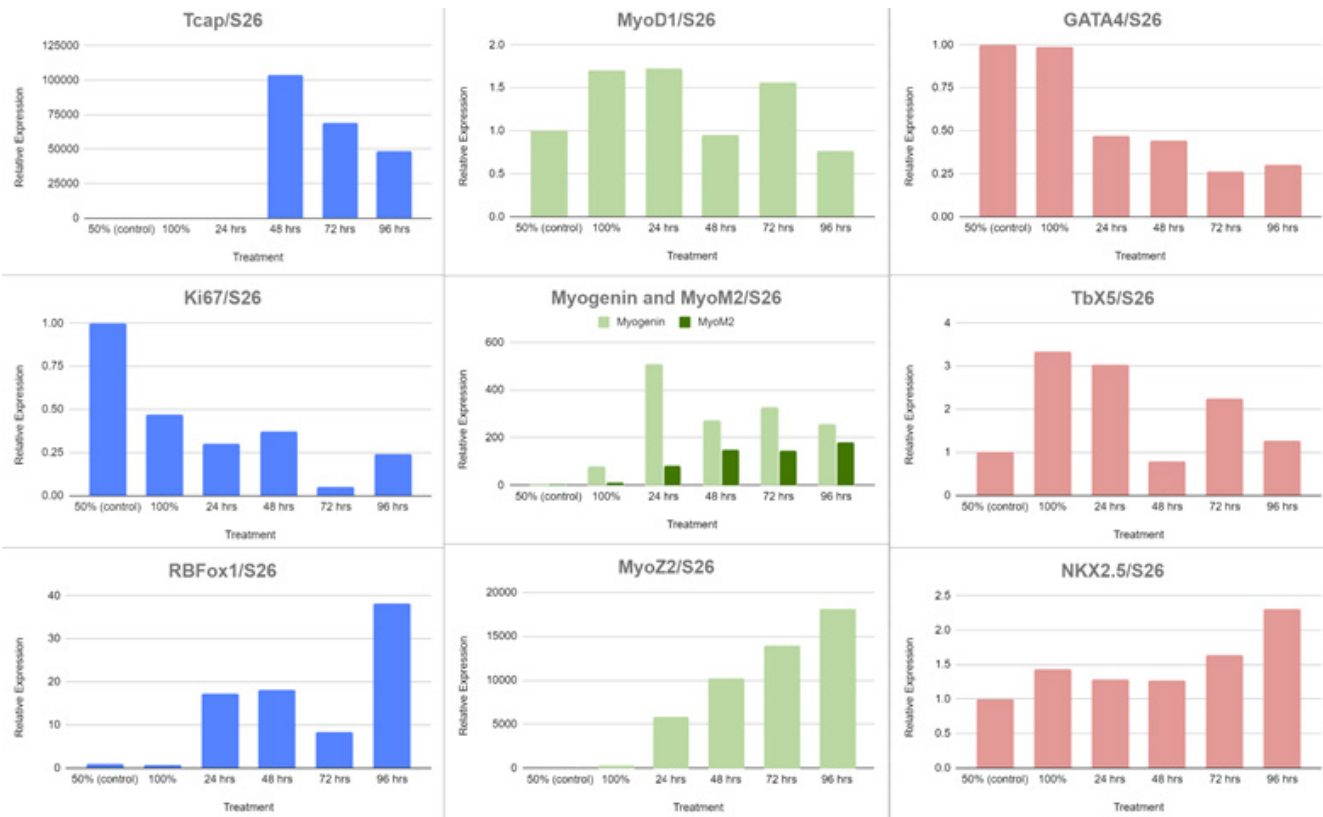


Figure 3

Images of cells at each of the six harvest time points



Note. Green color indicates genes involved in myogenesis (MyoD1, Myogenin, MyoM2, MyoZ2).

Red color indicates genes involved in cardiogenesis (GATA4, TbX5, and NKX2.5).

Figure 4

Tcap expression at different time points during differentiation

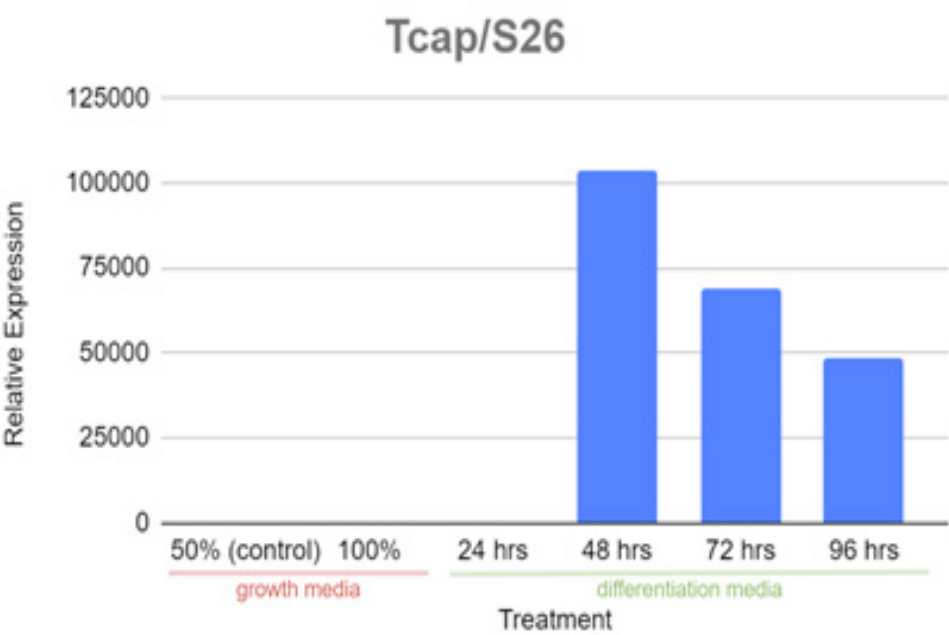


Figure 5

Ki67 expression at different time points during differentiation

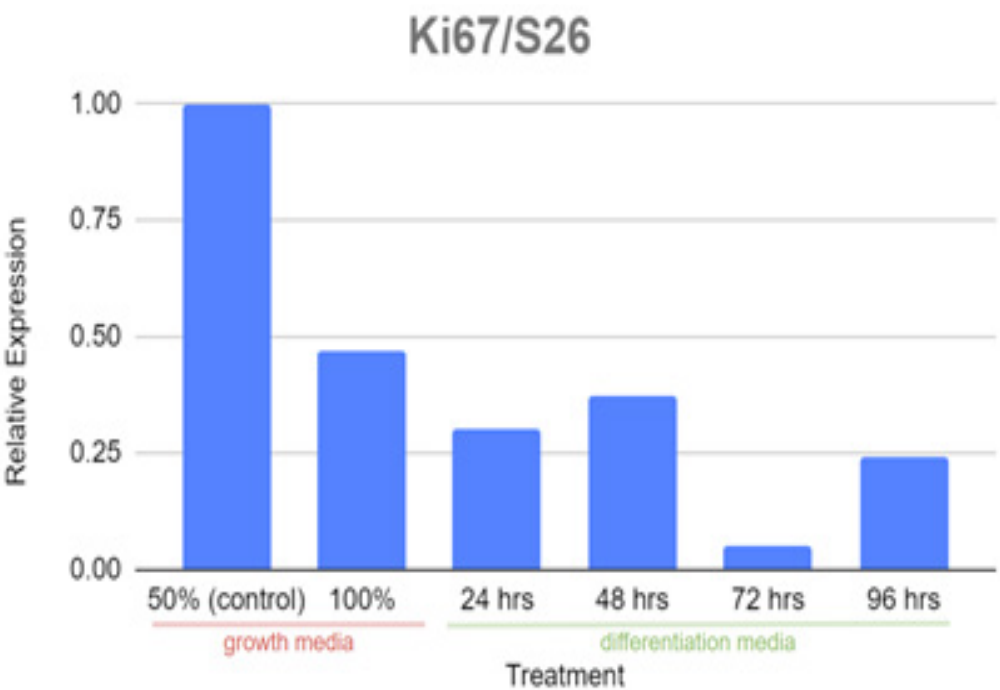


Figure 6

Myogenesis (MyoD1, Myogenin, MyoM2, MyoZ2) expression at different time points during differentiation

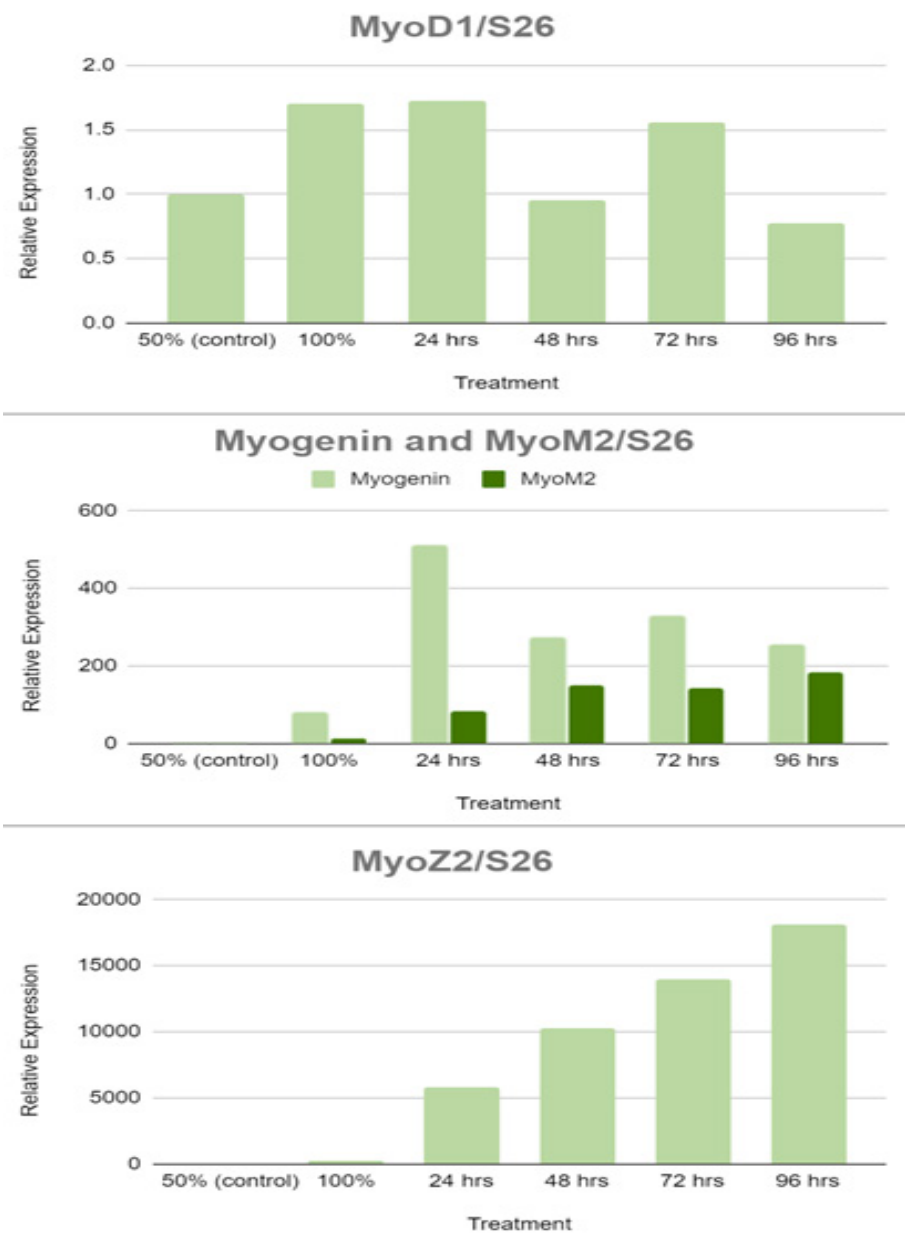


Figure 7

RBFox1 expression at different time points during differentiation

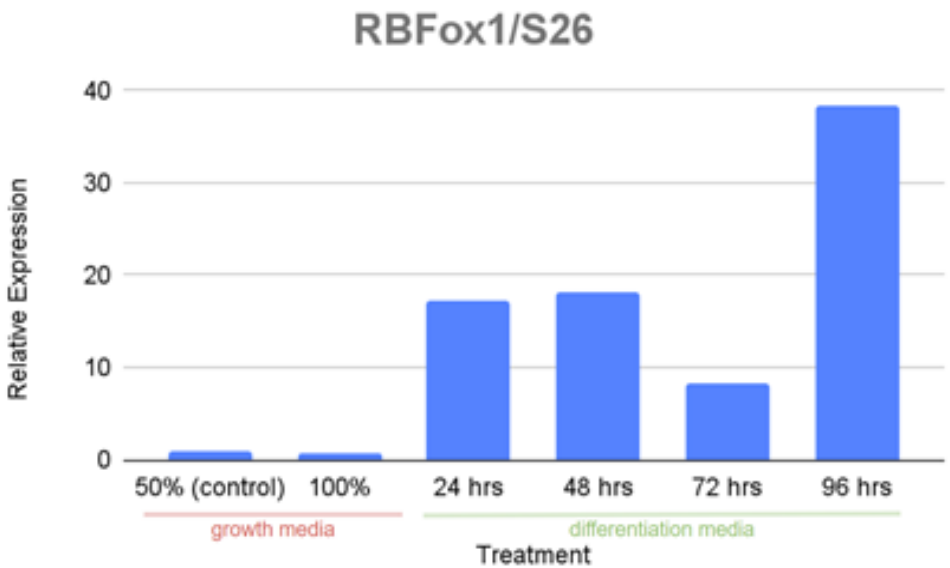
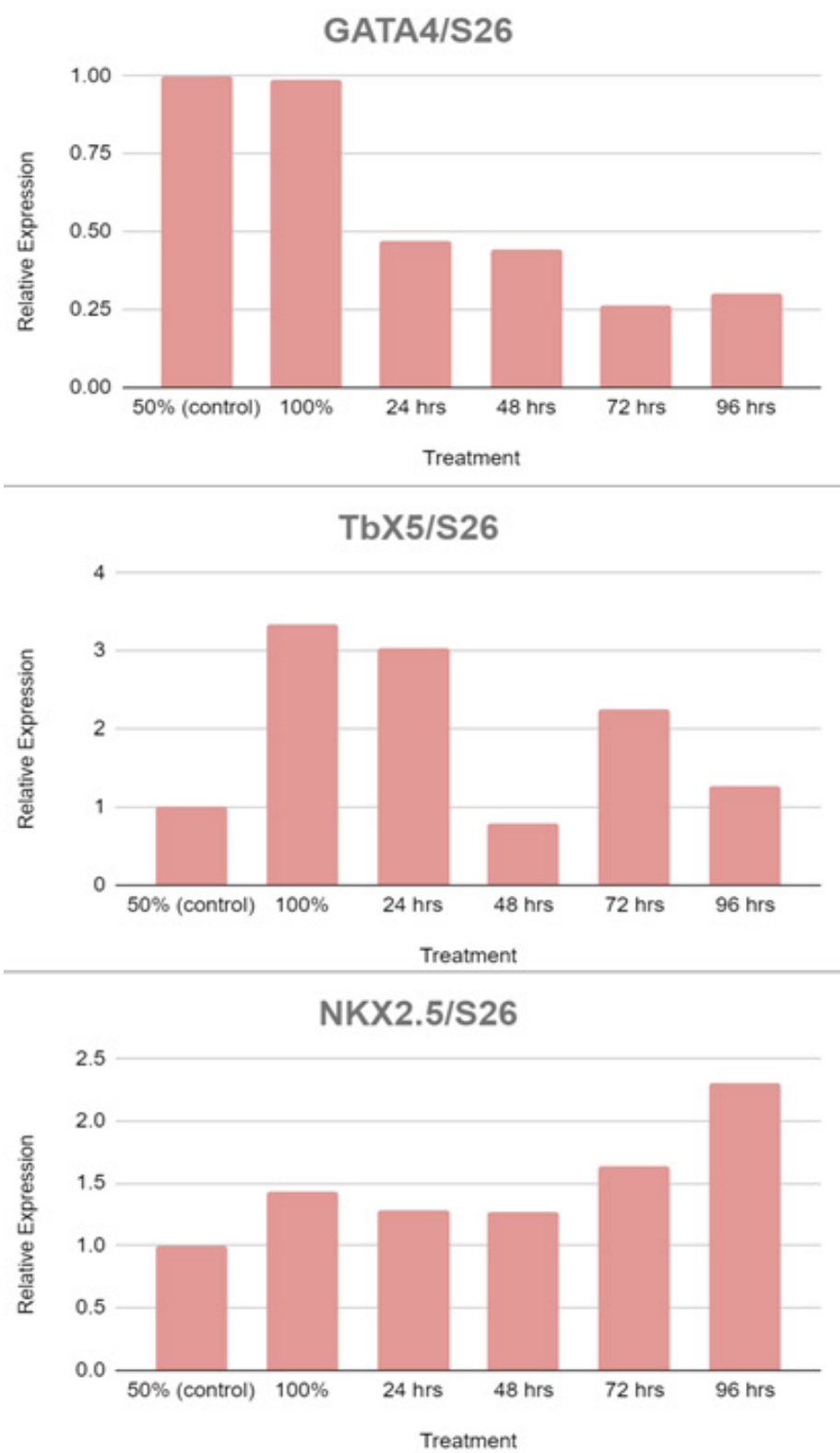


Figure 8
Cardiogenesis (GATA4, TbX5, and NKX2.5) expression at different time points during differentiation



Zoe Storaasli

About The Author

Majors: Medicine,
Health, and Society &
French

Minor: Chemistry
Class: 2025



Zoe has always loved science. This interest deepened in eighth grade after she was diagnosed with a heart abnormality and she found herself wanting to know more about how her heart worked.

Her first research opportunity was a summer at USC's Brain and Creativity Institute, where she looked at the different neurobiological foundations of mental qualities conducive to scholarly achievement.

She then spent a summer conducting independent research on the effectiveness of different brush removal methods as it pertains to forest fire prevention. That investigation led her to Russia, where she presented her work at the International Junior Forestry Competition as the US team and won third place. The following summer, Zoe was offered a spot in the LA-HIP program with Children's Hospital - Los Angeles. There,

she quantified data for a project examining the effect of e-cigarettes on heart regeneration in zebrafish.

The summer after her freshman year at Vanderbilt, she went back to Los Angeles under the LA-HIP 2.0 program to study the mechanisms of congenital heart disease via mouse and cell model systems.

The study outlined in this paper on gene expression during C2C12 differentiation was completed there, at the Touma Neonatal Congenital Heart Laboratory at UCLA David Geffen School of Medicine.

Currently, Zoe is in her second year at the Ayala Lab with VUMC, studying the gut-brain axis and how it pertains to appetite during obesity. Through her history with research, she's come to realize that she loves learning.