ENGINEERING THREE DIMENSIONAL CARDIOSPHERES FROM PLURIPOTENT STEM CELLS

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ENGINEERING THREE DIMENSIONAL CARDIOSPHERES FROM PLURIPOTENT STEM CELLS

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LIST OF ABBREVIATIONS

hPSC	Human pluripotent stem cell
hPSC-CM	Cardiomyocyte
EB	Embroyd Body

SUMMARY

Cardiovascular disease causes many lasting heart problems because when blood flow is blocked to an area of the heart, the cells in that area are damaged and could die. This dead area causes increased strain on the rest of the heart because the cells are unable to repair themselves. Human pluripotent stem cells have the ability to differentiate into the muscle cells of the heart, cardiomyocytes, which could be useful in treating the damaged areas of the heart. Currently, differentiation processes result in low yields of cardiomyocytes that additionally have an immature phenotype. Before these processes can be clinically relevant, the yield of cardiomyocytes must be improved. In original studies, it has been shown that 3D cardiospheres, or aggregated cardiomyocytes, enriched the cardiomyocyte populations and resulted in more mature cells compared to the 2D controls[1]. This study expands on the formation of cardiospheres by aggregating them at different time points during the protocol. Initial data was collected regarding the number of cells/well of the differentiation cultures, number of cells seeded per cardiosphere, and beating frequency for the cardiospheres made on Day -4 Day 0, Day 5, Day 9, and Day 14. This study proved it is possible to form beating cardiomyocytes at earlier time points in the differentiation. The size of the cardiospheres was variable between time points, and this may play an effect on the yield and maturity of cardiomyocytes.

CHAPTER 1

INTRODUCTION

Heart disease is responsible for 1 in every 4 deaths for both men and women in America, killing about 600,000 people annually[2]. The heart has little endogenous ability to repair itself, but stem cells have the potential to be used as therapeutics for the repair of damaged hearts. Human pluripotent stem cells (hPSCs) have the ability to differentiate into the muscle cells of the heart, cardiomyocytes (hPSC-CM), which could be useful in tissue engineering and cell-based therapies for damaged myocardium. Studies have demonstrated the ability to generate populations of cardiomyocytes by treating human pluripotent stem cells with growth factors such as Activin A and BMP4[3], using supporting extracellular matrices[4], or using small molecule directed protocols[5] in an attempt to achieve enriched, homogeneous cardiac differentiation. Further, hPSC-CMs have been used in tissue engineering approaches, such as forming a "biowire" out of the cells to use as a suture in the heart[6]. However, one current limitation with current differentiation protocols is that hPSC-CM efficiency is highly variable, illustrating a need for improvement. Additionally, the hPSC-CM yields an immature phenotype resembling that of embryonic hPSC-CM, not mature adult hPSC-CM. In order for cell-based cardiac therapies to realize their clinical potential, methods to enrich and mature the hPSC-CM populations are required.

In our previous work, we generated 3D cardiospheres from pre-differentiated hPSC-CM derived from human pluripotent stem cells. The formation of these 3D cardiospheres has enriched the hPSC-CM populations and resulted in early signs of a more mature cardiomyocyte phenotype compared to 2D controls[1]. The goal of this project is to identify at what stage during the pre-differentiation process we are able to

create cardiospheres with the highest yield of mature hPSC-CM in an attempt to create a more efficient protocol.

CHAPTER 2

LITERATURE REVIEW

There are many hurdles to jump before cardiomyocytes can become clinically relevant. The hPSCs need to differentiate with a high yield of cardiomyocytes, the maturation age of the cardiomyocytes and the methods for implantation needs to be optimized for transplant, and lasting effects of the implantation need to be characterized.

Previous Studies

Studies have demonstrated the ability to generate populations of cardiomyocytes by treating the cells with activin A and BMP4[3], using extracellular matrices on either side of the pluripotent cells[4] (matrigel sandwich), or using small molecule directed protocols[7] in an attempt to encourage more pure cardiac differentiation. In this aspect, the three different protocols had purity yields of 82.6%[3], 40%-92%[4], and 80%-98%[7] respectively. These percentages are too low or too wide of a range to produce efficient, dependable results, although the last protocol shows the most promise as an initial study to be expanded upon. In addition, all these protocols require a significant amount of time to produce the cardiomyocytes, making it harder to complete follow up studies in an expedited fashion.

Little is known regarding the maturation of the implanted cardiomyocytes in the mouse models. Purely embroyd-body (EB) derived transplants had only an 18% success in injured mouse heart grafts[3] when compared to a 90% success in uninjured mouse heart

grafts. All the hearts that contained the surviving EB grafts also displayed "a substantial component of noncardiac elements, including rare epithelium-lined cysts[3]." This poses a problem with regards to heart transplants because data must show a complete lack of teratomas, cysts, and anything not cardiac specific in order to be safe and applicable to human implants. This study then focused on changing the mechanics behind the graft implementation[1] in an attempt to prevent cardiomyocyte death after transplant. Another possible way to increase graft success is to increase the purity of the cardiomyocytes before implantation. This would lead to less noncardiac elements, implying a safer and more successful graft.

A different study formed a biowire out of cardiomyocytes to use as a suture in the heart[6]. Their EBs showed low levels of maturation, even when taken out to day 40[6]. The study hoped that by physically organizing the hPSCs spatially in a way that represented how the hPSCs would be organized in the heart, the cells would mature faster. After two weeks in culture, the cardiomyocytes on the biowire closely resembled adult cardiomyocytes[6]. This study provides a novel way to mature the cardiomyocytes and force them to have a more cardiac muscle-like appearance. While they did not implant these biowires in this study, it is highly plausible that the more mature cardiomyocytes would better integrate into the existing heart muscle.

Therefore, one current limitation with cardiomyocyte is that cardiomyocyte efficiency resulting from differentiation protocols is highly variable, and the purification needs improvement. Additionally, the cardiomyocyte yield an immature phenotype resembling that of embryonic cardiomyocyte, not mature adult cardiomyocyte. In order

for cell-based cardiac therapies to realize their clinical potential, methods to enrich and mature the cardiomyocyte populations are required.

Cardiospheres

In original studies, 3D cardiospheres, similar to EBs, have been generated from pre-differentiated cardiomyocytes derived from human pluripotent stem cells. The formation of these 3D cardiospheres has enriched the cardiomyocyte populations and resulted in more mature cells compared to the 2D controls[1]. Almost 100% of the cardiospheres showed spontaneous contraction, even when the 2D populations started with about 10%-40% cardiomyocytes[1]. These cardiospheres even had enhanced structural maturation when compared with the 2D controls[1]. This work shows that generating cardiomyocytes in a three dimensional way holds promise to mature the cells faster at a higher purity. Moving forward, this differentiation process should be further examined and altered in an attempt to further streamline the cardiomyocyte maturation.

CHAPTER 3

MATERIALS AND METHODS

The overall objective of these studies is to evaluate the cardiac differentiation process by forming aggregates at four different time points during the differentiation process. These four time points are illustrated in Figure 1, where the cells differentiate from pluripotent cells into hPSC-CM. The first time point corresponds to the initial seeding of the hPSCs for the experiment, before pluripotent culture (D-4). The second time point is prior to differentiation when the cells are still pluripotent (D0). The third time point is after directed mesoderm differentiation via modulation of the Wnt signaling pathway (D5). The fourth time point was chosen at the day spontaneous beating is first observed during the differentiation process (D9). The final time point is when the cells show robust beating (D14). At each of these time points 3D aggregates will be formed as shown in Figure 2 and will continue to be cultured for up to a total of 21 days before being evaluated.

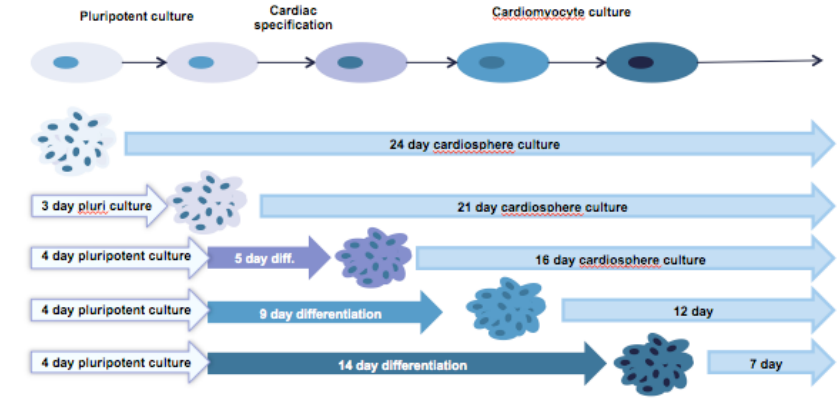

Figure 1- Overview of Experimental Design

The differentiation process for the hPSCs was followed using a previously established protocol[1]. In preliminary studies, seeding the hPSCs in 12-well plates at 200,000 cells/well optimized the protocol. The cells were cultured for three days with daily mTeSR medium changes until they reached confluence. Cardiac differentiation was

directed with the GsK3 inhibitor and Wnt inhibitor[1]. The small molecules modulated the Wnt signaling pathway over the course of the first 5 days of differentiation. Between days 7-9, small beating foci were observed in regions of the wells. By day 14 of differentiation, robustly beating cells were observed across all culture wells. Cells were used at different points in this differentiation timeline (D-4, D0, D5, D9, D14) to form cardiospheres. Cardiospheres were formed by trypsinizing cardiomyocytes into single-cell suspension, resuspending the cells at a concentration of 1500 cells/aggregate, and centrifuging the cells into Aggrewell inserts filled with 400um microwells (Figure 2). After 24 hours, the cardiospheres were removed from the wells and transferred to rotary culture. The cardiomyocytes remained in rotary culture until day 21 of the protocol.

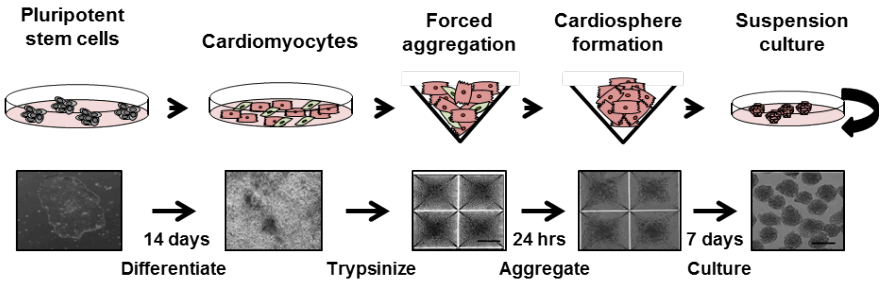

Figure 2- Process of Cardiosphere Generation

To assess the phenotype of the resulting hPSC-CM, we will utilize a variety of quantitative assays. To quantify the percentage of hPSC-CM in the cardiospheres generated at the different time points, flow cytometry will be used on cells stained for α-actinin. To evaluate the distribution of hPSC-CM within the cardiospheres, wholemount immunostaining for α-actinin combined with confocal microscopy will be used. Cardiospheres will be dissociated, replated, and stained for further immunostaining with both progenitor (nkx2.5) and mature (Troponin T, Connexin43) hPSC-CM markers to determine the maturity levels of the cardiospheres by staining. If differences are observed in the percentages or the maturity level of the cells from different treatment groups, follow-up studies can be done to more closely examine the maturation level of the cells.

CHAPTER 3

RESULTS

Initial data was collected regarding the number of cells/well of the differentiation cultures, number of cells seeded per cardiosphere, and beating frequency was recorded for the cardiospheres made on Day -4 Day 0, Day 5, Day 9, and Day 14.

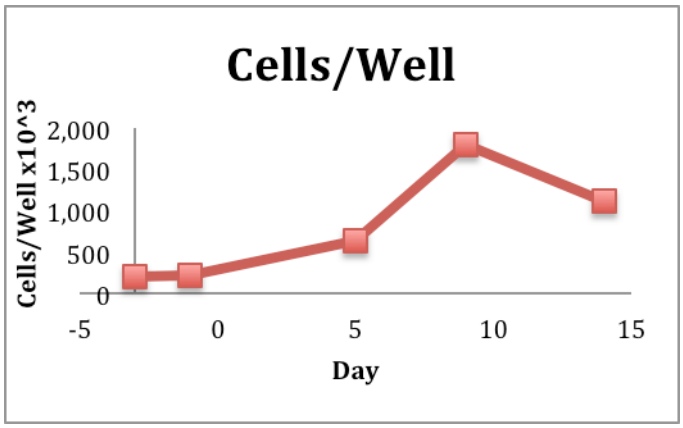

Figure 3-Cell Numbers Throughout Differentiation Process. Throughout the differentiaton protocol, cells proliferate while they differentiate.

The first cardiospheres were formed on Day -4 (group 1), which is the same day the rest of the hPSCs were seeded in the 12 well plates. Forming cardiospheres on Day -1 (group 2) instead of Day 0 ensures that the small molecule was not added during the aggregation process, but one day after instead. At Day 0 cell counts were performed to determine that there were 56,600 cells/cm^2 in the wells. The cardiospheres were formed in five 24 well Aggrewell inserts, making the total aggregate concentration 6000 cardiospheres per plate. No beating was recorded in any aggregates at this time point.

Similarly, cardiospheres were produced on Day 5 (group 3) of the protocol. The concentration of the cells at this point was 167,000-cells/ cm^2 in each well. Group 3 formed cardiospheres smaller than those formed in groups 1 and 2. Beating was observed on Day 17 in some of the aggregates, primarily in the smaller cardiospheres. Cardiospheres created on Day 9 (group 4) of the protocol contained a cell concentration

of 474,000-cells/ cm^2 in each well. These cardiospheres were also much smaller than the cardiospheres formed in groups 1 and 2.

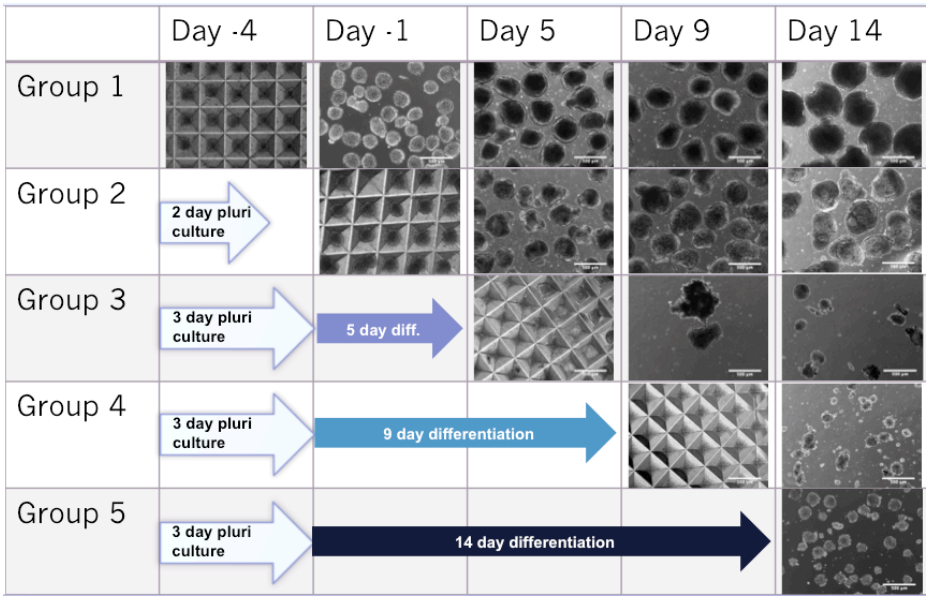

Figure 4- Cardiospheres Formed at Different Time Points. Images show phenotypes of cardiosphere formation throughout the differentiation proces. Groups 1 and 2 produce larger cardiospheres.

Finally, cardiomyocytes formed at Day 14 (group 5) had grown to a cell density of 293,000-cells/ cm^2 in each well. These cardiospheres were also much smaller than the cardiospheres formed in groups 1 and 2. Some beating was recorded on Day 18 in these cardiospheres.

CHAPTER 4

DISCUSSION

In conclusion, this study showed that it is possible to form beating cardiomyocytes at earlier time points in the differentiation. Size was an important theme in this study, as beating was shown primarily in smaller aggregates and, in addition, later time points formed smaller aggregates. Cardiospheres formed better before the differentiation process, groups 1 and 2 had larger aggregates that seemed to grow over time. Groups 1 and 2 also had higher yields of aggregates. Conversely, while the cardiospheres formed at later time points were smaller, they had a higher percentage of cardiomyocytes. This could be because when forming cardiospheres after the small molecule directed differentiation, only the cells that had committed to the cardiac lineage bound together. This would explain both the smaller aggregates formed and the higher percentage of cardiomyocytes in the cardiospheres. This data will be analyzed through Flow Cytometry, PCR, and wholemount and replated 2D immunostaining with a-actinin.

This study implies additional cell-to-cell interactions related to cardiomyocyte efficiency. Cardiosphere formation clearly affects cardiomyocyte purity and maturity, which is important for the clinical relevance of cardiomyocyte formation. By increasing the maturity and purity of the cardiomyocyte yields, these differentiation protocols step closer to use for tissue engineering and cell based therapies.

Limitations

Unfortunately, the 2D differentiation shows a relatively low yield of cardiomyocytes compared to previous attempts. This correlates to a lower yield of cardiomyocytes in the cardiospheres formed, which implies cardiosphere formation could show different results due to the phenotypes of the hPSCs during cardiosphere formation.

Future Studies

Since the cardiosphere size had the greatest effect on cardiomyocyte yield, future studies will be done with different seeding densities of cardiospheres. Smaller cardiospheres could possibly help mature the cardiomyocytes faster, but there is a risk that the cardiospheres will be too small to withstand the small molecule directed differentiation treatment. Forming cardiospheres at different time points had an unexpected affect on the resulting phenotypes of the cardiospheres. Group 1 seemed to be large and dense, increasing in size throughout the protocol. In addition, small dimples formed on a large number of the group 1 cardiospheres for unknown reasons. Group 2, while also growing in size, were not as dense and seemed to undergo pinching, where smaller cardiospheres would break off from larger ones. Further research needs to be done to understand these phenotypic differences and their possible implications for the differentiation protocol.

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