Spring 2024

Impact of HDAC Inhibitors on the Efficiency of Cardiomyocyte Differentiation

Lauren Mary Sargent

Follow this and additional works at: https://scholars.unh.edu/honors

Part of the Biochemistry Commons, and the Molecular Biology Commons

Recommended Citation
Sargent, Lauren Mary, "Impact of HDAC Inhibitors on the Efficiency of Cardiomyocyte Differentiation" (2024). Honors Theses and Capstones. 865.
https://scholars.unh.edu/honors/865

This Senior Honors Thesis is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Honors Theses and Capstones by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact Scholarly.Communication@unh.edu.
Impact of HDAC Inhibitors on the Efficiency of Cardiomyocyte Differentiation

Lauren Sargent
Department of Biochemistry, Molecular and Cellular Biology
Honors Thesis, Spring 2024
Advisor: Dr. Feixia Chu, Department of Biochemistry, Molecular and Cellular Biology

University of New Hampshire
Durham, NH
Table of Contents

Abstract...........................................................................................................................................2

Introduction...................................................................................................................................3

Materials and Methods..................................................................................................................7

Results and Discussion...................................................................................................................9

Conclusion...................................................................................................................................16

References....................................................................................................................................17
Abstract

Heart disease and injury is the leading cause of death worldwide, with much of its severity caused by the low regenerative capacity of cardiomyocytes (CMs). One of the strategies to counteract the poor regeneration of CMs is the production of CMs via differentiating stem cells. However, the low efficiency of obtaining CMs from stem cells is an issue because there are numerous inconsistencies in the process. Histone deacetylase (HDAC) inhibitors have been researched as ways to increase efficiency of CM differentiation by increasing histone acetylation, and thereby allowing differentiation to occur more efficiently. In this study, the HDAC inhibitor, trichostatin A (TSA), was investigated to analyze its effect on accelerating the process of CM differentiation by treatment with different concentrations. This was performed using the hanging drop method of differentiation to perform multiple differentiations, so the prevalence of beating morphology associated with CMs could be observed under different treatments. One trial was successful and demonstrated that the application of 10 nM TSA was an ideal concentration for efficiency of CM differentiation. While these results were promising, the complications with the first differentiation trials and issues with replication of the successful trial indicated that these results may be inconsistent. Further replications must be performed to ensure the results found in this study are accurate and reproducible.
Introduction

One of the leading causes of death in the United States is cardiovascular heart disease (CVD). Approximately 17.7 million people die due to CVDs every year, which constitutes around 31% of all global deaths, of which 85% were due to heart attack or strokes. CVDs refer to a group disorders of the heart and blood vessels which include coronary heart disease and rheumatic heart disease. The presence of any of these diseases can weaken the heart muscle over time and can lead to heart failure. This issue is especially prominent due to the limited capability of heart tissue to regenerate, because cardiomyocytes (CMs), or heart cells, are not replaced naturally by the body. Instead, after injury to the heart, they are replaced by stiff fibrotic scar tissue. This depletion of CMs leads to decreased cardiac contraction, which subsequently leads to pathological cardiac dilatation, additional CM loss, mechanical dysfunction, and finally culminates in heart failure. This is collectively known as cardiac remodeling.

Cardiac remodeling has been a primary target in preventing this progression into heart failure as once patients have developed end-stage heart failure, their only possible intervention is heart transplantation. Since heart transplantation is a long, arduous process that involves luck and waiting lists, strategies have focused on improving heart function before the damage can progress too far using either noncell-based or cell-based therapies. Noncell-based therapies aim at promoting heart repair via growth factors, microRNAs, and other secretory factors, but are limited by the lack of heart cell regeneration as well as the inefficient delivery of therapeutic factors. Alternatively, cell-based therapies are focused on potentially restoring contractile function of damaged heart through utilizing functional CMs derived from stem cells. This study investigates further into these cell-based therapies, specifically on the efficacy of obtaining CMs from stem cells.
Human pluripotent stem cells (PSCs) have been highly attractive sources for large-scale production of CMs in vitro. Their promise stems from their nearly unlimited self-renewal capability and their ability to exit from self-renewal and differentiate into all cell types of the three germ layers. If successful, patient-specific CMs can be resources in cell replacement therapy as well as in pharmaceutical drug testing and disease modeling for pathological studies. This strategy, however, has been noted to be impeded by inconsistencies in PSC-derived CM maturity, engraftment rates, and potential risk of arrhythmia. As such, the current investigation into these methods seeks to mitigate these inconsistencies and risks.

One type of PSC that has been investigated is the embryonic stem cell (ESC), which are derived from the inner cell mass of preimplantation embryos and can proliferate unlimitedly. It has also been demonstrated that transplanted ESC-derived CMs can synchronize their contraction with host myocardium in certain animal models. As such, being able to manipulate ESCs into producing healthy CMs is highly desirable. While the signaling pathways and intrinsic factors necessary for self-renewal have been investigated, scientific studies also suggest that epigenetic mechanisms have integral roles in maintaining stem cell identity and guiding differentiation and lineage specification of said cells. The chromatin of ESCs in particular has a characteristic structure that allows it increased accessibility compared to other cell types; this is due to the presence of fewer and more loosely bound histones as well as architectural proteins. With this knowledge, molecular techniques have been utilized to modulate epigenetic states—such as post-transcriptional modifications of histones—in an attempt to reveal their influence on stem cell renewal and differentiation.

Histone modifications not only influence stem cell maturation, but also the differentiation into cardiomyocytes specifically. Previous research has indicated that acetylation of lysine
residues on histones encourage the development of ESCs into CMs,\(^\text{11}\) supported by the known correlation between histone acetylation and increased transcription on genes.\(^\text{3}\) Thus, if the acetylation of histones in ESCs can be increased or maintained, differentiation into cardiomyocytes would be simpler and more efficient. However, the natural presence of histone deacetylases (HDACs) presents a challenge for this strategy. As the name suggests, HDACs remove the acetyl groups previously attached to histones, causing the chromatin to coil more tightly around the histones, repressing transcription of genes beneficial to differentiation.\(^\text{3}\) To counter these enzymes, agents called HDAC inhibitors have potential to be useful.

One HDAC inhibitor, trichostatin A (TSA), is a highly considered option to counter HDACs. TSA is a metabolic metabolite that has been noted to induce cell differentiation, cell cycle arrest, and reversal of transformed cell morphology.\(^\text{3}\) The inhibitor has been noted to specifically result in histone H4 hyperacetylation.\(^\text{10}\) TSA functions by mimicking the substrate of an HDAC and inducing chelation of zinc in the catalytic pocket of the enzyme by the hydroxamic group of the molecule.\(^\text{3}\) The structure of TSA is depicted in Figure 1 with the hydroxamic group located on the far right of the molecule.

![Figure 1. Molecular structure of trichostatin A.](image)
Previous research at the University of New Hampshire conducted by Dr. Nan Yang into the usage of TSA revealed that acetylation of histones was upregulated in CMs when compared to the same histones in murine ESCs (mESCs) after differentiation. Knowing this, Yang conducted an experiment applying the same concentration of TSA at different stages of CM differentiation to determine at which stage is TSA most efficient in producing CMs. The stages of differentiation investigated were the mesoderm stage (Day 0-2), the progenitor cell stage (Day 2-5), and the committed CM stage (Day 5-8; Day 8-11). In summary, Yang determined that when applying 3 nM TSA to differentiating mESCs, more CMs were produced earlier into differentiation than other treatments as well as differentiating mESCs that were not treated at all. Thus, the incorporation of TSA and its ability to upregulate histone acetylation can increase CM differentiation efficiency when applied during the progenitor cell stage.

It is with this knowledge that this study investigates if the efficiency of differentiation from ESC to CM is improved upon with the application of different concentrations TSA during differentiation. Through the induction of a mouse ESC line into CMs, a comparable investigation was performed, which can be comparable to if one was performed with human ESCs. To achieve this, a protocol similar to the one seen in Wang et al. was followed. This is regarded as the “Hanging Drop Protocol” in which a stem cell suspension is maintained without antidifferentiation factors in a droplet hanging from the lid of a tissue culture plate. This provides an optimized environment for forming embryoid bodies from the ESCs as the rounded bottom of the droplet allows for efficient aggregation. After transferring these droplets to plates for cultivation into CMs, TSA can be applied when changing the media to assess if increasing the concentration of TSA would also increase the amount of CMs as well as the rate in which they are formed.
Methods and Materials

Embryonic Stem Cell Maintenance and Storage

The mESC line, E14, was initially woken up from liquid nitrogen storage for cultivation. Cells were cultured in a 37 °C incubator with humidified atmosphere of 5% carbon dioxide. E14 cells were maintained in E14 culture media, composed of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1x non-essential amino acids, 2 mM L-glutamine, 50 U/mL penicillin/streptomycin, 0.12 mM beta-mercaptoethanol (BME), and 12.5 ng/mL leukemia inhibitory factor. The tissue culture plates were coated with 0.2% gelatin solution in Dulbecco’s phosphate-buffered saline (DPBS). After being woken up, E14 cells were maintained in E14 culture media (10 mL in 10 cm plate) until confluency. Around every 36 hours afterward, the cells were lifted from the plate with 0.05% trypsin-EDTA solution for 3.5 minutes at 37 °C, resuspended in fresh media, and passaged with 1.5 million cells in fresh E14 media (10 mL) onto another 10 cm dish.

After four passages, any remaining suspension not being used for other experimental purposes were collected via centrifugation and resuspended in fresh media supplemented with 10% DMSO into 1 mL aliquots of 3-5 million cells. Aliquots were frozen overnight in -80 °C and stored in liquid nitrogen.

Hanging-Drop Cardiomyocyte Differentiation

On the fourth passage, after resuspending the detached cells, the cells were collected via centrifugation and resuspended in fresh E14 media (10 mL). The cell suspension would then be plated on a gelatinized plate and incubated at 37 °C for 1 hour. Cells were collected via centrifugation and resuspended in differentiation media (10 mL), composed of DMEM
supplemented with 15% FBS, 1x non-essential amino acids, 2 mM L-glutamine, 50 U/mL penicillin/streptomycin, 0.12 mM BME. A portion of the cell suspension was diluted to a concentration of 450 cells per 20 μL for plating the hanging drops. The lids of two 10 cm tissue culture dishes were inverted and lined with 20 μL drops of cell suspension. Sterile DPBS (10 mL) was added to each dish and the lids were carefully placed back onto the dishes. The dishes were incubated at 37 °C for two days.

After the incubation, differentiation media (180 μL) was added to each well of a ultra-low retention flat-bottom 96-well plate. One drop was added to each well and incubated at 37 °C for 3 days. After two days, two 48-well tissue culture plates were coated with 300 μL of 0.2% gelatin solution in each well and incubated at 37 °C overnight. The gelatin was replaced with 500 μL of differentiation media in each well. From the 96-well plate, 80 of the healthiest EBs were transferred to the 48-well plates, leaving the two bottom rows blank for negative controls, then the plates were incubated for two days.

On the second day, the media was replaced with 600 μL of fresh media. For the two variable groups, the media was supplemented with the necessary concentration of TSA. From that day forward, the media was changed in the same manner every other day until Day 8. From Day 8 onward, the media was changed in the same manner every day until the end of the differentiation on Day 14. A summary of this process is summarized in Figure 2.
Results and Discussion

While Yang’s research demonstrated that applying TSA at the progenitor stage is most efficient, its statistical significance had not yet been determined. To confirm that these results are significant and that further differentiations using TSA should apply the drug at the progenitor stage, a two-way ANOVA statistical analysis was performed followed by post hoc comparisons with Tukey’s Honestly Significant Difference (HSD) test, the results of which are depicted in Figure 3. The average percentage of wells that contained pulsing CMs was calculated over time per condition of TSA application with a total of 11 wells per condition. From Figure 3, each condition can be seen increasing from Day 7-8 to the end of the differentiation on Day 13-14. This
falls in line with the expected results of a differentiation with there being a general increase in contracting CMs as time progressed. As previously described by Yang, application during the progenitor cell stage resulted in the highest percentage of pulsing wells, and this occurred for every time period of observation. Importantly, for each time period of observation, the progenitor cell stage shared no letters with any other conditions. Thus, for each observation except Day 7-8, it was statistically significant from all other conditions, including the “no treatment” condition. With assessment of the results overall, it can be concluded that the efficiency of TSA being applied at the progenitor cell stage is statistically significant and should be the time of application moving forward with future experiments.

![Application of 3 nM TSA](image)

**Figure 3.** Progression of hanging drop CM differentiation after application of 3 nM TSA at different phases of development over time. No treatment indicates that TSA was not applied to the wells. Different letters (‘a’, ‘b’, etc.) denote significant statistical differences by two-way ANOVA ($P = 8.78673 \times 10^{-10}$) followed by *post hoc* comparisons with Tukey’s HSD Test ($P < .05$).
With the optimal time of TSA application for differentiation of CM being confirmed, four differentiations of E14 cell to CM were performed with varying concentrations of TSA applied during the process. Unfortunately, only one trial out of the four produced viable results for analysis, although both scenarios will be further discussed.

For the successful trial of differentiation, 80 total wells potentially containing CMs were observed and categorized into three conditions: no treatment, 10 nM of TSA, and 15 nM TSA with 26 wells attributed to “no treatment” and the other conditions each getting 27 wells each. The goal was to determine a possible threshold for TSA application, where its HDAC inhibitory effects are optimal without inducing cell toxicity. The differentiation was observed over the course of 14 days for contraction, which is depicted in Figure 4A. As evident from the graph, the 10 nM TSA condition consistently had the highest percentage of pulsing wells. From this information, it can be inferred that applying 10 nM TSA at the progenitor cell stage of CM differentiation is most ideal. To support this claim, this information was analyzed for statistical significance via two-way ANOVA and a post hoc Tukey HSD test, which is depicted in Figure 4B. Observing the notation of statistical significance, the 10 nM TSA condition is near consistent in each time period of observation to be statistically significant from the other treatment conditions. The only exceptions to this statement are towards the beginning and end of the differentiation period where the number of pulsing wells is often lower. Therefore, based on the overall data from Figure 4, the application of 10 nM TSA is determined to be the ideal concentration of TSA to apply to differentiation to increase efficiency.
Figure 4. Differentiation of CMs using 10 nM and 15 nM TSA. (a) Progression of hanging drop CM differentiation over time. (b) Progression of hanging drop CM differentiation over time with statistically significant differences by two-way ANOVA ($P = 1.87688 \times 10^{-12}$) followed by post hoc comparisons by Tukey’s HSD test.
In addition to the number of contracting wells, the health of the cells as they differentiated was also observed. The morphology of the cells were recorded over time, and an example of each condition is provided in Figure 5. As observed in the figure, both TSA conditions had samples continue pulsing until the end of the differentiation whereas the “no inhibitor” treatment lost contractions in its wells. This calls into question whether TSA is capable of maintaining healthier CMs than the untreated condition. Observing the TSA conditions versus the untreated condition, the latter has much more black/dark spots present throughout the differentiation, especially on Day 14. These are distinct signs of cell toxicity, in which the dead cells congregate in a three-dimensional manner instead of distributing themselves flatly. From this, it can be inferred that applying TSA to differentiation maintained healthier samples than without applying it. This, however, is slightly odd given that TSA should accelerate the growth of the differentiating cells, thus reaching cell death more quickly. The opposite effect was observed, with TSA seemingly postponing the inevitable cell death that plagues the “no inhibitor”.

Between the two TSA conditions (10 nM and 15 nM), the 10 nM condition appears to foster the healthiest environment for CMs. This is apparent because of the presence of multiple CMs within the 10 nM TSA well in Figure 5 on Day 10. This phenomenon was present in 5 wells of the 10 nM TSA condition. In the 15 nM condition, only 1 well exhibited this behavior. As such, the use of 10 nM TSA appears as the most ideal concentration to use during CM differentiation.
There are two issues with these conclusions from this differentiation that relate to each other: (1) the positive control “no treatment” condition began contracting on Day 3, four days earlier than its expected time, and (2) this is only one trial’s worth of information and thus has not yet been reproduced. The first issue calls into question how confident a conclusion can be drawn that TSA accelerates CM differentiation more than “no treatment”. This issue is exacerbated by the second issue, with no replicated results to support it. Thus, a replication was performed with the same conditions to see if the information would be supported in future trials. Unfortunately, the following replication was not successful, and no pulsing was observed from the wells. Additionally, distinct signs of cell toxicity were present in all conditions. Examples of these wells can be seen in Figure 6. Similar instances of cell toxicity were present in the differentiations performed before the successful one described above.
As for why these issues were occurring, one potential reason is that the plates may have been exposed to the air during their periods of observation under the microscope. Before the successful differentiation, the plates were kept as is in the incubator and under the microscope. With the plate needing to be moved around constantly under the microscope, it is possible that the lid of the plate may have been slightly lifted, exposing the cells to a non-sterile environment. For the successful differentiation, the plates were wrapped in Parafilm. This appeared to rectify the issue with its results already discussed above. For the subsequent differentiation, however, the issue reappeared even with the use of Parafilm. Despite the precaution, it is possible that the lid of the well plates may have been slightly lifted when rewrapping the plates with Parafilm.

Another issue is with the percentage of pulsing wells. While there were clear differences between each condition, the maximum percentage of pulsing wells only reached approximately 30% as seen in the 10 nM condition of Figure 4A. For a cell-based therapy intended to produce CMs, the output of produced CMs from this experiment is not acceptably high. This combined with the issue of air exposure suggests that this type of experiment is not suitable for an academic laboratory setting. This type of research may be benefitted more if it was conducted in a professional setting where many of the conditions can be more highly controlled, especially when handling a spontaneous process such as CM differentiation.
Conclusion

This study intended to begin the research into determining the most ideal concentration of the HDAC inhibitor TSA to apply during CM differentiation that would produce the highest efficiency of CM production. If this research were to be successful, then it would become a highly viable strategy in cell-based therapies dedicated to restoring heart function in those with CVDs. The successful trial did provide some insight as to how 10 nM TSA benefits both the production and overall health of the CMs, however the issue that it is the only available trial with these conditions does not make this the most reliable results. More replications are necessary to corroborate the findings in this experiment or disprove them. The feasibility of this endeavor in an academic laboratory, however, is unlikely given the difficulty of reproducing differentiations due to the higher risk of exposure to a non-sterile environment. For future experiments, it is likely that the research should be conducted in a professional setting where trials can be systematically replicated within a controlled environment that minimizes exposure and reduces the chance of cell toxicity caused by external factors.
References


