Title
The Role of Bone Morphogenetic Protein 10 (BMP10) and Crossveinless 2 (CV2) in Cardiomyogenesis

Author
Cubberly, Mark Raymond

Publication Date
2013

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The Role of Bone Morphogenetic Protein 10 (BMP10) and Crossveinless 2 (CV2) in Cardiomyogenesis

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Mark Raymond Cubberly

2013

ABSTRACT OF THE THESIS

The Role of Bone Morphogenetic Protein 10 (BMP10) and Crossveinless 2 (CV2) in Cardiomyogenesis

by

Mark Raymond Cubberly

Master of Science in Physiological Science

University of California, Los Angeles, 2013

Professor Kristina Boström, Co-Chair

Professor Alan Garfinkel, Co-Chair

The formation of the heart, cardiomyogenesis, is highly regulated to ensure proper formation and function. The objective of this study was to examine the role of bone morphogenetic protein (BMP) signaling in cardiomyogenesis. In order to accomplish this, we used an adipocyte-derived multipotent cell model termed dedifferentiated (DFAT) fat cells which readily differentiate into cardiomyocytes. Specifically, we investigated the effects of treating DFAT cells with BMP10 and crossveinless-2 (CV2), a BMP inhibitor. BMP10 is expressed exclusively in the heart and activates the activin receptor-like kinase 1 (ALK1) which

is a BMP receptor that induces expression of cardiogenic factors such as Nkx2.5 through the SMAD signaling pathway. CV2 was investigated because it has been shown to modulate BMP signaling. We chose to examine BMP10's role in cardiomyogenesis because it is structurally similar to BMP9, which regulates vascular cell differentiation, and is expressed early in the developing heart. We hypothesized that BMP10 induced proliferation of cardiac progenitor cells and CV2 induced their differentiation.

We demonstrated that CV2 binds directly to BMP10 and inhibits BMP10 from initiating SMAD signaling using co-immunoprecipitation and a luciferase reporter gene assay. We showed that BMP10 treatment induced proliferation of DFAT cells using a ^{3}H-thymidine proliferation assay, whereas CV2 proliferation levels were similar to those produced by control treatments. However, CV2 induced differentiation of DFAT cells into cardiomyocytes, as demonstrated by the induction of expression of cardiomyocyte markers by real-time PCR. Expression levels of Nkx2.5 and troponin I, which are early and late cardiomyocyte markers, respectively, were studied. Treatment with BMP10 showed an increase in Nkx2.5 expression at Day 7 after treatment, whereas CV2 treatment showed an increase in troponin I expression at Day 14. These results were corroborated by immunohistochemistry studies using antibodies against troponin I, which indicates the presence of cardiomyocytes, and α-myosin heavy chain (α-MHC) which indicates the presence of muscle tissue. The results indicated that BMP10 increased the number of early cardiomyocyte progenitor cells, whereas CV2 induced differentiation of DFAT cells into cardiomyocyte-like cells that were electrically active and beating. Taken together, these results supported a model where cardiomyogenesis occurs via a two-step mechanism. First, BMP10 increases the pool of cardiac progenitor cells. Then, once the progenitor pool has been expanded sufficiently, CV2 inhibits BMP10 and triggers cardiomyocyte differentiation.

Our findings may improve our understanding of the mechanism involved in cardiomyogenesis and enhance our ability to generate cardiomyocytes from DFAT cells or other stem cells.

The thesis of Mark Raymond Cubberly is approved.

Mark Frye

Alan Garfinkel, Committee Co-Chair

Kristina Boström, Committee Co-Chair

University of California, Los Angeles, 2013

TABLE OF CONTENTS

LIST OF FIGURES vii

ACKNOWLEDGEMENTS viii

INTRODUCTION 1

METHODS 4
Isolation of Adipocytes 4
Culture of Mouse DFAT Cells 4
RNA Analysis 5
Proliferation Assay 6
Luciferase Assay 6

RESULTS 8
BMP10 Interaction with CV2 8
CV2 Inhibits BMP10 10
BMP10 Increases Proliferation of DFAT Cells 12
BMP10 Treatment Increases the Pool of Cardiac Progenitor Cells 14
Morphological and Immunohistochemical Analysis 16

DISCUSSION 20

REFERENCES 25

LIST OF FIGURES

Figure 1: CV2 binds BMP10 9
Figure 2: CV2 inhibits BMP10 signaling 11
Figure 3: BMP10 increases proliferation of DFAT cells 13
Figure 4: Nkx2.5 expression after 7 days of treatment 15
Figure 5: Troponin I expression after 14 days of treatment 15
Figure 6: Immunohistochemical staining of DFAT cells 17
Figure 7: Immunohistochemical staining of DFAT cells treated with BMP10 17
Figure 8: DFAT cells in control medium and visualized at 10X magnification 18
Figure 9: DFAT cells treated with BMP10 and visualized at 10X magnification 18
Figure 10: DFAT cells treated with CV2 and visualized at 10X magnification 18
Figure 11: DFAT cells treated with BMP10 for 5 days then CV2 for 5 days afterward and were visualized at 10X magnification 19

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my advisor, Dr. Kristina Bostrom for allowing me to work in her lab for the past two years. Her guidance and mentorship helped me grow as a scientist and learn the fundamentals of lab work as well as scientific writing. I will always be grateful for the time she took to help me in devising experiments and editing this thesis. I would also like to thank Dr. Alan Garfinkel and Dr. Mark Frye for agreeing to be members of my committee and their time in reviewing this manuscript.

I am also thankful for all of the members of the lab who have helped me throughout this project. I especially want to thank Dr. Medet Jumabay and Dr. Yucheng Yao for teaching me the fundamental experiments and techniques for conducting basic scientific research. I also want to thank Ana, Albert, Pierre, Jiayi, Jeremy, and Emma for their help and advising in my experiments.

Lastly, I want to thank my parents for their unwavering support and love throughout my entire Master's program. I would not be where I am today without them and I could not thank them enough for always being there and listening.

INTRODUCTION

In embryonic development, the heart is the first organ to form and function (Olson et al., 2004). The function of the heart is to pump blood and its corresponding nutrients to the tissues throughout the body. This task must carry on without cessation for the duration of the organism's life. Due to this essential role, the formation of the heart is highly regulated to ensure proper formation. We will specifically look at cardiomyocytes, which are derived from the mesodermal germ layer, and their formation (termed cardiomyogenesis) in our study. One class of growth factors involved in cardiomyogenesis are bone morphogenetic proteins (BMPs) (Jamali et al., 2001). Conversely, BMP inhibitors oppose the effects elicited by BMPs and also affect cardiomyogenesis (Walsh et al., 2010). The roles of these proteins have been examined in several cases, but it is their interaction that may be most essential in the formation of functioning cardiomyocytes.

The cells we will be using to model cardiomyogenesis are adipocyte derived stem cells (also referred to as dedifferentiated fat (DFAT) cells). These cells are easily generated from an adipocyte suspension and dedifferentiate into fibroblast-like cells (Jumabay et al., 2010). Because of their multipotent nature, DFAT cells can differentiate into several lineages such as, osteocytes, chondrocytes, adipocytes and myocytes. The cardiomyocyte lineage fate has been confirmed by previous studies that demonstrated beating cardiomyocytes derived from DFAT cells that were electrically active and expressed cardiomyocyte markers (Shen et al., 2011). Through PCR and morphological analysis, it has been established that DFAT cells are a useful model for cardiomyogenesis which will enable us to explore the mechanisms of BMP signaling in cardiomyogenesis.

Specifically, it has been shown that BMP9 can increase proliferation of endothelial cells (Suzuki et al., 2010), which are epithelial cells of mesodermal origin that line the inner wall of blood vessels. Because of this proliferative effect, we aimed to explore the proliferative effect of BMP10, a close relative to BMP9, which is specific to cardiac tissue (Chen et al., 2004). Crossveinless-2 (CV2) has been shown to be an extracellular modulator of BMP9 signaling and aimed to determine if it also modulated BMP10 (Yao et al., 2012).

BMP10 is the heart specific ligand of the activin receptor like kinase-1 (ALK1) receptor (David et al., 2007). It has been shown that BMP10 is produced exclusively in the trabeculae of the embryonic mouse heart (Chen et al., 2004). Importantly, BMP10 has been found to induce expression of cardiogenic transcription factors such as Nkx2.5 and MEF2C (Jamali et al., 2001). This regulation operates through the SMAD pathway. BMP10 binding to ALK1 causes a phosphorylation of SMAD 1/5/8, which then binds with SMAD4. This complex of SMAD1/5/8 and SMAD4 then translocates to the nucleus to upregulate transcription of Nkx2.5 (Jamali et al., 2001) and increase the pool of cardiac progenitor cells.

CV2 was first discovered as a modulator of BMP signaling in fruit flies. *In Drosophila melanogaster*, CV2 is required with BMP to activate the SMAD pathway, as can be seen by CV2 mutants having decreased phosphorylated MAD at the crossveins (Harada et al., 2008). The same study discovered the human homologue of CV2 and identified it as an extracellular inhibitor of BMP signaling. CV2 also acts to increase differentiation of cardiac cells. A previous study demonstrated that CV2 inhibits BMP signaling in early cardiogenesis and leads to the increased generation of cardiac cells (Binnerts et al., 2004). Thus, BMP10 as well as CV2 are important for cardiomyogenesis.

In this study, we will explore the relationship between BMP10 and CV2 in cardiomyogenesis. We demonstrate that BMP10 binds directly to CV2 and inhibits BMP10 from initiating SMAD signaling. We show that treatment with BMP10 increases the rate of proliferation in DFAT cells before their differentiation into cardiomyocytes. On the other hand, CV2 has the ability to induce cardiomyocytes differentiation in DFAT cells. Taken together, the data support a model where cardiomyogenesis occurs in a two step mechanism. First, BMP10 induces proliferation of cardiac progenitor cells. Then, once the progenitor pool has been expanded, CV2 inhibits BMP10 and triggers cardiomyocytes differentiation in these cells. This finding could improve our understanding of the mechanisms involved in cardiomyogenesis and how to more efficiently enhance our ability to generate cardiomyocytes from DFAT cells or other stem cells.

METHODS

Isolation of Adipocytes

Mice on C57BL6/J background were obtained from the Jackson Laboratory (Bar Harbor, ME), and the investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996). Adipose tissue was removed from wild type mice aged 4-5 weeks. To isolate adipocytes, an incision was made on the dorsal side from the neck to the tail of the mouse. Then, the inguinal and gonadal fat pads were removed. The fat was washed three times in phosphate buffered saline (PBS) solution before the tissue was used for adipocyte preparation. After washing, the fat was digested using 0.1% (w/v) collagenase at 37° C for 45 minutes on a shaker at 85 RPM. After filtration through a mesh strainer, the cells were centrifuged at 1000 RPM for 3 minutes (Yagi et al., 2004). To thoroughly wash the cells, the top floating layer of adipocytes was removed and placed in a new 15 ml centrifuge tubes with 10 ml of culture medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Mediatech, Manassas VA)), and centrifuged at 1000 RPM for one minute. This was repeated three times. The adipocytes were removed and the purity of the adipocytes was assessed as previously described in detail (Jumabay et al., 2012).

Culture of Mouse DFAT Cells

Purified adipocytes were placed in a 10 cm dish with 10 ml of culture medium and incubated for 24 hours (Jumabay et al., 2012). After 24 hours, the floating layer of adipocytes was removed from the dish. The 24 h incubation period had allowed potential pluripotent non-

adipocytes to detach from adipocytes and sink to the bottom of the well, further ensuring the purity of the adipocytes. Before seeding the adipocytes in 6 well dishes, 70 μm cell strainers are placed into each well. Then each well is filled with 5-6 ml of medium containing the desired treatment, which included, (control (culture medium), BMP10 (25 ng/μl), CV2 (25 ng/μl), BMP 10 (25 ng/μl) for 5 days followed by CV2 (25 ng/μl) for 5 days, BMP 10 (25 ng/μl) with CV2 (50 ng/μl) (all reagents were from R&D Systems, Minneapolis, MN). Finally, the adipocytes were placed into each well and cultured for 5 days. The adipocytes did not attach to any plastic surfaces, unlike in the previously used ceiling culture method (Matsumoto et al., 2008), which prevented stretching of the adipocytes. Our method allowed dedifferentiated fat (DFAT) cells without fat to fall through the filter, adhere to the bottom of the dish and be exposed to the treatment while excluding the remaining adipocytes from the newly generated DFAT cells. The filters were removed and treatment changed after 5 days. The experiment was stopped when actively beating cardiomyocyte-like cells were detected in any of the treated cells.

RNA Analysis

RNA was isolated from the cultured cells using the RNeasy kit (Qiagen) following the manufacturer's instructions. Briefly, 2 μg of total RNA was reverse-transcribed with random hexamers using an MMLV Reverse Transcription Reagents kit (Stratagene, La Jolla,CA) according to the manufacturer's protocol. Each amplification mixture (20 μl) contained 25 ng of reverse-transcribed RNA, 8 μM forward primer, 8 μM reverse primer, 2 μM dual-labeled fluorogenic probe (Applied Biosystems, Foster City, CA), and 10 μl of Universal PCR mix (Quantitect probe RT-PCR kit, Qiagen). The real-time PCRs were performed using an Applied Biosystems 7700 sequence detector (Applied Biosystems). The probes used were Troponin I and

Nkx2.5. The primers and probes (Troponin I and Nkx2.5) used for real-time PCR were pre-designed and obtained from Applied Biosystems as part of *Taqman*® Gene Expression assays.

Proliferation Assay

DFAT cells were seeded in a 24 well dish with an equal number of cells at low density and treated with culture medium for 24 hours. Then, the medium was replaced with the treatments described above under "Culture of mouse DFAT Cells". This medium was supplemented with 1 μCi/ml of [^{3}H]thymidine (Amersham Biosciences), and used in proliferation assays as previously described (Bostrom et al.,2004). As soon as one of the wells became confluent, the experiment was stopped. The medium was removed, the cells were washed once with PBS, and 300 μl of lysis buffer (1:100 dilution of β-mercaptoethanol with RLT Lysis Buffer) was added to each well. Each sample was mixed with 5ml of biograde counting cocktail and added to a 20 ml scintillation vial. Incorporation of ^{3}H thymidine was determined by scintillation counting.

Luciferase Assay

Transient transfections of BAECs were performed in triplicates in 24-well plates. BAECs were plated with culture medium 24 hours prior to transfection at 10,000-20,000 cells per well that yield target confluency of 40-80%. The cells were transiently transfected using 1.5 μl of FuGene6 reagent (Roche Molecular Biochemicals) and 500 ng of DNA per well. The cells were transfected with a BMP responsive luciferase reporter gene (Korchynskyi and Dijke, 2002) and *Renilla* (R&D Systems) for normalization. Cells were taken for analysis 24 hours after transfection. The cells were lysed in 100 μl of Passive Lysis Buffer (Promega, Madison, WI) per well. The cells were freeze-thawed twice and agitated for 15 min. Luciferase activity was

determined using an AutoLumat LB953 luminometer (PerkinElmer Life Sciences) and expressed as mean from triplicate transfections after normalization to *Renilla*.

RESULTS

BMP10 Interaction with CV2

In order to determine if BMP10 interacts with CV2, an immunoprecipitation was performed. Anti-BMP10 antibodies were used to precipitate BMP10 and were compared to a control immunoglobulin G (IgG). After the immunoprecipitation, an immunoblot was performed with antibodies against CV2 (anti-CV2). Figure 1 illustrates that the band for CV2 at 80 kDa is only present when paired with the anti-BMP10 immunoprecipitation. This suggests an interaction between BMP10 and CV2 because CV2 is bound to BMP10 when the immunoprecipitation with anti-BMP10 is performed (Figure 1). Then, when we perform an immunoblot for CV2, we see the band for CV2. If there were no interaction, as is the case with IgG, we would not see a band for CV2 when the immunoblot for anti-CV2 was performed. This is because CV2 was not initially bound to IgG when the initial immunoprecipitation for IgG was completed.

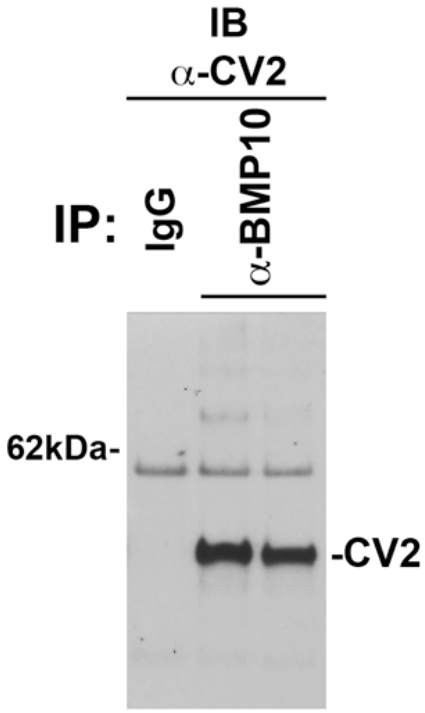

Figure 1: CV2 binds BMP10. Immunoprecipitation of IgG and anti-BMP10 followed by immunoblot for anti-CV2. Presence of a band at 80 kDa for CV2 indicates CV2's interaction with BMP10

CV2 Inhibits BMP10

Next, it was important to determine if the interaction between BMP10 and CV2 resulted in inhibition of BMP10 signaling. To determine this, a luciferase assay was performed. In this assay, BMP response elements from the promoter region of the Id-1 gene are transfected into bovine aortic endothelial cells (BAEC) in a luciferase reporter gene. BMP10 binding to its cell surface receptor induces the SMAD pathway and causes expression of the luciferase reporter gene, which results in an increase in luciferase activity. Different concentrations of CV2 were applied in order to determine if CV2 caused BMP10 inhibition. BMP10 inhibition was assessed by a reduction in luciferase reporter gene activation compared to control cells that were transfected with a control vector. Figure 2 demonstrates that CV2 inhibits BMP10 and that 200 ng/µl was the most efficient in inhibiting BMP10 in this experiment.

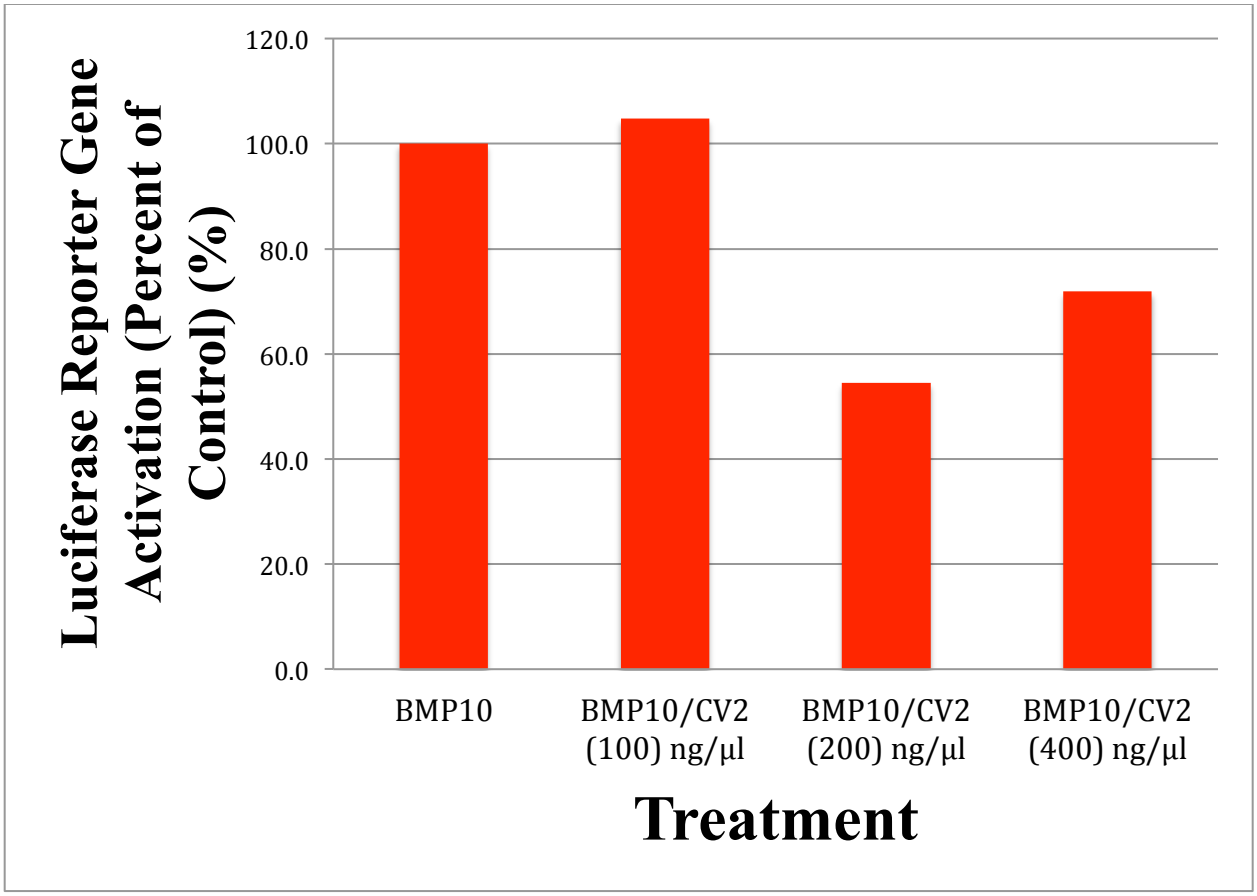

Figure 2: CV2 inhibits BMP10 signaling. Luciferase assay performed on bovine aortic endothelial cells (BAECs) (N=2). Increasing concentrations of CV2 were applied to determine the optimal concentration required for BMP10 inhibition, which appears to be at 200 ng/µl.

BMP10 Increases Proliferation of DFAT Cells

We then determined if BMP10 could increase the proliferation of DFAT cells. Proliferation is an increase in the number of cells due to cell division. This experiment was performed because we had noticed in earlier studies that BMP10 appeared to increase the number of cardiomyocytes relative to other treatments. To test this hypothesis, we performed a ^{3}H-thymidine proliferation assay. The assay involves incorporation of ^{3}H-thymidine into the DNA of DFAT cells. Then, upon cell division, the daughter cells will contain radioactive thymidine in their DNA as well. At the end of an incubation period of three days, the cells are lysed and placed in a counting solution inside of a scintillation vial. Then, the amount of radioactivity is determined. Cells from treatments that produce higher counts per minute (CPM) have incorporated more tritiated thymidine and thus, have a higher rate of proliferation. In our experiment, BMP10, as well as BMP9, provided an increase in proliferation over CV2 which produced the same amount of proliferation as control cells (Figure 3). BMP9 was used as a positive control because it had previously demonstrated its ability to induce proliferation (Suzuki et al., 2010). This enabled us to conclude quantitatively that BMP10 contributed to an increased rate of proliferation of DFAT cells.

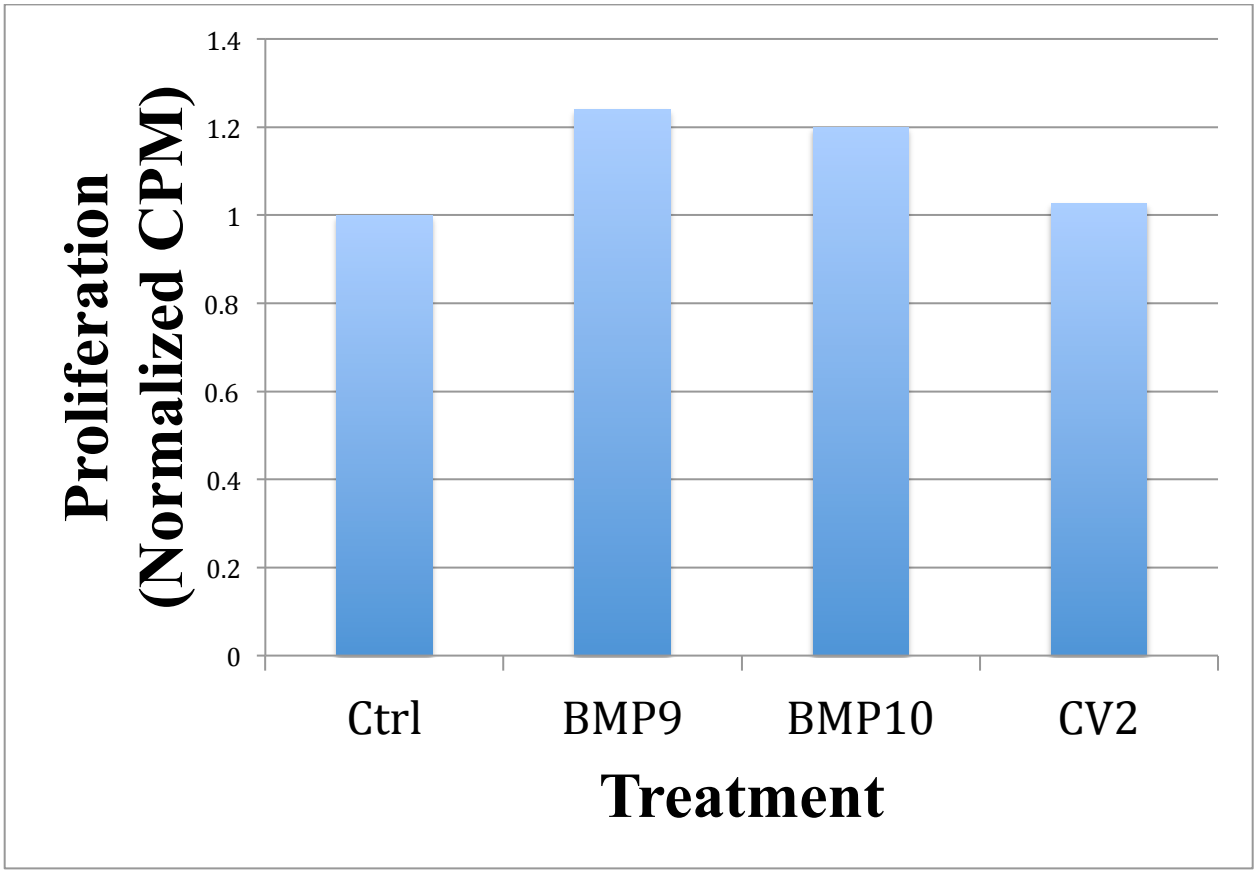

Figure 3: BMP10 increases proliferation of DFAT cells. ^{3}H-thymidine proliferation assay performed on DFAT cells (N=2). Cells were incubtaed with treatment for three days then measured with scintillation counting. BMP 9 and 10 enhanced proliferation whereas CV2 was similar to control cells.

BMP10 Treatment Increases the Pool of Cardiac Progenitor Cells

Next we aimed to determine the role of BMP10 and CV2 in cardiomyogenesis. To accomplish this, we examined the expression levels of Nkx2.5 and Troponin I by performing real-time PCR (qPCR). Both of these genes are involved in cardiomyogenesis. Nkx2.5 is a marker for immature cardiac progenitor cells, and Troponin I is a marker for mature cardiomyocytes. These experiments yielded some surprising results. They showed that BMP10 increased the expression of the immature cardiac progenitor marker, Nkx2.5 (Figure 4). This increase in Nkx2.5 indicates that BMP10 plays a role in proliferation of DFAT cells into cardiac progenitor cells. In addition, they demonstrated that CV2 treatment caused an increase in the mature cardiomyocyte marker Troponin I (Figure 5). This increase in cardiac progenitor cells could lead to an overall increase in the amount of cardiomyocytes after cardiomyogenesis is complete.

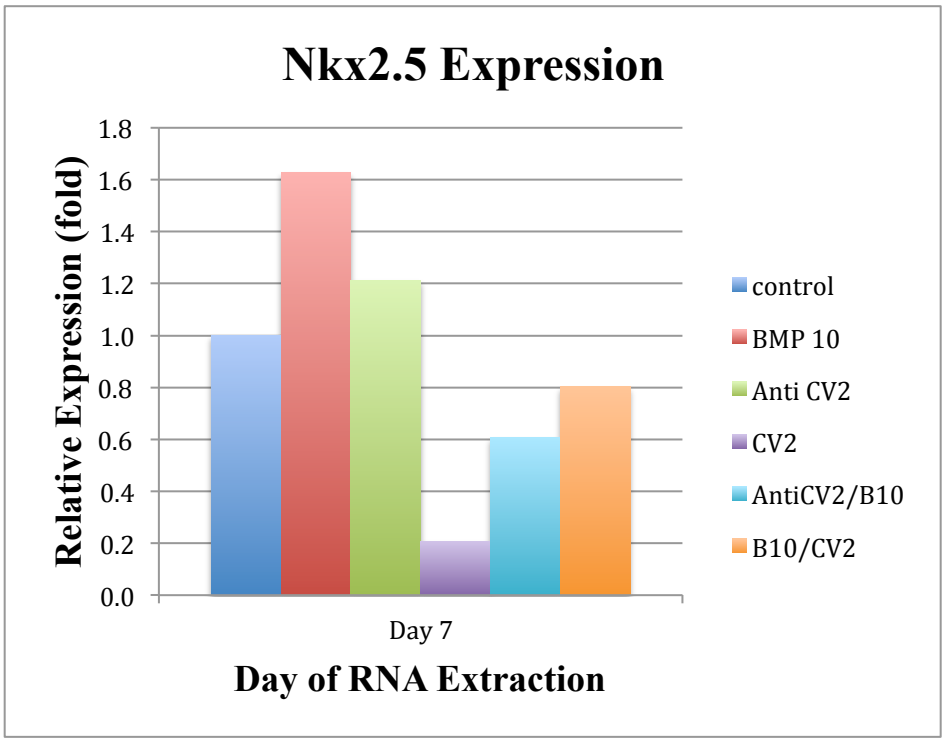

Figure 4: Nkx2.5 expression after 7 days of treatment. Expression was measured using real-time PCR and normalized to GAPDH.

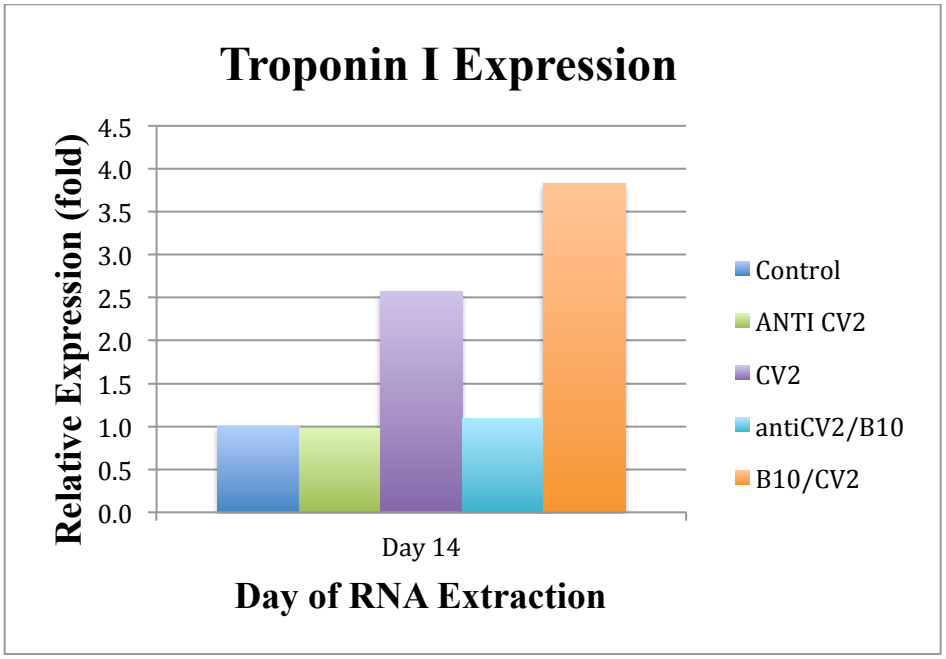

Figure 5: Troponin I expression after 14 days of treatment. Expression was measured using real-time PCR and normalized to GAPDH.

Morphological and Immunohistochemical Analysis

Finally, we wanted to ensure that we could chronicle the growth of DFAT cells and their transition from undifferentiated multipotent cells to cardiomyocytes. In order to accomplish this, we took photographs of the cells at 4x and 10x magnification on specific time points (5, 7, and 14 days). From morphological analysis, it appeared that CV2 treatment caused the earliest emergence of mature cardiomyocytes. Later however, BMP10 treatment produced a larger number of beating cardiomyocytes than CV2 treatment. Videos were also taken of beating cardiomyocytes using *image J software* to demonstrate that the cells had electrical activity and were beating.

These results were further confirmed by immunohistochemistry studies. Cardiomyocyte-like cells from BMP10 and CV2 treatments, as well as control cells, were stained with antibodies to Troponin I and α-myosin heavy chain (α-MHC) (Figure 6). α-MHC antibodies are used to visualize muscle tissue, and anti-Troponin I antibodies are used to specifically identify cardiac muscle. DAPI staining was used to visualize nuclei. Fluorescent images captured under fluorescent microscopy corroborate our earlier morphological findings. They showed a large increase in the number of mature cardiomyocytes from BMP10 treatment in relation to control cells (Figure 7). This supported our hypothesis that BMP10 increased the number of cardiac progenitor cells, and thus, the number of differentiated cardiomyocytes.

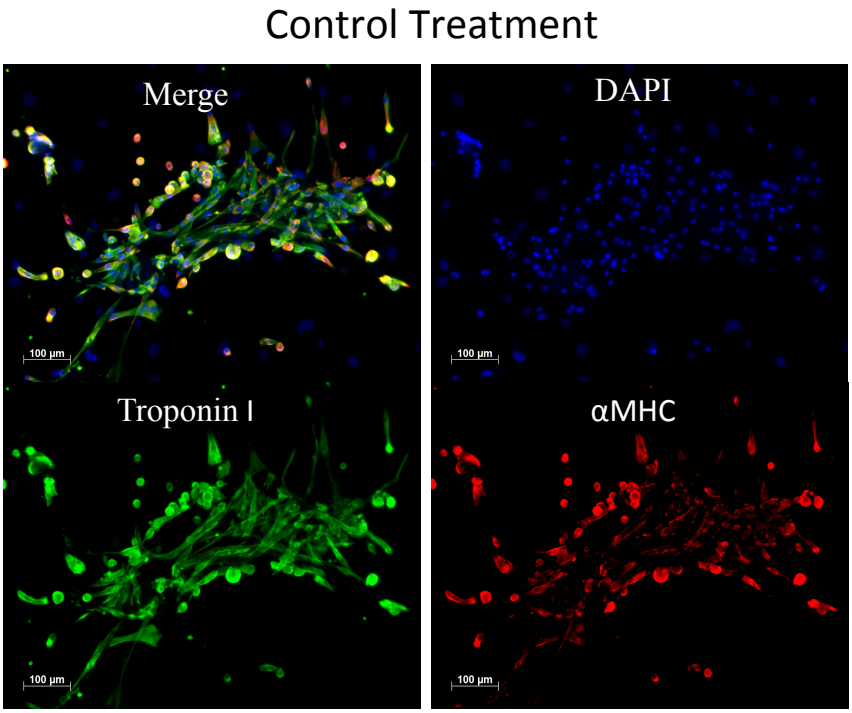

Figure 6: Immunohistochemical staining of DFAT cells. Cells from control wells were stained with antibodies to troponin I (green) to visualize cardiomyocytes and α-MHC (red) to visualize muscle tissue. DAPI (blue) staining visualized nuclei.

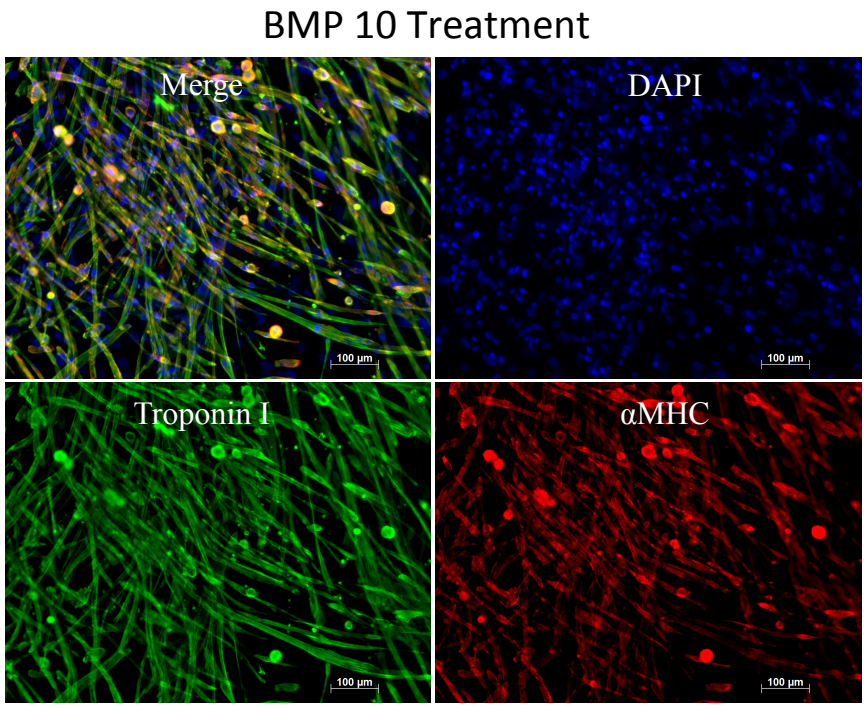

Figure 7: Immunohistochemical staining of DFAT cells treated with BMP10. Cells from control wells were stained with antibodies to troponin I (green) to visualize cardiomyocytes and α-MHC (red) to visualize muscle tissue. DAPI (blue) staining visualized nuclei.

Day 7 Day 14 Day 21

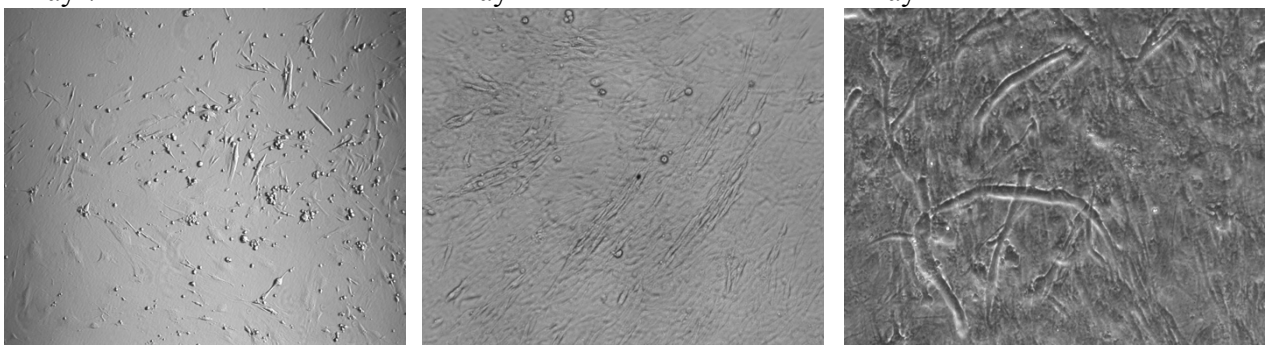

Figure 8: DFAT cells in control medium and visualized at 10X magnification. Images were taken 7, 14, and 21 days after treatment began. The appearance of cardiomyocytes began on Day 14.

Day 7 Day 14 Day 21

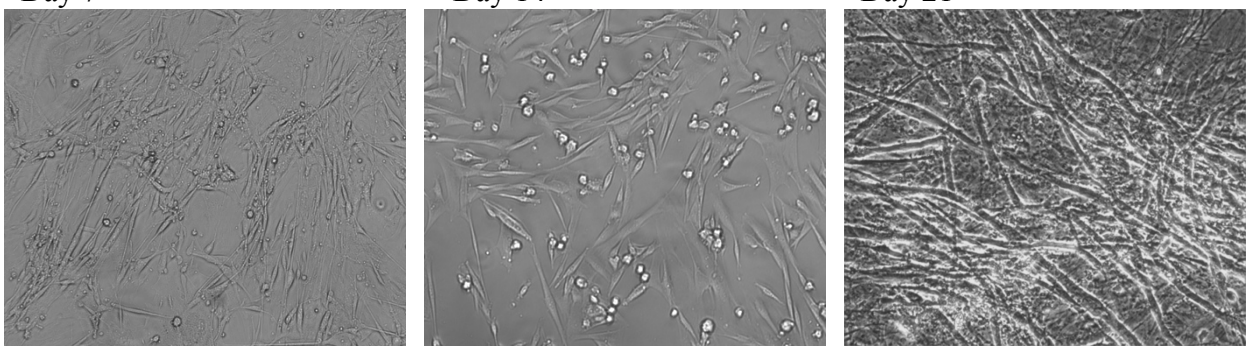

Figure 9: DFAT cells treated with BMP10 and visualized at 10X magnification. Images were taken at 7, 14, and 21 days after treatment began. Cardiomyocytes appear at Day 14 and have a greater number of mature cardiomyocytes at Day 21.

Day 7 Day 14 Day 21

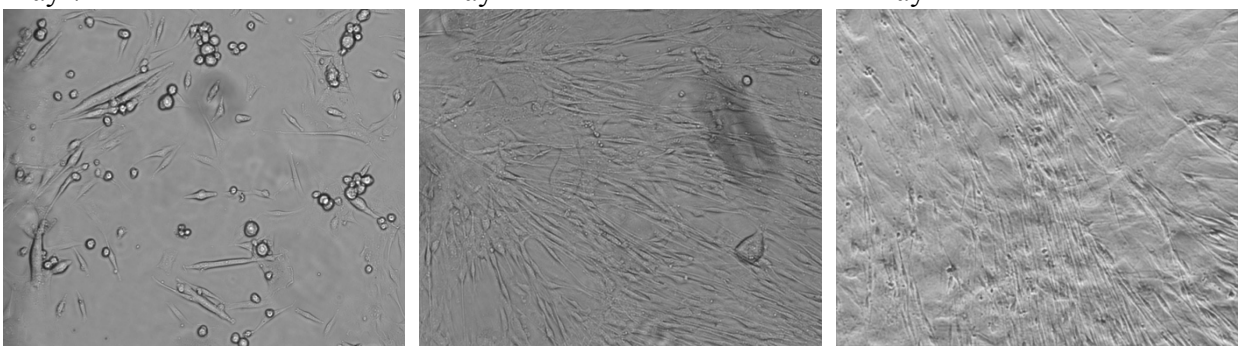

Figure 10: DFAT cells treated with CV2 and visualized at 10X magnification. Images were taken at 7, 14, and 21 days after treatment began. Immature cardiomyocytes appear at Day 7, indicating CV2 induced differentiation.

Day 7 Day 14 Day 21

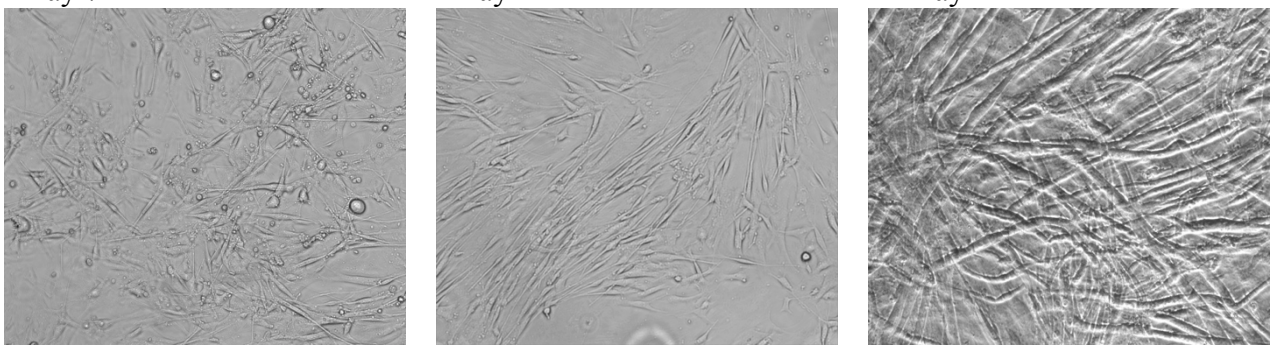

Figure 11: DFAT cells treated with BMP10 for 5 days then CV2 for 5 days afterward and were visualized at 10X magnification. Images were taken at 7, 14, and 21 days after treatment. There were the largest number of cardiomyocytes with this treatment regimen.

DISCUSSION

In this study, we showed that BMP10 and CV2 are involved in different processes of cardiomyogenesis in cells derived from white mature adipocytes. Our results suggested that cardiomyogenesis might occur via a two-step mechanism. First, BMP10 will increase the number of cardiac progenitor cells, then CV2 will induce differentiation of these progenitor cells into mature cardiomyocytes. Ultimately, these findings outline a novel approach to cardiomyogenesis that may be used as a model in the future.

Cardiomyogenesis was originally thought to occur prenatally, and the cardiomyocytes that were produced were said to be terminally differentiated (Nadal-Ginard and Mahdavi, 1989). Thus, cardiomyocytes respond to stress by hypertrophy or death and cannot regenerate on their own. Recently, this view was challenged by researchers who believe that cardiomyocytes can be replaced by a subpopulation of nonterminally differentiated myocytes entrance into the cell cycle or activation of a pool of progenitor cells who then differentiate into a myocyte lineage (Hierlihy et al., 2002). To expand upon this finding, it was observed that there were male cells in a female heart that was transplanted into a male patient (Anversa et al., 2006). Taken together, these findings demonstrated that the myocardium could undergo cell division.

This paradigm shift in viewing the heart as having cells that are able to regenerate rather than be permanently quiescent allowed the next major discovery in cardiac biology. In thinking the cells of the heart had regenerative abilities, researchers began looking for stem cells from the bone marrow or intrinsic to the heart that could provide this regenerative capability. This enabled them to discover cardiac stem cells (CSCs), which are found in niches in the myocardium and differentiate into functioning myocytes (Urbanek et al., 2005). Primitive cells leave the niche and

their growth, proliferation, and differentiation is controlled by these CSCs. Therefore, it was critical to identify the type of cells that left the niche and to determine if these cells could be derived from any other tissue in the body.

In our study, we used DFAT cells as a model of cardiomyogenesis. This was an ideal model because we had previously shown that these DFAT cells lost their adipocyte-specific markers but gained mutipotent characteristics. These characteristics enabled them to differentiate into a variety of lineages including chondrocytes, osteocytes, and most importantly for this study, cardiomyocytes (Jumabay et al., 2010). Because these cells can spontaneously differentiate and become a source of electrically active and beating cardiomyocytes, we explored the underlying processes of these cells.

We decided to investigate BMP10 in this study for a variety of reasons. The first reason being that it is known that BMP signaling is crucial for proper cardiac development. Secondly, in a recent article published by Huang et al. (2012), it was discovered that there was a BMP10/Myocardin pathway that was crucial for cardiac development and a block in BMP10 signaling produced a block in cardiomyocytes proliferation. This was a significant finding because our lab had also discovered that BMP9 was required for endothelial cell proliferation and proper endothelial cell layer formation (Yao et al., 2012). These facts, combined with previous work from David et al. (2007) that demonstrated BMP10 has the highest structural homology to BMP9 and that BMP10 was found exclusively in the myocardium, guided our decision to investigate BMP10's effect on cardiomyogenesis.

Due to the proliferative effect of BMP10 and its importance in cardiac development, we hypothesized that it would cause an increase in the amount of cardiac progenitor cells. These cells would be similar to those released by the niche found in the myocardium. To test this

hypothesis, we performed qPCR and examined the expression of Nkx2.5 in response to treating DFAT cells with BMP10 and CV2. Nkx2.5 expression was examined because it is a transcriptional activator which is involved in cardiomyogenesis that is initiated by BMP signaling via the ALK1 receptor and SMAD1/5/8 pathway (Jamali et al., 2001). Our data revealed that on day 7 after treatment, BMP10 treatment increased the expression of Nkx2.5, which represented an induction of cardiomyogenesis and an increase in cardiac progenitor cells. This is significant because it demonstrated BMP10s ability to initiate cardiomyogenesis through Nkx2.2 induction and cardiac progenitor proliferation.

We then chose to investigate CV2 because it had previously been shown by Yao et al. to inhibit BMP9 signaling. As BMP10 is structurally similar to BMP9, we hypothesized a similar inhibition would occur in cardiomyocytes. To test this hypothesis, we performed luciferase assays with varying concentrations of CV2 in order to elucidate the optimal concentration which exhibited maximal inhibition. This was assessed through the production of bioluminescence from the activation of a luciferase promoter gene. A decreased luminescence was observed when CV2 (200 ng/ml) was added to medium containing BMP10. We also had previous immunoprecipitation assays that showed CV2 binds to BMP10. Taken together, these findings illustrate CV2's ability to inhibit BMP10 signaling and thus, inhibit cardiomyogenesis. This theory was confirmed through our qPCR results which indicate that Nkx2.5 expression is markedly decreased when treated with CV2. This was in contrast to our qPCR results when DFAT cells were treated with BMP10 which produced an increased amount of Nkx2.5 expression.

Throughout each experiment, we took pictures of the cells in each treatment at specific time points (7, 14, and 21 days). We also recorded time-lapse video to prove we had functional

cardiomyocytes which was assessed by visualizing the beating cells. Lastly, we performed immunohistochemistry with antibodies against troponin I and α-MHC. Visualization of fluorescence from the labeled antibodies for these proteins indicated the presence of cardiomyocytes. All of this data combined proves that functional cardiomyocytes can be derived from DFAT cells.

An interesting finding related to CV2 was the fact that treatment with CV2 induced differentiation of cardiomyocytes (Figure 10). This was first observed in morphological analysis of CV2 treated cells. DFAT cells who underwent CV2 treatment differentiated into cardiomyocytes before any other treatment given (including BMP10). This was further confirmed through qPCR analysis which showed CV2 treatment resulted in a higher expression of troponin I (a mature cardiomyocyte marker) at day 7. This is a novel finding that demonstrated CV2's ability to induce differentiation of DFAT cells into cardiomyocytes.

There are certain limitations related to this study. First, additional proliferation and luciferase reporter gene assay experiments need to be conducted to confirm the results in this paper. One study that would be useful would be a time course experiment looking at a wider variety of time points to pinpoint the transition between proliferation and differentiation. In addition, a dose dependence experiment would be useful in determining optimal concentrations of treatments to use. In order to enhance the evidence of BMP10-CV2 protein interactions, cross-linking studies could also be performed. If these experiments are successful, then this model could be translated to other cell models such as embryonic stem cells. Lastly, in vivo experiments and 3D cultures could be preformed to expand upon these findings.

Overall, this study suggested that BMP10 and CV2 are intimately related in the onset of cardiomyogenesis. We proposed that cardiomyogenesis may occur in a two-step mechanism.

First, BMP10 acts to increase the number of cardiac progenitor cells, thus increasing the possible amount of cells differentiating into cardiomyocytes. Then, CV2 acts to induce differentiation of these cells into cardiomyocytes. This proposed mechanism is a novel finding and explanation for the induction of cardiomyogenesis. With this knowledge, more studies can be conducted to try to optimize and maximize the amount of cardiomyocytes produced which would have a profound clinical significance.

REFERENCES

Anversa, Piero, Jan Kajstura, Annarosa Leri, and Roberto Bolli. "Life and Death of Cardiac Stem Cells A Paradigm Shift in Cardiac Biology." *Circulation* 113, no. 11 (March 21, 2006): 1451–1463

Binnerts, Minke E, Xiaohui Wen, Kirsten Canté-Barrett, Jessica Bright, Huang-Tsu Chen, Vinod Asundi, Peter Sattari, et al. "Human Crossveinless-2 Is a Novel Inhibitor of Bone Morphogenetic Proteins." *Biochemical and Biophysical Research Communications* 315, no. 2 (March 5, 2004): 272–280

Chen, Hanying, Shu Shi, Lourdes Acosta, Weiming Li, Jonathan Lu, Shideng Bao, Zhuang Chen, et al. "BMP10 Is Essential for Maintaining Cardiac Growth During Murine Cardiogenesis." *Development* 131, no. 9 (May 1, 2004): 2219–2231

David, Laurent, Christine Mallet, Sabine Mazerbourg, Jean-Jacques Feige, and Sabine Bailly. "Identification of BMP9 and BMP10 as Functional Activators of the Orphan Activin Receptor-like Kinase 1 (ALK1) in Endothelial Cells." *Blood* 109, no. 5 (March 1, 2007): 1953–1961

Harada, Koichiro, Akiko Ogai, Tomosaburo Takahashi, Masafumi Kitakaze, Hiroaki Matsubara, and Hidemasa Oh. "Crossveinless-2 Controls Bone Morphogenetic Protein Signaling During Early Cardiomyocyte Differentiation in P19 Cells." *Journal of Biological Chemistry* 283, no. 39 (September 26, 2008): 26705–26713

Hierlihy, Andrée M, Patrick Seale, Corrinne G Lobe, Michael A Rudnicki, and Lynn A Megeney. "The Post-natal Heart Contains a Myocardial Stem Cell Population." *FEBS Letters* 530, no. 1–3 (October 23, 2002): 239–243

Huang, Jianhe, John Elicker, Nina Bowens, Xi Liu, Lan Cheng, Thomas P. Cappola, Xiaohong Zhu, and Michael S. Parmacek. "Myocardin Regulates BMP10 Expression and Is Required for Heart Development." *The Journal of Clinical Investigation* 122, no. 10 (October 1, 2012): 3678–3691

Jamali, Mina, Christina Karamboulas, Parker J Rogerson, and Ilona S Skerjanc. "BMP Signaling Regulates Nkx2-5 Activity During Cardiomyogenesis." *FEBS Letters* 509, no. 1 (November 30, 2001): 126–130

Jamali, Mina, Parker J. Rogerson, Sharon Wilton, and Ilona S. Skerjanc. "Nkx2–5 Activity Is Essential for Cardiomyogenesis." *Journal of Biological Chemistry* 276, no. 45 (November 9, 2001): 42252–42258

Jumabay, Medet, Rui Zhang, Yucheng Yao, Joshua I. Goldhaber, and Kristina I. Boström. "Spontaneously Beating Cardiomyocytes Derived from White Mature Adipocytes." *Cardiovascular Research* 85, no. 1 (January 1, 2010): 17–27

Kajstura, Jan, Konrad Urbanek, Shira Perl, Toru Hosoda, Hanqiao Zheng, Barbara Ogórek, João Ferreira-Martins, et al. "Cardiomyogenesis in the Adult Human Heart." *Circulation Research* 107, no. 2 (July 23, 2010): 305–315

Matsumoto, Taro, Koichiro Kano, Daisuke Kondo, Noboru Fukuda, Yuji Iribe, Nobuaki Tanaka, Yoshiyuki Matsubara, et al. "Mature Adipocyte-derived Dedifferentiated Fat Cells Exhibit Multilineage Potential." *Journal of Cellular Physiology* 215, no. 1 (2008): 210–222

Nadal-Ginard, B, and V Mahdavi. "Molecular Basis of Cardiac Performance. Plasticity of the Myocardium Generated through Protein Isoform Switches." *Journal of Clinical Investigation* 84, no. 6 (December 1989): 1693–1700.

Olson, Eric N. "A Decade of Discoveries in Cardiac Biology." *Nature Medicine* 10, no. 5 (May 2004): 467–474

Shen, Jie‐fei, Atsunori Sugawara, Joe Yamashita, Hideo Ogura, and Soh Sato. "Dedifferentiated Fat Cells: An Alternative Source of Adult Multipotent Cells from the Adipose Tissues." *International Journal of Oral Science* 3, no. 3 (July 2011): 117–124

Suzuki, Yuka, Noritaka Ohga, Yasuyuki Morishita, Kyoko Hida, Kohei Miyazono, and Tetsuro Watabe. "BMP-9 Induces Proliferation of Multiple Types of Endothelial Cells in Vitro and in Vivo." *Journal of Cell Science* 123, no. 10 (May 15, 2010): 1684–1692

Urbanek, Konrad, Marcello Rota, Stefano Cascapera, Claudia Bearzi, Angelo Nascimbene, Antonella De Angelis, Toru Hosoda, et al. "Cardiac Stem Cells Possess Growth Factor-Receptor Systems That After Activation Regenerate the Infarcted Myocardium, Improving Ventricular Function and Long-Term Survival." *Circulation Research* 97, no. 7 (September 30, 2005): 663–673

Walsh, David W., Catherine Godson, Derek P. Brazil, and Finian Martin. "Extracellular BMP-antagonist Regulation in Development and Disease: Tied up in Knots." *Trends in Cell Biology* 20, no. 5 (May 2010): 244–256

Yao, Yucheng, Medet Jumabay, Albert Ly, Melina Radparvar, Anthony H. Wang, Raushan Abdmaulen, and Kristina I. Boström. "Crossveinless 2 Regulates Bone Morphogenetic Protein 9 in Human and Mouse Vascular Endothelium." *Blood* 119, no. 21 (May 24, 2012): 5037–5047