A Pilot Proteomic Analysis: The Study of P19 Cells in Cardiac Differentiation

by

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Abstract

This study utilized a proteomics-based approach for the purpose of better understanding cardiomyocyte differentiation. In particular, the study concentrated on those proteins involved in differentiation of P19 cells into cardiomyocytes. This type of research is vital to the medical field where there is an immense need for novel therapies that involve the use of an individual’s own stem cells for generating new cardiomyocytes. The P19 cell line was used as a model for gaining more insight into those proteins possibly involved in differentiation of human adult stem cells into new cardiomyocytes. To accomplish this task, P19 cells were cultured in a 1% DMSO medium to induce differentiation and harvested at specific time points. The harvested samples were then subjected to a Modified Bradford Assay, which determined protein concentration. After determination of each samples’ protein concentration, the samples were subjected to two-dimensional gel electrophoresis (2DGE) which produced a protein profile for each sample. To visualize these protein “maps”, gels were initially stained with Coomassie Brilliant Blue stain. Upon obtaining preliminary data, 2DGE was carried out again, this time using the highly sensitive SYPRO Ruby protein gel stain to visualize proteins. The SYPRO stained gels were subjected to analysis using PDQuest software for in-depth comparison of the protein profiles on each gel. Resultant data suggested a change in the protein profiles at specific time points during differentiation. This change in proteins expressed by the P19 cells signifies a change in gene expression and thus a change in cell function. Future work includes excising those differentially protein spots and subjecting them to mass spectrometry for exact identification of those proteins possibly involved in cardiomyocyte differentiation.
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CHAPTER I:

INTRODUCTION
The Human Heart:

The human heart functions as a pump, propelling blood through numerous blood vessels supplying tissue with nutrients and taking away wastes. This circulation of blood allows for oxygen as well as nutrients to be dispersed to cells within the body while carbon dioxide (CO₂) and other wastes are carried away and then expelled. The human heart beats approximately 70 beats/min, and over 3 billion times during an average human lifespan (Severs, 2000; Wildmaier et al., 2004). Additionally, the heart pumps approximately 5 L of blood through 100,000 miles of blood vessels per minute when the body is at rest (Severs, 2000; Wildmaier et al., 2004). Since the total blood volume within the human body is also approximately 5 L, all the blood is pumped through the entire circuit once each minute (Widmaier et al., 2004). During exercise, however, the heart may pump as much as 35 L/min, which would allow the entire blood volume to be pumped around the entire circuit seven times a minute.

The human heart is a muscular “pump” enclosed within the pericardium (a fibrous sac) that is located within the chest (thorax) (Widmaier et al., 2004). The epicardium, another fibrous membrane, is closely attached to the heart and is separated from the pericardium by a very narrow space that is fluid filled. This fluid serves as a lubricant as the heart moves within the sac. Myocardium is cardiac muscle that forms the walls of the heart. The inner surface of the cardiac chambers and the inner walls of all blood vessels are lined by a thin layer of endothelial cells. The heart can be divided longitudinally into two functional halves, known as right and left halves. Each half has two chambers, the atrial chamber which is the upper chamber and the ventricular chamber, the lower chamber. The atrioventricular valve (AV) is located between the atrium and the ventricle.
on both the right and left halves. The AV permits blood flow between the atrium to ventricle but not vice versa. The right AV valve is the tricuspid valve and the left AV valve is called the bicuspid valve and is commonly called the mitral valve. When blood pressure within the atrium is greater than in the ventricle, the valve opens and blood flows into the ventricle. Once the ventricle is filled, the AV valve is subsequently forced shut. The superior and inferior vena cava collect blood from above and below the heart, respectively. Blood from the superior and inferior vena cava then flows into the right atrium through the tricuspid valve and then into the right ventricle. Blood then flows through the pulmonary valve, into the pulmonary trunk, which divides into the pulmonary arteries. One pulmonary artery supplies the left lung and the other pulmonary artery supplies the right lung. Once in the lungs, the arteries branch into capillaries that then fuse to form venules and eventually veins. It is within the capillaries that the blood becomes oxygenated. The oxygenated blood leaves the lungs by four pulmonary veins, all of which empty into the left atrium. When in the left atrium, the blood flows through the bicuspid valve, into the left ventricle, through the aortic valve, and finally into the aorta. The oxygenated blood is then dispersed to all organs and tissues other than the lungs.

During the development of an embryo, specific organs such as the eyes, limbs, and heart begin to develop, and this phenomenon is known as organogenesis (Wolpert et al., 1998). Organogenesis is a very important stage of development that allows the embryo to eventually become a fully functioning organism. During organogenesis, the heart is one of the first structures to form (Wolpert et al., 2002). It is mesodermal in origin and begins as a single tube made up of two epithelial layers. The inner layer is
known as the endocardium, and the outer layer is the myocardium, which is contractile. The tube eventually becomes divided longitudinally into the atrial and ventricular chambers. Looping and additional partitioning allow for the four-chambered heart seen in humans. According to J-Marc Schleich, by day 15 of human embryonic development, gastrulation, the process of establishing the three primary germ layers (ectoderm, endoderm, and mesoderm) occurs. During this time a crescent is formed and the main regions of the heart (atrium and ventricle) are already specified. Around day 21, a primitive linear heart tube is formed when the two arms of the crescent fuse (Schleich, 2002). At day 28, this linear heart tube then loops and regions of the heart are arranged around their ultimate positions. At day 50, valves are formed such as the AV (Lamers et al., 1995).

The myocardium is the contractile layer of the heart, and it consists of cardiac muscle cells that are responsible for its pumping ability. Cardiac muscle cells are striated which results from the arrangement of myosin (thick) and actin (thin) filaments. They are relatively short and are joined to neighboring cells by intercalated disks (Widmaier et al., 2004). Adjacent to the intercalated disks are gap junctions, which are protein channels that link together the cytosols of neighboring cells (Widmaier et al., 2004). This channel allows ions and other small molecules to flow between cells.

Myogenesis and Cellular Differentiation:

Myogenesis is the process of skeletal muscle formation (Kalthoff, 2001). Studies have found that the MyoD gene, a muscle specific protein, is capable of reprogramming the process of cell differentiation by genetically transforming cells of one type using
genes that are associated with the differentiation of cells of another type. For example, when connective tissue was transfected with cDNA encoding MyoD, the connective tissue converted to muscle cells (Davis et al., 1987). These muscle cells then underwent myogenesis and formed skeletal muscle fibers. Thus, it was found that the MyoD gene was capable of quite a phenomenon: redirecting a cell and forcing it into a new pathway of differentiation. Also, the MyoD family of regulatory factors directs the activation as well as the maintenance of skeletal muscle-specific gene transcription (Olson, 1993). The MyoD gene structure contains a basic helix-loop-helix (HLH) motif that is necessary for dimerization as well as DNA binding. Since the MyoD gene has only been discovered in skeletal muscle, it seems that cardiac muscle cells must express their own cell type-specific helix-loop-helix proteins, or utilize a separate regulatory strategy that leads to the start of cardiac muscle transcription (Olson, 1993). In the same gene family as MyoD, there are three other genes, MRF-4+, myf-5+, and myogenin+. All of these genes can induce muscle differentiation in fibroblasts as well as non-muscle cells. The presence of MyoD is significant since it would help to distinguish whether cells are cardiac muscle cells or skeletal muscle cells.

Cellular differentiation is the process by which embryonic cells start to become functionally and structurally different from each other, ending up as distinct cell types with specialized functions (Wolpert et al., 1998). Cellular differentiation deals with the appearance of cell types that have a definite identity in the adult, such as muscle cells and nerve cells. In the first stages of cellular differentiation, cell identity is determined by their developmental potential. For example, the mesoderm of somites produces muscle, cartilage, dermis, and vascular tissue and will not give rise to any other cell types. Cell
differentiation is directed by many external signals such as cell-surface proteins, secreted polypeptide cytokines, and molecules of the extracellular matrix (Wolpert et al., 1998). Cell differentiation is a gradual process that takes place over consecutive cell generations, and each generation becomes increasingly more differentiated. When muscle cells become fully differentiated, they no longer divide or proliferate.

Heart Disease:

Ischemic heart disease is a serious problem caused by inadequate circulation of blood to the heart muscle (American College of Cardiology, 2002). Ischemic heart disease is also called coronary artery disease (CAD) or coronary heart disease. Blood flow to the heart is blocked by obstructions of heart arteries by cholesterol deposits also known as plaque. Overtime, the arteries harden and narrow due to the buildup of plaque within their inner walls (Michaels and Chatterjee, 2002). This buildup of plaque is known as atherosclerosis. As plaque continues to buildup, the amount of blood flow to the heart is greatly decreased and the heart no longer receives adequate oxygen rich blood flow. CAD can cause damage to heart muscle that can result in heart failure (Jurt et al., 2002). Approximately 14 million Americans have ischemic heart disease and are therefore at a greater risk for angina, myocardial infarction, and sudden death (American College of Cardiology, 2002). The medical term for a heart attack is myocardial infarction. A myocardial infarction occurs when a blood clot abruptly develops in a heart artery and produces a blockage. This sudden clot usually occurs when the surface of cholesterol plaque breaks off (American College of Cardiology, 2002). Once this clot forms within the plaque-ridden coronary artery, most or all of the blood supply is cut off to an area of
heart muscle (National Heart Lung and Blood Institute, 2006). The cells within the heart muscle begin to die since they are no longer receiving adequate oxygen-rich blood.

As noted previously, CAD can result in heart failure. Other causes of heart failure include hypertension, idiopathic, infections, inherited, as well as many others (Jurt et al., 2002). Moreover, epidemiologic studies have found that congestive heart failure is a chief reason why those 65 years and older are hospitalized (Min et al., 2005). Studies have shown that in humans, the aging process brings about a considerable loss of cardiac myocytes, mainly in the left ventricle (Olivetti et al., 1991). Heart failure symptoms as well as heart function may be oftentimes be treated by medicine, surgery, or a combination of both (Jurt et al., 2002). However, there are cases when patients cannot be treated by medicine or surgery, leaving a heart transplant as an option for treating their heart failure symptoms and reduced heart function. It is stressed that a heart transplant is not a cure, but only a treatment with a one-year survival rate of 80% with an average length of survival of 9.1 years (Jurt et al., 2002). These statistics seem promising, but the number of patients who need a heart transplant in comparison to available hearts must also be taken into consideration. At any given time, there are approximately 4,000 patients on the national patient waiting list for a heart transplant, while only 2,300 donor hearts become available each year (The Texas Heart Institute, 2006). In addition to the fact that heart availability is low, heart transplantation is costly and heart transplant patients may experience many complications due to the immunosuppressive drugs they must take (Davani et al., 2005). Some of the complications from the immunosuppressive drugs may be so great that they have a major affect on the quality of one’s life. This is a
major problem since the purpose of a heart transplant is to improve the length and quality of one’s life.

Currently, there is no effective medicine or procedure for replacing damaged or partially functioning myocardial tissue with fully functioning cardiac tissue, therefore, there is a great need for novel therapies that can regenerate normal cardiomyocytes (Orlic et al., 2002). There have been attempts to repair myocardial scar tissue in animal models with limited but encouraging success. It has been found that the most promising results were acquired when bone marrow was transplanted and mobilized to the site of the myocardial infarction. More specifically, in a study conducted on mice with myocardial infarcts, researchers used bone marrow in hopes of promoting myocardial repair (Orlic et al., 2001). It appears that recent damage to the heart, as well as a large number of circulating stem cells are two major factors that are essential for the colonization and transdifferentiation of bone marrow cells (BMC) into specific tissues (Lagasse et al., 2000; Brazelton et al., 2000; Bjornson et al., 1999; Mezey et al., 2000). Based on that finding, the researchers utilized stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) with the expectation that a significant number of BMC would mobilize to the infarcted heart and begin cardiac repair (Orlic et al., 2001). It was found that BMC mobilization to the heart was increased by SCF and G-CSF. This increase in BMC mobilization to the heart resulted in an increase in myocardial regeneration, and therefore a dramatic increase in survival of infracted mice (decreased mortality by 68%) (Orlic et al., 2001). These results demonstrate that there is a need for more research to be done on stem cell plasticity and its potential in myocardial therapy.
Stem Cells:

Stem cells are immature tissue precursor cells that have the ability to proliferate and differentiate into specific cell types while under proper conditions (Zhang et al., 2006). Stem cells are classified into four different categories: (1) Totipotent, (2) Pluripotent, (3) Multipotent, and (4) Monopotent or oligopotent. In general, totipotent cells have the potential to differentiate into the ectodermal, endodermal, or mesodermal layers. Only zygotes and blastomeres at the early stage of cleavage are totipotent cells. As differentiation continues, zygotes form into the outer and inner layers of the cells (Zhang et al., 2006). The cells within the inner layer can then produce every cell type within the body and are referred to as pluripotent stem cells. The outer layer, on the other hand, develops into the placenta. The pluripotent stem cells continue to divide and eventually become the progenitors of special tissues and are then referred to as multipotent. At this stage, they are able to differentiate into numerous types of cells in a specific organ.

Stem cells can be classified as either embryonic stem cells (ES) or adult stem cells (Zhang et al., 2006). ES cells are pluripotent whereas adult stem cells are multipotent. Thus, it is believed that ES stem cells have greater potential in generating any cell type whereas adult stem cells have the ability to generate limited cell types. Using human ES cells for scientific/medical purposes is currently non-existent in the United States since many believe the use of ES in the medical field is unethical. Therefore, more research must be done on adult stem cells and their potential generating new cardiac myocytes.

Adult stem cells have only recently been considered promising in cardiac repair, since their potential has only been studied a little over a decade (Eisenberg and Eisenberg, 2004). For many years, the adult heart was believed to be a postmitotic organ.
It was always understood that the endothelial, smooth muscle, and fibroblast cells of the heart proliferate, but the myocardial cells were believed to be terminally differentiated and consequently no longer able to proliferate. However, recent research seems to show that new cardiac myocytes are continually being generated throughout the life of an adult (Eisenberg and Eisenberg, 2004). Also, current research has indicated that some adult cell populations are able to generate differentiated cell phenotypes other than the cell types of the source tissue (Krause 2002; Poulsom et al., 2002; Verfaillie et al., 2002). For instance, mesenchymal stem cells (MSCs) (presumptive multipotential progenitors of the bone marrow stroma) have the ability to supply the blood, lung, liver, and intestine (Pittenger et al., 1999; Jiang et al., 2002). Additionally, stem cell populations found in the brain, skin, and adipose tissue exhibit a formerly unsuspected multipotency (Bjornson et al., 1999; Clarke et al., 2000; Zuk et al., 2001; Liang and Bickenbach, 2002). Thus, it should be plausible to assume that extracardiac stem cells can generate new cardiac myocytes (Eisenberg and Eisenberg, 2004).

**Stem Cell Plasticity:**

There is still the question on whether or not adult stem cell plasticity, also referred to as transdifferentiation, actually occurs (Eisenberg and Eisenberg, 2004). Stem cell plasticity is described as the phenomenon of adult stem cells becoming “plastic “or “adaptable” in their differentiation capacities (Poulsom, 2002). This plasticity property allows stem cells already committed to generating a specialized set of cells to switch and begin to generate a new and different set of specialized cells (Poulsom, 2002). While there may be several reports that support the idea of adult stem cell plasticity, it is still
viewed by some to be an unproven occurrence that needs greater scientific proof. There are several suggested criteria that help determine if stem cell plasticity exists. One suggested criterion is that “transplanted stem cells should give rise to robust and sustained regeneration of the target tissue” (Anderson et al., 2001). Research has been done which suggests that circulating hematopoietic stems cells (HSCs) produce replacement cardiac myocytes in the adult heart (Eisenberg and Eisenberg, 2004). HSCs are self-generating multipotent cells found in the bone marrow and can generate mature blood cell types found in the blood such as basophils, monocytes, red blood cells, etc. (Alberts et al., 1998). These HSCs would only create enough new myocytes to preserve homeostasis of the myocardium since generating a “robust” amount would be unnecessary in the adult myocardium. Therefore, it seems as if stem cell plasticity is actually occurring, but the HSCs are not meeting the criterion of “robustness”.

Critics of adult stem cell differentiation argue that the occurrence of cell fusion can explain the cases of adult stem cell differentiation that have been previously described (Vassilopoulos and Russell, 2003). More specifically, while some researchers may believe transdifferentiation is occurring, others have argued that it is actually cell fusion that is taking place. One notable experiment reports of hybrid cell formation from co-cultures of tissue derived stem cells and embryonic stem (ES) cells within mice (Eisenberg and Eisenberg, 2004; Terada et al., 2002; Ying et al. 2002). However, Eisenberg and Eisenberg explain that there was extreme ambiguity within that specific published report, enough to prevent the conclusion that cell fusion does occur while transdifferentiation does not (Eisenberg and Eisenberg, 2004). A significant point was the fact that within this experiment, the tissue derived stem cells and ES cells were cultured
in extreme conditions in which the only way to survive was to fuse. Specifically, the ES cells were antibiotic-sensitive and could not survive in puromycin or G418 alone. Therefore, it was vital for the antibiotic-sensitive ES cells to fuse with the tissue-derived stem cells containing an antibiotic resistance gene.

Eventually, it is believed that the stem cells can be administered intravenously where they then travel to the site of ischemia and begin to generate new cardiomyocytes in replace of the damaged cardiomyocytes. It has been found that injected exogenous endothelial progenitor cells were hardly ever noticed in non-ischemic regions (Zhang et al., 2005). This finding may suggest that that there is some type of “homing mechanism” that guides the injected cells mainly to the ischemic areas. It is believed that increased permeability of vascular endothelium, secretion of chemokines, and appearance of adhesion molecules may have a significant part in the homing mechanism (Zhang et al., 2005). Also, another option for restoring heart function or enhancing heart muscle function is to use the patient’s stem cells to engineer myocardial tissue in vitro which can then be engrafted into the patients heart (Zimmermann et al., 2006).

When the mouse ES cell line ES-D3 was injected intramyocardially into aging rats, an improvement of myocardial function was seen (Min et al., 2005). In this study conducted by Min and others, the ES cells were first transfected with green fluorescent protein (GFP), which is a marker that identifies the engrafted cells. Adult rats (24 months) and aging rats (3 months) received intramyocardial injections of ES cells. After six weeks, there was an increase in the left ventricular myocardial perfusion in aging rats compared to those aging rats that did not receive an intramyocardial injection. It was also found that when ES cells were injected into the hearts of aging rats, there was a partial
restoration in the number of myocytes within the left ventricle compared to those aging rats without ES cell injection. This study revealed that the hearts of aging rats have considerably less myocytes than aged rats, but the number of myocytes within the aged hearts could be restored back towards normal values with the help of ES cells. Also, it was confirmed by GFP-positive tissue that the ES cells that were injected were still alive 6 weeks later. These ES cells had since differentiated into cardiac tissue, which could be determined by positive staining to cardiac-specific α-MHC (Min et al., 2005).

P19 Cell Line:

The P19 line is a pluripotent embryonic carcinoma that is derived from a *Mus musculus* (mouse) (McBurney and Rogers, 1982). The P19 line of embryonic carcinoma cells may represent a line of cells that under the control of certain chemicals/drugs are able to form specific tissues that usually comprise the fetus (McBurney et al., 1982). More specifically, these embryonic carcinoma cells can differentiate into neural and glial like cells in the presence of retinoic acid and while in the presence of dimethylsulfoxide (DMSO), the cells differentiate into cardiac and skeletal muscle-like elements (McBurney et al., 1982). Therefore, specific chemicals/drugs can be used to produce two completely different embryonic tissue types from the same embryonic carcinoma cells.

In Dr. Walker’s lab, we propose that the P19 line can be considered a pseudo-stem cell since they have the ability to express several different types of cells. It is believed that the P19 line, while undergoing differentiation into cardiomyocytes, can be used as a good representation of adult stem cells as they undergo differentiation into cardiomyocytes. While the P19 line is differentiating into cardiac myosin, the proteins
expressed during myogenesis will be intensely studied. The main objective is to understand the changes of protein expression that occur at any point in the life of a cell. More specifically, the changes of protein expression that occur while the P19 line differentiates into new cardiomyocytes and what these proteins are will be studied. This process of studying changes in protein populations expressed at any point within the lifetime of a cell is referred to as proteomics.

Some specific objectives of this project are to compare the protein profiles between the P19 cell line and the DMSO treated P19 cells (new cardiomyocytes). We will also want to determine protein profiles at various stages in the process of differentiation. We will do this by running two-dimensional gels (2-D gels), which will show us the different protein profiles that arise at specific stages of myogenesis. Next, we will take these protein profiles and compare to the protein profiles produced from C2C12 cells. C2C12 cells (Mus musculus) are skeletal muscle stem cells that are characterized by rapid differentiation, the formation of contractile myotubes, and expression of muscle-specific proteins (Yang and Goldspink, 2002). We will be analyzing these proteins to compare their protein profiles with the protein profiles from the P19 cells. Since cardiac muscle cells are similar but not identical to skeletal muscle, comparison of the protein profiles will allow us to observe the different proteins each cell line expresses. Some specific proteins that we expect to be expressed from the P19 cell line include cardiac myosins and cardiac specific tropomyosins. These two proteins will be used as tissue specific markers. Our specific aims are to use two-dimensional proteomic analysis to discover which proteins are important in the overall process of
cardiac muscle differentiation, as well as to compare the difference between cardiac specific proteins and skeletal muscle proteins.

The C2C12 line has been continuously studied over the last several years in the Walker lab. Specifically, the effects of dexamethasone (a glucocorticoid) on the C2C12 cell line were studied (Lariviere, 2002; Sobecki, 2006). Lariviere studied the affects of dexamethasone on differentiation capacity as well as its affects on the expression of cdk1 and ERK1 proteins in the C2C12 cell line. The results from Lariviere’s study concluded that unlike previous reports, dexamethasone increased rather than decreased the rate of myotube formation from C2C12 cells (Lariviere, 2002). As determined from Western blot and immunoblot analysis, Lariviere was able to conclude that dexamethasone did not affect expression of cdk1 and ERK1 in myotubes (Lariviere, 2002). Sobecki’s study allowed for a greater understanding of the role that glucocorticoids play in myogenesis. Similar to the current P19 study, Sobecki utilized a proteomic approach, and studied the changes in protein expression in response to dexamethasone. With the use of 2DGE, Sobecki was able to determine that changes in protein expression occurred as a result of the addition dexamethasone to media of C2C12 mouse myoblast cells (Sobecki, 2006). In the Walker lab in 2005, Dolphin studied “The Effects of Testosterone on Protein Expression in C2C12 Myoblasts During Differentiation” (Dolphin, 2005). During C2C12 differentiation, testosterone was added to the myogenic cultures and samples were harvested at specific time points. Dolphin then subjected the samples to 2DGE in order to study effects of testosterone on protein expression. Dolphin found that differential proteins were expressed at different points of differentiation, therefore suggesting changes in gene expression (Dolphin, 2005).
CHAPTER II:

MATERIALS AND METHODS
Experimental Approach to P19 Differentiation:

The P19 cell line was used to observe the proteins involved in differentiation of cardiac myocytes. P19 cells were grown in complete growth media until appropriate confluency. Upon proper confluency, the complete growth media was replaced with differentiation media and treated samples were collected in a time-dependent manner at time 0 h, 24 h, 48 h, and 72 h following initiation of differentiation. Triplicate samples from each time point were obtained to ensure accurate results. All samples were analyzed using two-dimensional gel electrophoresis (2DGE). PDQuest 2-D Analysis Software was then used to detect protein changes possibly involved with P19 differentiation into cardiac myocytes.

Experimental Approach to C2C12 Differentiation:

The C2C12 cell line was used to examine the proteins possibly associated with differentiation of skeletal myocytes as well as to compare skeletal muscle stem cells to cardiac muscle stem cells. C2C12 cells were grown in complete growth media until appropriate confluency. Upon suitable C2C12 cell confluency, differentiation media replaced the complete growth media and treated samples were collected in a time-dependent manner at time 0 h, 24 h, 48 h, and 72 h following initiation of differentiation. All four samples were analyzed using two-dimensional gel electrophoresis (2DGE).

P19 Culture (Mus musculus)/ C2C12 Culture (Mus musculus)

It should be noted that many of the methods utilized for this study were obtained from Dolphin (Dolphin, 2005). First, a vial of the P19 mouse teratocarcinoma cell line,
strain C3H/He, was purchased from American Type Culture Collection (ATCC). Once the vial containing frozen P19 cells was received, it was quickly thawed in a 37°C water bath by gentle agitation. After the P19 cells were completely thawed, the vial was dipped in 70% ethanol to ensure decontamination. The vial was then placed under a laminar flow hood that was previously sterilized by ultraviolet light (UV) and 70% ethanol. Under the sterile laminar flow hood, the contents of the vial were transferred to a sterile centrifuge tube that contained 9.0 mL of complete culture medium. This tube was then spun at 760 rpms for 6 minutes. The supernatant was discarded and the cell pellet was re-suspended in approximately 5 mL of filter-sterilized complete growth medium. The cell suspension was then transferred to a vented-cap culture flask (75 cm²) that contained approximately 15 mL of filter-sterilized complete growth medium. This vented-cap culture flask containing the filter-sterilized complete growth medium had just been removed from the incubator, where it was placed for 15 minutes to allow the medium to reach its normal pH of 7.0 to 7.6 (ATCC). The growth medium consisted of 90% Alpha Minimum Essential Medium with ribonucleosides and deoxyribonucleosides, 7.5% Fetal Bovine Serum (FBS) and 2.5% Fetal Calf Serum (ATCC). Before use, the growth medium was subjected to vacuum filtration (pore size 0.2 μm) under the laminar hood to ensure sterilization of the medium. The cells were incubated at 37°C in 5% CO₂. The growth medium was changed every two days using proper sterile techniques. Using an Olympus T041 microscope connected to a monitor, the cells were checked daily. Characteristics such as cell density, cell removal, and cell morphology were observed.

The P19 cells were sub-cultured following a series of steps. Sub-culturing was carried out upon 70-80% confluency. First, the cells were quickly rinsed with
approximately 5 mL of 0.25% (w/v) Trypsin-0.53 mM EDTA solution. The rinsing was done to remove any serum that contained trypsin inhibitor, thereby ensuring the full effectiveness of trypsin solution. Next, 3.0 mL of trypsin-EDTA solution was added to the flask and swirled for approximately 10 minutes, or until cells were noticeably detached from the flask. Next, 8.0 mL of complete growth medium was added to the flask and appropriate aliquots (approximately 2 mL) of the cell suspension were dispersed into 4 new culture flasks each containing approximately 16 mL of pre-incubated complete medium. The flasks were then placed in an incubator at 37°C at 5% CO2.

To harvest P19 cells, the cells were gently rinsed with 5 mL of 0.25% (w/v) Trypsin-0.53 mM EDTA solution and then eluted with 9 mL of 0.25% (w/v) Trypsin-0.53 mM EDTA solution. The flasks were then incubated for approximately 10 minutes or until the cell layer is visibly dispersed from the flask. The cell suspension was transferred to a centrifuge tube and vortexed at 2000 rpm for 10 minutes. The supernatant was then removed and the cells were washed 3 times with 10 mL of sterile 1X Trans Buffered Saline (TBS) by centrifugation at 2000 rpm for 10 minutes per wash. After the final TBS wash, the TBS was completely removed from the pelleted cells. The dry pelleted cells were then stored in -80°C freezer for later use. Additional cells were also harvested to guarantee cells for future cultures. These cells were harvested as described above. However, the pelleted cells were placed in 1 mL of 5% (v/v) dimethylsulfoxide (DMSO) instead of 2DGE sample buffer. They were frozen in the -80°C freezer for 60 minutes and then placed in a liquid nitrogen tank for long-term storage.

To induce P19 cells to differentiate into cardiac muscle-like elements, the cells must be in the cultured in the presence of 0.5% to 1.0% dimethylsulfoxide (DMSO).
Therefore, the appropriate amount of DMSO was added to the complete growth medium to create a 1.0% DMSO medium. The treated (experimental) and untreated (control) cells were then harvested at several different time points after exposure to 1% DMSO. These time points included t=0 h, t=24 h, t=48 h, and t=72 h.

Additionally, a vial of the C2C12 mouse myoblastic cell line, strain C3H, was purchased from American Type Culture Collection (ATCC). The C2C12 cells were cultured under the exact conditions stated above for the P19 cell line, however the complete growth medium differed. The complete growth medium for the C2C12 cell line consisted of 90% Dulbecco’s Modified Eagle’s Medium (DMEM), and 10% fetal bovine serum (FBS). To induce differentiation of C2C12 myoblasts, the growth medium was reduced to a 1% FBS/DMEM solution.

In order to accurately analyze the different proteins that the P19 cell line expresses during differentiation into cardiac muscle-like cells, triplicate samples were collected. Using triplicate samples while carrying out this experiment allows us to pinpoint numerous proteins of interest that are expressed at different points of differentiation. It is important that each protein of interest be present in each of the three triplicates in order to erase any doubt of ambiguous spots and to demonstrate consistency, thus proving accuracy.

**Protein Preparation and Quantitative Protein Analysis:**

The previously frozen pelleted cells were resuspended in 1mL of two-dimensional gel electrophoresis (2 DGE) sample buffer and sonicated 3X on ice with the Cell Disrupter for 15 seconds per sonication with a 60 second delay between sonications.
Lysed cells were microcentrifuged at 2000 rpm for five minutes and the supernatant was recovered.

A modified Bradford Assay was then used to quantify protein in solution. The modified Bradford Assay is a quick technique that determines the total protein concentration (μg) of a sample by utilizing Coomassie brilliant blue dye (0.4% Coomassie, 50% methanol, 10% acetic acid) that binds to proteins, mainly at arginine residues, in a sample (Bradford, 1976). Protein concentrations of our sample were determined by comparing the absorbance of the protein-dye binding complex at 595 nanometers (nm) to a bovine serum albumin (BSA) standard (Bradford, 1976). A spectrophotometer (Hewlett-Packard 8453 UV-Visible System) was used to detect the shift in absorbance of the protein-dye binding complex. The absorbances of the BSA and unknown samples were measured using UV-Visible light at 595 nm. Using the X-Y scatter plot in Microsoft’s Excel program, a standard concentration curve was created by plotting the known protein per unit volume (μg/μL) of the BSA standard on the x-axis versus the change in absorbency at 595nm on the y-axis. The resulting R² value of the standard concentration curve must be 0.95 or higher. The Excel program also produces an equation that fits the curve. This equation, along with our sample’s change in absorbance at 595nm obtained from the spectrophotometer is used to determine our sample’s unknown concentration.

As mentioned previously, a standard BSA solution was used to carry out the Modified Bradford Assay. Eight standards containing 10, 15, 20, 25, 30, 35, and 40 micrograms (μg) of BSA solution were prepared in individual test tubes. Also, in separate test tubes, 5 μL and 10 μL from each sample was pippetted into the appropriate tubes. In
all the test tubes, 80 μL of Deionized (DI) H2O, 10 μL 0.1 M Hydrochloric acid (HCl),
10 μL 2-DE buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, 25 mM spermine base, 50
mM dithiothreitol) was added. A blank containing all of the above except for protein was
also prepared. The standards, samples, and blank sat for 5 minutes and then 4 mL of
Bradford Dye was added to each tube. The tubes were mixed and then incubated for
another 5 minutes. The contents from the blank were transferred to a plastic cuvette, and
the cuvette was placed in the holder of the spectrophotometer. The machine was then
“blanked” by clicking on the “0 ABS 100% T” button on the spectrophotometer. The
“blank” solution was then discarded, the contents of the standard tube with 10 μg of BSA
was transferred to the cuvette, the cuvette was placed back in the spectrophotometer, and
its absorbance was then displayed. This process was repeated for all the standards and
then the samples.

**Two-dimensional gel electrophoresis (2DGE):**

Two-dimensional gel electrophoresis separates the proteins within in a sample by
differences in charge as well as mass. In the first dimension, isoelectric focusing (IEF)
separates proteins solely on the basis of their net charge (Twyman, 2004). Electrophoresis
is carried out in a pH gradient, permitting proteins to migrate to their isoelectric point.
The isoelectric pH of a protein, also known as pI, is the pH at which the number of
negative charges is equal to the number of positive charges, thus making the protein
electrically neutral. When the proteins reach their isoelectric point (i.e., protein is neutral)
the protein stops migrating. In the second dimension, sodium dodecyl sulfate
polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins based on
mass (Twyman, 2004). Using this technique, sodium dodecyl sulfate (SDS), an anionic detergent, binds to proteins in a 1:1 ratio leaving all proteins with the same charge to mass ratio. Basically, SDS imparts a negative charge to all proteins, and when current is applied they will be forced to run to the opposite, positively charged end. Additionally, the proteins are denatured, giving all proteins a random coil configuration. Therefore, proteins must sieve through a porous acrylamide via diffusional movement. Using a constant voltage and an immobilized pH gradient (IPG) strips (obtained from the first dimension), the proteins will diffuse through the gel, with the larger proteins moving more slowly than the smaller proteins. Two-dimensional gel electrophoresis is indisputably one of the most powerful methods for studying the proteome of cells (Wittmann-Liebold et al., 2006). This is due to the fact that 2DGE has the ability to quickly separate the proteins within a proteome. More specifically, the 2DGE technique can simultaneously separate thousands of proteins on one 2D gel. For each protein resolved on the gel, its isoelectric point, molecular weight (MW), and the relative quantity can be calculated (Beranova-Giorgianni, 2003).

Passive Rehydration and Isoelectric Focusing-

1st Dimension:

The appropriate concentration of sample load for the passive rehydration process was established based on 100 µg of protein for 7 cm immobilized pH gradient strips (IPG) and 250 µg of protein for 17 cm immobilized pH gradient strips (IPG). Keeping the above statement in mind, the appropriate amount of sample to be loaded on the IPG strip for passive rehydration was determined using the results from the Modified Bradford
assay. For the 7cm IPG strips, the 3 -10 pH gradient range was used while the 4-7 pH gradient range was used on the 17 cm strips. To begin passive rehydration, the samples were taken out of the -80°C freezer and thawed. The total volume of each sample to be loaded into the IPG tray was 125 μL for the 7 cm IPG strips and 300 μL for the 17 cm IPG strips. This total volume consisted of the protein sample (in μL for a concentration of 100 μg for 7 cm/ 250 μg for 17 cm strips) plus the amount of Modified sample buffer needed to bring the total volume up to 125 μL for 7 cm/ 300 μL for 17 cm, and 2 μL of Bromophenol Blue.

For both the 7 cm and 17 cm IPG strips, the proper volume of sample was loaded into the respective lane of the IPG tray. Using tweezers, the ReadyStrip™ IPG strip (Bio-Rad) was placed gel-side down on top of the sample. It was made sure that the sample was in complete contact with the IPG strip and that all bubbles were removed. After 10 minutes, a layer of mineral oil was placed on top of the IPG strip to prevent dehydration. A cover was placed over the tray and the strips were left overnight. The following day, electrode wicks were placed over the electrodes in the Bio-Rad PROTEAN® IEF focusing tray, and each electrode wick was moistened with 8 ml of water. The IPG strip was placed gel-side down in the Bio-Rad PROTEAN® IEF focusing tray, making sure to contact the electrodes. It was made sure that the plus end of the strip was at the anode. The strips were then overlaid with mineral oil to prevent dehydration, placed into the PROTEAN® IEF cell. For 7 cm strips, the IEF cell was programmed for linear focusing conditions to 40,000 volt-hours (V-hr) with a maximum current of 50 μA per strip and a temperature of 20°C. For 17 cm strips, focusing conditions were set linearly at 60,000 V-hr, with maximum current of 50 μA per strip, and temperature of 20°C. The IPG strips
were held at 500 V upon completion of isoelectric focusing. The strips were then frozen to later be subjected to SDS-PAGE for further analysis.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)- 2\textsuperscript{nd} Dimension:**

After the 1\textsuperscript{st} dimension (IEF) was complete, the IPG strip was carefully drained with KIM wipes to remove excess mineral oil. The IPG strip was then placed in a loading tray gel side up and 3.0-6.0 mL of Equilibration buffer I (6 M Urea, 0.375 M, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT) was loaded on top of each the IPG strips. The tray containing the strips and Equilibration buffer I was then shaken on an orbital shaker for 10 minutes on speed two. After 10 minutes, Equilibration buffer I was drained, and 3.0-6.0 mL of Equilibration buffer II (6 M Urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, and 2.5% (w/v) iodoacetamide) was loaded on the strips. The tray was again placed on the orbital shaker for another 10 minutes. The strips were removed and blotted onto a KIM wipe to remove excess buffer. It was then dipped into 4 mL of 1x Tris-glycine-SDS (TGS) running buffer and added to a loading tray. Next, an agarose overlay (5% agarose) was heated in a microwave until it was fully liquefied. The warm liquid agarose was then pipetted over room-temperature 12 % polyacrylamide second dimension gels while concurrently inserting the gel strip onto the glass gel plate. This process had to be done very quickly to endure that the agarose not solidify before the strip was inserted. It was made sure that bubbles were removed and the agarose was given time to solidify. The 12% polyacrylamide gels were made 24 hours before the 2\textsuperscript{nd} dimension procedures (Appendix 1).
A 1X TGS buffer was added to both the inside and outside of the electrophoresis chambers. For 7 cm strips, Bio-Rad’s Mini-PROTEAN® 3 Electrophoresis chamber was used while the PROTEAN II XL Electrophoresis chamber was used for 17 cm strips. The gels were run at constant current of 16 mA per gel for 7 cm gels and 10 mA for 17 cm gels at 200 volts. When the dye front created by the Bromophenol Blue (was added to the agarose overlay) reached the bottom of the gels, electrophoresis was finished. The run time for 7 cm gels was approximately an hour and a half and the run time for 17 cm gels was 8 hours. After electrophoresis, the gels were gently detached from the glass gel plates and specific corners were scored for identification as well as orientation purposes. The gels were then subjected to different staining processes.

**Coomassie and SYPRO® Staining:**

In order to create protein maps, gels were either submerged in Coomassie Brilliant Blue (5% Coomassie brilliant blue G-250, 45% methanol, 10% acetic acid) or SYPRO Ruby protein gel stain (1X pre-mixed solution). When using SYPRO Ruby protein gel stain, the protein maps could be visualized using an ultraviolet light. When staining with Coomassie, the gels could only be visualized using epi-white light.

Gels stained with Coomassie were completely submerged in a plastic container and left overnight on an orbital shaker. The following day, the gels were destained using High Destaining (40% methanol, 6% acetic acid) for approximately 60 minutes. After 60 minutes, the high destain was then removed and new destain was added. The gels remained in the fresh destain until the background of the gel was clear. The gels were
then submerged in double distilled water (ddH2O) overnight and imaged the next morning.

Before the gels were stained with SYPRO® Ruby protein gel stain, they were first submerged in SYPRO® Fixing Solution/High Destain (40% methanol, 10% acetic acid) for 60 minutes. Next, the gels were submerged in SYPRO® overnight. Care was taken to fully submerge the 17 cm gels completely in the SYPRO staining. The next day, the gels were washed with deionized/distilled H2O on an orbital shaker for 30 minutes before imaging.

**PDQuest™ Imaging and Analysis:**

In order to image and analyze all the gels within this research project, the Bio-Rad Gel Doc system as well as PDQuest™ 2-D Analysis Software was used. The Bio-Rad Gel Doc system includes a CCD camera that captures images in real time. This feature allows for more accurate positioning of the gel as well as more precise focusing of the image. Additionally, the Bio-Rad Gel Doc system has the ability to image chemiluminescent, fluorescent, colorimetric samples. All gels were placed onto the Gel Doc, positioned into desired place, scanned, and then focused. For those gels stained with SYPRO®, which were all the 17 cm gels, the UV box and Trans-UV light were used. For gels stained with Coomassie blue, which were all of the 7 cm gels, the white tray and Epi-White light were utilized. To find the best-fit image for each gel, Auto Exposure of the gel was necessary. All gels that were scanned into the computer using the Gel Doc system could then be analyzed using the PDQuest™ 2-D Analysis Software. Once the
raw scanned images were seen in PDQuest program, they were immediately saved all under one file.

The PDQuest™ 2-D Analysis Software allowed for in-depth analysis of the protein patterns amongst gels. The raw images scanned into the computer by the Gel Doc system were then cropped to contain the main portion the gel containing the protein spots while excluding the dye fronts. Once the very first gel was cropped, its crop settings were saved and then applied to all the other gels of the same condition. This is a necessary step in order to create a Primary MatchSet for all gels of the same condition. Next, the Automated Detection and Matching button was pressed and the PDQuest program auto-selected the master image for the Primary Matchset of each condition. Next, manual guidance of the master gel was done. During this step a small, faint, and large spot was picked from the master gel. The Process All Gels button was pressed, the matchset was named, and the matchset was then brought up on the screen. Protein spots on the gel were identified and quantified using The Spot Detection Wizard. The Edit Spot tool allowed for deleting streaks that the PDQuest software recognized as spots as well as adding spots that the PDQuest software did not recognize. Also, the PDQuest software did not identify spots that were clearly matched in the other gels. In this case, the proteins that were not matched initially had to be manually matched. Using the Primary Matchsets from each condition (t=0 h, t=24 h, t=48 h, t=72 h) multiple Higher Level MatchSets were created. This allowed for more thorough comparison of the different time courses. For example, t=0 h could be compared to t=72 h to investigate the difference in proteins as the P19 line differentiated for 72 hours.
CHAPTER III:

RESULTS
Sample Preparation:

As mentioned previously, both P19 cells as well as C2C12 were cultured in their appropriate complete media until 70-80% confluency. Upon 70-80% confluency, the P19 and C2C12 cells were cultured in their respective differentiation media and then harvested at specific time points: t=0 h, t=24 h, t=48 h, and t=72 h. Since the C2C12 cell line was simply used for protein expression comparison purposes between a skeletal muscle cell line (C2C12) and a cardiac muscle cell line (P19), only one trial was carried out for the C2C12 cell line. Under the microscope, as well as to the unaided eye, all four C2C12 cell cultures appeared to proliferate and differentiate without any complications. By visualizing the C2C12 cell line under the microscope, it was possible to observe characteristics such as rapid cell proliferation.

Unlike the C2C12 cell cultures, some problems arose during culturing of the P19 cell line. Several cultures of the P19 cell line were needed to accommodate the three trials and four time points for each trial. Additionally, extra cultures were created for future use. Some of these cultures, approximately 5 flasks, experienced “floaters.” In these particular P19 culture flasks, white particles were observed floating in the medium. Upon visualization with a microscope, cell detachment from the bottom of the flask was observed. It was then assumed that the white particles floating in the medium were the dead cells that were no longer adherent to the flask. Some flasks experienced greater cell detachment and subsequently more white particles floating in the medium than others. In particular, the cultures from Trial 1 experienced this problem, especially t=72 h. When it came time to harvest this particular culture (Trial 1, t=72 h), very few cells were still attached to the flask. Since only a small amount of viable cells appeared to be present in
the culture, it was questionable whether or not enough protein was present to produce results using 2DGE. The cultures for Trial 2 and Trial 3 appeared to be free of any problems. These cultures appeared to maintain viable cells that proliferated as expected.

After all protein samples were harvested, they were then subjected to the Modified Bradford Assay. Using a BSA standard with a known amount of concentration, a standard curve was created. Table 3.1 demonstrates the absorbance (at 595 nm) registered by spectrophotometer in response to increasing concentrations of BSA (μg). The absorbance at 595 nm for the increasing amounts of BSA standards ranged from 0.112-0.509. Using the Excel program, these values were then used to assemble a linear Standard BSA Curve necessary to determine the protein concentrations of the C2C12 and P19 samples. From the linear Standard BSA Curve, an equation that best fits the curve along with its R^2 value was produced. A linear \(y = mx + b\) equation was produced with \(y\) = the absorbance and \(x\) = the unknown amount of protein (in μg). The R^2 value for the Modified Bradford Assay was found to be approximately 0.997. As mentioned previously, the resulting R^2 value must be 0.95 or higher in order to ensure the treadline is consistent. The closer the R^2 value is to 1, the more reliable the treadline is. Consequently, the R^2 value of 0.997 was satisfactory and therefore protein concentrations of the samples could be deduced.

To determine protein concentrations for the P19 and C2C12 samples, the equation from the linear Standard BSA Curve was utilized. The samples’ absorbance at 595nm was plugged into \(y\), and samples’ protein concentrations were then able to be determined. The protein concentrations determined from the Modified Bradford Assay for the C2C12 cells are displayed in Table 3.2. Table 3.2 exhibits the protein concentrations determined
by the Bradford assay for the C2C12 cells cultured in differentiation medium at t=0 h, t=24 h, t=48 h, and t=72 h. The protein concentrations for the C2C12 samples ranged from 1.19 μg/μL to 2.04 μg/μL. All four of these protein concentrations are adequate for 2DGE. This table also displays the amount of C2C12 sample load volume (in μL) needed for 100 μg of protein. Table 3.3 displays the protein concentrations determined by the Bradford Assay for the three trials of P19 cells cultured in differentiation medium at t=0 h, t=24 h, t=48 h, and t=72 h. The protein concentrations for the P19 samples ranged from 0.494 μg/μL to 6.66 μg/μL. The P19 sample with the protein concentration of 0.494 μg/μL was obtained from Trial 1, t=72 h. Due to the lack of viable cells, this sample had an extremely low protein concentration. Even though this sample had an extremely low protein concentration, the sample was still subjected to 2DGE with the expectation of attaining results. All eleven other P19 samples had sufficient, or in some cases, extremely sufficient protein concentrations.

Bradford assay results were used to determine sample load volumes for 2DGE. The sample load volumes displayed in Table 3.2 and Table 3.3 provide the information required for 2DGE. More specifically, Tables 3.2 and 3.3 exhibit the amount of each sample needed for 100 μg and 300 μg of protein, which is the amount of protein needed for a 7 cm and 17 cm IPG strip, respectively. For 7 cm IPG strips, a total volume of 125 μL should be loaded on to the IPG strips. For the 17 cm IPG strips a total volume of 300 μL is required. The volumes (in μL) of Modified Sample Buffer and C2C12 sample load needed to obtain a total volume of 125 μL for 7 cm strips are depicted in Table 3.4. Table 3.5 presents the volumes of Modified Sample Buffer and P19 sample load needed to obtain a total volume of 125 μL for 7 cm IPG strips. In Table 3.5, the P19 sample load
volume for Trial 1 (t=72 h) is 125 μL with 0 μL of Modified Sample Buffer. The Modified Sample Buffer was not added to this particular P19 sample since the protein concentration of this sample was extremely low. Finally, Table 3.6 illustrates the volumes of Modified Sample Buffer and P19 sample load needed to obtain a total volume of 300 μL for 17 cm IPG strips. Similar to Table 3.5, Table 3.6 displays the P19 sample load volume for Trial 1 (t=72 h) as 300 μL with 0 μL of Modified Sample Buffer.

For more in-depth results of P19 differentiation, 17 cm IPG strips were utilized. The 17 cm strips were employed for improved visualization of proteins. Moreover, PDQuest Analysis can be fully utilized with the use of 17 cm gels. The 17 cm strips that were used had a pH range of 4-7. In comparison, the 7 cm IPG strips that were initially used had a pH range of 3-10. Hence, the pH range for the 17 cm strips was much more narrow compared to the 3-10 pH range on the 7 cm strips. This narrowing of the pH range allowed for enhanced visualization of proteins.

Skeletal Muscle Stem Cell (C2C12) Comparison with Cardiac Muscle Stem Cell (P19)

During the 1st dimension, IPG strips were passively rehydrated at 40,000V-hours using 100 μg of C2C12 or P19 protein per 7 cm strip, pH 3-10. Next, the 2nd dimension involved gel electrophoresis, carried out on a 12% polyacrylamide gels. Electrophoresis was performed in Bio-Rad’s Mini-PROTEAN® 3 Electrophoresis chamber. Each Bio-Rad Mini-PROTEAN® 3 Electrophoresis chamber held two gels, so two Bio-Rad Mini-PROTEAN® 3’s were run concurrently at 16 mA per gel, for a total of 64 mA. All gels were stained with Coomassie Brilliant Blue and then visualized with Bio-Rad’s ChemiDoc. The 7 cm C2C12 and P19 gels were manually compared since Matchsets for
the 7 cm gels could not be easily produced using the PDQuest software. All gels in Figures 3.2-3.5 were arranged from left to right with an increasing pH range as well as top to bottom by decreasing molecular weight (MW).

Protein profiles of differentiated C2C12 cells appear to show a typical pattern. Figure 3.2 displays the four gels that were produced from the C212 cell line. Gel 1 in Figure 3.2 depicts the C2C12 proteins at t=0 h, i.e., the proteins in the C2C12 line at 70-80% confluency, harvested while in the presence of complete growth medium. Gel 2 in Figure 3.2 presents the C2C12 proteins at t=24 h, i.e., the proteins in the C2C12 cell line harvested after 24 hours in 1% FBS medium (differentiation medium). Gel 3 in Figure 3.2 illustrates the protein profile of the C2C12 cells at t=48 h, i.e., the proteins in the C2C12 cell line harvested after 48 hours in 1% FBS medium. Lastly, Gel 4 in Figure 3.2 shows the proteins in the C2C12 line at t=72 h, i.e., the proteins in the C2C12 cell line harvested after 72 hours in 1% FBS medium.

The protein profiles of differentiated P19 cells from the three trials are displayed in Figures 3.3, 3.4, and 3.5. Gel 1 in Figure 3.3 depicts the P19 proteins at t=0 h for trial 1, i.e., the proteins in the P19 line at 70-80% confluency, harvested while in the presence of complete growth medium. Gel 1 in Figures 3.4 and 3.5 depicts P19 at t=0 h for trials 2 and 3, respectively. Gel 2 in Figure 3.3 presents the P19 proteins at t=24 h for trial 1, i.e., the proteins in the P19 cell line harvested after 24 hours in 1% DMSO (differentiation medium). Gel 2 in Figures 3.4 and 3.5 presents P19 at t=24 h for trials 2 and 3, respectively. Gel 3 in Figure 3.3 illustrates the protein profile of the P19 cells at t=48 h for trial 1, i.e., the proteins in the P19 cell line harvested after 48 hours in 1% DMSO medium. Gel 3 in Figures 3.4 and 3.5 presents P19 at t=48 h for trials 2 and 3,
respectively. Finally, Gel 4 in Figure 3.3 shows the proteins in the P19 line at t=72 h, i.e., the proteins in the P19 cell line harvested after 72 hours in 1% DMSO medium. Gel 4 in Figures 3.4 and 3.5 presents P19 at t=72 h for trials 2 and 3, respectively.

When a manual comparison between the undifferentiated C2C12 cells (t=0 h, control) and undifferentiated P19 cells (t=0 h, control) was carried out, there appeared to be several protein spots between the two gels that were similar as well as several protein spots that were unique to each cell line. Figure 3.6 shows the most obvious similar protein is the one large protein located around the middle of both the C2C12 gel and the P19 gel. In order for proper identification of the two protein spots that appear to be similar, the spots would have to be excised and then subjected to mass spectrometry. Due to the streaking in the P19 gel, manual comparison was difficult, especially in certain areas of the gel. Figure 3.7, displays the comparison between differentiated C2C12 at t=72 h and differentiated P19 cells at t=72 h. Much alike Figure 3.6, both of the differentiated cells lines in Figure 3.7 appear to have a large protein in the middle of gel. Both figures display a large “streak” on the right side of both the C2C12 and P19 gels. This streak appears to be a very large molecular protein common to both cell lines that was too large to diffuse through the gel. The P19 gel in this figure also has streaking which made it difficult to accurately manually compare the two gels. The two differentiated gels displayed in Figure 3.7 seem to have more protein dissimilarities than the two undifferentiated gels in Figure 3.6.

These protein similarities as well as protein dissimilarities between the C2C12 line and P19 cell line are expected. Protein similarities are expected since the two cell lines once differentiated, are both muscle cells. Conversely, protein dissimilarities
between the two cell lines are also expected since the C2C12 line is derived from a *Mus musculus* (mouse) myoblast while the P19 line is derived from an embryonal carcinoma induced in a *Mus musculus* (mouse). Another reason for protein dissimilarities includes the fact that the C2C12 cell line differentiates into skeletal muscle cells while the P19 cell line differentiates into cardiac muscle cells. For instance, the MyoD protein, a skeletal muscle-specific protein, should appear in the differentiated C2C12 gel (e.g., C2C12, t=72 h) but should not be present in the differentiated P19 gel (e.g., P19, t=72 h).

**Time Course of P19 Differentiation**

Manual comparison of the different time points of P19 differentiation from each trial, e.g., comparison between t=0 h and t=48 h from Trial 2, proved to be difficult and not very accurate due to streaking and the numerous amount of protein spots on each gel. Once preliminary data was obtained with the use of the 7 cm strips, the P19 samples were then subjected to 2DGE using 17 cm strips. During this 2DGE, 17 cm strips, pH 4-7, were used and the gels were stained with SYPRO® Ruby protein gel stain. This change in procedure permitted greater resolution of proteins. SYPRO® Ruby protein gel stain was used to detect proteins that could not be visualized using Coomassie Blue Staining. In the 1st dimension 12 IPG were passively re-hydrated at 60,000V-hours using 300 μg of P19 protein per 17 cm strip, pH 4-7. For the 2nd dimension, gel electrophoresis was carried out on a 12% polyacrylamide gels. Electrophoresis was performed in Bio-Rad’s PROTEAN II XL with a running power of 200V. All gels were stained with SYPRO® Ruby protein gel stain, scanned with Bio-Rad’s ChemiDoc, and then analyzed with PDQuest. The
results obtained from the PDQuest analyses are located in the PDQuest Analysis Results section.
Table 3.1: **Modified Bradford Assay Concentrations and Absorbances**

Known concentrations of BSA in micrograms with their accompanying absorbances at 595nm of UV-Visible light. These values were then used for the construction of a linear Standard BSA curve (Figure 3.1) required for resolving the unknown concentrations of the samples.
### Modified Bradford Assay Concentrations and Absorbances

<table>
<thead>
<tr>
<th>Standard BSA Concentrations (μg)</th>
<th>Absorbance (at 595 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.112</td>
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<tr>
<td>15</td>
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</table>
Figure 3.1: *Standard BSA Curve*

The known concentration of the BSA (in μg) is on the x-axis while the absorbance of the BSA standards at 595 nm as determined by the spectrophotometer is on the y-axis. This creates a linear Standard BSA curve necessary for uncovering the samples’ protein concentration. A known protein concentration of each sample is necessary for proper protein sample load onto ReadyStrip™ IPG strips.
Figure 3.1

Bradford Assay

\[ y = 0.0129x - 0.0059 \]

\[ R^2 = 0.997 \]
Table 3.2: **C2C12 Protein Concentrations**

Figure 3.2 displays the absorbances at 595 nm for the C2C12 cell line at different time points of differentiation. The figure also displays the unknown protein concentrations in μg/μl for each of the C2C12 samples as determined by the Standard BSA Curve (Figure 3.1). Additionally, this figure demonstrates the Sample Load values for 7 cm IPG strips based on the practice of loading 100 μg of protein per 7 cm IPG strip.
Table 3.2

**C2C12 Protein Concentrations**

<table>
<thead>
<tr>
<th>C2C12 Sample (Time (h))</th>
<th>Absorbance at 595 nm</th>
<th>Concentration (μg/μl)</th>
<th>Sample Load 7cm IPG Strip (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T=0</td>
<td>0.202</td>
<td>1.56</td>
<td>64.1</td>
</tr>
<tr>
<td>T=24</td>
<td>0.149</td>
<td>1.19</td>
<td>84.0</td>
</tr>
<tr>
<td>T=48</td>
<td>0.212</td>
<td>2.04</td>
<td>49.0</td>
</tr>
<tr>
<td>T=72</td>
<td>0.168</td>
<td>1.41</td>
<td>70.9</td>
</tr>
</tbody>
</table>
Table 3.3: P19 Protein Concentrations

Table 3.3 represents the absorbances at 595 nm for the P19 cell line for 3 separate trials at different time points of differentiation. The table also displays the unknown protein concentrations in μg/μl for each of the P19 samples as determined by the Standard BSA Curve (Figure 3.1). Additionally, this figure demonstrates the Sample Load values for 7 cm IPG strips based on the practice of loading 100 μg of protein per 7 cm IPG strip. Figure 3.3 also exhibits Sample Load values for 17 cm strips based on loading 300 μg of protein per 17 cm IPG strip.
Table 3.3

**P19 Protein Concentrations**

<table>
<thead>
<tr>
<th>P19 Sample (Time (h) /Trial)</th>
<th>Absorbance at 595 nm</th>
<th>Concentration (μg/μl)</th>
<th>Sample Load 7cm IPG Strip (μl)</th>
<th>Sample Load 17cm IPG Strip (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T=0 Trial 1</td>
<td>0.143</td>
<td>1.18</td>
<td>84.7</td>
<td>211.8</td>
</tr>
<tr>
<td>T=24 Trial 1</td>
<td>0.289</td>
<td>2.57</td>
<td>38.9</td>
<td>97.2</td>
</tr>
<tr>
<td>T=48 Trial 1</td>
<td>0.307</td>
<td>2.13</td>
<td>46.9</td>
<td>117.3</td>
</tr>
<tr>
<td>T=72 Trial 1</td>
<td>0.040</td>
<td>.494</td>
<td>202.4</td>
<td>300</td>
</tr>
<tr>
<td>T=0 Trial 2</td>
<td>0.395</td>
<td>3.80</td>
<td>26.3</td>
<td>65.7</td>
</tr>
<tr>
<td>T=24 Trial 2</td>
<td>0.360</td>
<td>3.95</td>
<td>25.3</td>
<td>63.2</td>
</tr>
<tr>
<td>T=48 Trial 2</td>
<td>0.251</td>
<td>2.60</td>
<td>38.5</td>
<td>96.1</td>
</tr>
<tr>
<td>T=72 Trial 2</td>
<td>0.303</td>
<td>3.30</td>
<td>30.3</td>
<td>75.7</td>
</tr>
<tr>
<td>T=0 Trial 3</td>
<td>0.319</td>
<td>3.41</td>
<td>29.3</td>
<td>73.5</td>
</tr>
<tr>
<td>T=24 Trial 3</td>
<td>0.418</td>
<td>4.40</td>
<td>22.7</td>
<td>56.8</td>
</tr>
<tr>
<td>T=48 Trial 3</td>
<td>0.617</td>
<td>6.66</td>
<td>15.0</td>
<td>37.3</td>
</tr>
<tr>
<td>T=72 Trial 3</td>
<td>0.491</td>
<td>6.05</td>
<td>16.4</td>
<td>50.0</td>
</tr>
</tbody>
</table>
Table 3.4: C2C12 Total Load Volumes for 7 cm IPG Strips

Figure 3.4 displays the Total Load Volumes for 7 cm IPG Strips. This table includes the amount of Modified Sample Buffer and C2C12 sample load, both in micrograms, needed for a total load volume of 125 μL per 7 cm IPG strip.
<table>
<thead>
<tr>
<th>C2C12 Sample (Time (h))</th>
<th>C2C12 Sample Load (μl)</th>
<th>Modified Sample Buffer (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T=0</td>
<td>64</td>
<td>61</td>
</tr>
<tr>
<td>T=24</td>
<td>83.5</td>
<td>41.5</td>
</tr>
<tr>
<td>T=48</td>
<td>49.0</td>
<td>76.0</td>
</tr>
<tr>
<td>T=72</td>
<td>71.1</td>
<td>53.9</td>
</tr>
</tbody>
</table>
Table 3.5: P19 Total Load Volumes for 7 cm IPG Strips

This figure presents the Total Load Volumes for 7 cm IPG Strips. This table includes the amount of Modified Sample Buffer and P19 sample load, both in micrograms, needed for a total load volume of 125 μL per 7 cm IPG strip.
### Table 3.5

#### P19 Total Load Volumes for 7 cm IPG Strips

<table>
<thead>
<tr>
<th>P19 Sample (Time (h)/Trial)</th>
<th>P19 Sample Load (µl)</th>
<th>Modified Sample Buffer (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T=0/Trial 1</td>
<td>84.7</td>
<td>40.3</td>
</tr>
<tr>
<td>T=24/Trial 1</td>
<td>38.9</td>
<td>86.1</td>
</tr>
<tr>
<td>T=48/Trial 1</td>
<td>46.9</td>
<td>78.1</td>
</tr>
<tr>
<td>T=72/Trial 1</td>
<td>202.4</td>
<td>0.0</td>
</tr>
<tr>
<td>T=0/Trial 2</td>
<td>26.3</td>
<td>98.7</td>
</tr>
<tr>
<td>T=24/Trial 2</td>
<td>25.3</td>
<td>99.7</td>
</tr>
<tr>
<td>T=48/Trial 2</td>
<td>38.5</td>
<td>86.5</td>
</tr>
<tr>
<td>T=72/Trial 2</td>
<td>30.3</td>
<td>94.7</td>
</tr>
<tr>
<td>T=0/Trial 3</td>
<td>29.4</td>
<td>95.6</td>
</tr>
<tr>
<td>T=24/Trial 3</td>
<td>22.7</td>
<td>102.3</td>
</tr>
<tr>
<td>T=48/Trial 3</td>
<td>15</td>
<td>110.0</td>
</tr>
<tr>
<td>T=72/Trial 3</td>
<td>16.4</td>
<td>108.6</td>
</tr>
</tbody>
</table>
Table 3.6: P19 Total Load Volumes for 17 cm IPG Strips

This figure shows the Total Load Volumes for 17 cm IPG Strips. This table includes the amount of Modified Sample Buffer and P19 sample load, both in micrograms, needed for a total load volume of 300 $\mu$L per 7 cm IPG strip.
Table 3.6

**P19 Total Load Volumes for 17 cm IPG Strips**

<table>
<thead>
<tr>
<th>P19 Sample (Time (h)/Trial)</th>
<th>P19 Sample Load (µl)</th>
<th>Modified Sample Buffer (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T=0/Trial 1</td>
<td>211.8</td>
<td>88.2</td>
</tr>
<tr>
<td>T=24/Trial 1</td>
<td>97.2</td>
<td>202.8</td>
</tr>
<tr>
<td>T=48/Trial 1</td>
<td>117.3</td>
<td>182.7</td>
</tr>
<tr>
<td>T=72/Trial 1</td>
<td>300</td>
<td>0.0</td>
</tr>
<tr>
<td>T=0/Trial 2</td>
<td>65.7</td>
<td>234.3</td>
</tr>
<tr>
<td>T=24/Trial 2</td>
<td>63.2</td>
<td>236.8</td>
</tr>
<tr>
<td>T=48/Trial 2</td>
<td>96.1</td>
<td>203.9</td>
</tr>
<tr>
<td>T=72/Trial 2</td>
<td>75.7</td>
<td>224.3</td>
</tr>
<tr>
<td>T=0/Trial 3</td>
<td>73.5</td>
<td>226.5</td>
</tr>
<tr>
<td>T=24/Trial 3</td>
<td>56.8</td>
<td>243.2</td>
</tr>
<tr>
<td>T=48/Trial 3</td>
<td>37.3</td>
<td>262.7</td>
</tr>
<tr>
<td>T=72/Trial 3</td>
<td>50</td>
<td>250</td>
</tr>
</tbody>
</table>
Figure 3.2: Time Point Comparison of Differentiating C2C12 Cells

The protein profiles of differentiated C2C12 cells are displayed in this figure. Gel 1 in Figure 3.2 depicts the C2C12 proteins at T=0 h. Gel 2 in the figure presents the C2C12 proteins at T=24 h. Gel 3 in Figure 3.2 illustrates the protein profile of the C2C12 cells at T=48 h, while Gel 4 in Figure 3.2 shows the proteins in the C2C12 line at T=72 h.
Figure 3.2

C2C12 Gel 1
T=0 h

C2C12 Gel 2
T=24 h

C2C12 Gel 3
T=48 h

C2C12 Gel 4
T=72 h
Figure 3.3: Time Point Comparison of Differentiating P19 Cells (Trial 1)

Protein profiles of differentiated P19 cells obtained from Trial 1 are displayed in Figure 3.3. Gel 1 in Figure 3.3 depicts the P19 proteins at T=0 h. Gel 2 in Figure 3.3 displays the P19 proteins at T=24 h. Gel 3 in this figure illustrates the protein profile of the P19 cells at T=48 h. Lastly, Gel 4 in Figure 3.3 shows the proteins in the P19 cell line at T=72 h.
Figure 3.3

P19
T=0 h/Trial 1

P19
T=24 h/Trial 1

P19
T=48 h/Trial 1

P19
T=72 h/Trial 1
Figure 3.4: **Time Point Comparison of Differentiating P19 Cells (Trial 2)**

Protein profiles of differentiated P19 cells obtained from Trial 2 are displayed in Figure 3.4. Gel 1 in Figure 3.4 depicts the P19 proteins at T=0 h. Gel 2 in Figure 3.4 displays the P19 proteins at T=24 h. Gel 3 in this figure illustrates the protein profile of the P19 cells at T=48 h. Lastly, Gel 4 in Figure 3.4 shows the proteins in the P19 cell line at T=72 h.
Figure 3.4

P19
T=0 h/Trial 2

P19
T=24 h/Trial 2

P19
T=48 h/Trial 2

P19
T=72 h/Trial 2
Figure 3.5: Time Point Comparison of Differentiating P19 Cells (Trial 3)

Protein profiles of differentiated P19 cells obtained from Trial 3 are displayed in Figure 3.5. Gel 1 in Figure 3.5 depicts the P19 proteins at T=0 h. Gel 2 in Figure 3.5 displays the P19 proteins at T=24 h. Gel 3 in this figure illustrates the protein profile of the P19 cells at T=48 h. Lastly, Gel 4 in Figure 3.5 shows the proteins in the P19 cell line at T=72 h.
Figure 3.5

P19
T=0 h/Trial 3

P19
T=24 h/Trial 3

P19
T=48 h/Trial 3

P19
T=72 h/Trial 3
Figure 3.6: Manual Comparison of Undifferentiated C2C12 and P19 Cells (t=0h)

Figure 3.6 displays a manual comparison between the undifferentiated C2C12 cells (t=0 h, control) and undifferentiated P19 cells (t=0 h, control). The proteins from the undifferentiated C2C12 cells are in the gel on the left of the figure while the proteins from the undifferentiated P19 cells are in the gel on right side of the figure. There appears to be several similar as well as several dissimilar proteins between the two undifferentiated cell lines. Proteins that appear to be similar are circled in yellow and proteins that appear to be dissimilar are circled in red.
Figure 3.6

Key:
- Yellow = Similar protein spots
- Red = Dissimilar protein spots

C2C12
T=0h

P19
T=0h
Figure 3.7: **Manual Comparison of Differentiated C2C12 and P19 cells (t=72h)**

Figure 3.7 presents a manual comparison between the differentiated C2C12 cells (t=72 h) and differentiated P19 cells (t=72 h). The proteins from the differentiated C2C12 cells are in the gel on the left of the figure while the proteins from the differentiated P19 cells are in the gel on right side of the figure. There appears to be several similar as well as several dissimilar proteins between the two differentiated cell lines. Proteins that appear to be similar are circled in yellow and proteins that appear to be dissimilar are circled in red.
Figure 3.7

Key:

= Similar protein spots

= Dissimilar protein spots

C2C12
T=72h

P19
T=72h
PDQuest Analyses:

PDQuest analyses were applied to the 17 cm gels for enhanced understanding of those proteins possibly involved in P19 differentiation. Particularly, PDQuest is a valuable tool that was used to study qualitative and quantitative changes that occurred during P19 differentiation into cardiac muscle cells. To carry PDQuest analyses out, all 17 cm gels were first scanned into the computer using Bio-Rad’s ChemiDoc. The images were then opened with PDQuest software and then cropped using the saved cropped settings. Triplicate samples of differentiating P19 cells were studied at four different time points, \( t=0 \) h (control), \( t=24 \) h, \( t=48 \) h, and \( t=72 \) h. First, “Primary MatchSets” were generated from the triplicate samples from each time point. For instance, triplicate samples from \( t=0 \) h were used to generate a Primary “MatchSet” for \( t=0 \) h (control). Primary “MatchSets” were also generated for \( t=24 \) h, \( t=48 \) h, \( t=72 \) h. The PDQuest analysis software automatically detected protein spots on each of the gels. It was necessary to manually edit the gels to guarantee removal of speckling, artifacts, horizontal and vertical streaking, and false spots. A considerable amount of false spots had to be manually removed around the edges of the gels and where tearing had occurred. Alternatively, it was required to manually edit the gels to add any protein spots that were not detected by the PDQuest Software. Along with the Primary “Matchset” produced for each time point, PDQuest also constructed a Matching summary. The matching summaries contained detailed information such as the number of spots on each gel that were detected and the number of matched spots in comparison to the “Master.” These numbers were then converted into percentages seen as “Match Rate 1” and Match Rate 2”. “Match Rate 1” describes the percentage of matched spots on the gel relative to the
total number of spots on the gel. “Match Rate 2” indicates the percentage of matched spots on the gel to the total number of spots on the “Match Set Master.” A “Master” image is a synthetic image that contains all of the protein spots from all of the gels in the Primary “MatchSet.” The “Master” images from each Primary “MatchSet” were needed to generate “Higher Level MatchSets.” The “Higher Level MatchSets” allowed for comparison of protein spots from different time points, e.g. t=0 h vs. t=72 h.

The MatchSets from t=0 h, t=24 h, t=48 h, and t=72 h are depicted in the next figures. Each Matchset contains four quadrants. The upper left quadrant contains the Master image and is clearly noticeable due to its white background, the upper right quadrant represents the gels from Trial 1, the lower left quadrant contains the results from Trial 2, and the lower right quadrant displays the gels from Trial 3. Figure 3.8 displays the MatchSet summary generated from t=0 h (control). The MatchSet summary for t=24 h (experimental) is seen in Figure 3.9. Figure 3.10 demonstrates the resulting MatchSet summary for t=48 h (experimental). Finally, Figure 3.11 shows the MatchSet summary for t=72 h (experimental). In this figure, the gel in the upper right quadrant contains noticeably lighter protein spots compared to the other protein spots in the other three gels in the figure. Problems with cell culture during that specific time point (t=72 h) and trial (Trial 1) resulted in lower than expected protein concentration therefore resulting in more subtle protein spots. Particularly, the majority of cells in the culture flask for Trial 1, t=72 h were floating and longer adherent to the bottom of the culture flasks, indicating a problem with cell culture.

In theory, for each Matchset, the “Match Rate” percentages should be 100% since the gels in each Matchset are from the same cells and cultured under the same conditions.
The Matchset summary data in Figures 3.8-3.11 show that the “Match Rate” percentages are much lower than 100%, but still indicate that there are varying degrees of match percentages for each MatchSet. For example, the Matchset for t=48 h (Figure 3.10) produced the highest matching percentages, with a “Match Rate 1” of 64%, 60%, and 100%. In contrast, the Matchset for t=72 h (Figure 3.11) yielded the lowest matching percentages with a “Match Rate 1” of 100%, 32%, and 26%. As mentioned before, the gel match percentages in theory should be 100%, however the actual MatchSet data for each time point was much lower. This could be due to the PDQuest software not detecting appropriate spots or from the software detecting spots that were not actually spots (e.g., streaking). For example, in each of the Matchset gels, there appears to be a very high molecular weight protein located on the right side of the gel. This high molecular protein is seen a thick “streak” on each gel. The PDQuest software detected the entire streak as many different protein spots. These “protein spots” had to be manually removed since they were not actually different protein spots but rather appeared to be remnants from the high molecular protein at the top of the gel. Also on the same streak, there appeared to be actual protein spots coming off the sides or coming out from under the streak. These spots were manually added since the PDQuest software did not detect them. Additionally, PDQuest oftentimes did not detect matching spots between gels that were clear to the human eye. The most obvious matching spots that were un-detected by PDQuest were manually matched in the PDQuest program.

Using the results from the Primary MatchSets, Higher Level Matchsets were then generated. In Figure 3.12, a HLM is manufactured from t=0 h (Control), t=24 h (experimental), t=48 h (experimental) and t=72 h (experimental), with t=0 h designated
as the Master image. Figures 3.13-3.15 represent HLMs created from the same time points but with sequentially different Masters. Results from the Higher Level Matchsets seem to infer that as time increases (in days), the more differentiated the P19 line becomes. Figure 3.12 shows a HLM using \( t=0 \) h (control) as the Master image for comparison. The Matching Summary Report in the figure reveals that when the gel image from \( t=24 \) h (experimental) is compared to the Master \( t=0 \) h (control) image, 71 out of 220 spots were matched with a Match Rate 1 of 32% and a Match Rate 2 of 55%. When the gel image from \( t=48 \) h (experimental) is compared to the Master \( t=0 \) h (control), 65 out of 220 spots matched with a Match Rate 1 of 43% and a Match Rate 2 of 50%. Finally, when the gel image from \( t=72 \) h (experimental) is compared to the Master \( t=0 \) h (control), only 45 out of 167 spots were matching with a Match Rate 1 of 26% and a Match Rate 2 of 35%. Hence, it appears that the P19 cells are increasingly differentiating as they are cultured longer in 1% DMSO (differentiation medium). A change in cellular proteins accompanies changes in cellular function.
Figure 3.8 PDQuest Analysis- T=0 (Control) MatchSet

Displays the MatchSet summary data for three trials of T=0 h (control). The computer automatically chose Trial 1 of T=0 h (Gel 1) for the Mater Image. The upper left quadrant contains the Master image, the upper right contains Trial 1 of T=0 h (Gel 1), the lower left quadrant displays Trial 2 of T=0 h (Gel 5), and the lower right quadrant shows Trial 3 of T=0 h (Gel 9).
Figure 3.8
Figure 3.9 PDQuest Analysis-T=24 h (Experimental) MatchSet

Displays the MatchSet summary data for three trials of T=24 h (experimental). The computer automatically chose Trial 2 of T=24 h (Gel 2) for the Mater Image. The upper left quadrant contains the Master image, the upper right contains Trial 1 of T=24 h (Gel 2), the lower left quadrant displays Trial 2 of T=24 h (Gel 6), and the lower right quadrant shows Trial 3 of T=24 h (Gel 10).
Figure 3.9
Figure 3.10 PDQuest Analysis-T=48 h (Experimental) MatchSet

Represents the MatchSet summary data for three trials of T=48 h (experimental). The computer automatically chose Trial 3 of T=48 h (Gel 11) for the Mater Image. The upper left quadrant contains the Master image, the upper right contains Trial 1 of T=48 h (Gel 3), the lower left quadrant displays Trial 2 of T=48 h (Gel 7), and the lower right quadrant shows Trial 3 of T=0 h (Gel 11).
Figure 3.10

Matching Summary for "mo 529-08 148":

<table>
<thead>
<tr>
<th>Gel Name</th>
<th>Replicate Group</th>
<th>Spots</th>
<th>Matched</th>
<th>Match Rate 1</th>
<th>Match Rate 2</th>
<th>Corr Coeff</th>
<th>Detected parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>mega gel B17c...</td>
<td>not assigned</td>
<td>148</td>
<td>95</td>
<td>64%</td>
<td>64%</td>
<td>-1.40C</td>
<td>mega148</td>
</tr>
<tr>
<td>mega gel 717...</td>
<td>not assigned</td>
<td>172</td>
<td>104</td>
<td>62%</td>
<td>70%</td>
<td>0.82T</td>
<td>mega148</td>
</tr>
<tr>
<td>mega gel 111...</td>
<td>not assigned</td>
<td>148</td>
<td>148</td>
<td>100%</td>
<td>100%</td>
<td>-1.40C</td>
<td>mega148</td>
</tr>
</tbody>
</table>

Master is marked with * Correlation coefficient based on mega gel 111 c...

MatchSet information
Spots matched to every member: 79
Overall mean coefficient of variation: 38.98

Repeate group and classes information
Replicate Group/Classes | Member | Matched to all | Mean CV |
---|--------|----------------|---------|
all not assigned | 3      | 79             | 36.88   |
Figure 3.11 **PDQuest Analysis-T=72h (Experimental) MatchSet**

Represents the MatchSet summary data for three trials of T=72 h (experimental). The computer automatically chose Trial 1 of T=72 h (Gel 4) for the Mater Image. The upper left quadrant contains the Master image, the upper right contains Trial 1 of T=72 h (Gel 4), the lower left quadrant displays Trial 2 of T=72 h (Gel 4), and the lower right quadrant shows Trial 3 of T=72 h (Gel 12).
Figure 3.12 Higher Level MatchSet- T=0 h Master

Represents Higher Level Matchset data (HLM) generated from the Primary MatchSets’ Masters. The T=0 h (control) Master was chosen as the Master image for this HLM. The upper left quadrant contains the Master image, the middle upper area represents the T=0 h Master image, the upper right box displays T=24 h Master image, the lower left box represents T=48 h Master image, and the middle lower box indicates T=72 h Master image.
Figure 3.12
**Figure 3.13 Higher Level MatchSet- T=24h Master**

Represents Higher Level Matchset data (HLM) generated from the Primary MatchSets’ Masters. The T=24 h (experimental) Master was chosen as the Master image for this HLM. The upper left quadrant contains the Master image, the middle upper area represents the T=0 h Master image, the upper right box displays T=24 h Master image, the lower left box represents T=48 h Master image, and the middle lower box indicates T=72 h Master image.
Figure 3.13

Matching Summary for "new MO HL matchset 124":

Member information

<table>
<thead>
<tr>
<th>Cell Name</th>
<th>Replicate Group</th>
<th>Spots</th>
<th>Matched</th>
<th>Match Rate 1</th>
<th>Match Rate 2</th>
<th>Core Coeff</th>
<th>Detect param</th>
</tr>
</thead>
<tbody>
<tr>
<td>mo 5:22 08 L</td>
<td>not assigned</td>
<td>120</td>
<td>65</td>
<td>50%</td>
<td>20%</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>tea 5:22 08 L</td>
<td>not assigned</td>
<td>220</td>
<td>220</td>
<td>100%</td>
<td>100%</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>mo 5:29 08 L</td>
<td>not assigned</td>
<td>149</td>
<td>79</td>
<td>50%</td>
<td>95%</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>mo 5:29 08 L</td>
<td>not assigned</td>
<td>167</td>
<td>62</td>
<td>37%</td>
<td>28%</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Master is marked with * Correlation coefficient based on N/A

MatchSet information

| Spots matched to every member | 21 |
| Overall mean coefficient of variation: | N/A |

Replicate group and classes information

<table>
<thead>
<tr>
<th>Replicate Group/Classes</th>
<th>Members</th>
<th>Matched to all</th>
<th>Mean CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>all not assigned</td>
<td>4</td>
<td>21</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 3.14 Higher Level MatchSet- T=48h Master

Represents Higher Level Matchset data (HLM) generated from the Primary MatchSets’ Masters. The T=48 h (experimental) Master was chosen as the Master image for this HLM. The upper left quadrant contains the Master image, the middle upper area represents the T=0 h Master image, the upper right box displays T=24 h Master image, the lower left box represents T=48 h Master image, and the middle lower box indicates T=72 h Master image.
Figure 3.14
**Figure 3.15 Higher Level MatchSet- T=72h Master**

Represents Higher Level Matchset data (HLM) generated from the Primary MatchSets’ Masters. The T=72 h (experimental) Master was chosen as the Master image for this HLM. The upper left quadrant contains the Master image, the middle upper area represents the T=0 h Master image, the upper right box displays T=24 h Master image, the lower left box represents T=48 h Master image, and the middle lower box indicates T=72 h Master image.
Figure 3.15
CHAPTER IV:

DISCUSSION
This study was carried out with the idea that the P19 cell line could possibly serve as a model for human stem cell differentiation. By utilizing a proteomics-based approach, the proteins involved in the P19 differentiation process into cardiac cells could be intensely studied with the notion that some of those same proteins may play a role in further investigating cardiac cell differentiation. It is anticipated that studying this cell line may allow for better understanding of how to direct pluripotent or multipotent cells to cardiac lineage. Previous to carrying out this study, consideration of the fact that the P19 cell line is not an exact representation of human adult stem cells had been taken into account. It was determined that although the P19 cell line may have some limitations due to it origin (embryonal carcinoma), use of this cell line to further study cardiac cell differentiation had many strengths. First, the P19 cell line is a well-established cell culture system for differentiation, thus pertinent information such as cell culture instructions was readily and easily available. Also, generation of new cardiomyocytes via P19 cell differentiation is a fairly easy task since its only requirement for differentiation is 1% DMSO.

Although manual comparison of the 7 cm P19 gels allowed for some identification of protein spots involved in P19 differentiation, the PDQuest™ 2-D Analysis Software was the main tool for studying the proteins that play a role in differentiation of P19 cells into new cardiomyocytes. The PDQuest™ 2-D analysis Software was able to pick out protein spots from one gel at a specific time point of differentiation and then compare to protein spots found another gel from a different specific time point of differentiation. In other words, PDQuest™ analysis allowed for
preliminary identification of proteins involved in differentiation of P19 into cardiomyocytes.

After obtaining the results from our gel, mass spectrometry (MS) can be used to identify certain proteins of interest by creating a “peptide mass fingerprint” as well as fragmenting selected peptides into product ions (Beranova-Giorgianni, 2003). When a “peptide mass fingerprint” is created, the mass of the peptides within the protein is revealed. When the selected peptides are fragmented into product ions, a segment of the amino acid sequence of the peptide, also referred to as a “sequence tag”, can be determined (Beranova-Giorgianni, 2003). “Spots” on the gel can be cut out and then sent to The Ohio State University for MS. Once our results (“peptide mass fingerprints”, product-ion data, or sequences tags from our proteins of interest) come back, they can be compared to theoretical data by employing database search programs such as SWISSPROT, MS-Fit, etc. (Beranova-Giorgianni, 2003). Comparison of our data to theoretical data will ultimately allow us to identify proteins of interest.

During the preliminary stages of the study, some problems arose while culturing of the P19 cells. When the cells were received from the ATCC, they were immediately thawed and cultured under appropriate conditions. Upon 70-80% confluency, the cells were subcultured in order to produce enough cell cultures for three different trials, with each trial consisting of four different time points (0 h, 24 h, 48 h, and 72 h). It was observed that the longer the P19 cells were cultured (in days) and the more times they were split, the more likely the cells were to detach from the bottom of the cell culture flask, therefore making them no longer viable. In response to this problem, the cells were subcultured to the minimum number of times necessary for all three trials. To further try
to prevent this problem, the P19 cells were cultured in 1% DMSO (differentiation medium) for a maximum of 3 days (72 hours) even though previous published studies cultured the P19 cells in 1% DMSO for 4-5 days (McBurney, et. al, 1982).

The P19 cell line is an extremely suitable cell line to study cardiomyocyte protein differentiation since it already has been broadly characterized. Studying the P19 cell line has already lead to extensive knowledge into areas such as the role of cardiac specific transcription factors, signaling pathways, and functional ion channel expression (van der Heyden and Defize, 2003). A study to greatly consider in the near future is to culture embryonic P19 derived cardiomyocytes with isolated adult cardiomyocytes in order to gain knowledge into the processes that are occurring after cardiomyocyte cell transplantation (van der Heyden and Defize, 2003). The direct interaction between the two different cell types (embryonal vs. adult) as well as the exchange of secreted proteins can be intensely studied (van der Heyden and Defize, 2003). The results from the study could possibly determine the role stem cells play in therapeutic purposes for damaged heart muscle following a myocardial infarction. Also, since P19 derived cardiomyocytes are embryonic in nature, further research should be carried out to further study the processes involved in heart development as well as maturation (van der Heyden and Defize, 2003).

As a result of studying the P19 cell line, a “panoramic view” of changes in protein expression involved in cardiomyocyte differentiation was obtained (Wen et al, 2007). In a recent study, the P19 cell line consistently expressed 16 different proteins when treated with DMSO (Wen et al, 2007). The proteins were identified and then categorized into classes based on their functions. The majority of the proteins were involved in cellular
metabolism, signal transduction, cellular organization, and possibly even maintenance of the cardiac state. By identifying the 16 differential proteins, further insight into the workings of cardiomyogenesis was attained. However, to fully describe the functions of the differentially expressed proteins more research must be conducted. If complete details of the differentially expressed proteins can be discovered, the development of innovative therapies for generation of new cardiomyocytes to replace damaged cardiomyocytes can be one step closer. Additionally, if stem cell plasticity is found to have even greater potential than once believed (i.e., specialized cell of one tissue lineage is found to transdifferentiate and thus generate a specialized cell of a different type), it could be said that adult stem cells have pluripotent characteristics (Verfaillie, 2002). This finding would be extremely significant in the medical world. If stem cell plasticity is proven true, and an effective way to transdifferentiate adult stem cells is created, then the findings from this study may even have greater implications.

Future work on this study would include excising proteins of interest. Any proteins within the P19 gels that are possibly linked to inducing differentiation of P19 cells into cardiomyocytes need to be excised and sent to the Ohio State University for more in-depth analysis. Also, a more thorough investigation as to why problems with the culturing of the P19 cells occurred would be helpful for any future work. Finding a resolution to this problem would allow for longer culture time, more trials, and perhaps even greater results.

The information provided in this study is a beginning and important step to determining which proteins play a role in cardiomyocyte differentiation. The basis for this study, proteomics, has allowed for greater understanding of the proteins involved in
P19 differentiation. A proteomics-based approach allowed for comparison of the proteomes before and during P19 differentiation, which in turn allowed for detection in any changes of biological processes. It is anticipated that once the proteins involved in cardiomyocyte differentiation are identified, that they can one day be used to induce human stem cells to become newly formed cardiac cells.
Appendix 1a and 1b Polyacrylamide Gel Components

Appendix 1a and 1b details the recipe used for making 100 ml of 12% polyacrylamide gel-making solution and 800 ml of 12% polyacrylamide gel-making solution.

Approximately 12 7 cm gels could be made with 100 ml of 12% polyacrylamide gel-making solution and 12 17 cm gels could be made with 800 ml of 12% polyacrylamide gel-making solution. The components were added in the order listed. The 10% ammonium persulfate was made immediately before making the gels. Additionally, 10 mL of H₂O-saturated butanol was added previous to the gel components for 7 cm gels while 32 mL of H₂O-saturated butanol was added previous to the gel components for 17 cm gels. All gels were made approximately 24 hours before the 2nd dimension was resolved.
### Appendix 1a

#### 12% Gel Components (100mL)

<table>
<thead>
<tr>
<th>Solution Components</th>
<th>100mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>48</td>
</tr>
<tr>
<td>40% acrylamide</td>
<td>25</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>25</td>
</tr>
<tr>
<td>10% SDS</td>
<td>1</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>1</td>
</tr>
<tr>
<td>TEMED</td>
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</table>
Appendix 1b

12% Gel Components (800mL)

<table>
<thead>
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<th>Solution Components</th>
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</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>344</td>
</tr>
<tr>
<td>40% acrylamide</td>
<td>240</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>200</td>
</tr>
<tr>
<td>10% SDS</td>
<td>8</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>8</td>
</tr>
<tr>
<td>TEMED</td>
<td>.320</td>
</tr>
</tbody>
</table>
CHAPTER 4:

REFERENCES


Lariviere, Andrew. (2002). Dexamethasone increases differentiation capacity in C2C12 cells and its effects on Cdk1 and ERK1 expression. 757.


Zimmermann W., Didié M., Döker S., Melnychenko I., Naito H., Rogge C., Tiburcy M.,

Zuk P.A., Zhu M., Mizuno H., Huang J., Futrell W., Katz A.J., Benhaim P., Lorenz H.P.,