ADIPOSE-DERIVED STEM CELLS AND THEIR CARDIOGENIC DIFFERENTIATION

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Heart diseases are leading causes of death in the world. The inability of heart muscles to regenerate or restore the lost function makes the use of stem cells an attractive therapeutic option for patients with heart diseases. Stromal cells from fat tissue, namely adipose-derived stem cells (ADSCs), exhibit properties of mesenchymal stem cells and can be differentiated towards several cell types. That adipose tissue is abundant and easily harvested from patient's own body makes it an ideal source for patient specific stem cells. Yet, less is known about the capacity for self-renewal and stability properties of mesenchymal ADSCs during expansion in culture. Furthermore, efficient differentiation of these cells towards cardiogenic lineage has not been established.

We first characterized human ADSCs cultured for varying times. Flow cytometry indicated that ADSCs maintained expression of mesenchymal markers (CD29, CD44 and CD90) for extended expansion over 20 passages. In contrast, mRNAs for pluripotent markers, such as Nanog, Oct4 and Sox2, were significant only in early passages, but were dramatically declined during culture. Serum removal increased mRNA levels for various cardiogenic genes, such as Mef2C, cardiac actin and troponin. Moreover, the protein kinase activator phorbol ester (phorbol myristate acetate PMA, 10 nM) caused further increases in these cardiac mRNAs. The upregulation of cardiac mRNAs by serum removal and PKC activation were constant in ADSCs cultured for various times. The use of various inhibitors specific for PKC subtypes suggested

that the novel PKC theta/delta isoforms mediate this upregulation. RT-PCR revealed that ADSCs express significant mRNA for PKC delta, but not theta isoform. Overexpression of cDNA for PKC delta resulted in marked increases in cardiac mRNA expression. These results indicate that activation of PKC delta induces expression of multiple cardiogenic genes freshly-prepared and expanded ADSCs.

These findings demonstrated that human ADSCs maintain the expression of mesenchymal markers and the ability to exhibit cardiac gene expression for extended expansion. They also suggest that a specific signaling molecule is involved in the transdifferentiation towards cardiogenic lineage.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

It was fall in the year of 2000 when I joined a biological laboratory. In 2008, I am standing on this site as a bioengineering Ph.D candidate. Success is not a destination but a journey, and I have walked through all kinds of beauty on the road to a Ph.D. I wish to take this attitude to embrace and enjoy the process, not just a title or goal, in future.

I would like to specially thank my advisor Dr. Amit Patel for his continues mentoring and letting me work on an independent project. In addition, it was a great honor to have the support and best kindness from the committee members: Dr. Sanjeev Shroff, Dr. Partha Roy and Dr. Peter Rubin.

Finally, I truly, deeply appreciate the continuous encouragement and help for completing this dissertation from colleagues, friends, family members, Ms. Karanee Leelavanichkul and Dr. Koichi Takimoto.

This dissertation is dedicated to my mom Ms. Soo Hee Jung.

1.0 INTRODUCTION

Adipose tissue has gained much attention as a source of adult stem cells, because it is abundant and easily harvested from the patient's own body. Adipose tissue is derived from the embryonic mesoderm and contains a heterogeneous stromal cell population. These cells possess a potential to differentiate to multiple cell types and thus called adipose-derived stem cells.

Differentiation of stem cells into certain cell types is directed, in part by external cues. Drugs that influence chromatin state and cellular signaling have been shown to induce differentiation of embryonic stem cells and fibroblasts towards cardiomyocytes. In addition, electrical stimulation has been shown to increase the interconnectivity and contractile function of neonatal myocytes. Thus, electric stimulation may also be a useful tool to stimulate differentiation of stem cells towards cardiac lineages.

This thesis investigates the characteristics of human adipose-derived stem cells expanded in culture. Further, it evaluates the abilities of various culture conditions, drugs and electrical stimulation to differentiate human adipose-derived cells towards cardiomyocytes.

1.1 GOAL

The overall goal of this thesis is to identify conditions and mechanisms for cardiomyogenic differentiation of human adipose-derived stem cells (ADSCs).

Heart diseases place an enormous burden on health care systems and the economy of the country. The facts that ADSCs can be easily harvested from patients in a large quantity and that they can be differentiated towards cardiomyocyte lineage suggest a great prospect of using these cells for restoring function to damaged hearts. Therefore, this project was initiated to develop methods for the effective and consistent cardiomyogenic differentiation of ADSCs. Furthermore, we wished to elucidate the nature of ADSCs which may be associated with variable responses to differentiation strategies being developed. Hence, this study focused on characterizing human ADSCs expanded in culture and to evaluate various culture conditions and external stimuli for cardiomyogenic differentiation of these cells using cell biological, molecular and biochemical methods.

1.2 OVERVIEW

We hypothesize that exogenous stimuli induce cardiac differentiation of ADSC, depending on their culture and endogenous conditions. We tested this hypothesis and accomplished the objective of the thesis work by pursuing the following three aims.

1.2.1 Aim 1: Characterize adipose-derived stem cells expanded in culture

Mesenchymal stem cells are defined as multipotent cells originating from adult organs. Isolation of mesenchymal stem cells is usually performed by trypsinization of tissue, followed by attachment to plastic surface of culture dishes. Therefore, the isolated cells are often referred as to stromal cells, instead of stem cells, and may represent a mixture of various cell types. Human

ADSCs are also prepared similarly based on trypsinization and attachment to plastic surface, following surgical isolation of subcutaneous or visceral adipose tissue. Thus, ADSCs used in this study may also contain various cell types. Moreover, human ADSCs may significantly differ in their properties due to the differences in gender, age, and physiological and pathological states of donors. Hence, we first characterize ADSCs used in this study.

1.2.2 Aim 2: Examine chemical drugs and electrical stimulation for cardiac differentiation of adipose-derived stem cells

Chemical drugs that influence chromatin status have been shown to direct embryonic and mesenchymal stem cells towards cardiogenic lineage [1-5]. Furthermore, inhibition of protein kinase Cs has been known to block the Wnt11-induced cardiac differentiation of stem cells. This suggests that direct activation of these enzymes may facilitate the cardiac differentiation process. Therefore, we tested the effects of DNA methylase inhibitor (RG108), deacetylase inhibitor (Trichostatin A: TSA) and PKC activator (phorbol-12 myristate-13 acetate: PMA) on cardiogenic differentiation of ADSCs.

Electrical field or activity is known to act as an essential differentiation cue for the developing heart *in vivo* [6]. As a tissue, precursor myocytes or stem cells are in contact with each other while stimulated synchronously by induced or intrinsic electrical activity. Thus, we also test if similar electrical environments created in a culture setting might elicit differentiation of ADSCs towards cardiomyocyte lineages

1.2.3 Aim 3: Identify the roles of PKC isoforms in cardiac differentiation of adipose-derived stem cells

Protein kinase Cs (PKC) positively and negatively influences cardiomyogenic differentiation in the developing embryo [7-9]. We found that phorbol myristate acetate (PMA), an activator of protein PKCs increases cardiomyogenic gene expression in ADSCs. Furthermore, specific inhibition of classical PKCs caused marked increases in several cardiac genes [10]. These findings suggested that distinct roles of PKC isoforms in regulating cardiac gene expression in ADSCs. We hypothesize that novel PKC (nPKC) isoforms induce cardiogenic gene expression in ADSCs, whereas classical PKC (cPKC) isoforms block this induction. Hence, the purpose of this aim is to test this hypothesis and identify specific PKC isoforms mediating these effects.

2.0 BACKGROUND

2.1 HEART DISEASES

Cardiovascular diseases (CVD) are the leading causes of mortality and morbidity in the developed world. The American Heart Association reported that cardiac failure was responsible for 38% of all deaths in 2005, or approximately 930,000 people in the United States [11]. Of this heart failure, 53% were directly caused by myocardial infarction, 18% by stroke, and 6% due to congestive heart failure. The heart's inability to heal itself is the primary factor contributing to the high incidence in mortality. Unlike most other cell types, cardiac myocytes, the functional components of the heart muscle, are incapable of replication, or do so on a very small scale [12]. As a result, when the heart is damaged, as in the case of a myocardial infarction, there is limited internal mechanism for repair and the patient is left with decreased cardiac function which rarely improves. Drug treatments help to alleviate or prevent additional damage from some of the factors which contributed to the initial damage, such as hypercholesterolemia and atherosclerosis, however none actually conducts cardiac repair. Another intervention for cardiac diseases is a total heart replacement or mechanical implants. Heart transplantation and artificial hearts are effective treatments for loss of organ and tissue function. These treatment, however, are associated with serious problems such as a critical shortage of donor organs, rejection, the need for life-long immunosuppression, and unstable biocompatibility. These limitations have led to

the exploration of biologic options through cell-based therapy using stem cells to repair damaged cardiac tissue.

2.2 REGENERATIVE MEDICINE

Medical researchers have attempted to harness the potential of embryonic and adult cells, as well as new materials, to rebuild the lost function of damaged organs and tissue. This field is often defined as "Regenerative Medicine" [13]. It is often interchangeably used with stem cell-based therapy; however, when widely defined, it includes approaches using differentiated cells, and even artificial devices and materials to repair or restore the lost function to damaged organs and tissues. On the contrary, "Stem cell therapy" usually indicates the replacement of the lost cells with stem cell-derived mature cells. Thus, regenerative medicine represents a broadly-defined approach and might involve various mechanisms to restore the lost function to a damaged organ and tissue.

2.2.1 Cell transplantation

The aim of cell transplantation is to repair damaged tissues and organs. General approaches for cell delivery have involved the use of direct injection of single cell suspensions into the target tissues and implantation of a scaffold with a population of desired cells in the tissues [14-16]. Despite the recent progress in material science and *in vitro* production of bioactive peptide and other molecules, the availability of replacement cells remains a major challenge [17]. Cell transplantation heavily depends on a sample of autologous cells from the

disease organ of the patient; biopsies from patients with extensive end-stage organ failure may not yield enough normal cells. In these situations, stem cells are envisioned as being an alternative source.

2.3 STEM CELL THERAPY

Stem cells are characterized by self-renewal and potency to differentiate towards certain cell types. Their potency largely varies, ranging from pluripotency, the capacity to differentiate to cells from any of the three germ layers, to unipotency, the ability to produce only one cell type. Stem cells are divided into two major classes: embryonic and adult stem cells. The former cells derived from the inner cell mass of a blastocyst or morula stage embryos and exhibit pluripotency. The latter cells are found in most tissues of the adult body, but possess a limited capacity to differentiate into various cell types.

It is hoped that stem cell-based therapies will help people who have suffered devastating diseases. The use of stem cells has been featured in several untreatable conditions, such as spinal cord injury and heart diseases [18-20]. There have been many clinical trials for stem cell therapy of heart diseases. The NIH's clinical trial website currently lists more than 150 on-going or planned trials for stem cell therapy for heart diseases [21]. Although some may be proven to be useful or improve cardiac functionality and extend the length and quality of life, the difficulty in evaluating mechanisms in human studies poses for some questions in identifying the best method for various cases in future.

2.3.1 Embryonic stem cells and pluripotent markers

Since human pluripotent stem cells were first isolated in 1998 [22], scientific researchers and medical doctors venture making the stem cells remedy to restore a lost or damaged organ in the human body. The establishment of human embryonic stem cell lines in theory enables us to generate all tissues, and provide opportunities for drug screening and gene manipulation as a research tool. However, present legislation surrounding human embryonic stem cells makes their use problematic. In addition, allogeneic nature of embryonic stem cells forces the use of immunosuppressant for the life time of patients.

Embryonic stem cells are also characterized by the presence of several transcription factors, such as Oct4, Nanog and Sox2 [23-44]. These transcription factors, known as pluripotent markers, constitute the core transcription network that ensures the suppression of genes for differentiation and the maintenance of pluripotency. Recent studies also demonstrated that these transcription factors are capable of turning some adult cells to regain pluripotency [25-26]. Therefore, these markers are not simple signs of embryonic stem cells, but are fundamental reasons for pluripotency.

2.3.2 Adult stem cells and mesenchymal markers

Tissue-regenerating stem cells are present in a large number of tissues in the adult body with various differentiating capacities. Many tissues contain a small number of stem cells that may be differentiated into one or several mature cell types that constitute their own tissue when it is damaged or lost. In contrast, some other tissues appear to include stem cells that can be turned into multiple cell types, some of which are unrelated to their source tissue [27-30]. For example,

bone marrow is known to contain mesenchymal stem cells (MSCs) that can be directed towards diverse cell types [31-34]. MSCs are a subpopulation of stromal cells in connective tissues of mesodermal origin. They are characterized by a high proliferation potential and the capacity to differentiate into progenitor cells for distinct tissues in culture [35]. Several cell surface markers, such as Stro-q, CD29, CD44 and CD90, are known to identify MSCs [36-38]. These cell surface markers are widely used for the isolation and characterization of less-committed cells from the adult tissue. MSCs from bone marrow have been shown to develop into various terminally-differentiated cells and tissues including bone, cartilage, fat, muscle, tendon and neural tissue [39-40]. However, the clinical use of MSCs from bone marrow have presented problems, including pain, morbidity, and low cell number upon harvest [41-43]. Therefore, it is desirable to have an abundant source of MSCs for the use in clinical applications.

2.3.3 Adipose-derived stem cells

Adult stem cells similar to those in bone marrow are found in fat tissues and are termed adipose tissue-derived stem cells (ADSCs). ADSCs share many of the characteristics with their counterparts in bone marrow. ADSCs are a subpopulation of stromal cells in subcutaneous and visceral fat tissues [44]. These cells exhibit high proliferation capacity and a potential to differentiate towards multiple lineages [45-47]. Advantages of using ADSCs over bone marrow MSCs include their simple harvest with local anesthesia and the high density of adult stem cells in fat tissue. The number of ADSCs in harvested fat is approximately 40-times higher than that of bone marrow MSCs [44]. Many studies have shown that ADSCs can be directed towards the three lineages: adipogenic, osteogenic and myogenic cells [48-50]. While differentiation towards the former two lineages have been achieved in a large population of isolated cells using

fairly well-established manipulations, many attempts have so far yielded very low percentages of cells or colonies with myogenic properties. Furthermore, ADSCs from experimental animals and human patients/volunteers may not similarly respond to the same stimulus for differentiation. For example, 5-azacytidine induced cardiac differentiation of mouse ADSCs, but was ineffective in turning human cells towards cardiac lineage [51-53]. This may be due to species difference, age or other conditions. In any case, there is no effective method to direct ADSCs towards cardiac lineage at this moment. Moreover, since ADSCs from human patients are associated with some differences, it is important to elucidate these differences that may influence their capacity to differentiate towards cardiac lineage.

2.4 CARDIOGENIC DIFFERENTIATION OF STEM CELLS

It is considered that differentiation of stem cells may be similar to the normal developmental process of cellular speciation. Thus, various internal changes that occur in progenitors during cardiogenic speciation can be used as markers for differentiating stem cells. Moreover, factors that control cellular speciation in the early development may be employed to induce cardiogenic differentiation of stem cells in culture. Other approaches that facilitate cadiogenic differentiation of stem cells include the use of drugs interfering with chromatin status.

2.4.1 Early heart development

The heart is generated from a small number of progenitor cells that are derived from mesoderm during gastrulation [54-55]. These progenitors undergo complex and dynamic

processes, migrating, expanding and diversifying to acquire a linear heart tube, the primitive form of a functional heart. During these processes, progenitors acquire cardiogenic phenotypes, initially general cardiac progenitors and then those with more specific properties, such as atrial and ventricular cells [56-57].

Factors that are essential in early cardiac speciation include fibroblast growth factors (FGFs) [58], bone morphogenic proteins (BMPs) [59-60] and wingless-related proteins (Wnt) [61]. FGF signaling is involved in the induction and formation of the mesoderm in all vertebrate species. More specific role in the generation of cardiac progenitors is seen with FGF8 in mice. FGF8 is secreted by the endodermal cells to induce cardiac progenitors in the primitive streak [62]. Other factor that is secreted by endodermal cells and control myocardial speciation of mesodermal cells include BMPs. BMP2 is endodermally expressed and ectopic injection of BMP2 induces expression of several cardiac genes [63]. The lack of BMP2 causes reduced myocardial progenitors and abnormal heart field formation. FGF8 is capable of restoring the lack of BMP2 to some extent, suggesting that FGF8 and BMP4 act together to induce myocardial speciation of mesodermal cells.

Distinct Wnt members appear to influence cardiogenic speciation differently. Wnt family proteins act on G protein-coupled receptors. Two classes of Wnt members control myocardial speciation in positive and negative ways: Wnt members that activate canonical β–catenin signaling inhibit cardiac speciation, whereas others, such as Wnt11, activate this process [64-65]. The latter Wnt 11 effect is mediated by activation of protein kinase C [66]. These findings suggest that several external factors may be capable of stimulating cardiogenic differentiation of adult stem cells.

2.4.2 Cadiomyocyte differentiation of stem cells

Stem cell differentiation is driven by complex processes that are only beginning to be understood. The natural developmental process provides a great deal of information. Several growth factors and hormones are used to mimic these processes [67-68]. Yet, the complexity of interactions between various cell types and their temporal changes makes it difficult to replicate this process. On the other hand, non-natural or semi-toxic chemical drugs are often used to reset the cell's preprogrammed status with some success.

Various factors and other strategies have been used for differentiation of adult stem cells towards cardiac lineages. Wnt11 has been shown to increase the expression of cardiac genes in mesenchymal stem cells of the bone marrow origin [69]. However, Wnt11-treated cells exhibited partial differentiation or transdifferentiation, in which only some cardiac genes were increased whereas others were depressed without clear functional or morphological changes. Other methods include the use of chemical drugs that interfere with chromatin status [1]. Indeed, differentiation of ADSCs into cardiomyocyte lineage was first observed with embryonic carcinoma cells using 5-azacytidine, a nucleotide derivative that causes demethylation of DNA. However, this study used weeks of culture in the presence of multiple factors in addition to the non-specific methylase inhibitor, and isolated a small fraction of formed colonies. More recent study failed to induce cardiogenic differentiation of human ADSCs with the same drug [70]. In addition to the methylase inhibitor, the blocker of histone deacethylase, tricostatin A has been shown to increase the expression of cardiac transcription factors, such as GATA4, in embryonic stem cells [71]. Thus, these and other drugs that alter chromatin status may be useful in cardiac differentiation of adult stem cells. Yet, it is necessary to identify cellular conditions and treatment paradigm that effectively stimulate cardiac differentiation.

2.4.3 Markers for cardiogenic differentiation

One of markers for early cardiac progenitors is Nkx2.5 [56]. It is detected cardiac crescent, a structure formed by migrated cardiac progenitors prior to heart tube formation. Nkx2.5 is a transcription factor that controls the expression of downstream cardiac genes. Indeed, the role of Nkx2.5 is conserved from insects to mammals, and located on the top of gene regulatory network for cardiac development. GATA4 is the other transcription factor that is detected in cardiac crescent and plays important roles in cardiac development [72]. GATA4 gene knockout study demonstrated the essential role of this factor in cardiac development and coordination of the expression of downstream cardiac genes [73]. However, the role of GATA4 in myocardial speciation may be less clear than Nkx2.5. In flies, only one GATA transcription factor (Pannier) is genetically located downstream of Nkx2.5 and essential for cardiac speciation of progenitors, whereas two related transcription factors GATA5 and GATA6 are also expressed in developing cardiac progenitors in vertebrates. Other transcription factors important for cardiac development include Mef2C that controls the expression of contractile proteins in both cardiac and skeletal muscles [74]. Hence, these cardiac transcription factors are considered useful markers in detecting differentiating stem cells towards cardiogenic lineage.

The contractility is a hall mark of cardiac myocytes. Many contractile proteins are also uniquely or predominatly expressed in cardiac myocytes, but not in other muscle cells. These proteins include several troponins, cardiac actins and myosins. Troponin is a complex of three regulatory proteins (Troponin C, T and I) that is integral to contraction in myocardium. Cardiac troponin I (TNNI3) and I (TNNT2) are very sensitive and specific indicators of damage to the heart muscle. Actins and myosins constitute contractile fibers. Cardiac α–actin (ACTC1), myosin light chains (MYL2), α-myosin heavy chains (MYH6) and β–myosin heavy chain

(MYH7) are predominantly expressed in the heart, but not in other muscles [75]. The expression of these and other contractile proteins is developmentally regulated. For example, some skeletal-type contractile proteins are significant in neonatal myocytes. Similarly, reappearance of these neonatal-type genes is well-documented in hypertrophied and failing hearts [76-78]. Since cardiac contractile proteins constitute the essential cardiac contractile function, we also use several contractile gene expressions as markers for cardiogenic differentiation of ADSCs.

Other cardiac-specific genes include ones for channels. In particular, gap junction-forming proteins, connexins, are commonly used as cardiogenic markers. In particular, two subunits, connexin 40 and connexin 43, are known to express predominantly in cardiomyocytes. Thus, expression of the two connexin genes also represents cardiogenic differentiation of ADSCs.

2.5 PROJECT CONTRIBUTION

The biggest barrier for clinical use of stem cells resides in the uncertainty of what and how cellular and molecular signals drive stem cells into specialized cell types. Many studies with different cell sources, species and treatment regimes revealed that cell type-specific differentiation depends on source of stem cells [79-82], species, and other conditions [83-84], some of which remain unclear. Thus, it was necessary for stem cell researchers to devise more specific and reproducible ways for identifying cellular context and differentiation methods. This thesis provides more information for fundamental understanding of cardiogenic differentiation of adipose stromal cells. It investigates the characteristics of human ADSCs cultured for extended expansion and cardiogenic gene expression in these cells, so that obtained information can be used to elucidate fundamental biological questions and processes, such as the capacity of self-

renewal and the causes of differentiation, tissue repair, and even aging. As we learn more about the essential properties of stem cells, it may be truly valuable to use stem cells not only for treating diseases, but also for screening drugs and understanding developmental disabilities.

3.0 MATERIALS AND METHODS

3.1 ADIPOSE-DERIVED STEM CELL S

Two sources of ADSCs were used in this study. One was prepared in our laboratory, whereas the other was from a commercial provider. For own preparation, subcutaneous adipose tissue was harvested from three individual patients during elective adbominoplasty. The subcutaneous adipose tissue was minced and then digested in Hank's Balanced Salt Solution containing 1 mg/ml type II collagenase and 3.5% fatty acid-free BSA in a 37 °C in a shaking water bath until the mixture was homogeneous. The digested tissue was filtered through a double-layered gauze (350 μm) and centrifuged at 1000 rpm for 10 minutes. After centrifugation, the resulting pellet was treated with Erythrocyte Lysis Buffer, vortexed, and centrifuged at 1000 rpm for 10 minutes. The cells were resuspended in regular cell culture media (DMEM/F12 50:50, supplemented with 10% FBS and antibiotics) and plated at a density of $5x10^3$ cells/cm^2 at 37 °C under 5% atmosphere. Cell culture media was changed every two days until confluence. The cells attached to plastic surface in the initial isolation were considered as the passage 0. ADSCs from the commercial source (ScienCell Research laboratories, Carlsbad, CA) were also used in this project. A frozen stock provided by the company was set at the passage 1. According to the company, these cells were obtained using procedure similar to ours and were propagated less than 3 passages.

3.2 FLOW CYTOMETRY

ADSCs were harvested and analyzed with fluorescence-activated cell sorter (FACS) (Becton Dickinson, Rockville, MD). Monoclonal antibodies against human CD44 (Invitrogen, , Carisbad, CA), CD29 (Chemicon), CD90 (Invitrogen), CD34 (Chemicon), and CD45 (abcam, Cambridge, MA) were used. Following primary antibodies, cells were labeled with goat anti-mouse IgG conjugated with Donkey anti-mouse Alexa Fluor-488 (Molecular Probes, Invitrogen) with 1:10,000 dilutions. Analysis was performed with 500,000 cells per sample and the positive expression was defined as the level of fluorescence greater than 99% of the corresponding unstained cell sample.

3.3 CHEMICALS

Tricostatin A (TSA) and RG108 were obtained from Calbiochem (EMD Chemicals, Gibbstown, NY). Stock solutions were made with dimethyl sulfoxide (DMSO) and were added to culture medium at the final solvent amount less than 1/1000.

3.4 ELECTRICAL STIMULATION

An electrical stimulator (C-Pace, Ion Optix Corporation, Milton, MA) was used to generate field electrical stimulation in cultured ADSCs. This device generates bipolar square pulses with various time settings. ADSCs were seeded on plastic dishes at 5,000/cm^2. One day

after seeding, cells were subjected to electrical stimulation. We used two voltage pulses to 10 and 40 V. The lower voltage would be expected to elicit a larger Ca^{2+} influx through voltage-gated Ca^{2+} channels, whereas the change in voltage by itself may produce Ca^{2+} entry-independent effects. Various pulse durations and intervals, as well as different stimulation periods, were used to stimulate cultured ADSCs.

3.5 RT-PCR

Total RNA was extracted from ADSCs using RNeasy Mini RNA extraction kit (Qiagen Inc., Alameda, CA) according to the manufacturer's protocol. Human heart and brain RNAs were obtained from a commercial source (Ambion, Austin, TX) and were used as positive controls. First-strand cDNA was synthesized with total RNA using Thermostable reverse transcriptase (Thermoscript, Invitrogen). cDNA samples is subject to PCR amplification with primers for cardiac-specific genes (Table 1). PCR reaction was performed with 0.2-2 μL of cDNA template using a commercially-available pre-mixed solution (GenChoice, Shawano, WI) in 25 μL. Standard PCR was performed at 30 cycles each consisting of 94 °C for 5 seconds, 58 or 62 °C for 5 seconds and 72 °C for 30 seconds with a final extension at 72 °C for 4 minutes. The PCR products were size-fractionated by 2% agarose gel electrophoresis.

Signals with EtBr staining was detected and quantified using a CCD camera-based instrument (UVP, Upland, CA). For semi-quantitative measurement, various cycle numbers and amounts of cDNAs were used to test the linearity of PCR reactions. The level of mRNA was estimated from the data that were in the linear range with respect to the amount of cDNA used. Levels of cardiac mRNAs were normalized using GAPDH mRNA for comparison.

3.6 IMMUNOBLOT ANALYSIS

Cells were washed with cold phosphate-buffered saline (PBS). Washed cells were lyzed with 100 μL RIPA buffer (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail (1:100 dilution, Pierce). Protein concentration was determined using BSA protein assay reagents (Pierce) using BSA as a control. Equal amounts of protein from each sample were loaded on a 10% SDS-PAGE gel (Pierce) for gel electrophoresis. The separated proteins were transferred to nitrocellulose membrane. The membrane was coated with Blocking solution (Pierce) at room temperature for at least 1 hours, and probed with monoclonal antibody specific for PKC-alpha (Upstate, Millipore, Milford, MA) at 1 μg/ml in Blocking solution at 4 °C for 2 hours. After washing with PBA supplemented with 0.1% Tween 20 three times, the membrane was incubated with secondary donkey anti-mouse IgG conjugated with peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1/10,000 in Blocking solution for 1 hour. The membrane was washed four times with Tween-containing PBS each for 2 minutes. Immunoreactive signals were detected with chemiluminescent reagents (Pierce) using a CCD camera-based instrument (UVP).

3.7 IMMUNOFLUORESCENCE STAINING

Cells were washed once with ice-cold PBS and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. After washing twice with PBS, fixed cells were incubated with PBS supplemented with 0.1% Triton-X for 15 minutes, and then non-specific binding was blocked using Normal Goat Serum (NGS). Monoclonal antibody for cardiac actin (Biodesign

International, Saco, ME) and troponin T/I (Biomedia, Beaufort, SC) was applied at 4 °C overnight. Cells were washed with PBS, followed by incubation with fluorescent-tagged secondary antibody Alexa Fluor-488 (Invitrogen) for 1 hour. After subsequent washes with PBS, stained cells was viewed by fluorescent microscopy (Olympus Provis, OLYMPUS Optical Co., Led., Tokyo). To provide a quick assessment of cell distribution, 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (0.6 μg/mL) was used to stain the nuclei of cells.

3.8 MOLECULAR ANALYSIS

Small interfering RNAs (siRNAs) were obtained from commercial sources. siRNA duplex oligo targetin PKC-alpha (on-TARGET plus SMART pool) and non-targeting duplex oligo were obtained from Dharmacon (Chicago, IL). siRNAs for PKC-delta and the corresponding control were obtained from Novus Biologicals (Littleton, CO) and IDT (Integreated DNA Technologies, online). Mouse cDNA for PKC-delta was obtained as an EST clone form Research Genetics (Invitrogen). The full-length insert was subcloned into pcDNA3 (Invitrogen).

siRNA and cDNA were transfected into ADSCs using cationic lipid reagents. Briefly, ADSCs were plated at a density of $10 \times 10^3/cm^2$ in a 100-mm dish, 24 hour prior to transfection. Cells were transfected with expression vector (2 μg) or siRNA (100 nM) using Lipofectamin Plus reagents (Invitrogen). The ratio used for the transfection was (plasmid/siRNa:Lipofectamin:PLUS = 1:2:4). Cells were treated with the mixture for 2 hours using serum/antibiotics-free medium in a CO_2 incubator. The mixture was removed and further cultured in the standard medium for one or 3 days.

For cDNA overexpressoin, we cotransfected with a small amount of GFP-C1 (100ng/100-mm dish) to mark transfected cells. After transfection, transfected ADSCs were separated using flow cytometry. Briefly, cells were detached from culture dishes using 0.25% trypsin/EDTA and washed with PBS twice. The collected cells were suspended in PBA supplemented with 0.1% serum contain at 10^6/mL and were sorted using a FACS caliber (Becton Dickinson).

3.9 STATISTICAL ANALYSIS

We used mRNA levels for cardiac genes relative to the control value (10% FBS or vehicle) in one set of experiment for statistical comparison. Statistical analysis of one data set to the control value was done using Wilcoxon singed rank test. Two data sets with variables were statistically analyzed using two-tailed paired t test. The threshold for statistical significance was set at $p<0.05$ in both analyses.

We used two types of statistical analysis in this study. We have set experiments for mRNA measurement to include 10% FBS or vehicle as a control. For example, we compared serum conditions by setting 10% FBS, 5% calf serum and no serum (0.1% BSA). Since no absolute mRNA level can be determined in this assay, cardiac mRNA levels were given as the values relative to 10% FBS (i.e. no serum/10% FBS). Thus, the situation provided one numerical valuable, and statistical comparisons to 10% FBS (control) were done using Wilcoxon-singed rank test.

When testing the effect of drug on PMA-induced change, each experimental set included four groups. For instance, we tested the nPKC inhibitor for the PMA-induced increase in cardiac

gene expression with four groups: vehicle-vehicle (A), vehicle-PMA (B), nPKC inhibitor-vehicle (C) and nPKC inhibitor-PMA (D). Relative increase by PMA were given B/A in the presence of vehicle and D/C in the presence of nPKC inhibitor. Since the PMA-induced increase in cardiac gene expression largely varied in one preparation to another, we chose to use two-tailed paired *t*-test for statistical comparison for the inhibitor effects.

Since we limited the use of ADSCs in certain passage numbers limited experimental sets could be produced. Therefore, we corrected all the data from the experiment sets that included the target experimental groups for statistical comparison. The threshold for statistical significance was set at $p<0.05$ in both analysis.

Table 3-1 Primers designed for RT-PCR

Name	GenBank Accession#	Primer Sequence	Position	Tm	Length (bp)
Cardiogenic					
GAPDH	NM_002046.3	GTCAACGGATTTGGTCGTATTG CATGGGTGGAATCATATTGGAA	124-262	58	139
GATA-4	NM_002052.2	TCCCTCTTCCCTCCTCAAAT TCAGCGTGTAAAGGCATCTG	1851-2044	58	193
Nkx2.5	NM_004387.2	AGCCCTGGCTACAGCTGCA TGGGAGCCCCTTCTCCCC	969-1230	62	262
Mef2C	NM_002397.2	AGTGGTTTCCGTAGCAACTCCT TAGTGCAAGCTCCCAACTGACT	1283-1512	62	228
α-cardiac actin	NM_005159.3	TCTATGAGGGCTACGCTTTG GCCAATAGTGATGACTTGGC	677-936	58	260
Troponin-T	NM_000364.2	AGAGCGGAAAAGTGGGAAGA CTGGTTATCGTTGATCCTGT	672-906	58	235
beta-myocin heavy chain (βMHC)	NM_000257.1	CGAGGCAAGCTCACCTACAC CATTAACAGCCTCCACGGCC	3993-4311	62	319
Connexin43	NM_000165.2	CAAGGTTGCCCAAACTGATGGT TGATGTGGGCAGGGATCTCTTT	546-791	62	244
Pluripotency					
Oct-4	NM_203289.2	GGGGTTCTATTTGGGAAGGTA GTTGTGCATAGTCGCTGCTTGA	330-699	62	370
Rex1	NM_174900.3	GGAATGTGGGAAAGCGTTCGTT TTTGCATGCGTTAGGATGTGGG	1068-1310	62	241
Nanog	NM_024865.1	ATAGCAATGGTGTGACGCA GATTGTTCCAGGATTGGGTG	698-916	58	217

Table 3-1 (Continued)

Name	GenBank Accession#	Primer Sequence	Position	Tm	Length (bp)
PKC isoforms					
PKC-alpha	NM_002737.2	TCCAACAACCTTGACCGAGT TTTGTCAAGCAGGGCCAAGA	1026-1232	58	207
PKC-beta	NM_002738.5	ACGCTGCAGAAATTGCCATC TCCAAATGCCCACCAATCCA	1512-1759	58	248
PKC-gamma	NM_002739.3	AAGTTCACCGCTCGCTTCTT TATGCAGGCGGAACTTGTGT	391-601	58	211
PKC-delta	NM_006254.3	TGTAACGCTGCCATCCACAA ATGCCGCAGTCTTCACACTT	927-1150	58	224
PKC-epsilon	NM_005400.2	TTCGTCACCGATGTGTGCAA TTCCCTGAACACACGCTCTT	402-644	58	243
PKC-eta	NM_006255.3	TCGTCCATAAACGCTGCCAT ACACTGAAGTCCTTGTCGCA	931-1133	58	203
PKC-theta	NM_006257.2	CCGACAATGCAATGCAGCAA TGCACATTCATGCCACATGC	645-884	58	240

4.0 CHARACTERIZE ADIPOSE-DERIVED STEM CELLS EXPANDED IN CULTURE (AIM I)

4.1 INTRODUCTION

Adipose-derived stem cells (ADSCs) represent an attractive source of mesenchymal stem cells for patient-oriented cell therapy, because of their abundance and easy harvest with less-invasive surgical operations. Several cell surface markers, such as Stro-1, CD29, CD44 and CD90, are known to identify mesenchymal stem cells [85-86]. Previous studies have shown that ADSCs express these cell surface markers, CD29, CD44 and CD90 [45-47]. Yet, only a small fraction of these cells seem to show differentiated phenotypes upon treatment with drugs and growth factors. For example, only a small number of spontaneously-beating cardiomyocyte-like cells were obtained by treatment with 5-azacytidine [87-88]. Furthermore, the induction of differentiated phenotypes usually requires extended culture of cells for weeks or months. Thus, we wondered whether mesenchymal marker expression represents the differentiation potential of ADSCs. We used human ADSCs cultured for varying periods of time to examine the changes in expression of mesenchymal and pluripotent markers.

4.2 EXPERIMENTAL PROCEDURE

4.2.1 Preparation of ADSCs

ADSCs prepared in our laboratory and from a commercial source were used for experiments in this part. ADSCs prepared in our laboratory originated from three female patients, whereas the one from a commercial source was from a 44 years-old female patient. ADSCs were expanded in monolayer with serial passages at confluence. For ADSCs prepared in our laboratory, the passage number 0 indicates those obtained by attachment to plastic dishes in the isolation procedure. For ADSC from a commercial source, the passage number 1 represents the initial culture form a frozen stock provided by the company. According to the information provided by the company, these cells were expanded in culture for less than 3 passages.

4.2.2 Morphology

Cell morphology was checked with pictures taken under phase-contrast images using Nikon microscope (Melville, NY). We compared the size and several characteristics, such as the lengths, number and shape of extrusions and cell shape. The karyotype was evaluated by CellLine Genetics LLC. (Madison, WI). In addition, cell proliferation was determined by directly counting cell numbers in various days after plating.

4.2.3 Mesenchymal markers

Flow cytometry was used to determine the distribution of mesenchymal cell surface markers (CD29, CD44 and CD90) in ADSC populations [36-38, 85-86]. Cells were detached

from culture dishes using 0.25% trypsin and 1 mM EDTA. After washing with PBS, cells were incubated with primary antibody against mesenchymal markers (1:250 dilutions) for 1 hour. Cells were washed twice with PBS and incubated with goat anti-mouse IgG conjugated with Alex Fluor 488 (Invitrogen). After eliminating unbound antibody by washing, marked cells were subjected to cell sorter (Becton Dickinson, Rockville, MD). Cell surface marker proteins was examined in ADSC preparations from the two sources over several passages (4, 7-9 and 15-16 passages).

4.2.4 Pluripotent markers

RT-PCR was used to detect mRNAs for mRNAs for pluripotent genes (Nanog, Oct4, Sox2 and Rex1). GAPDH mRNA was detected for normalization purpose. ADSCs from the two different sources over multiple passages (2-3, 5-6, 10-11 and 15-16 passages) were examined for expression of pluripotent genes using RT-PCR, as described in Materials and Methods. .

4.3 RESULTS

4.3.1 Morphology and karyotypes

Figure 4-1 shows a phase contrast picture of ADSCs prepared in our laboratory. A majority of cells in this preparation exhibited multiple extensions from a relatively round cell body, whereas a small number of cells (less than 5%) showed a flat and extended cell body with

shorter and less obvious extensions. Almost all ADSCs from a commercial source were morphologically identical to the former, with a very small number flat cells (<1%). We detected no apparent morphological changes in ADSCs cultured over 20 passages. Karyotype analysis of ADSCs from the two preparations at passage 4 and 5 demonstrated a normal female karyotype of 46 chromosomes with no aneuploidy, tetraploidy or other visible abnormalities. ADSCs prepared in our laboratory appeared to have a doubling time of ~1.6 days in DMEM/F12 with 10% FBS, whereas purchased ADSCs proliferated a little slower with a doubling time of ~2.4 days.

4.3.2 Expression of mesenchymal markers in ADSCs

Flow cytometry indicated that a large fraction of human ADSCs prepared in our laboratory expressed the three tested mesenchymal markers (CD44, CD29 and CD90) at passage 4 (Figure 4-2A and Table 4-1). Although less clear, the cells were positive with the other marker CD34. In contrast, ADSCs lacked the hematopoietic marker, CD45. Purchased ADSCs at the passage 3 were also positive for these mesenchymal surface markers with less negative populations (Figure 4-2B and Table 4-1). The distribution of these cell markers at this early stage indicates that this ADSC preparation consists of at least two distinct population. In particular, the expression of CD44 and CD29 clearly exhibited two peaks: the major marker-positive population (~75%) and the minor marker-negative population (~25%). Thus, a large fraction of ADSCs at the earliest passage is positive for many mesenchymal markers.

ADSCs at higher passages displayed elevated expression of mesenchymal markers with more uniform distributions (Figure 4-2A and Table 4-1). The peaks of CD44, CD29 and CD34 were shifted to right while being sharper. These changes were associated with a reduction in marker-negative populations. Similar increased and more-uniform expression patterns of

mesenchymal markers were seen in ADSCs from a commercial source at later passages, although CD34 expression seemed to decline at the passage 22 in these cells (Figure 4-2B and Table 4-1). These findings indicate that ADSCs maintain mesenchymal surface markers for extended expansion. However, CD45, a marker for hematopoietic stem cells, appeared in later passages of ADSCs in both preparations. Since a majority of ADSCs were positive with CD45, ADSCs appeared to possess both mesenchymal and hematopoietic markers.

4.3.3 Expression of pluripotent marker genes in ADSCs

It is well-established that pluripotent transcription factors play pivotal roles in differentiation potentials. Yet, less is known about their expression adult stem cells. In ADSCs at earlier passages, Nanog, Oct4 and Rex1 mRNAs were significantly expressed, whereas we failed to detect Sox2 message (Figure 4-3 for ADSCs prepared in our laboratory). Purchased ADSCs at the passage 3 were also positive for Nanog and Rex1; however, no detectable Oct4 or Sox2 mRNAs were seen (Figure4-3B). The levels of mRNAs for the former three pluripotent marker genes decreased as the passage progressed. Semi-quantitative measurement indicated that the levels of pluripotent marker mRNAs were ~half at 5-10 passages compared to those at the passage 2. Nanog and Rex1 mRNAs were similarly decreased in ADSCs from a commercial source during culture (Figure 4-3B). Thus, ADSCs relatively rapidly lose pluripotent marker expression. These data suggest that ADSCs at earlier passage may be more suitable for differentiation.

4.4 DISCUSSION

4.4.1 ADSCs from different sources

One of the potential problems associated with the use of patient-oriented ADSCs is the variability in age, sex and pathological and physiological status of patients. These possible differences could significantly influence differentiation in culture. This study compared ADSCs from two different sources. A large portion of cells in the two preparations appeared similar in morphology and mesenchymal surface marker expression. Furthermore, both preparations maintained expression of these markers over many passages. These observations indicate that mesenchymal marker-positive ADSCs with identical properties are present in a large quantity in different preparations.

4.4.2 Distinct expression patterns of pluripotent and mesenchymal markers

Recent studies demonstrated that pluripotent transcription factors are capable of turning some adult cells to stem cells [25-26]. We found that ADSCs cultured for a short period express mRNAs for several pluripotent markers. ADSCs prepared in our laboratory expressed significant mRNAs for Oct4, Nanog and Rex1, whereas purchased ADSCs were positive with the latter two. In both preparations, pluripotent marker mRNAs were decreased as the passage progressed. Thus, ADSCs appear to contain several pluripotent transcription factors, but lose their expression during culture. Since ADSCs at early passages contain a significant fraction of mesenchymal marker-negative cells, it is possible that pluripotent transcription factors are present in these marker-negative cells.

Although mesenchymal markers are commonly used to identify multipotent adult cells, they are not considered to play any direct role in maintenance of multipotency. As culture passage of ADSCs progressed, the peak position for mesenchymal markers tended to shift towards more positive or stronger expression direction. In addition, the peak seemed to become sharper. These observations could indicate that ADSCs at later passages are better suited for differentiation. However, the hematopoietic marker CD45 became apparent at later passages, suggesting that ADSCs might alter their original properties or might have committed to non-myogenic lineage. Thus, although ADSCs retain mesenchymal markers over extended expansion, they might change their properties, affecting their potential to differentiate towards cardiogenic lineage.

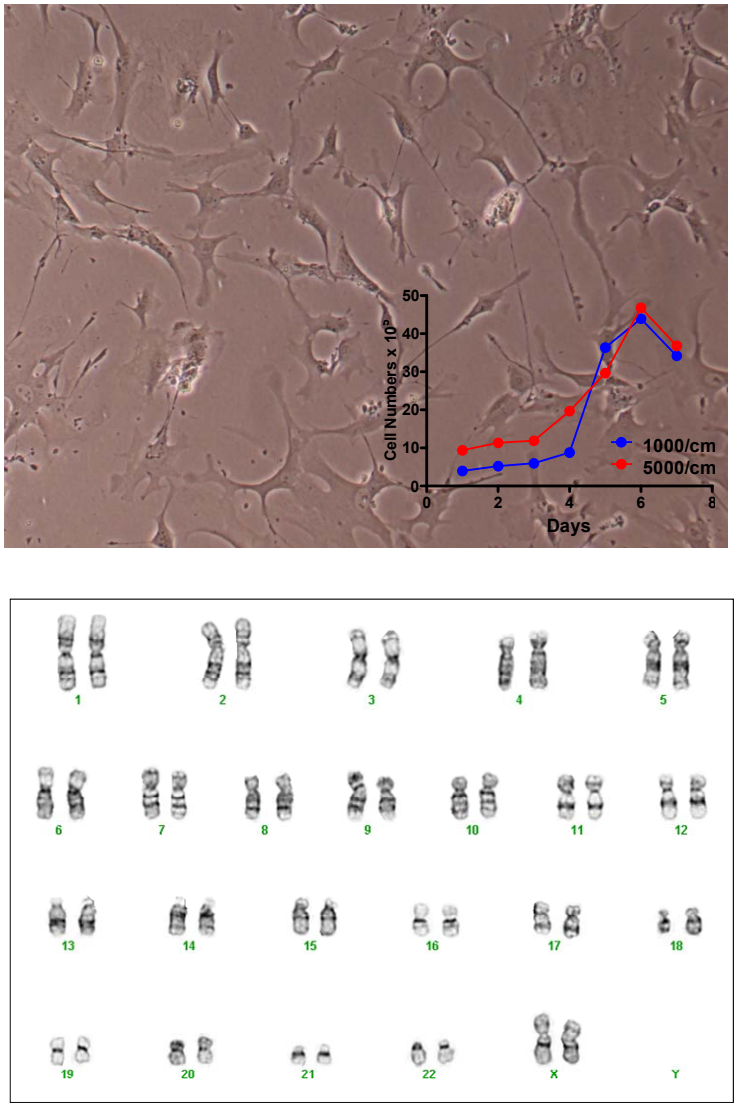


Figure 4-1 Morphology, karyotype and proliferation of human ADSCs in culture

Human ADSCs prepared in our laboratory were cultured in the standard serum-containing medium 10% FBS for 3-4 passages. A picture was taken under phase contrast microscope (top panel). The insert represents cell proliferation when plated at two different initial densities. The doubling time estimated from a logarithmically-growing period was ~1.6 and ~2.2 days at the high and low initial platings. The bottom panel represents normal female karyotype of ADSCs. Note that similar morphology, doubling time and normal karyotype were seen in ADSCs from a commercial source.

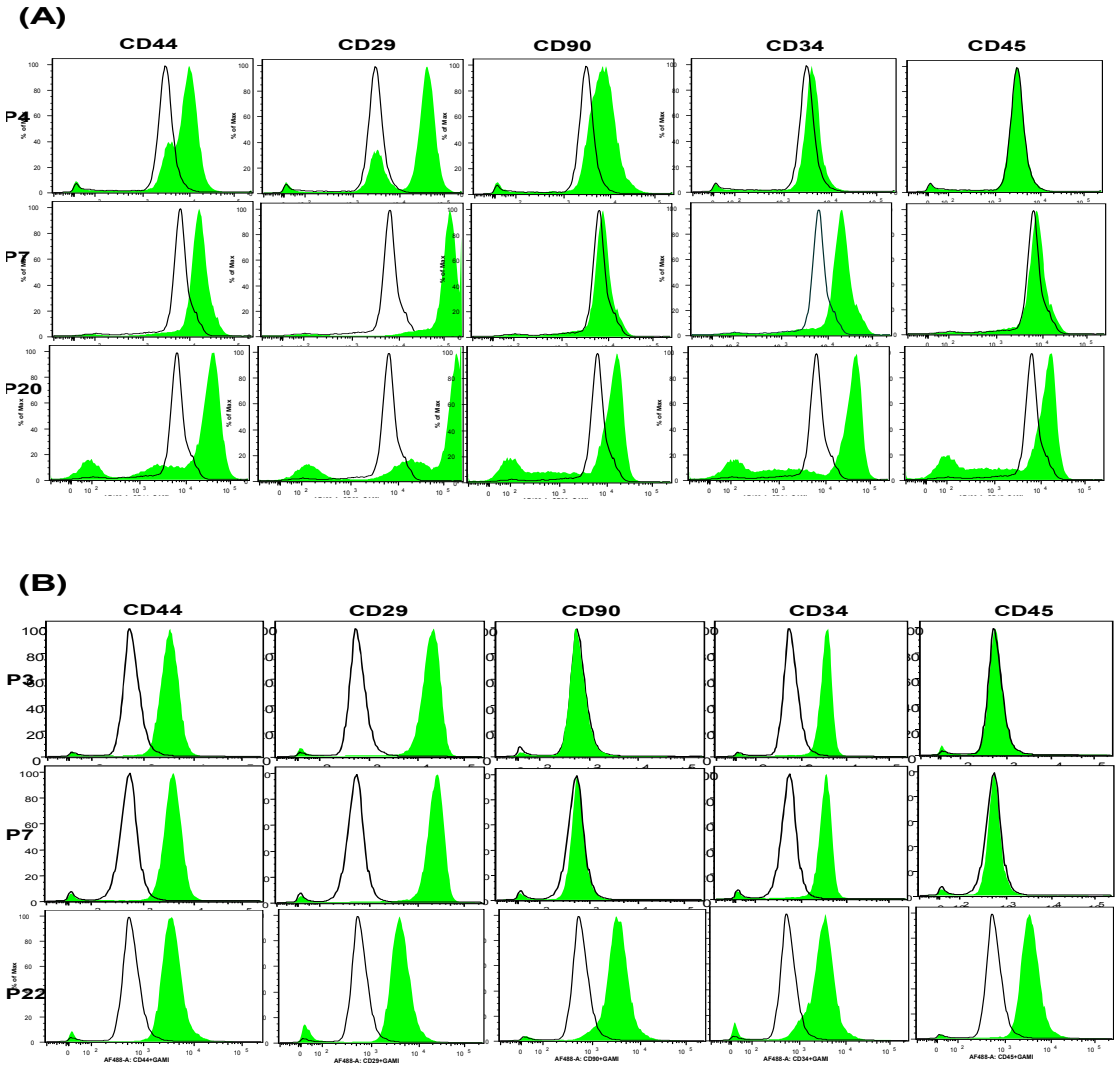


Figure 4-2 Expression of mesenchymal stem cells surface markers by flow cytometry

Flow cytometric analysis for several mesenchymal cell surface markers was performed with ADSCs cultured for various passages. **(A)** CD44, CD29 and CD90 are considered mesenchymal markers, whereas CD45 often marks hematopoietic progenitors. Top: passage 4 (P4); Middle passage 7 (P7); and Bottom: passage 20 (P20). Black lines represent control patterns obtained without primary antibody, whereas green areas with antibody against the indicated mesenchymal markers. **(B)** Commercial ADSCs showed similar patterns and shifts during increasing passages with less mesenchymal marker-negative cells at the beginning.

Table 4-1 Summary for flow cytometric analysis

Marker	Passage Number	Others (%)	Peak-1 (%)	Peak-1 Position	Peak-2 (%)	Peak-2 Position
CD44	P4	0.5	25.5	1250	74.0	10100
	P7	1.5	-	-	98.5	10800
	P20	5.3	-	-	94.7	12200
CD29	P4	0.5	24.8	1230	74.7	46000
	P7	0.2	-	-	99.8	105000
	P20	0.2	-	-	99.8	108000
CD90	P4	0.6	-	-	99.4	1600
	P7	0.2	-	-	99.8	1650
	P20	0.2	-	-	99.8	10200
CD34	P4	1.7	-	-	97.8	1300
	P7	3.8	-	-	93.8	13000
	P20	6.2	-	-	93.8	125000
CD45	P4	1.0	99.0	1200	-	-
	P7	3.7	-	-	96.3	1700
	P20	5.8	-	-	94.2	10300

Marker	Passage Number	Others (%)	Peak-1 (%)	Peak-1 Position	Peak-2 (%)	Peak-2 Position
CD44	P3	1.8	-	-	98.2	1230
	P7	3.9	-	-	96.1	1280
	P22	3.4	-	-	96.6	1220
CD29	P3	0.7	-	-	99.3	10100
	P7	1.3	-	-	98.7	10500
	P22	6.3	-	-	93.7	10600
CD90	P3	0.8	-	-	99.2	150
	P7	3.2	-	-	96.8	150
	P22	3.9	-	-	96.1	150
CD34	P3	2.9	-	-	97.1	1230
	P7	3.6	-	-	96.4	1270
	P22	4.2	-	-	89.0 (6.8*)	1230 (1010*)
CD45	P3	0.8	99.2	130	-	-
	P7	4.2	95.8	130	-	-
	P22	4.3	-	-	95.7	1250

Only major peaks containing more than 5% of total cells were analyzed ("-" less than 5%). Peak-1 represents the one with a peak position that is considered identical to the control without primary antibody. Peak-2 indicates the largest one that appeared at the position distinct from the Peak-1. We included overlapped minor peaks with less 5% of total cells detected by Gaussian distributions in Peak-1 or -2. Left table: ADSCs prepared in our laboratory; Right table Commercial source of ADSCs. *CD34 in P22 cells exhibited an additional peak, which appears as a shoulder on the right side of the main peak.

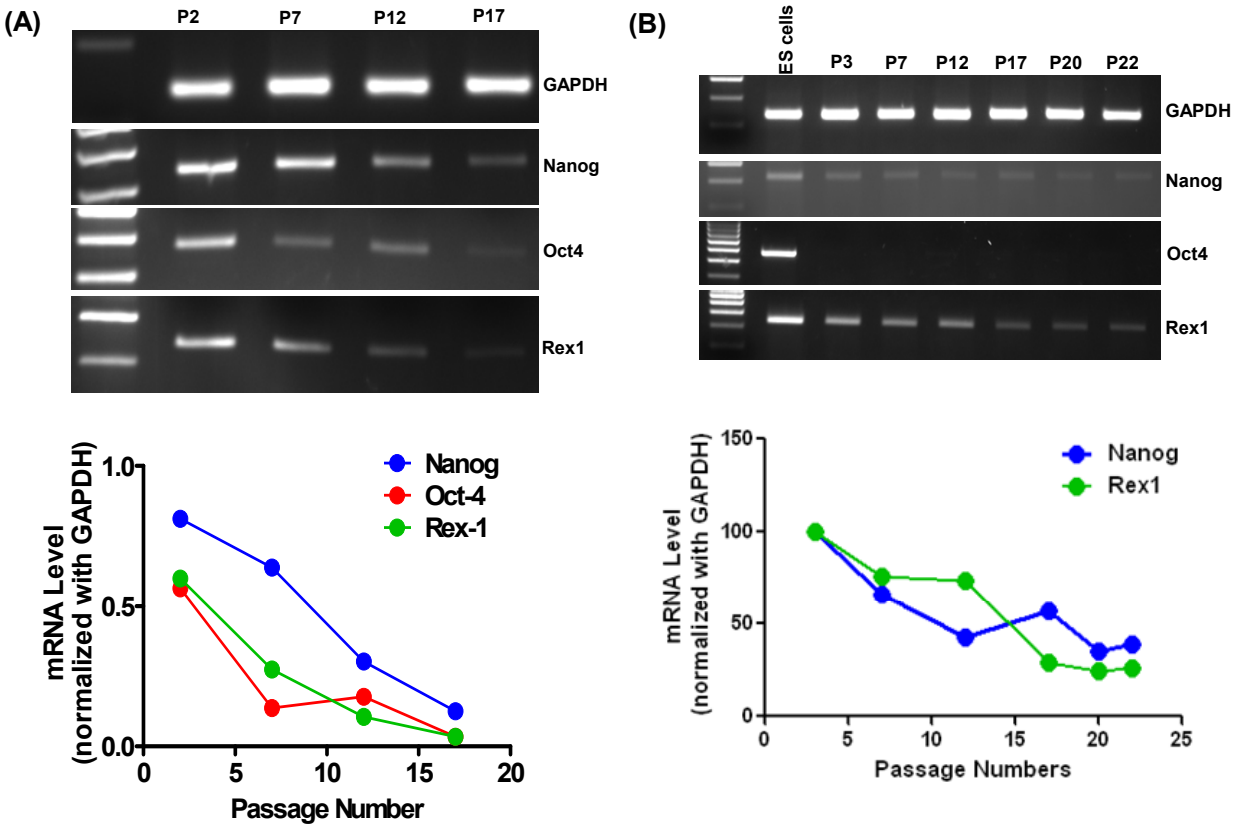


Figure 4-3 Pluripotent mRNA expression in ADSCs at different passages

(A) RT-PCR was used to detect mRNAs for the indicated pluripotent marker genes in ADSCs prepared in our laboratory (top). The mRNA levels normalized with GAPDH mRNA are shown. Note that commercial ADSCs contained significant mRNAs for Oct4 and Nanog, but not Rex1. **(B)** Different source of ADSCs also declined the Nanog and Rex1 genes but Oct4 gene was not present in this preparation.

5.0 EXAMINE CHEMICAL DRUGS AND ELECTRICAL STIMULATION FOR CARDIAC DIFFERENTIATION OF ADIPOSE-DERIVED STEM CELLS (AIM II)

5.1 INTRODUCTION

Cardiomyocytes are known to arise spontaneously from embryonic stem cells when they form aggregates called embryonic bodies with the presence of serum [78, 89]. However, the efficiency of this process is extremely low. Studies indicated that serum removal increased 24-fold the number of contraction areas of differentiated cardiomyocytes, compared with those obtained in the presence of 20% serum [78, 90]. In addition, like the normal developmental process, cardiac commitment within the mesoderm requires endoderm-secreted factors devoid of other general growth-promoting milieu [91-92]. Stimulating effects of serum-free culture on cardiomyocyte differentiation was also observed with several human embryonic stem cell lines [93]. Thus, serum deprivation might provide a better condition for differentiation of ADSCs towards cardiogenic lineage.

Cardiomyogenic differentiation of mesenchymal stem cells was first suggested with the DNA-methylating agent, 5-azacytidine, with an immortalized cell line obtained from bone marrow stromal cells [94-98]. Similar results were reported with human mesenchymal cells using 5-azacytidine [99-102]. Furthermore, inhibition of histone deacetylation by trichostatin A (TSA) has been shown to activate cardiac transcription factors [1]. These findings support the

utility of chromatin-modifying reagents in differentiation of ADSCs towards cardiogenic lineage. The other potential target for differentiation towards cardiac muscle cells is protein kinase C (PKC). Wnt11 signaling is known to direct the fate of cells towards cardiomyogenic lineage during early heart development and in experiments with embryonic and mesenchymal stem cells [66]. Wnt11 effects for cardiac differentiation are prevented by inhibition of PKC. Thus, we tested the ability of chromatin-modifying and PKC-altering chemicals to induce cardiogenic differentiation of ADSCs.

Finally, electrically active cells, such as cardiac myocytes and neurons, respond to direct stimulation with electric current to alter their morphology and function during normal development and in culture [103]. Electrical stimulation has been shown to increase interconnectivity and contractile function of neonatal myocytes [104]. This increased intercellular communication is mainly due to the increased activity of L-type calcium channel [105]. Thus, electric stimulation may also be a useful tool to induce differentiation of stem cells towards cardiac lineages. Taken together, we use several culture conditions, chemical drugs and electrical stimulation individually or in combination to identify the conditions that effectively induce cardiomyogenic differentiation of ADSCs.

5.2 EXPERIMENTAL PROCEDURE

ADSCs at passage number between 3-10 were used for all experiment in this section. ADSCs were plated at 5,000/cm^2 for experiments which require further proliferation in 10% FBS-containing medium, and at 10,000/cm^2 for a cardiogenic induction in serum-free medium. One day after seeding, cells were subjected to various treatments for cardiogenic differentiation.

5.2.1 Serum deprivation

To test the effect of serum concentration on cardiac lineage differentiation, ADSCs were cultured in various culture media: 0.1% BSA (no serum), 1% FBS, 5% Calf serum and 10% FBS for 5 days. Calf serum was used since this serum is known to contain significantly less growth-promoting factors than fetal serum, while it maintains other factors that are essential for survival and preserving differentiated state. The expression of mRNAs for transcription factors (GATA4, Nkx2.5 and Mef2C), gap junction protein (connexin 43), and contractile proteins (cardiac actin, troponin, β-MHC) was determined by RT-PCR analysis.

5.2.2 Chemical drugs

To examine the chemical drugs for cardiomyocyte induction, we tested the effects of DNA methylase inhibitor (RG108), deacetylase inhibitor (Trichostatin A; TSA) and PKC activator (phorbol-12myristate-13 acetate; PMA) on cardiogenic differentiation of ADSCs. The mRNA levels for cardiac genes were determined as described above. We also conducted the flow cytometry and immunostaining for several target proteins.

5.2.3 Electrical stimulation

To evaluate the impact of electrical stimulation on cardiac differentiation of ADSCs, we set up 4 distinct stimulation paradigms, as follow: a) Pulses with 20 ms at 10 or 40 mV at 1 Hz for 24 hours, followed by culturing cells without stimulation for 4 days; b) Pulses with 5 ms at 10 or 40 mV at 0.5 Hz for 24 hours, followed by culturing cells without stimulation for 4 days; c)

Pulses with 5 ms at 10 or 40 mV at 0.5 Hz for 1 hour everyday for 5 days; and d) Pulses with 5 ms at 10 or 40 mV at 0.5 Hz for 10 minutes 3 times spaced with a 10-minutes resting period everyday for 5 days.

5.3 RESULTS

5.3.1 Serum deprivation induces the expression of several cardiac genes

ADSCs from the two preparations showed significant mRNAs for several cardiac genes including Mef2C and cardiac actin in the standard serum-containing medium, whereas those for some others including Nkx2.5 and β-MHC were very low or undetectable levels. Serum-free and low-serum culture conditions were accompanied by increased level of mRNAs for several cardiac genes (Figure 5-1A). Statistical analysis revealed that mRNA levels for Nkx2.5, cardiac actin and troponin were significantly higher in ADSCs cultured in serum-free medium than those maintained in 10% FBS-containing medium (Figure 5-1B, $p<0.05$, n=9-11, Wilcoxon singed rank test). GATA4 and Mef2C mRNA levels also tended to increase upon serum removal (p=0.0674 for GATA4 mRNA and p=0.0547 for Mef2C). However, no apparent expression of β–MHC was detected in any culture conditions. Both 1% FBS and 5% calf serum resulted in less than 2-fold changes in any measured cardiac mRNAs in two independent experiments. These data indicate that low-serum formulas may provide a better condition for other stimuli to promote cardiomyocyte differentiation of ADSCs.

5.3.2 Phorbol ester (PMA) increases the expression of several cardiac genes

We next examined whether genome- and signaling-modifying chemicals might influence cardiac gene expression. Since the previous set of serum withdrawal experiments was performed by culturing ADSCs for 5 days, we treated ADSCs with each chemical for the initial 24 hours and kept cultured cells for additional 4 days in the absence of the chemicals (Figure 5-2A). In the presence of 10% FBS, these chemicals produced only minor effects on the expression of cardiac genes (Figure 5-2B left). Similarly, DNA methylase inhibitor (RG108, 100 nM) and deacetylase inhibitor (TSA, 10 or 100 nM) did not produce clear changes in mRNA levels for cardiac genes in serum-free culture medium (Figure 5-2B right). In contrast, the PKC activator phorbol ester (PMA, 10 nM) caused marked increases in mRNAs for several cardiac marker genes. Statistical analysis indicated that Mef2C, cardiac actin and troponin mRNA levels were significantly higher in PMA than vehicle control (Figure 5-2C, $p<0.05$, $n=9$-11, two tailed paired t test). Similarly to serum removal, PMA tended to increase mRNA levels for other cardiac genes. Thus, treatment with phorbol ester increases the expression of cardiac genes only in the absence of serum.

5.3.2.1 Time course and dose response of PMA effects

Phorbol ester rapidly activates PKCs in a time scale of minutes to a few hours, whereas a long-term treatment for more than a few hours is known to cause down-regulation of these kinases. Therefore, the observed changes in cardiac gene expression might be due to activation or down-regulation of PKCs. To further evaluate the effects of PMA, we examined time course changes in cardiac mRNA levels upon treatment with PMA (Figure 5-3). PMA appeared to produce distinct time course changes in different mRNAs. The level of GATA4 mRNA rapidly

increased upon PMA treatment and kept high until the end of the experimental period (Figure 5-3 left). On the contrary, the expression of troponin mRNA remained low for the initial day or two and gradually increased in later days. Rapid and slow onsets of changes in the expression of GATA4 and troponin mRNAs were also seen upon serum withdrawal (Figure 4-3 right). Thus, the induction of cardiac gene expression by PMA may involve multiple mechanisms.

To test the specificity of PMA, we tested several different concentrations of PMA for the expression of cardiogenic genes (Figure 5-4). The dose dependency of PMA effect was consistent with its PKC activation (Kd for PKC activation ~5 nM). Thus, the PMA-induced upregulation of cardiac mRNAs are mediate by its effects on PKCs.

5.3.2.2 Changes in cardiac protein expression

The detected increase in cardiac mRNA might occur in a small population of cells. Alternatively, a large population of cells might similarly respond to serum removal and following PMA treatment to support the observed increases. To differentiate between these possibilities, we first used immunostaining of cells. Antibodies against cardiac actin and troponin showed positive staining of ADSCs (Figure 5-5). Immunoreactive cardiac actin and troponin exhibited fiber-like structure, reminiscent of cytoskeleton. The immunoreactivities were seen in the entire ADSC populations in all culture conditions. If only a small population of cells differentiated to express cardiac genes, then the variability in staining intensity would be larger in PMA-treated ADSCs than control vehicle-treated cells. Although we did not quantitate the staining intensity, no apparent difference in the staining variability between vehicle-treated and PMA-treated ADSCs. These observations suggest that serum removal and activation of PKCs enhance cardiac gene expression in the entire population of ADSC.

Furthermore, we were able to detect cardiac actin using flow cytometry technique. ADSCs were cultured in the presence of 10% FBS, 0.1% BSA for 5 days or 10 nM PMA for 24 hours, followed by culturing 4 additional days in serum-free medium were subjected to flow cytometric analysis for cardiac actin. ADSCs in all three preparations exhibited a single-peaked distribution for cardiac actin (Figure 5-6). Serum removal shifted the peak to right without apparent changes in the height or width of distribution. PMA treatment also caused a slight right shift without altering the shape of distribution. No apparent widening of the distribution pattern or appearance of a shoulder was seen by either serum withdrawal or PMA treatment. These results support that all ADSCs increase the expression, rather than a subpopulation of cells respond to these stimulation.

5.3.3 Electrical stimulation causes complex changes in the expression of cardiac genes

Next we tested whether electrical stimulation might induce cardiogenic differentiation of ADSCs. Several stimulation paradigms were tested (Figure 5-7 top, see Experimental Procedures). Strong electrical stimulation to 40V-, at high frequency and/or for a longer stimulation period appeared to damage cells, especially in the absence of serum. In all other stimulation paradigms, electrical stimulation caused an apparent increase in the level of cardiac actin mRNA (Figure 5-7 bottom for paradigm (d), others not shown). However, it suppressed the expression of other cardiac genes, such as GATA4 and troponin. Thus, electrical stimulation itself might not be sufficient to induce cardiogenic differentiation of ADSCs.

5.3.4 Combination of PMA treatment and electrical stimulation

Previous our own experiments found that mild electrical stimulation in 10-minutes interval to 10 V for one hour every day is most effective inducing cardiac actin mRNA without causing apparent cell damages. Therefore, we combined PMA treatment with this electrical stimulation in various ways (Figure 5-8). We observed that PMA, regardless of electrical stimulation, produces consistent increases in several cardiac mRNAs, such as Mef2C, cardiac actin and troponin. Electrical stimulation did not cause further increases in the effects produced by PMA. Thus, electrical stimulation is less effective at inducing cardiogenic differentiation by itself or in combination with activation of PKCs.

5.4 DISCUSSION

5.4.1 Cardiogenic gene expression by serum removal and activation of protein kinase Cs

We have evaluated culture conditions, chemical drugs and electrical stimulation for the induction of cardiac gene expression in human ADSCs. These cells seemed to show some deviations in the basal mRNA levels for several cardiac genes, notably cardiac actin and Mef2C. Despite these deviations, serum removal caused significant increases in mRNA levels for several cardiac genes while tended to raise the expression of other tested cardiac genes. Furthermore, activation of PKCs further increased the mRNA levels for these cardiac genes in the absence of serum. The responses to serum removal and PMA were consistently seen in the two preparations

of ADSCs. Thus, serum removal and following activation of PKCs facilitate cardiac gene expression in ADSCs.

The effect of serum removal might be due to elimination of specific factors. Alternatively, a reduced cell proliferation resulted from serum deprivation might secondarily induce the expression of cardiac genes. Since 5% calf serum did not support the upregulation of cardiac genes, it is less likely that specific factors present in FBS prevent the increases in cardiac mRNA levels. Rather, the observed changes in cardiac mRNA levels seemed to be consistent with known potencies of FBS and calf serum to promote cell proliferation. Thus, serum removal may increase cardiac gene expression by shifting from a growing state to a differentiation state.

Activation of PKCs appeared to increase the mRNA levels for many cardiac genes in the absence of serum, but not the presence of serum. Distinct PKCs are known to control cardiac speciation during the normal development and influence cardiogenic gene expression in mesenchymal stem cells from bone marrow. The requirement of serum deprivation might be due to changes in the expression pattern of distinct PKC isoforms. Alternatively, other changes in cellular context associated with serum removal might prime ADSCs for the response to PKC activation. Other interesting finding in this section is that activation of PKCs causes markedly-different time course changes in different cardiac mRNAs. For example, troponin mRNA expression takes one to two days following PMA treatment to raise its level, whereas GATA4 mRNA was rapidly increased upon PMA treatment. PMA treatment is known to result in rapid upregulation followed by downregulation of PKCs. Furthermore, the degree and speed of the upregulation and downregulation differs in distinct PKC isoforms. Hence, the PMA-induced increase in different cardiac mRNAs might involve distinct mechanisms triggered by the drug treatment.

The application of physical stimuli such as chronic stretch and electrical currents have been explored on neonatal and mature myocytes from rats and mice for the induction of maturation and differentiation. Electrical stimulation, as a physical or non-biologic stimulus common to all species, is expected to be more directly translatable across species than cell line- or species-specific growth factors. Furthermore, developmental biologists have demonstrated the presence and possible function of electrical currents setup by ion gradients in the developing embryo. Therefore, we expected that similar electrical environments created in a culture setting might elicit differentiation of ADSCs towards cardiomyocyte lineages. However, electrical stimulation in various settings failed to promote consistent cardiogenic gene expression in ADSCs. The lack of obvious effects might reflect the internal conditions of these cells or external factors that are necessary for this physical stimulus to exert a differentiating effect.

5.4.2 Differentiation vs. Transdifferentiation

The observed increase in cardiac gene expression might arise from two scenarios: (1) a small fraction (population) shifts towards cardiomyogenic phenotype to express cardiac genes, whereas the rest shows no change; and (2) the entire population moderately responds to stimulus to express cardiac genes (Figure 5-9). Immunostaining showed that almost all cells are positive for troponin and cardiac actin. If only a small population of cells differentiated to express cardiac genes, then the variability in staining intensity would be larger in a PMA-treated ADSC population than a control vehicle-treated group. Although we did not quantitate the staining intensity, no apparent difference was observed in the staining variability between vehicle-treated and PMA-treated ADSC population. Moreover, flow cytometry revealed a single-peaked distribution for cardiac actin. Serum removal and PMA treatment slightly shifted these peaks to

right without altering the shape of distribution. There was no apparent widening of the distribution pattern or appearance of a shoulder in these peaks.

These findings support the second possibility: PMA similarly albeit modestly increased expression of cardiac genes in almost all ADSCs. Taken together with the consistent morphology and mesenchymal marker expression in the population of ADSCs, these cells are quite homogenous in their responses to serum removal and PMA to express cardiomyogenic genes. On one hand, our results imply that almost all ADSCs are capable of transdifferentiating towards cardiogenic lineage to express multiple genes. On the other hand, fully-differentiating towards beating cardiomyocytes may require events which are not induced by standard drug treatment strategies.

5.4.3 Non-specific inhibition of deacetylation and methylation for cardiogenic differentiation

In this study, we used TSA, an inhibitor of histone deacetylases (HDAC) for its impact on cardiogenic gene expression in ADSCs. Histone acetylation is generally associated with gene activation. Therefore, inhibiting HDAC is expected to increase acetylation and gene expression. However, we found that the global inhibition of HDAC produced no apparent effects on cardiac gene expression in ADSCs. It is known that HDAC is recruited by other transcription factor Mef2C has been shown to recruit HDAC to control transcription of the downstream genes in embryonic stem cells [117]. Thus, the lack of TSA on cardiac gene expression may be due to other factors that act as a linker to the regulatory regions of these genes.

Methylation inhibitors (5-azacytidine, RG108) are considered to be useful agents in redirecting differentiation pathways of stem cells, because of their impact on the major

epigenetic mechanism. However, the use of these inhibitors has yielded various results. It was first reported that 5-azacytidine induced the generation of beating myocytes from murine mesenchymal stem cells from bone marrow [35]. However, this induction method did not produce the same results in several groups [51, 53, 106]. Similarly, the present study indicated that RG108 is ineffective in increasing cardiogenic gene expression in ADSCs. Moreover, these drugs non-selectively influence the genome structure. They are associated with various toxicities including the potential increase in the incidence of tumor formation. These findings demote the utility of these drugs in cardiogenic differentiation of mesenchymal stem cells.

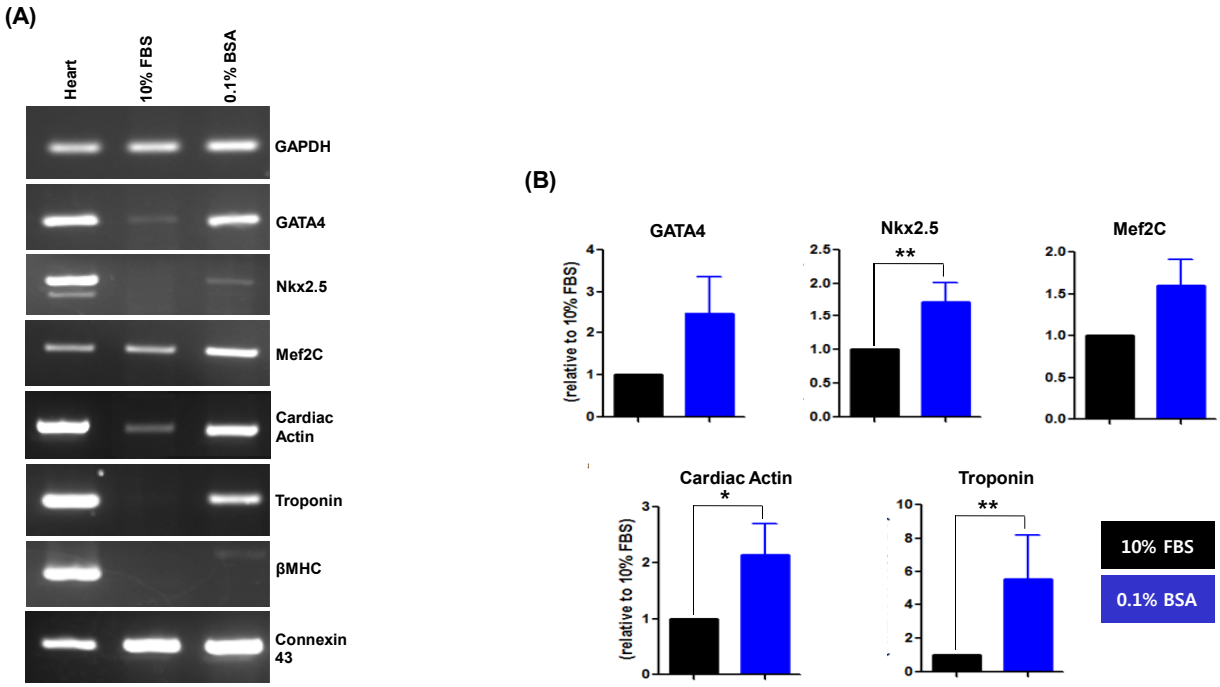
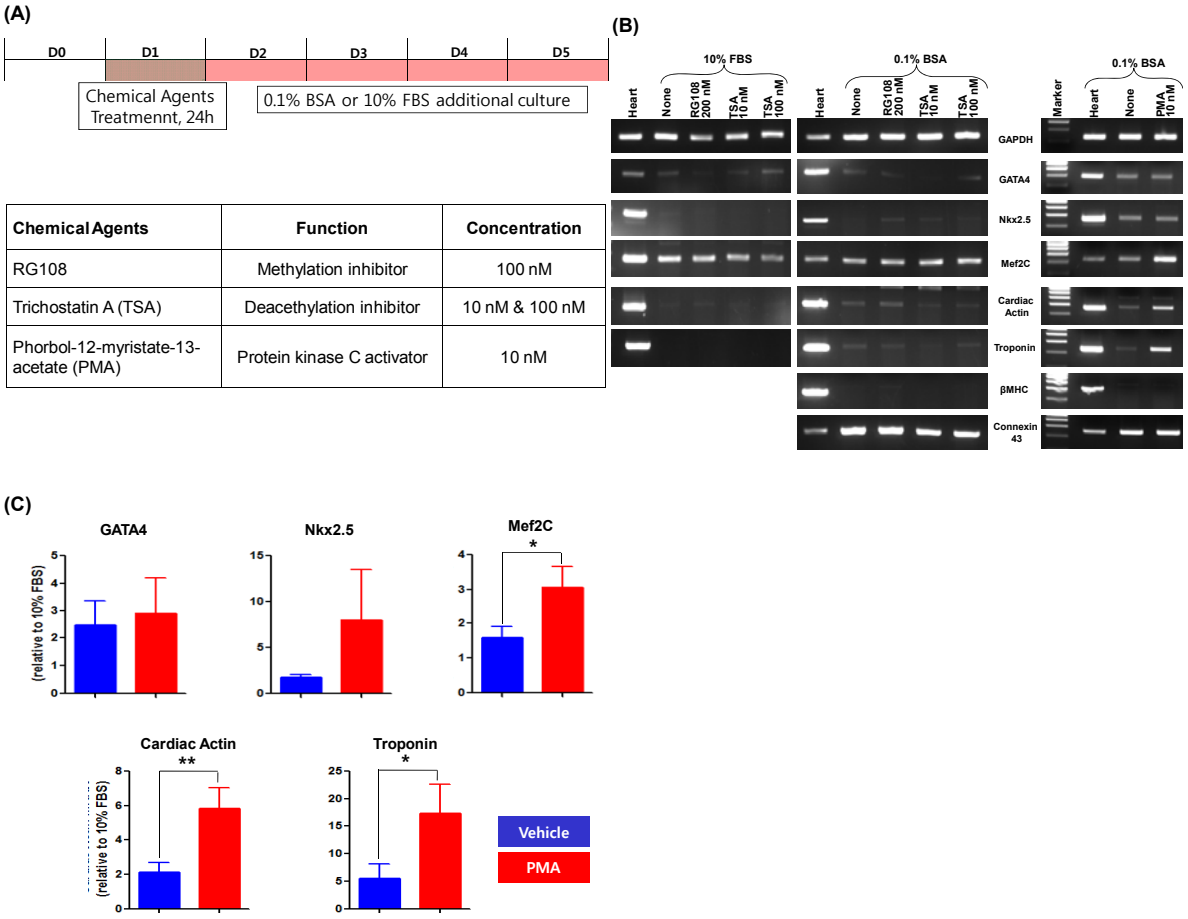


Figure 5-1 Changes in expression of cardiac mRNAs by serum conditions

(A) ADSCs were cultured for 5 days in various serum conditions. RT-PCR was used to detect mRNAs for cardiac genes. **(B)** The mRNA levels were estimated semi quantitatively using GAPDH as internal controls and are presented as a relative value to 10% FBS. *p<0.05 and **p<0.01 (n=9-11, Wilcoxon singed rank test).



Chemical Agents	Function	Concentration
RG108	Methylation inhibitor	100 nM
Trichostatin A (TSA)	Deacethylation inhibitor	10 nM & 100 nM
Phorbol-12-myristate-13-acetate (PMA)	Protein kinase C activator	10 nM

Figure 5-2 Changes in cardiac mRNA expression by chemical reagents

(A) A diagram shows a paradigm for drug treatment (top). ADSCs were plated on plastic dishes in the standard serum-containing medium on Day 0. One day later, serum was removed (pink) and drug was added (dark pink) for 24 hours. Following drug treatment, cells were further cultured in the absence of serum. A table summarizes chemical agents and their concentrations used in this study (bottom). **(B)** A representative RT-PCR data are shown. **(C)** The mRNA levels relative to 10% FBS are shown. *p<0.05 and **p<0.01 (n=9-11, paired t test)

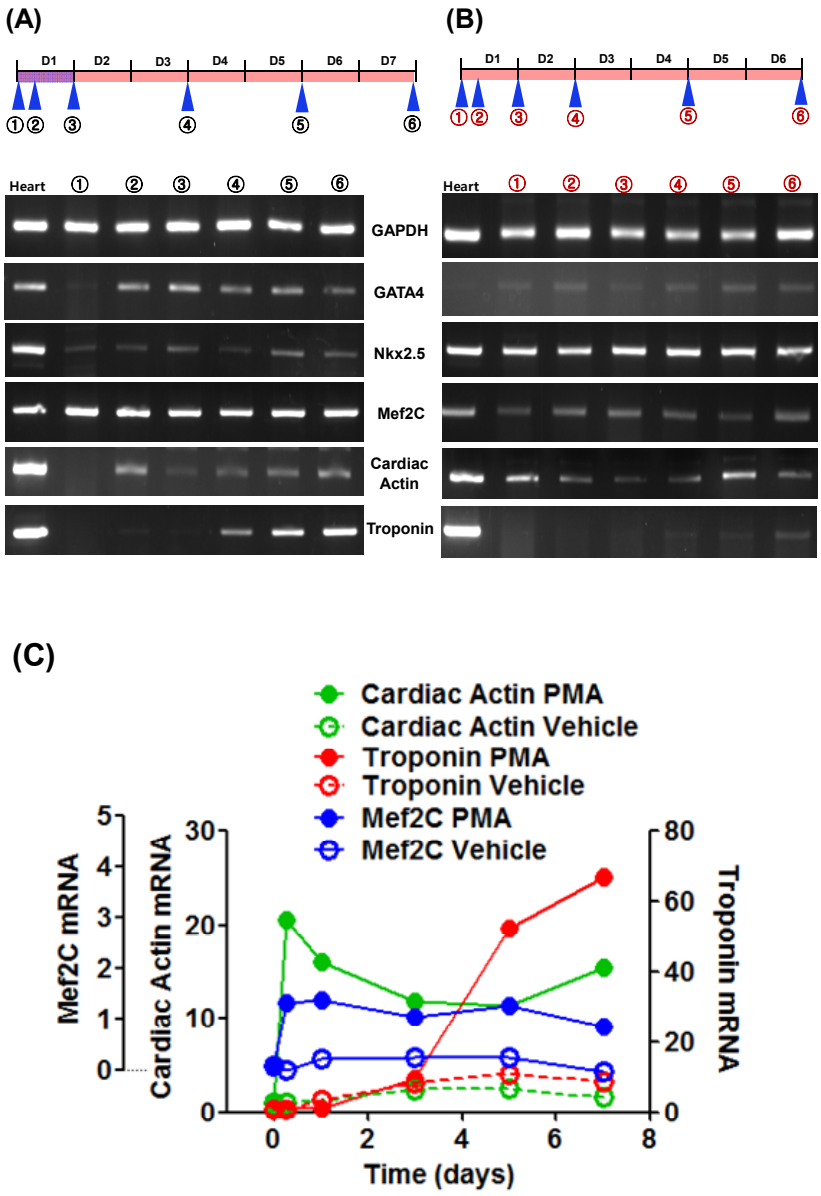


Figure 5-3 Time course changes in cardiac mRNAs upon PMA treatment

ADSCs were treated with PMA for various periods (top). We added PMA at various time points and harvested cells at the same time such that cells were cultured for the same length of time. RT-PCR was used to detect mRNAs in the presence of PMA **(A)** or vehicle **(B).** **(C)** Semi-quantitative measurement was used to obtain mRNA levels relative to control (Time 0). Points represent the mean of two independent experiments.

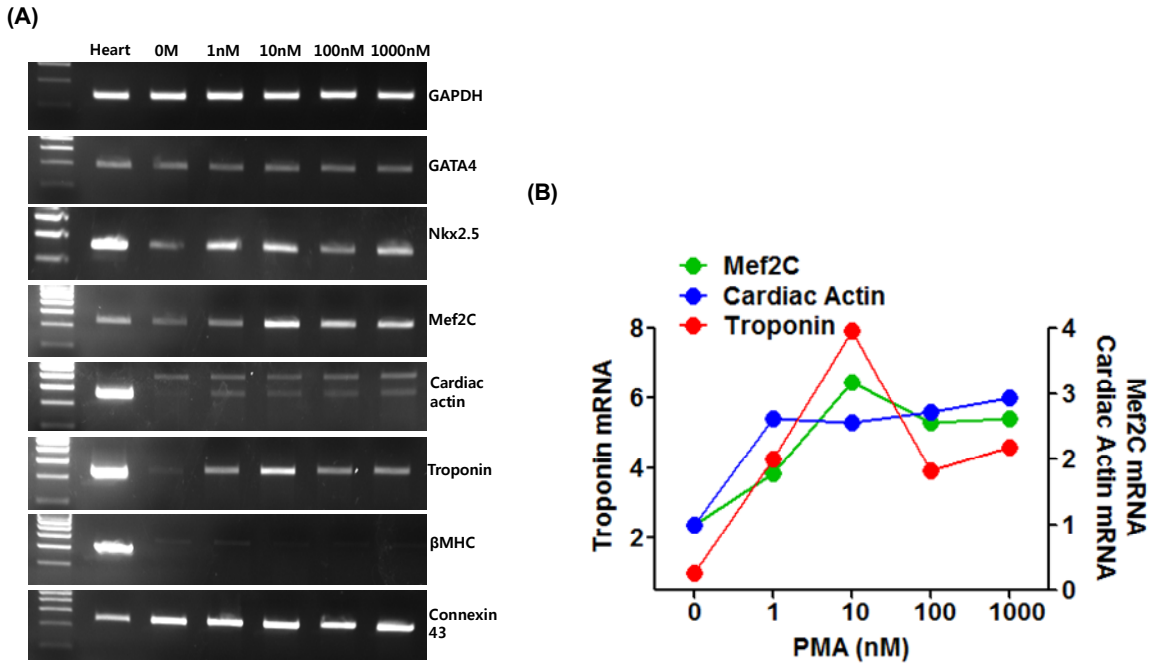


Figure 5-4 Dose-dependent PMA effects on cardiac mRNA expression

ADSCs were treated with the indicated concentrations of PMA or vehicle (0 M) for one day and further cultured for 4 more days in the absence of the drug. **(A)** RT-PCR was used to detect mRNAs. **(B)** Semi-quantitative measurement was used to obtain mRNA levels relative to vehicle without PMA. Points represent the mean of two independent experiments.

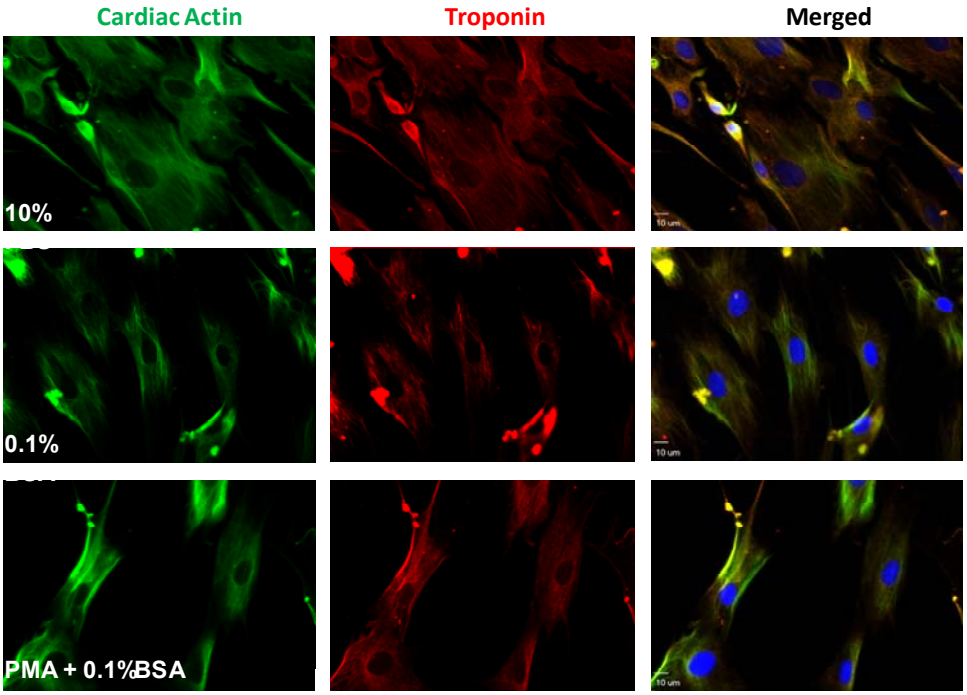


Figure 5-5 Immunofluorescent staining of ADSCs

ADSCs were cultured in standard medium (10% FBS) or serum-free medium (0.1% BSA) for 5 days. PMA (PMA) treatment was done for the first 24 hours in the absence of serum. Fixed cells were stained with the indicated antibody.

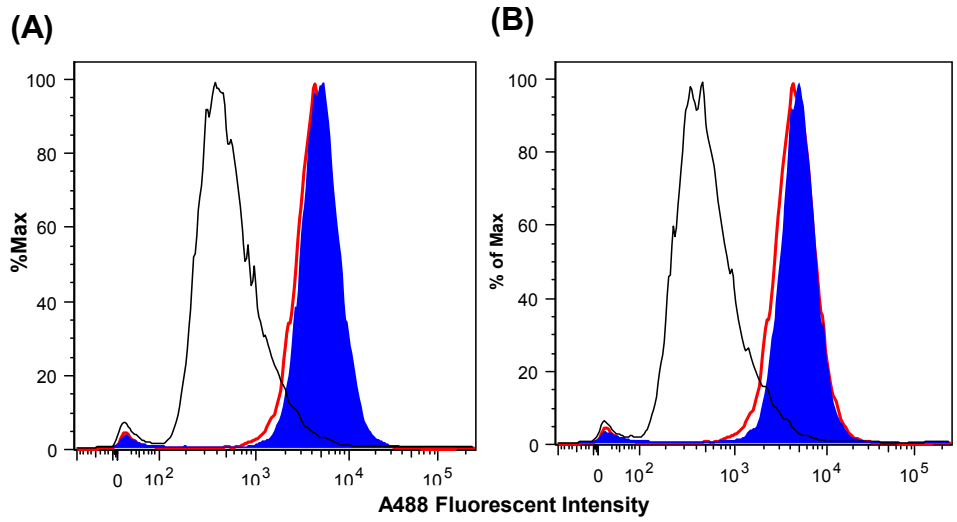


Figure 5-6 Flow cytometry for cardiac protein

Black lines represent negative control without primary antibody. Red line shows the expression of cardiac actin in the standard medium containing 10% FBS medium, whereas blue areas indicate the spread of cardiac actin in **(A)** serum-free culture and **(B)** PMA treatment in serum-free medium. The distribution of cardiac actin-positive cells on flow cytometry exhibited a Gaussian pattern with identical parameters in all treatment conditions. However, the peak position slightly differed: 10% FBS, 1330; 0.1% BSA, 1410; 0.1% BSA+10 nM PMA, 1450. These results indicate that serum removal and following treatment with PMA increased the cardiac actin level in the entire cell population.

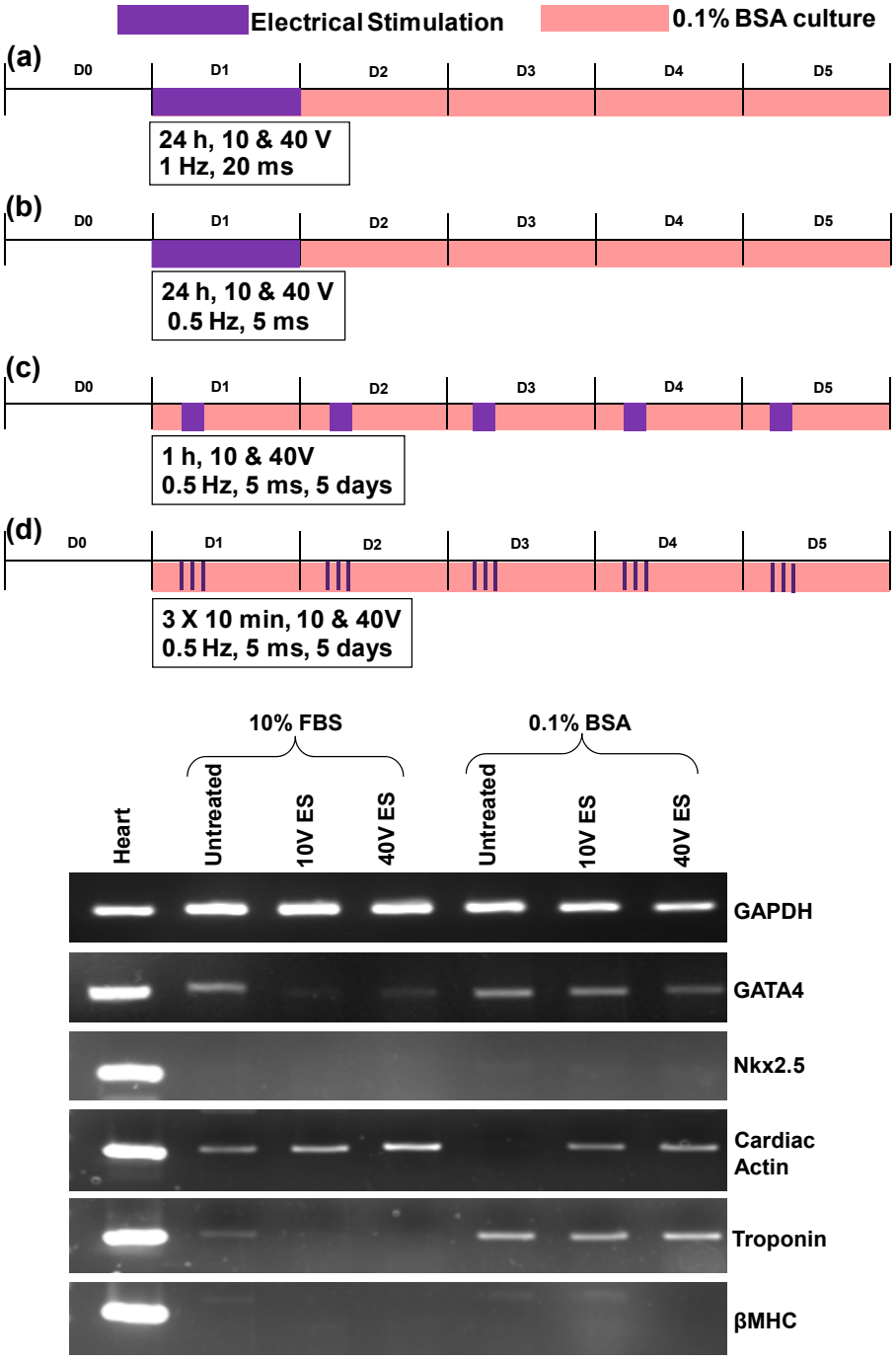


Figure 5-7 Changes in cardiac mRNA expression by electrical stimulation

ADSCs were subjected to electrical stimulation with various settings in the presence of absence of serum (top). RT-PCR results for Paradigm (d) are shown (bottom). Note that the condition (a) and (b) resulted in marked cell death and damage.

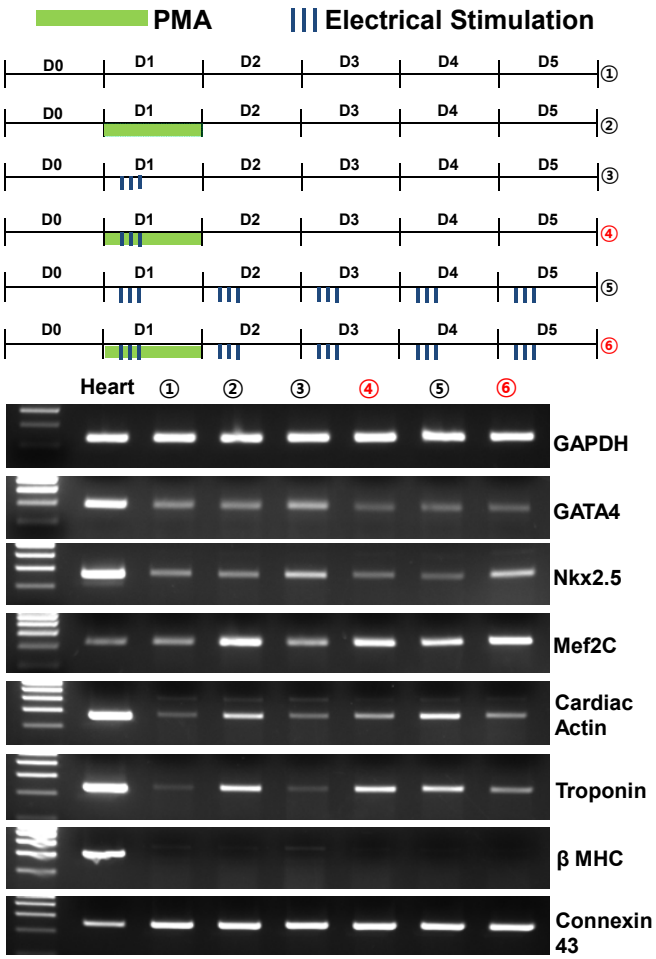


Figure 5-8 Changes in cardiac mRNAs by electrical stimulation and PMA

Electrical stimulation and PMA treatment were combined in various ways (top). RT-PCR data are shown (bottom).

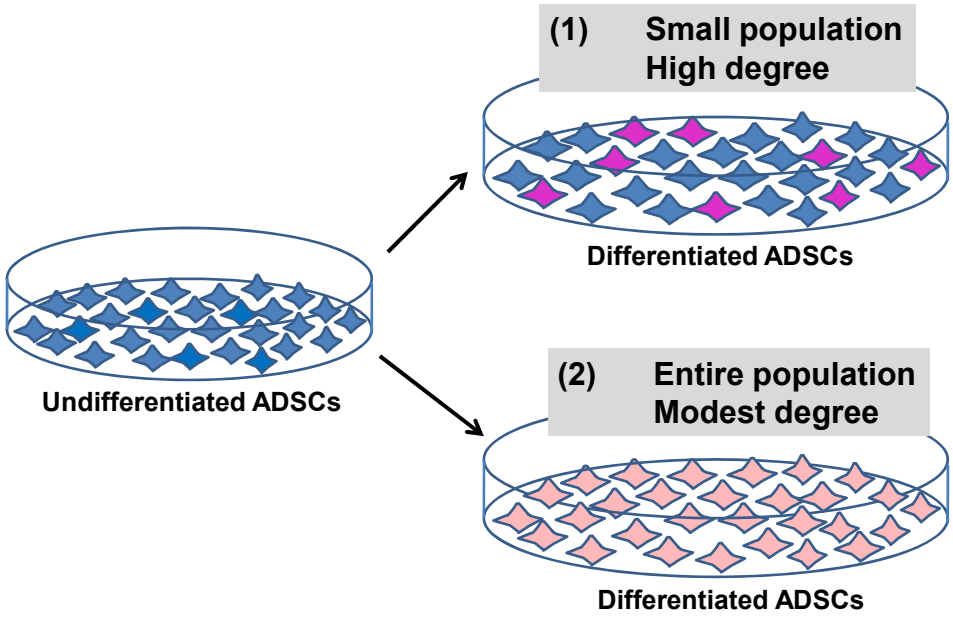


Figure 5-9 Possible changes in ADSC population by serum removal and PMA

A small fraction of ADSCs responds to serum removal or PMA to increase cardiac gene expression (purple, top). Alternatively, the entire population of ADSCs moderately increase gene expression (pink, bottom).

6.0 THE ROLES OF PKC ISOFORMS IN CARDIAC DIFFERENTIATION OF ADIPOSE-DERIVED STEM CELLS (AIM III)

6.1 INTRODUCTION

PKC isoforms are classified into three classes: classical, novel, and atypical. Classical PKCs (alpha-α, beta-β, and gamma-γ) depend upon phospholipids-, and calcium for their activation [107-108]. Novel PKCs (delta-δ, epsilon-ε, eta-η, theta-θ and mu-μ) rely on phospholipids only, but not calcium, while atypical PKCs (zeta-ζ, iota-ι, and lambda-λ) require neither. Because PMA act as phospholipids, it should activate cPKCs and nPKC, in which PMA-induced stimulation of the cPKC involves reducing their binding constant for calcium.

Virtually all tissue types have been shown to contain a diverse array of PKC isoforms, each of which has been implicated in regulating certain cellular events. Preadipogenic murine cell lines are known to express various PKC isoforms including epsilon, eta, delta and zeta. During differentiation from preadipocytes to mature adipocytes, the expression of PKC-epsilon and –zeta increase. Neonatal cardiomyocytes also express multiple PKCs, such as PKC-alpha, -beta I, -beta II, - gamma, -delta, -epsilon, -lambda, and –theta. Increased expression of PKC-alpha was seen during osteoblastic differentiation of bone marrow mesenchymal stem cells [108-110]. However, nothing is known about the expression of PKC isoforms in human ADSCs.

Our initial experiments raised the possibility that different PKC classes play distinct roles in regulating cardiogenic gene expression in ADSCs. Based on these data we propose the hypothesis that nPKC mediates cardiogenic gene expression in ADSCs, while cPKCs inhibits this nPKC action. In this section, we test this hypothesis using various drugs and molecular tools.

6.2 EXPERIMENTAL PROCEDURE

ADSCs at passage numbers from 3-11 were used for experiments. Various drugs selective for PKC subtypes, and siRNA-mediated suppression and overexpression of cDNA were employed to define the involvement of each enzyme in cardiogenic gene expression of ADSCs.

6.2.1 PKC isoforms in ADSCs

RT-PCR analysis was used to detect mRNAs for individual PKC isoforms in ADSCs. Primers for PCR will be chosen to give a 200 – 250 bp product with 20 -24 nucleotides in length using a software (PrimerQuest, IDT), followed by testing for their uniqueness using nucleotide Blast search. (Table 1)

6.2.2 PKC inhibitors

Inhibitors used in this section include chemicals and peptides corresponding to a functional region of individual PKCs (Table 2). These peptides mimic their translocation signal or substrates, and thus competitively block their membrane translocation or substrate binding.

Two substrate mimics are myristoylated to increase their membrane permeability, since they contain many charged amino acids. The PKC theta inhibitor is expected to inhibit PKC delta, in addition to the original target, since the two PKCs share significant sequence identity (information provided by the drug provider, Calbiochem; and Dr. Qiming Wang, Department of Pharmacology University of Pittsburgh). Thus, we call this inhibitor, PKC theta/delta inhibitor in this thesis.

6.2.3 siRNA suppression and cDNA overexpression

siRNA was introduced to ADSCs to suppress the targeted PKC isoform as described in Materials and Methods. We tested expression of the target and other PKC mRNA levels various days after transfection. The target PKC mRNA levels were lower at 3-4 days after transfection than 2 days. Thus, the analysis was done at 3-4 days after transfection unless otherwise stated. For overexpression of cDNA, ADSCs were transfected with expression constructs with the cytomegalovirus promoter and GFP-C1 which contains SV40 promoter-derived fluorescent protein expression unit, followed by isolation of GFP-positive cells.

6.3 RESULTS

6.3.1 PKC isoforms in ADSCs

We first examined which PKC isoforms are present in ADSCs using RT-PCR. Total RNAs was isolated from ADSCs cultured in the presence of 10% FBS or 0.1% BSA for 5 days. We also treated ADSCs with 10 nM PMA for 24 hours followed by culturing cells for 4 additional days in the absence the drug. As controls, adult brain RNAs was used. As expected, brain RNA gave significant products for all isoforms (Figure 6-1). In contrast, only PKC alpha mRNA was significant among cPKC members in ADSCs, whereas RT-PCR detected mRNAs for multiple nPKCs, such as delta, epsilon and eta. Serum removal seemed to decrease the mRNA levels for all detected isoforms, while PMA caused moderate increases in these messages. Either serum removal or PMA treatment appeared to induce mRNAs for the isoforms not seen in cells cultured in 10% FBS or 0.1% BSA.

6.3.2 Identification of inhibitory cPKC isoform

The initial experiment to identify the role of PKC isoforms was performed using two inhibitors with distinct specificities: BIM I, non-selective PKC inhibitors; and Gö6976, specific for cPKCs. If cPKC might mediate the PMA effects, then the latter drug, as well as the non-selective PKC inhibitor, would prevent the PMA-induced increases in mRNAs for cardiac genes. While this experiment failed to reproduce clear PMA effects on cardiac gene expression, the cPKC-selective inhibitor resulted in marked increases in mRNAs for several cardiac genes, such as Mef2C, cardiac actin and troponin (Figure 6-2). The dose dependencies of Gö6976 and

inhibitor specific PKC alpha were consistent with their inhibition of PKC alpha (Figure 6-4). Moreover, other sets of experiments demonstrated that nPKC is required for the PMA-induced increases in mRNAs for cardiac genes (see the next nPKC section). These initial observations raised the possibility that activation of nPKC induces cardiac gene expression, whereas cPKC prevents this upregulation.

We further tried to clarify the inhibitory role of PKC alpha in cardiac gene expression in ADSCs. We found that inhibition of cPKC or PKC alpha resulted in marked increases in mRNAs for several cardiac genes in some preparations, but not others. Statistical analysis of all the obtained data failed to show significant effects by Gö6976 or PKC alpha inhibitor (Figure 6-4). In addition, we successfully reduced expression of PKC alpha with siRNA (Figure 6-5). Transfection with PKC alpha siRNA reduced PKC alpha mRNA and protein levels to less than 20% and ~30% of those obtained with control siRNA. However, PKC alpha siRNA did not result in increased expression of cardiac genes. Therefore, we failed to confirm the inhibitory role of cPKCs in the regulation of cardiac gene expression in ADSCs.

6.3.3 Identification of stimulatory nPKC isoform

ADSCs appeared to express three nPKC isoforms, delta, epsilon and eta. To determine whether nPKCs isoforms are essential for the increase in cardiac gene expression, inhibitory peptides specific for each nPKC isoform was employed (Table 2 bottom). ADSCs were stimulated with PMA (10 nM) for 24 hours in the presence or absence of each nPKC inhibitor (10 μM) or vehicle. ADSCs were further cultured in the absence of any drugs for 4 additional days. If one nPKC inhibitor prevents the PMA-induced increase in cardiac gene expression, then nPKC targeted by this inhibitor would be considered to mediate the PMA effects. PMA

increased mRNA levels for several cardiac genes, such as Mef2C, cardiac actin and troponin in the presence of inhibitor for PKC epsilon or eta, as well as vehicle without any drug (Figure 6-6A). In contrast, inhibitor for PKC theta/delta prevented the PMA-induced increases. Statistical analysis demonstrated that PMA was no longer capable of inducing cardiac mRNA expression in the presence of this inhibitor (Figure 6-6B). Since ADSCs express PKC delta, but not theta, these results indicate that PMA induces cardiac gene expression by activating PKC delta.

To demonstrate the stimulatory role of PKC delta, we used siRNA for PKC delta and overexpression of its cDNA. We obtained three different siRNAs targeted PKC delta from two commercial sources. Transfection with these siRNAs, however, failed to decrease PKC delta mRNA level (data not shown). Indeed, PKC delta siRNA caused a transient increase in the target mRNA, followed by a small but sustained decrease (~20%). We then tried overexpression of PKC delta cDNA. Because transfection efficiency of plasmid DNA might be low in ADSCs, we cotransfected a GFP construct with PKC-delta expression construct or empty vector, and isolated GFP-positive cells by flow cytometry for analysis (Figure 6-7A). Overexpression of PKC-delta cDNA resulted in marked increases in mRNAs for several cardiac genes, such as GATA4, Mef2C, cardiac actin and connexin43 (Figure 6-7B). However, we failed to detect troponin mRNA, which is upregulated by PMA. This lack of upregulation of troponin mRNA might be due to damage caused by transfection and flow cytometry, since transfectioin with empty vector also resulted in low mRNA levels for troponin and others. Thus, these findings demonstrate that PKC-delta induces the upregulation of a subset of cardiac genes in ADSCs.

6.4 DISCUSSION

6.4.1 Regulation of PKCs towards cardiomyocyte in ADSCs

PKCs comprise of over 10 members and play pivotal roles in cellular differentiation and signaling. Almost all cell types express multiple isoforms, which play distinct roles in the cellular event. The present study shows that human ADSCs also express multiple PKC isoforms, such as cPKC alpha, and several nPKCs isoforms including delta, epsilon and eta. Our data also indicate that the nPKC delta is essential for expression of a subset of cardiogenic genes in ADSCs. The use of inhibitor specific for PKC theta/delta and overexpression of the delta isoform strongly support the involvement of this isoform in this process. These results partially prove the initial hypothesis that nPKCs simulates cardiogenic gene expression in ADSCs, whereas cPKCs inhibits this activator action of nPKC. They demonstrated the stimulatory role of nPKCs likely due to the dalta isoform. In contrast, the inhibitory role of cPKCs require further clarification.

Treatment with the inhibitor specific for cPKCs or PKC alpha caused marked increases in mRNAs for several cardiac genes in several sets of experiments. However, we failed to reproduce this result with later experiments. The variability associated with inhibition of cPKC or PKC alpha was not due to differences in the two ADSC preparations used in this study, as marked upregulation or its lack were seen in both preparations. Likewise, the passage number did not appear to account for this inconsistency, as we used limited passage numbers for the experiments in this section. Therefore, we are unable to pinpoint the cause for this problem at this moment. One possibility is that the basal levels of PKC isoforms present in different preparations might influence the outcome. Although we did not extensively measure expression

of PKC isoforms, PKC alpha mRNA level seem to differ from one experiment to another. In addition, we observed apparent changes in PKC alpha mRNA level by transfection procedure. Therefore, the balance between distint PKC isoforms may influence cardiogenic gene expression in ADSCs.

Table 6-1 PKC inhibitors used in this project

Name	Chemical name/structure	IC_{50} and specificity	Working concentrations
Bisindolemaleimide I	2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide	PKCs: ~10 nM	100 nM
Gö6976	12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole	cPKCs: 2-8 nM nPKCs and aPKCs: »1μM	10 nM
PKC-beta inhibitor	3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione	PKC-betaI/II: 5-20 nM PKC-alpha: 330 nM PKC-gamma/delta: »1μM	50 or 100 nM
PKC-epsilon Translocation Inhibitor Peptide	H-GAVSLKPT-OH	Selective for PKC-epsilon	10 μM
PKC-eta Pseudosubstrate Inhibitor, Myristoylated	Myr-TRKRQRAMRRRVHQING-NH_2	Selective for PKC-eta	10 μM
PKC-theta Pseudosubstrate Inhibitor, Myristoylated	Myr-LHQRRGAIKQAKVHHVKC-NH_2	Selective for PKC-theta	10 μM

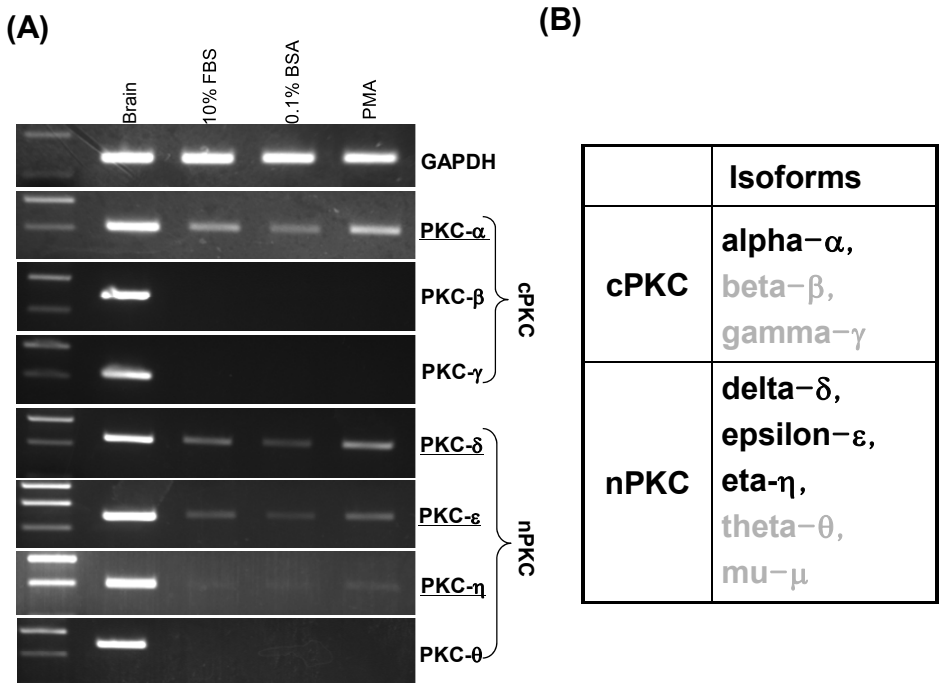


Figure 6-1 Expression of PKC isoforms in ADSCs

ADSCs were cultured with 10% FBS, 0.1% BSA (serum free) or PMA (10 nM) in serum-free medium. **(A)** PKC isoform mRNAs were detected with RT-PCR. **(B)** Only one cPKC isoform and three nPKC enzymes were detected.

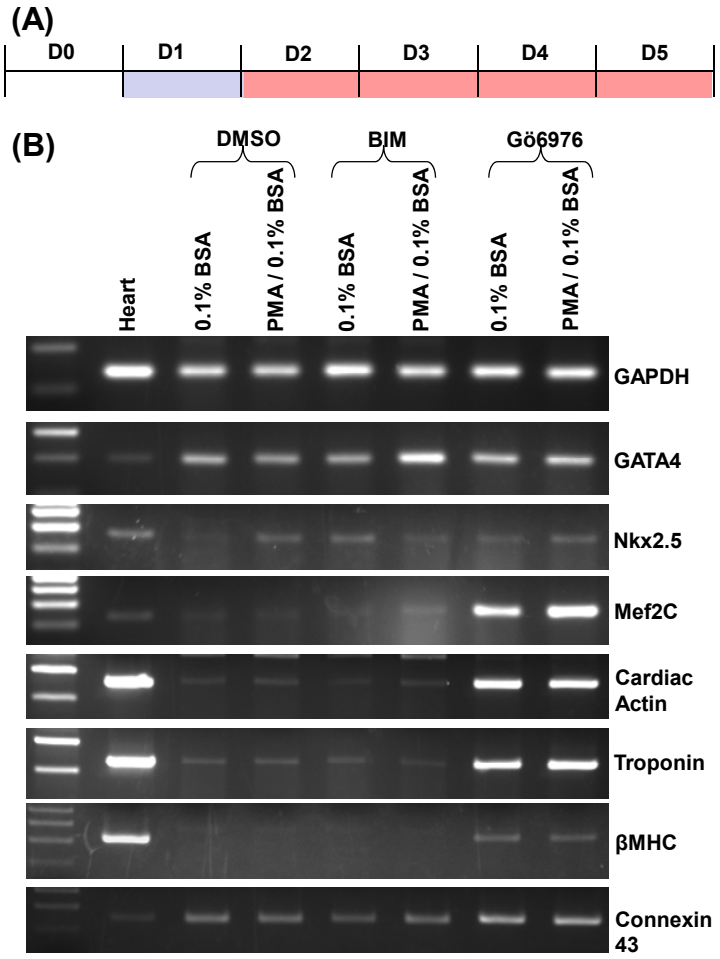


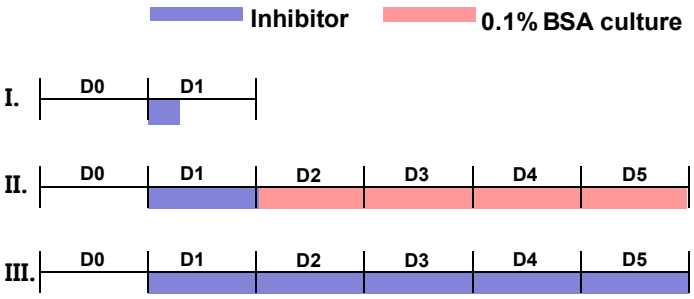
Figure 6-2 Induction of cardiac mRNAs by inhibition of cPKCs

ADSCs were treated with BIM I (non-selective PKC inhibitor), Gö6976 (cPKC-selective inhibitor), or vehicle (DMSO) for 24 hours in the absence of serum and further cultured for 4 additional days (top). Cardiac mRNAs were detected with RT-PCR (bottom).

(A)

Name	Specificity	Concentration
Go6976	All cPKC Blocker	1, 10, 100nM
Alpha Inhibitor	Alpha Blocker	500nM

(B)



(C)

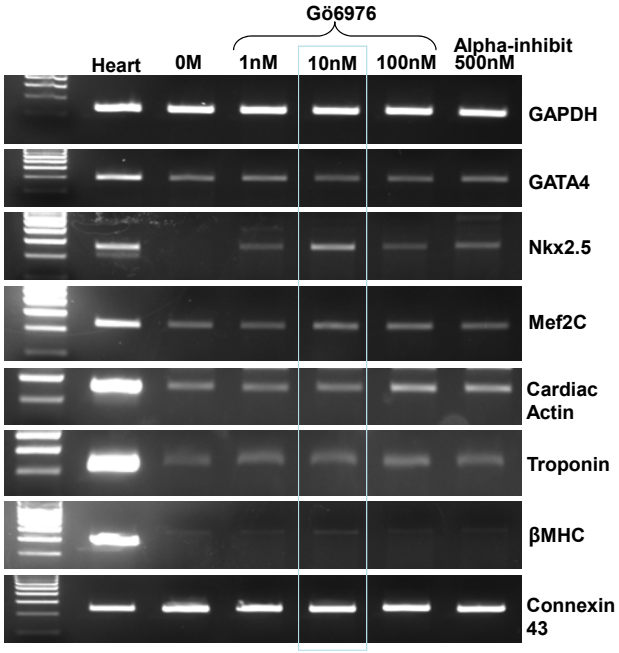


Figure 6-3 Changes in cardiac gene expression by cPKC inhibitors

(A and B) ADSCs were treated with Gö6976 (cPKC inhibitor), PKC-alpha pseudopeptide (PKC-alpha inhibitor) at various concentrations or vehicle for the indicated periods in serum-free medium. **(C)** RT-PCR was used to detect mRNAs for cardiac marker genes.

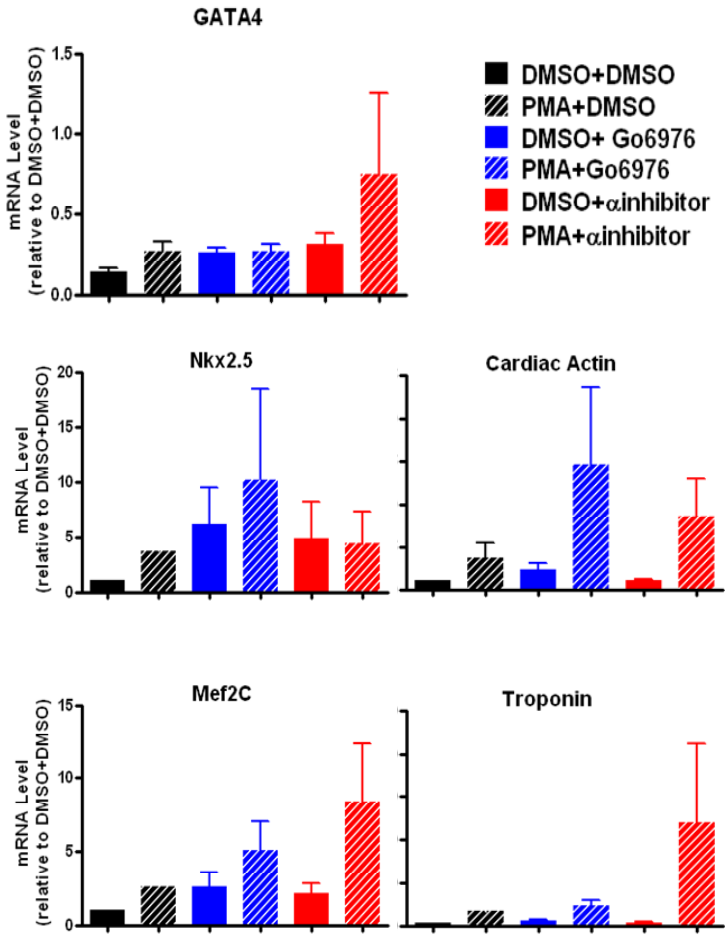


Figure 6-4 Inhibition of cPKC or PKC alpha caused variable effects on cardiac gene expression in ADSCs

ADSCs were treated with PMA or vehicle (DMSO) for 24 hours in the presence of the indicated PKC inhibitor or vehicle (DMSO). Cardiac mRNA levels relative to DMSO+DMSO are shown with error bars indicating SEM. N=12-14 for Gö6976; n=4-7 for alpha inhibitor.

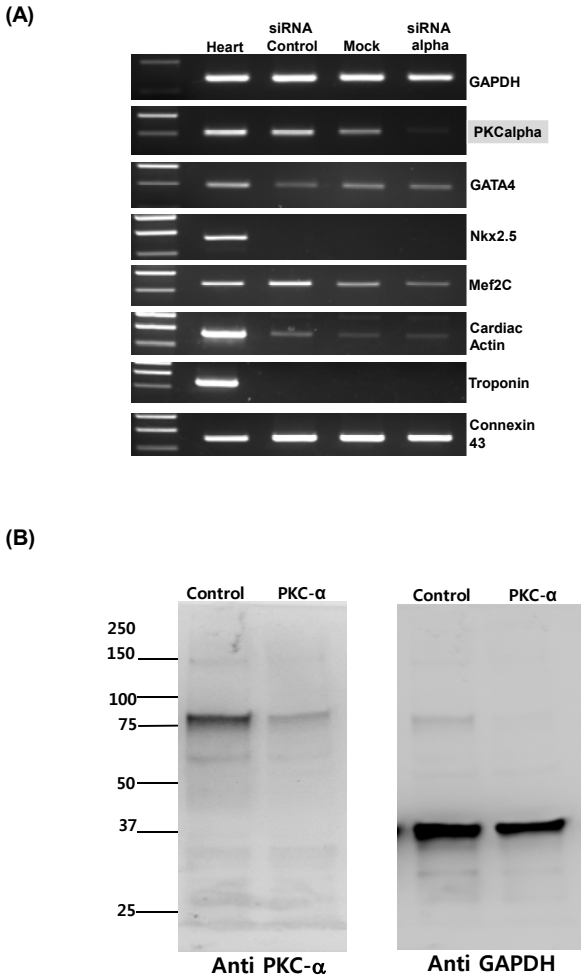


Figure 6-5 Reduced expression of PKC alpha by siRNA does not increase cardiac gene expression in ADSC.

ADSCs were transfected with siRNA targeted to PKC alpha or control siRNA. **(A)** Three days after transfection, mRNAs for PKC alpha and cardiac genes were detected with RT-PCR. **(B)** PKC alpha proteins were detected by immunoblot analysis (left). The same membrane was reprobed with anti-GAPDH antibody. Molecular weights for PKC alpha and GAPDH are ~77 kDa and ~36 kDa, respectively. Note that the signal at ~77 kDa in GAPDH blot is due to anti-PKC alpha antibody. A CCD camera-based quantitation indicated that PKC alpha protein level/GAPDH protein level was 1/3 in siRNA for PKC alpha than control.

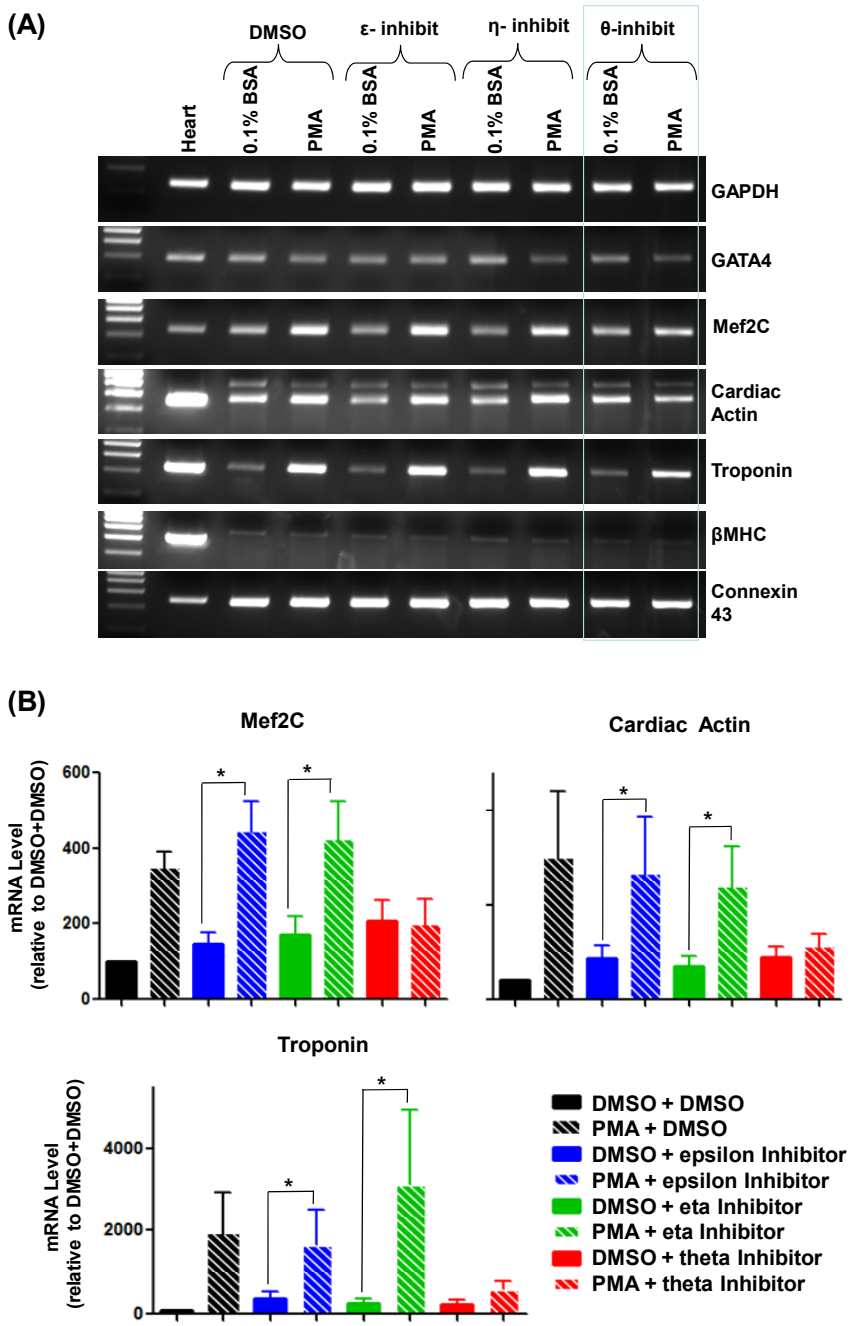


Figure 6-6 PKC theta/delta mediates cardiac gene expression by PMA

(A) ADSCs were treated with PMA or vehicle (DMSO) for 24 hours in the presence of the indicated PKC inhibitor or vehicle (DMSO). A representative RT-PCR data are shown. **(B)** Cardiac mRNA levels relative to DMSO+DMSO are shown with error bars indicating SEM. *p<0.05 (two-tailed paired t test, n=3)

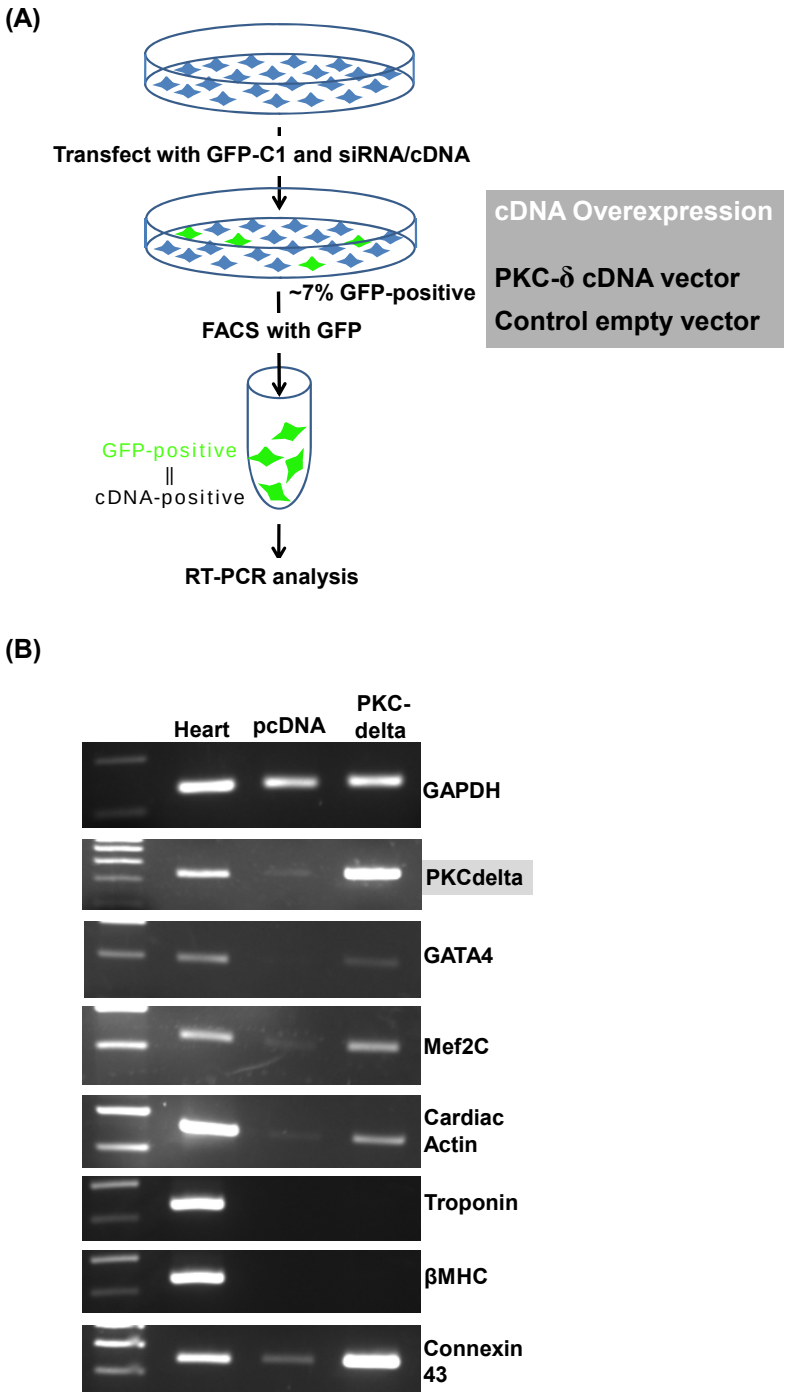


Figure 6-7 Cotransfection strategy for overexpression of PKC delta cDNA

(A) PKC-delta cDNA-transfected cells were isolated using cotransfected GFP. **(B)** isolated transfected cells were examined for expression of PKC delta and cardiac genes by RT-PCR.

7.0 PROJECT SUMMARY AND DISCUSSION

7.1 SUMMARY

ADSCs have attracted many clinicians and basic researchers as a source for patient oriented cell therapy, because of its abundance and easy collection from the body. Yet, little is known about their maintenance during extended expansion in culture. Furthermore, no effective differentiation towards cardiogenic lineage has been established. This project evaluated expression of mesenchymal and pluripotent genes, two of the commonly-used markers for stem cells. It also tested various chemical drugs and electrical stimulation for cardiogenic differentiation of ADSCs. Our study shows for the first time that ADSCs express several pluripotent genes. However, the expression of these genes rapidly declined in culture, while mesenchymal markers are stably present in ADSCs for extended expansion. Our data also establishes that ADSCs exhibit expression of various cardiogenic genes upon serum removal and activation of protein kinase Cs. The novel PKC isoform delta appears to mediate, at least in part, cardiogenic gene expression in these cells. These findings indicate that ADSCs expanded in culture retain the mesenchymal phenotype and are capable of exhibiting some cardiogenic differentiation.

7.2 DISCUSSION

7.2.1 Timing of extracellular stimuli for cardiogenic differentiation of stem cells

Normal cellular speciation and *in vitro* differentiation of embryonic stem cells indicate that differentiation towards mature cardiomyocytes is a complex step by step process. They require cell-cell interaction and other extracellular signals at the right timing. In the developmental embryo, sequential external signals are imposed on progenitors by the migration of progenitors to a new environment and simultaneous changes in surrounding cells. Cardiogenic differentiation of embryonic stem cells *in vitro* also requires extracellular signals at the timing. Aggregation of ES cells into spheres called embryoid bodies triggers their differentiation into all three germ layers: ectoderm, mesoderm, and endoderm. Neonatal myocyte-like cells are spontaneously generated from the embryonic body. However, this process is inefficient. Many hormones and cellular kinases have been shown to influence this process. It is conceivable that differentiation of ADSCs towards cardiomyocytes also requires multiple stimuli in an appropriate sequence. We found that activation of PKCs require serum deprivation to exert its action to increase cardiogenic gene expression in ADSCs. Moreover, the timing and length of treatment with the PKC activator PMA seemed to be important to induce cardiogenic gene expression. For example, continuous presence of PMA for days was less effective at increasing expression of cardiac genes than treatment for 24 hours, followed by culturing cells in its absence. Although we failed to identify appropriate conditions with electrical stimulation, this physical stimulus may also be useful at the right conditions to further facilitate cardiogenic differentiation of ADSCs. Hence, refining the timing, length and order of multiple stimuli may be required to uncover the full potential of ADSCs for cardiogenic differentiation.

The importance of the timing of extracellular stimuli likely reflects internal changes of stem and progenitor cells that allow the given stimulus to exert its differentiation effect. A simplest example is that a hormone to produce its effects requires the presence of its receptors and signal transduction machinery in the cells. In this study, we used the conditioned medium of *Wnt11*-expressin cells for cardiac differentiation of ADSCs. The *Wnt11*-containing medium was failed to induce any changes in cardiac gene expression. In contrast, direct activation of the downstream mediator PKCs with PMA caused significant increases in expression of several cardiac genes. The failure of *Wnt11* to produce similar changes might be due to the lack of its receptors or signaling molecules that mediate the receptor stimulation to PKC activation. Furthermore, this study identified that removal of serum is required for PMA to induce cardiac gene expression. Although the molecular changes associated with serum removal are unknown, it is likely that serum removal influences the downstream mediators of PKC delta for cardiac gene expression. PKC delta is involved in cardiac hypertrophy, oxidative stress and metabolism [111-113]. It has been shown that MAP kinases 1 and 2 mediate PKC delta-induced hypertrophy. Therefore, MAP kinases may be involved in the PKC delta-mediated expression of cardiac genes. These findings also suggest that the introduction of a gene for the mediator(s) may be explored for cardiogenic differentiation of adult stem cells.

7.2.2 Cardiogenic differentiation potential of ADSCs

The hall mark of cardiomyocytes is a self-enforcing rhythmic beating or electrical activity. In addition, the formation of well-organized muscle fibers as demonstrated by the appearance of striation is also used as a defining characteristic of cardiomyocytes. In these respects, cardiogenic differentiation of adult-origin mesenchymal stem cells has been limited to a

very small number of cells or isolated clones after extended culture. Bone marrow-derived mesenchymal stem cells have been shown to turn to cardiac myocytes as xenografts following experimentally-induced infraction or scar, resulting in the improvement of cardiac function [114-116]. However, these studies were associated with very small numbers of donor cells in the recipient myocardium, suggesting that the observed improvement of cardiac function is not due to the myocardial differentiation of mesenchymal stem cells. In this study, we showed that ADSCs maintain mesenchymal surface markers for extended expansion. Morphology and immunostaining suggested the homogeneity of ADSCs cultured over many passages. Consistent with this observation, ADSCs cultured for different times exhibited similar responses to serum removal and activation of PKCs to increase expression of a subset of cardiogenic genes. Thus, mesenchymal marker-positive ADSCs may possess a potential to exhibit some cardiogenic properties upon extracellular stimuli. Yet, these differentiated cells appear to express only a subset of cardiogenic genes. In addition, the expression levels of cardiac genes are substantially lower in these cells than cardiac tissues. Furthermore, no apparent morphological changes or spontaneous beating were associated with these changes in cardiac gene expression. These situations raise the issue of whether the entire mesenchymal cell population may possess a potential to differentiate towards mature myocytes.

In contrast to mesenchymal markers, little attempts have been made on evaluating expression of pluripotent genes in adult stem cells. This is probably because of the assumption that pluripotent transcription factors are only expressed in uncommitted cells only seen in the early development, but not in stem cells of the adult origin. In the present study, it is demonstrated that pluripotent genes are found in ADSCs at early passages. It remained whether the same cells express mesenchymal and pluripotent markers. However, almost all ADSCs from

a commercial source appeared to be positive for mesenchymal markers, supporting that mesenchymal marker-positive cells possess pluripotent mRNAs. It should be noted that ADSCs from the two sources differ in the expression patterns of pluripotent genes. ADSCs prepared in our laboratory were positive for Nanog, Oct-4 and Rex-1, whereas ADSCs from a commercial source lacked Oct-4. Flow cytometric analysis for mesenchymal markers indicated that a significant fraction of the former ADSCs was negative for mesenchymal markers. Therefore, it is possible that Oct4 might be present in mesenchymal marker-negative genes. Yet, it remains unclear whether mesenchymal marker-positive ADSCs possess all the pluripotent genes that may be required for the ability to differentiate towards various cell types.

Pluripotent marker genes encode transcription factors that set gene expression profiles necessary for self-renewal and keep the cells in undifferentiated state. Recent studies have shown that pluripotent transcription factors are capable of providing the embryonic stem cell-like differentiation potential to some adult cells. These findings suggest that pluripotent marker-positive ADSCs at early passages may be better source for differentiation towards cardiomyocytes. They also imply that mesenchymal marker-positive ADSCs may have a limited potential to differentiate towards mature cardiomyocytes.

7.2.3 Implications of this study in clinical applications

By facing patients without any therapeutic options and/or waiting for heart transplant, clinicians wish to use cell-based therapy as soon as possible. Numerous attempts with adult-origin stem cells from bone marrow and other sources are now being evaluated. Some may appear to be effective at improving cardiac function for a significant period of time in future. This study showed that ADSCs are stably expanded in culture for over ten passages, further

supporting the safety and easy expansion of these cells for clinical applications. Thus, ADSCs possess a great potential as a tool for cell-based therapy for cardiac and other disorders.

On the other hand, the present work reemphasized the difficulty in turning mesenchymal stem cells towards functional cardiac myocytes. This study was initiated by the motivation to identify culture conditions and external stimuli for effective generation of cardiac myocytes *in vitro.* However, the changes are limited to increases in a subset of cardiogenic genes without apparent morphological and functional characteristics of cardiac myocytes. These changes are often called "transdifferentiation" which represents abnormal changes that are obtained when the course of normal development is altered by external stimuli. Although 'transdifferentiated cells" may be useful for cell-based cardiac therapy, it poses a risk of the abnormal coordination or communication with other resident cells in the recipient heart.

7.3 FUTURE STUDIES

This project demonstrates that ADSCs are stably expanded for a long period of time in culture. It identified several conditions to promote cardiogenic gene expression in these cells. Yet, this work, as well as previous studies, revealed that standard drug treatment or physical stimuli may only achieve limited and partial differentiation (transdifferentiation) towards cardiogenic lineage. Various virus vectors are now employed in the field of stem cells. Thus, it is considerable to incorporate this approach to introduce desired genes into these cells. ADSCs are easily obtained in a large quantity and stably maintained in culture. Thus, the use of virus-mediated gene transfer can easily be achieved in these cells. This approach may also uncover the

full potential of the identified culture conditions and activation of PKCs to induce robust cardiogenic differentiation of ADSCs.

The present study identified PKC delta as an important mediator for cardiac gene expression in ADSCs. However, the molecular pathway underlying the PKC delta-mediated changes remain unknown. Further studies should aim at elucidating the link between PKC delta and a subset of cardiogenic genes. In addition, we were unable to confirm the roles of other PKC isoforms. In particular, our data suggested the inhibitory action of classical PKCs. The reason for this inconsistency should also be understood. Elucidating the PKC pathways and context under which these kinases produce their effects will provide insights into the development of a better strategy to differentiate ADSCs towards cardiogenic lineages.

Much effort has been focused on producing mature fully-differentiated cells in the field of stem cells. For instance, many studies have been aimed at generating spontaneously-beating cardiomyocytes with well-organized muscle fibers. However, these well-differentiated myocytes may not be suitable in real cell-based therapy. It is desirable to have cells with the adaptability in injected area of the heart to become a part of the heart muscle. Thus, future studies may require not only identifying better differentiation conditions, but also keep the adaptability of cells when injected to a damaged organ.

APPENDIX A

ACRONYM, ABBREVIATION AND SYMBOL DEFINITIONS

ADSCs	Adipose-derived stem cells
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenetic protein-4
BSA	Bovine serum albumin
CD	Cluster differentiation
cDNA	complementary DNA
cPKC	classical PKC
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
β-MHC	betaMyosin heavy chain

mRNA	Messenger ribonucleic acid
NGS	Normal Goat Serum
nPKC	novel PKC
Oct-4	Octamer-4
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PMA	Phorbol myristate acetate
PKC	Protein kinase Cs
siRNAs	Small interfering RNAs
TSA	Trichostatin A

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