Specific differentiation of bone marrow-derived mesenchymal stem cells into cardiogenic cells by PKC regulation

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Specific differentiation of bone marrow-derived mesenchymal stem cells into cardiogenic cells by PKC regulation

Directed by Professor Yangsoo Jang

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December, 2007

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ABSTRACT

Specific differentiation of bone marrow-derived mesenchymal stem cells into cardiogenic cells by PKC regulation

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Under proper stimulation, mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into myocytes, chondrocytes, adipocytes, osteoblasts, and tenocytes, both *in vivo* and *in vitro*. Despite the promising results of myogenic differentiation induced by 5-azacytidine, the molecular mechanisms underlying myogenic differentiation from MSCs remain poorly understood. To achieve effective regeneration of injured myocardium, it is important to find a more physiological way of improving the *in situ* myogenic

differentiation of MSCs. Protein Kinase C (PKC) is one of the signal transduction components involved in the myogenic differentiation of embryonic stem cells. In this study, we investigated the effect of phorbol myristate acetate (PMA), a PKC activator, on myogenic differentiation of MSCs. To confirm the effect of PMA, 1μM PMA-treated MSCs were grown for each two, five or nine days. In sandwich ELISA, the expression of cardiac-specific markers (cardiac troponin T, myosin light chain, myosin heavy chain, NK2 transcription factor-related, locus 5, Myocyte-specific enhancer factor 2) was elevated at nine days. MHC expression in PMA-treated MSCs was also demonstrated by immunocytochemistry. Though it has been known that few α_{1A} receptors exist in MSCs, α_1-adrenergic receptor subtypes were preferentially expressed in PMA-treated MSCs. Moreover, expression of the β-adrenergic and muscarinic receptors was increased 2-fold in PMA-treated MSCs compared to normal MSCs. The mRNA level of Ca^{2+}-related factor (SERCA 2a; sarcoplasmic reticulum Ca^{2+}-ATPase, LTCC; L-type Ca^{2+} channel) in treated MSCs was similar to that in cardiomyocytes. In the hypertrophic response to norepinephrine, activation of ERK1/2, a hypertrophic marker, was time-dependently augmented in PMA-treated MSCs. In conclusion, PMA, a PKC

activator, might induce the myogenic differentiation of MSCs. This result suggests that regulation of protein kinase could be an alternative method for differentiation of stem cells.

Key words: mesenchymal stem cells, PKC activator, myogenic differentiation

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I. INTRODUCTION

Congestive heart failure (CHF) and ischemic heart disease (IHD) are serious conditions that can result in morbidity and mortality[1]. IHD, also called myocardial ischemia, is a disorder of cardiac function caused by insufficient blood flow and oxygen supply to the heart muscle. These results can lead to heart attack, and it develops myocardial infarction (MI) and heart failure. Revascularization through either a catheter-based or a surgical approach often improves ischemic symptoms and cardiac performance and

reduces the risk of sudden death, but cardiac function is not clearly repaired in some cases[2]. The formation of new blood vessels to perfuse an ischemic heart and the generation of new myocardium to replace scar tissue represent the ultimate objectives of biologic myocardial regenerative therapy. These therapies can be broadly classified into two categories[3]. The first is the use of angiogenic growth factors using protein or gene-based delivery methods[4]. Angiogenesis describes the sprouting of new capillaries from post-capillary venules and involves an intricate molecular signaling pathway. Many cytokines are involved in the angiogenic pathway have been identified, including members of the fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF) family, platelet-derived growth factor (PDGF) family, and angiopoietins. The second therapeutic category is the use of cell-based therapy[5]. Researchers have used diverse cell types to induce functional improvement in ischemic myocardium. The first work began with committed cells such as skeletal myoblast, but currently, cell types, such as bone marrow cells, endothelial progenitor cells (EPC), mesenchymal stem cells (MSC), residence cardiac stem cells, and embryonic stem cells (ESC), expand to exploitation. Among these cells, lately the most useful cell type is stem cells.

Stem cells (Figure 1) are candidate cells for tissue engineering because of

their ability to self-renew and give rise to committed progenitors. Stem cells can be divided into two branches, embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs offer a highly promising therapeutic approach because they have totipotency. In this regard, they are characterized by their capacity to proliferate in an undifferentiated state while maintaining their capacity to differentiate into cell lines of all three embryonic germ layers. However, the use of ESCs has been hampered due to ethical and moral issues. The risks of teratoma formation and immune rejection upon transplantation are other problems associated with their use. Therefore, adult stem cells are currently the only cells suitable for use, as differentiated somatic cells possess neither the ability to proliferate nor to differentiate into any other cell type. ASCs are an undifferentiated cell found among differentiated cells in a tissue or organ which can renew themselves and differentiate to yield the major specialized cell types of the tissue or organ. The primary role of the ASCs in a living organism is to maintain and repair the tissue in which they are found. Some scientists now use the term ‘somatic stem cell’ instead of ‘adult stem cell’.

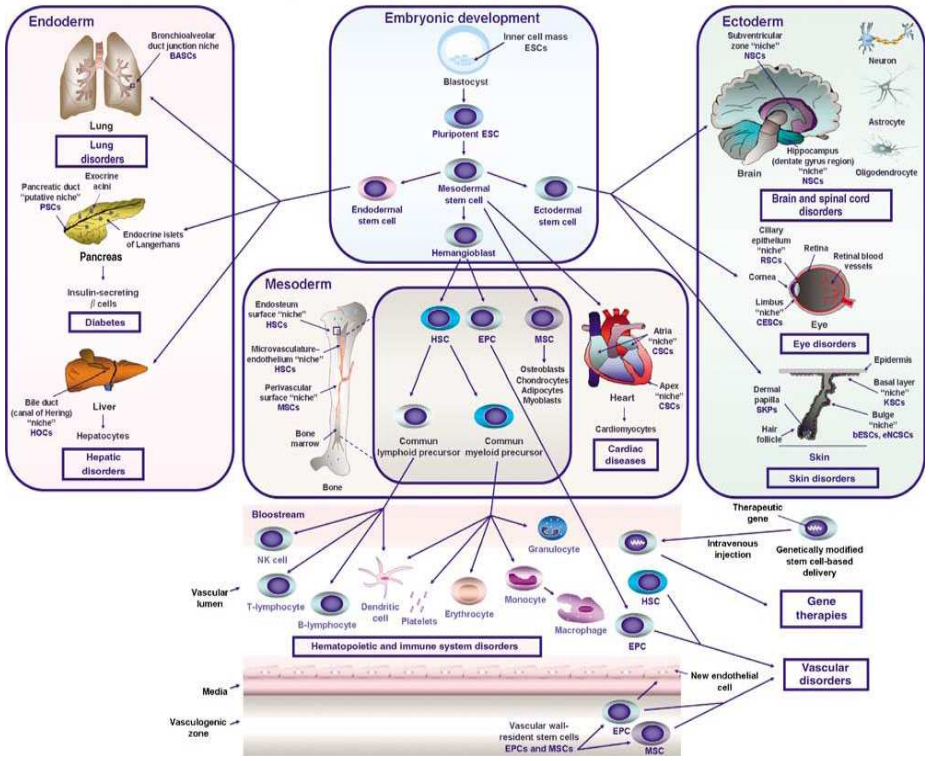

Figure 1. Schematic diagram of potential stem cell application. The embryonic development derived from blastocyst-stage multipotent adult stem cells arising from 3-dermal germ layers is shown. The pathological disorders and diseases that might benefit from ESCs- and ASCs-based therapies are indicated. Abbreviations: BASCs, bronchioalveolar stem cells; bESCs, bulge epithelial stem cells; CESCs, corneal epithelial stem cells; CSCs, cardiac stem cells; eNCSCs, epidermal neural crest stem cells; ESCs, embryonic stem cells; EPC, endothelial progenitor cell; HOCs, hepatic oval cells; HSCs, hematopoetic stem cells; KSCs, keratinocyte stem cells; MSCs, mesenchymal stem cells; NSCs, neuronal stem cells; PSCs, pancreatic stem cells; RSCs, retinal stem cells; SKPs, skin-derived precursors.

MSCs, first characterized by Friedenstein and colleagues in 1987, are multipotent cells capable of differentiating into several lineages, including the following: cartilage, bone, muscle, tendon, ligament and adipose tissue[6,7], both *in vivo* and *in vitro*, under appropriate culture conditions. MSCs originate mainly in either the cambium layer of the periosteum or bone marrow, although other sources such as muscle, fat, and synovium provide a limited amount of cells. *In vitro*, even cell colonies exhibit heterogeneity with respect to cell phenotype, colony size, and differentiation potential. Four types of cells are observed in the colonies: spindle-shaped cells, large-flattened cells, star-shaped cells, and small-round cells. Because of this variation, there have been attempts to isolate more homogeneous populations on the basis of surface and adhesion markers. For example, a few monoclonal antibodies (mAbs) against surface proteins expressed on MSCs have been raised, including SH-2 (CD105, endoglin)[8] and SB-10 (CD166, ALCAM), and the cell-surface antigens CD29 (β1-integrin), CD44 (H-CAM), and CD90 (Thy-1), but the prospective isolation of a homogenous population has still not been achieved. MSCs are also characterized by their negativity of the hematopoietic markers CD34 and CD45 and have been found to be human leukocyte antigen (HLA) class I positive and HLA class II negative[9]. Thus, these cells are hypoimmunogenic. That is, they do not

elicit an immune response and are suitable for allotransplantation, even between mismatched individuals.

As mentioned above, stem cells are able to differentiate into many cell types. Particularly, stem cells can differentiate into cardiomyocyte-like cells (CLCs) or cardiomyogenic (CMG) cells and develop into functional phenotypes of myocardial cells, which express myosin heavy chain (MHC) and cardiac troponin T (cTnT). Many studies have demonstrated that cardiomyocytes can differentiate from various multipotent stem cells such as ESCs and embryonic carcinoma cells[10, 11]. Moreover, if MSCs were co-cultured with cardiomyocytes, then they differentiate into functional cardiac phenotypes in the cardiac microenvironment[12, 13]. Researchers have reported that cardiomyocytes could be generated from bone marrow stromal cells and BM MSCs (Bone marrow-derived mesenchymal stem cells) *in vitro* with the use of 5-azacytidine (5-AZ)[14, 15, 16]. These cells appeared to possess a spontaneous heart-like beating and expressed NK2 transcription factor-related locus 5 (Nkx 2.5), Myocyte-specific enhancer factor 2 (MEF 2A), and MEF 2C, which is a cardiac specific marker and transcription factor. These cells were immunostained with anti-sarcomeric myosin, anti-actinin and anti-desmin antibody, and the rhythmic Ca^{2+} fluctuations were recorded by confocal laser scanning microscopy. Also, different cell types and various

methods have led to differentiation into CMG cells[17, 18].

We used a substance that would differentiate MSCs into CLCs. This substance is the well-characterized hypertrophic agonist phorbol myristate acetate (PMA)[19, 20, 21] that acts downstream of membrane receptors to activate both conventional and novel Protein kinase C (PKC) isoenzymes. PKC isozymes are activated in response to Ca^{2+} influx via voltage-gated L-type Ca^{2+} channels (LTCC) and by $G\alpha_q$ subunits of G protein-coupled receptors[22, 23].

In this study, we hypothesized that MSCs might differentiate into CLCs by PMA. To prove this hypothesis, we repeatedly screened MSCs that began differentiating after exposure to PMA. Screening was conducted by sandwich enzyme-linked immunosorbent assay (sandwich ELISA) after MSCs were treated with PMA for two, five, and nine days. Expression of cardiac-specific marker and neurohumoral-related receptor was compared with normal MSCs and neonatal rat cardiomyocyte. Finally, the sympathetic nerve ending transmitter was treated with differentiated MSCs. This finding may represent an important methodology in understanding stem cells differentiation into cardiomyocytes specialize.

II. MATERIALS AND METHODS

1. Materials

A. Reagents

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum and penicillin-streptomycin were all bought from the same corporation (Gibco BRL, Grand Island, NY, USA). For PCR, the oligonucleotide was synthesized by Bioneer (Bioneer, Korea), RNA was extracted with chloroform and 2-propanol (Sigma-Aldrich, USA), reverse transcription for cDNA synthesis was used by RT-&GO^{TM} (MP biomedicals, Solon, OH, USA), and we experimented with Real-Hi DNA Polymerase in the PCR using a 10 mM dNTP mix and a 10X reaction buffer (RBC, Taiwan). The following antibodies were used for ELISA, western blot analysis, flow cytometry and immunocytochemisty: mono/polyclonal troponin T-C (C-19), mono/polyclonal myosin light chain2 (E-4), mono/polyclonal myosin heavy chain (H-300), mono/polyclonal MEF-2 (B-4), monoclonal extracellular signal-related kinase 1 (ERK1), anti-phospho ERK, CD14, CD34, CD71, CD90, CD105, CD106 and ICAM-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mono/polyclonal anti-human Nkx-2.5 (R&D Systems, Minneapolis, MN,

USA), and β-actin (Sigma-Aldrich, USA). The secondary antibody usage can be divided into two types. One is western blot analysis (Horse-radish peroxidase-conjugated); mouse or rabbit (Stressgen, Ann Arbor, MI, USA), and goat (Santa Cruz Biotechnology), and the other is immunocytochemistry (Fluorescein isothiocyanate-conjugated); mouse, rabbit, and goat (Jackson Immunoresearch Laboratories, West Grove, PA, USA). The western blotting detection system was from Amersham Biosciences (Uppsala, Sweden).

B. Animals

BM MSCs were isolated from the bone marrow of 4-week-old Sprague-Dawley male rats. NRVCMs were isolated from the neonatal (1~2 day-old) Sprague-Dawley rats. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care Committee.

2. Methods

A. Cell culture

(A) Isolation and primary culture of BM MSCs

Isolation and primary culture of BM MSCs from the femoral and tibial bones of donor rats were performed. After induction of anesthesia with

10 ㎎/㎏ Zoletil (Virbac Laboratoires, France) and 5 ㎎/㎏ Rompun (Bayer, Korea) by intramuscular injection, the bones of rat legs were branched off. Then, BM MSCs were collected from the aspirates of the tibias and femurs of 4-week-old Sprague-Dawley male rats (80 ~ 130 g) with 10 ㎖ DMEM-Low Glucose supplemented with 10% FBS by using an 18 gauge syringe. Flushed medium was centrifuged at 1600 rpm for five minutes and resuspended in serum-supplemented medium. Next, the medium was loaded into a 4 ㎖ Ficoll (Amersham biosciences, USA) density gradient centrifugation per 3 rats at 1600 rpm for 30 minutes. Mononuclear cells recovered from the middle interface of the Ficoll-separated bone marrow and blood were washed twice and resuspended in PBS (Gibco BRL, USA). They were then suspended in fresh DMEM by adding 10 % FBS and 100 U/㎖ penicillin/streptomycin and plated at 1 rat/100 ㎜ plate. Cells were maintained in a 37 ℃ humidified atmosphere chamber containing 5% CO_2. After 72 hours, non-adherent cells were discarded, and fresh medium was added and replaced every three or four days for about 10 days. Upon attaining 80~90 % measurement, primary BM MSCs were typsinized (Gibco BRL, USA) and accompanied sub-culture.

(B) BM MSCs characterization

Immunocytochemical characterization of BM MSCs is demonstrated below. Cells were cultured in a 4-well slide chamber, washed with PBS and incubated in 1% paraformaldehyde solution (Sigma, USA) for 10 minutes. The cells were then washed twice with PBS before being permeated in 0.1% Triton X-100 for seven minutes. After this, the cells were blocked for one hour (blocking solution: 2% bovine serum albumin, 10% horse serum in PBS) and adhered with the following antibodies: CD34, CD71, CD90, CD105, and intracellular adhesion molecule (ICAM)-1. FITC-conjugated mouse, rabbit and goat (Jackson Immunoresearch Laboratories) were used as secondary antibodies. Then, they were detected with confocal microscopy (Carl Zeiss, Germany).

BM MSCs was performed according to the protocol for fluorescence-activated cell sorting (FACS) staining described below. Briefly, cells were detached from the plate with 10% trypsin-EDTA (Gibco BRL), washed in PBS and fixed in 70% ethanol at 4 °C for 30 minutes with agitation. Cells were washed twice in PBS and resuspended at 2×10^6 cells/㎖ in blocking buffer (1% BSA, 0.1% FBS) containing the following antibodies: CD14, CD34, and CD90; In the case of CD14, we used normal rabbit IgG as a negative isotype control. After staining,

cells were washed twice and then labeled with rabbit or mouse-FITC conjugated IgG for 20 minutes in the dark. After two more washes, flow cytometric analysis was performed on a FACS Calibur system (Becton Dickinson, CA, USA) using CellQuest™ software (Becton Dickinson, USA) with 10,000 events recorded for each sample[24].

(C) Isolation and primary culture of NRVCMs

NRVCMs were isolated from neonatal rat hearts, as previously described. This heart tissue was washed with pH 7.4 Dulbecco's phosphate-buffered saline (D-PBS; Gibco BRL) in order to remove red blood cells. After depletion of the atria, the hearts were sliced up approximately 0.5 ㎟ sized pieces and treated with 4 ㎖ collagenase Ⅱ (1.4 ㎎/㎖, 270 units/㎎; Gibco BRL) solution for five minutes. The supernatant was removed, and the pellet was suspended in collagenase Ⅱ. After cells were incubated in a 37 ℃ humidified atmosphere chamber containing 5 % CO_2 for five minutes, gained supernatant was mixed with alpha minimum essential medium (α-MEM; Gibco BRL, USA) then centrifuged at 2000 rpm for two minutes. The remaining tissue was treated with fresh collagenase Ⅱ solution for five minutes. This incubation procedure was repeated until the tissue was completely

digested. The gathering cell pellet was resuspended in α-MEM and attached to a culture dish at 37 ℃ in 5 % CO_2 chamber. Two hours later, adherent cells turned into fibroblasts and non-adherent cells became NRVCMs. The NRVCMs were washed twice and replated on 100 ㎜ plates with α-MEM plus 10 % FBS at 37 ℃ in 5 % CO_2 chamber. To eliminate fibroblast expansion, we used 0.1 mM 5-bromo-2'-deoxyuridine (Brd-U; Sigma-Aldrich, USA)[25].

B. BM MSCs differentiation

BM MSCs were seeded on 60 ㎜ plates at 2×10^5 cells/ml. To induce differentiation, BM MSCs were treated with 1 μM of PMA at an interval of three days and changed fresh 10 % FBS contained DMEM.

C. Sandwich ELISA

60 ㎜ plates of 80~90% filled with BM MSCs treated with PMA were washed twice with PBS and lysed in 1X cell lysis buffer (10X cell lysis buffer: Promega, Medison, WI, USA, 1 mM phenylmethylsulphonyl fluoride; PMSF, Sigma, 4 mM 3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate; 0.1 % CHAPS, USB, Cleveland, OH, USA) for 30 minutes at 4 ℃. After the lysate was centrifuged at

12000 rpm in a 4 ℃ environment for 10 minutes, the supernatant was collected and protein concentration was detected by BCA protein assay reagent (Pierce Biotechnology, Rockford, IL, USA).

The capture antibody (monoclonal) was bound to the bottom of the 96-well high binding plates, and mixture of 300 ng/㎠ rate was incubated overnight at 4 ℃. The plate was washed twice with PBS (Gibco, USA) and blocked with 3% BSA (Sigma, USA) in PBS for 2~3 hours at 37 ℃. After washing the plate twice with PBS, a sample was added to each well, and the plate was incubated in a humid atmosphere for two hours. The plate was washed four times with PBS containing 0.02% tween-20 (Sigma, USA). After adding the detector antibody (polyclonal), the plate was incubated for two hours in a humid atmosphere. The plate was incubated again after the addition of peroxidase-conjugated secondary antibody for one hour at 37 °C. Finally, the plate was treated with 100 ㎕ of tetramethylbenzidine (TMB; Sigma, USA) as substrate and 50 ㎕ of 0.1 M H_2SO_4 as stop buffer, then detected quickly at 450nm on an ELISA plate reader (Biorad, Hercules, CA, USA).

D. RT-PCR analysis

(A) Isolation of total RNA

Total RNA was extracted with 500 ㎕/ 60 ㎜ plate Tri-reagent (Sigma, USA). 100 ㎕ of chloroform was poured over the Tri-reagent and voltexing a sample about 10 seconds. Then, the sample was centrifuged at 12000 g and 4 ℃ for 15 minutes. After this, three layers appeared in the tube, and the upper transparency layer was collected in new tubes. Thereafter, 250 ㎕ 2-propanol was added to the sample, and the mixture was again voltex for about 30 seconds. Centrifugation was then carried out at 12000 g and 4 ℃ for 10 minutes. Next, the supernatant was discarded, and the pellet was washed with 75% ethanol (Duksan, Korea) mixed with diethylpyrocarbonate (DEPC; Sigma) water. At this point, centrifugation was done at 7500 g and 4 ℃ for five minutes, and the pellet was dried at room temperature for about seven minutes. Finally, 30 ㎕ nuclease free water (NFW) was added. The quality and quantity of the RNA was detected OD_{260}/OD_{280} value with DU 640 spectrophotometer (Effendorf, Hambug, Germany)

(B) cDNA synthesis

Complementary DNA (cDNA) was synthesized with RT-&GO™. 1 ㎍ total RNA was added to 1 ㎕ anchored primer $(dT)_{25}V$, 2 ㎕ dithiothreitol (DTT) and NFW, totaling 9 ㎕ of volume. To prevent

secondary structures, mixture was incubated for five minutes at 70 ℃ and 8 μℓ of RT-&GO^{TM} mastermix was added. Thereafter, the sample was incubated at 42 ℃ for one hour. At the conclusion, the sample was inactivated with the reverse transcriptase at 70 ℃ for 15 minutes. Finally, the sample was detected with a DU 640 spectrophotometer at OD_{260}/OD_{280}.

(C) PCR analysis

1 μg cDNA, each 10 pmol primer (forward and backward), 0.1 mM dNTP mixture, 1.25 U of Taq polymerase, and 10X reaction buffer were mixed with NFW, totalling 25 μℓ. PCR condition was fixing as fellow. A cycle of denaturing at 94 ℃ for three minutes was followed by 35 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 48 ℃ to 60 ℃ for 30 seconds, and elongation at 72 ℃ for 30 seconds[26]. Then, the sample was maintained at 72 ℃ for 10 minutes. Primers are listed in Table 1. When the PCR assay finished, the product was separated by electrophoresis in a 1.2% agarose gel (Biorad, USA), and Gel-Doc (Biorad, USA) was used to visualize after staining with ethidium bromide (EtBr; Sigma, USA).

Table 1. PCR primers used in this study

Gene	Primer	Size (bp)	Temp.
α_{1A}-adrenergic receptor	gag aat tcc gag gcc tca agt ccg gcc t ttg aat tct cgg gaa aac ttg agc ag	169	59
α_{1B}-adrenergic receptor	ctg ggg aga gtt gaa aga tgc c ccg aca gga tga cca aga tgt t	158	58
α_{1D}-adrenergic receptor	ttg aat tcc tac aga gac cca cga ccc ag cgg aat tct taa atg tca gtc tcc cgg ag	229	58
β_1-adrenergic receptor	acg ctc acc aac ctc ttc at agg ggc acg tag aag gag ac	440	53
β_2-adrenergic receptor	cct cat gtc ggt tat cgt cc ggc acg tag aaa gac aca atc	519	53
M1-muscarinic receptor	ctg gtt tcc ttc gtt ctc tg gct gcc ttc ttc ttc ttg ac	641	47
M2-muscarrinic receptor	ggc aag caa gag tag aat aaa gcc aac agg ata gcc aag att	552	47
SERCA 2a	tcc atc tgc ctg tcc at gcg gtt act cca gta ttg	196	42
LTCC	tgt cac ggt tgg gta gtg aa ttg agg tgg aag gga ctt tg	346	49
GAPDH	acc aca gtc cat gcc atc ac tcc acc acc ctg ttg ctg ta	450	49

E. Immunocytochemistry

Immunocytochemistry methods were the same as those for BM MSCs characterization. The primary antibody was MHC (1:100), and the secondary antibody was FITC-conjugated goat-anti rabbit (1:50). Cells were coated with Vectashield (Mounting medium with DAPI; Vector Laboratories, Burlingame, CA, USA) and a cover slide, and they were detected using confocal microscopy (Carl Zeiss, Germany).

F. Western blot analysis

A 60 ㎜ plate of 80~90 % filled with BM MSCs treated with PMA was

scrapped with a scrapper (Sigma, USA) and centrifuged at 12000 rpm and 4 ℃ for three minutes. Gathered cells were washed in PBS and lysed in 1X cell lysis buffer (10X cell lysis buffer; Promega and 1 mM PMSF, Sigma) at 4 ℃ for 25 minutes. After the lysate was centrifuged at 12000 rpm and 4 ℃ for 10 minutes, supernatants were collected. The protein concentration was detected by BCA protein Assay reagent (Pierce Biotechnology, Rockford, IL, USA). Like the detected value, the sample was prepared to the same amount. With 5X dye (0.225 M Tris·Cl, pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 0.25 M DTT), the quantitative protein sample was denatured at 95~100 ℃ heat block for five minutes. The sample was quickly placed in ice for three minutes. Then, it was loaded into 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and taken down separating (80V, 30 minutes) and running (120V, about one hour) gels. Protein in a running gel was then transferred onto a membrane (Millipore, Bedford, MA, USA) and blocked in blocking buffer (10% skim milk in PBS-t; tween 20; Becton, Dickinson and Company, Sparks, MD, USA) for 1 hour. The membrane was incubated with primary antibodies to ERK 1 (1:2000), phospho-ERK 1/2 (1:1000 diluted in 2 % BSA) and β-actin (1:4000) overnight at 4 ℃. Samples were then washed with PBS-t 3 times and then

incubated in secondary antibodies of HRP-conjugated rabbit and mouse for one hour. The samples were then washed with PBS-t 4 times and visualized with enhanced chemiluminescence (ECL; Amersham biosciences, USA) reagent. Specific bands were quantified using the Phospho-Image System (Kodak, Rochester, NY, USA).

G. Statistical analysis

Results are expressed as mean±SEM. Statistical analysis was performed using student's t-test. Relationships were considered statistically significant when the p value was less than 0.05.

III. RESULTS

1. Primary culture and characterization of BM MSCs

BM MSCs were first isolated from mixed cultures with hematopoietic cells based on their attachment to the culture plate. Three days later after mixed cultures seeded, we tapped a plate, old culture medium was discarded, and fresh 10% FBS containing DMEM was poured into a plate. Over time, the cells became adherent, elongated and spindle-shaped (Figure 2), and we yielded 3×10^6 cells within 2 weeks of culture with 95% purity. The BM MSCs maintained a fibroblastic morphology through repeated passages, and their identity was confirmed by immunocytochemistry and FACS analysis. Cultured BM MSCs expressed CD71, CD90, CD105, CD106, and ICAM. They expressed neither the hematopoietic marker CD34 nor CD14 (Figure 3).

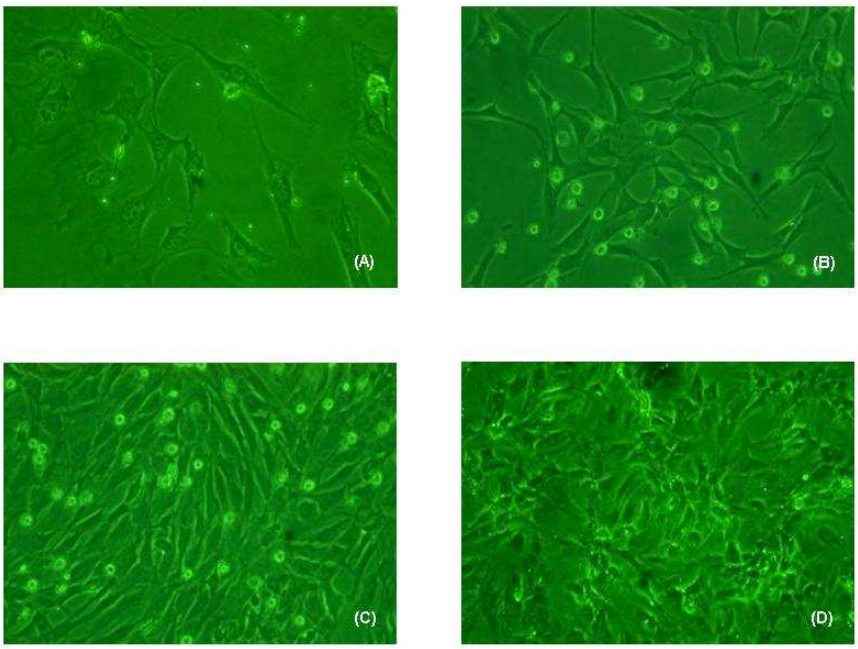

Figure 2. Isolation and culture of rat BM MSCs. Electron micrographs of isolated BM MSCs 3 days (A), 5 days (B), 7 days (C) and 10 days (D) after primary culture (magnification: X100). (A) BM MSCs proliferated to form a small colony after three days. (B) Large colony composed of densely distributed spindle-like and triangle-shaped BM MSCs after five days. (C) Flattened cells were surrounded by quickly proliferating spindle- or triangle-like cells after seven days. (D) Center of a large colony with several overcrowded layers of BM MSCs after 10 days.

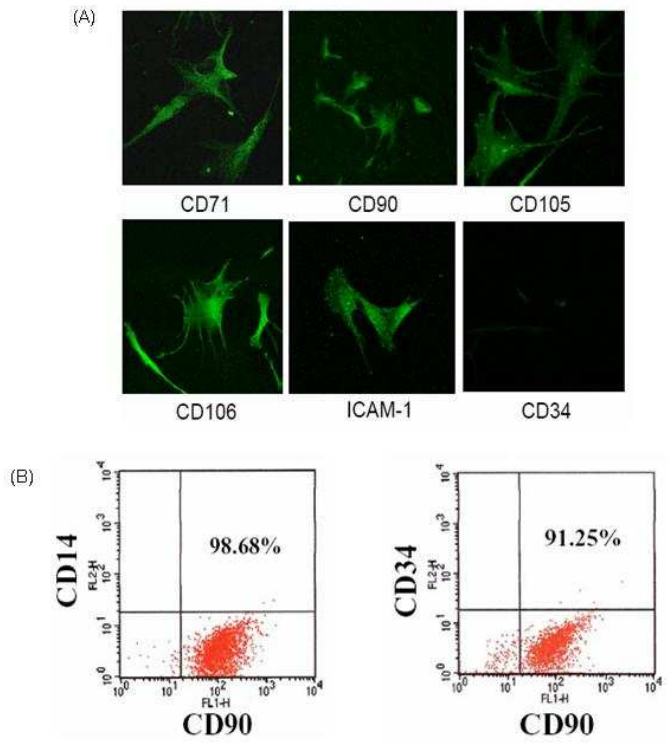

Figure 3. Characterization of rat BM MSCs. BM MSCs were primarily cultured in a PVC plate for 10 days. (A) BM MSCs transferred to a 4-well slide were executed before immunocytochemistry through a confocal microscopy was conducted. These cells were positive for CD71, CD90, CD105, CD106, and ICAM-1 but negative for CD34. (B) To confirm BM MSCs, cells were analyzed by CD14, CD34, and CD90 by the FACS Calibur system (magnification: X400).

2. Culture of BM MSCs differentiated with PMA

Primary culture BM MSCs were treated with 1 mM PMA (final concentration was 1 μM) according to the experimental group. This group was separated at harvest day, 2 days, 5 days and 9 days. Treatment with PMA was every 3 days. Because the dilution base for PMA was dimethylsulfoxide (DMSO, Sigma), the control group was also treated with DMSO of the same volume. Normal BM MSCs were spindle-shaped until 9 days, but BM MSCs treated with PMA changed into a stick-like or branching-out appearance at 5 days and 9 days (Figure 4).

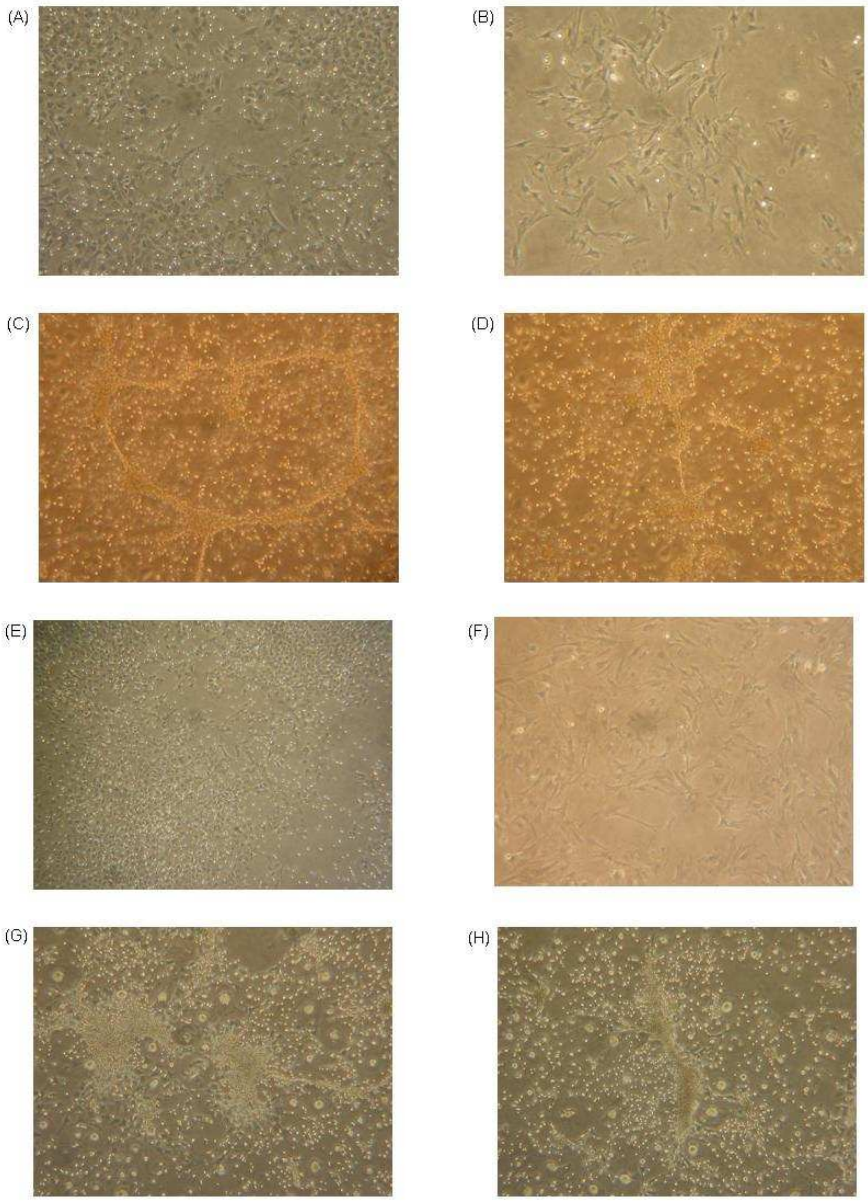
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)

Figure 4. Effect of PMA on morphology of BM MSCs. Electron micrographs of BM MSCs were taken after five days (A), (B), (C), and (D), and nine days (E), (F), (G), and (H). Also, (A), (B), (E), and (F) are normal BM MSCs and (C), (D), (G), and (H) were BM MSCs treated with PMA. (B), (D), (F), and (H) are magnified (A), (C), (E), and (G) magnified, respectively, up to 2.5 times. BM MSCs treated with PMA changed morphology to cardiomyocyte-like and fibroblast-like aggregated long-branches compared to their counterparts. Magnification: (A), (C), (E), and (G), X40; (B), (D), (F), and (H), X100.

3. Sandwich ELISA-based analysis with cardiac-specific marker expression of BM MSCs treated with PMA

We isolated protein from cultured BM MSCs treated with PMA using cell lysis buffer (Composition of lysis buffer was described in methods section). Capture antibody, detect antibody and lysed samples were treated and HRP-conjugated secondary antibodies were bound to the detect antibody. These substrates were excited by TMB, then stop solution halted the reactions[27]. Distinct colorimetry was detected by a microplate reader. Antibodies to cardiac specific markers[28, 29] were chosen, which selectively bound to BM MSCs treated with PMA. Each sample was repeated 3 times, and the score was averaged. Expression of most of the specific markers was elevated by 2 days and peaked around 9 days (Figure 5).

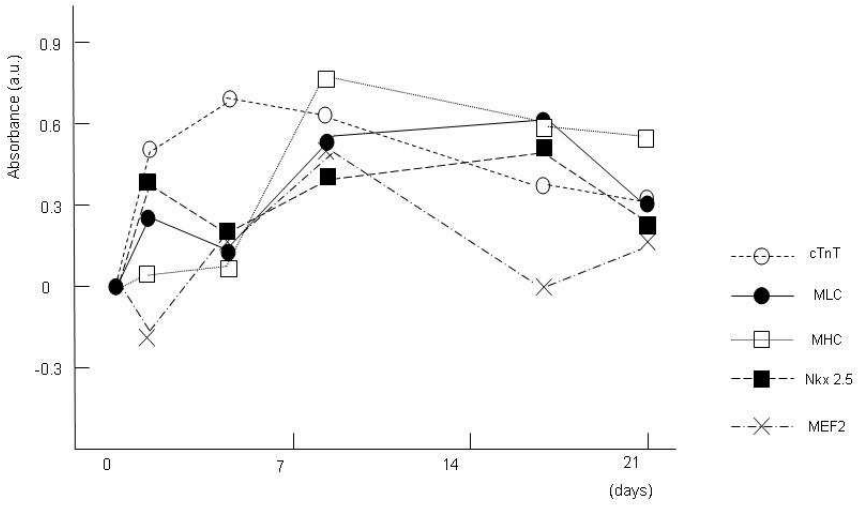

Figure 5. Change of cardiac specific marker in BM MSCs treated with PMA. This change was detected by sandwich ELISA. Samples of protein were lysed to BM MSCs treated with PMA each harvest day (2, 5, 9, 17 and 21 days). This experiment was repeated three times, and each standard deviation was 0.05. Cardiac-specific markers (cTnT, MLC, MHC, Nkx 2.5 and MEF2) were elevated from two days to 21 days, especially day nine, which highly expressed in five markers.

4. Immunocytochemistry detection of cardiac MHC expression in BM MSCs treated with PMA.

BM MSCs treated with PMA express multiple sarcomeric proteins associated with NRVCMs. To examine where MHC-expressing cells are located in differentiated BM MSCs, immunostaining was performed. Clearly, CLCs had significant morphological changes with positive immunocytochemical analysis for anti-MHC, but normal BM MSCs showed neither significant morphological changes nor cardiac-specific protein expression of MHC during the 9 day period of observation with the same treatment (Figure 6).

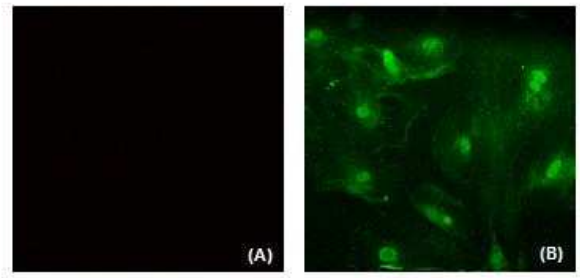

Figure 6. MHC change in BM MSCs treated with PMA. Immunocytochemistry was detected for cardiac MHC in BM MSCs treated with PMA. Representative photos of normal BM MSCs as positive control (A) and BM MSCs treated with 1 μM PMA (B) are shown (Magnification: X400). Cardiac MHC visualized by FITC is located on the stress fibers (B).

5. Temporal expression of adrenergic and muscarinic receptor subtype mRNA in BM MSCs treated with PMA

To begin to address whether receptor-mediated subtypes are involved in modulating the function of BM MSCs treated with PMA, we conducted the following experiment. Total RNA and cDNA was extracted from the cell lysis with Tri-reagent. cDNA was measured by a spectrophotometer. PCR with 1 μg cDNA was achieved at various temperatures, and DNA electrophoresis was accomplished. Finally, we watched the expression level of receptor subtype mRNA. As a result, BM MSCs treated with PMA were expressed more so than their counterparts **(Figs. 7, 8, 9)**.

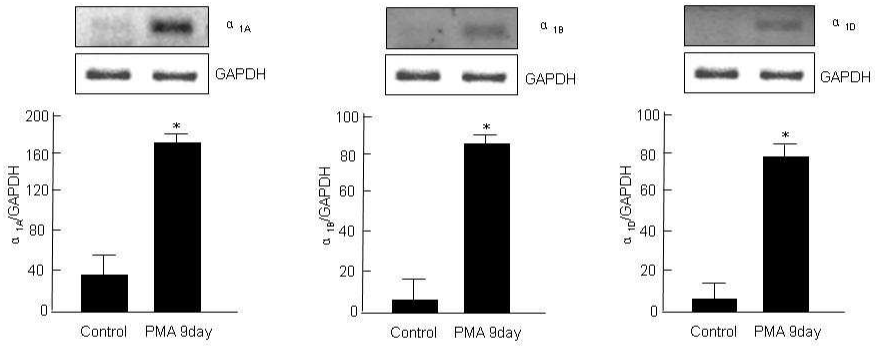

Figure 7. The change of α-adrenergic receptor subtype in BM MSCs treated with PMA. Each panel shows RT-PCR products for expression of α_{1A}-adrenergic receptor, α_{1B}-adrenergic receptor and α_{1D}-adrenergic receptor. Bar-type densitometry showed that all subtypes were more expressive than normal control. The mRNA expression of genes was established by DNA electrophoresis and visualized by EtBr. Data are presented as arbitrary units over controls. Values are mean±SEM. **P*<0.01 vs. controls.

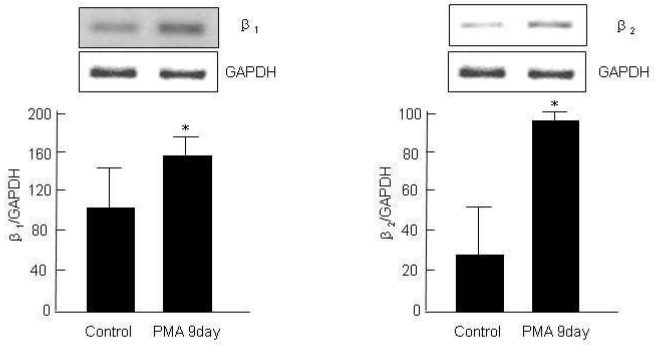

Figure 8. The change of β-adrenergic receptor subtype in BM MSCs treated with PMA. Each panel shows RT-PCR products for expression of β_1-adrenergic receptor and β_2-adrenergic receptor. Experiments were run as

described in Figure 7. Bar-type densitometry showed that all subtypes were more expressive than normal control. The mRNA expression was established by DNA electrophoresis and visualized by EtBr. Data are presented as arbitrary units over controls. Values are mean±SEM. *P<0.01 vs. controls.

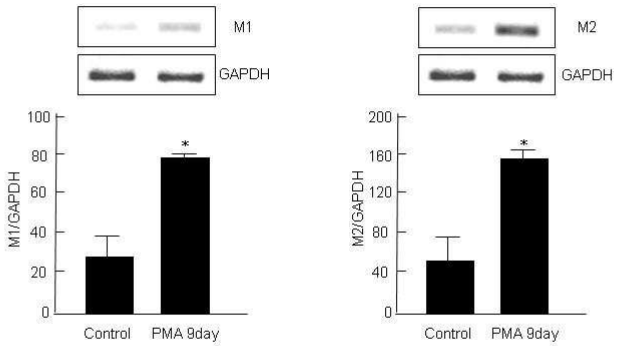

Figure 9. The change of muscarinic receptor subtype in BM MSCs treated with PMA. Each panel shows RT-PCR products for expression of M1 and M2 muscarinic receptors. Bar-type densitometry showed that all subtypes were more expressive than normal controls. The mRNA expression was established by DNA electrophoresis and visualized by EtBr. Data are presented as arbitrary units over controls. Values are mean±SEM. *P<0.01 vs. controls.

6. Changes in Ca^{2+} handling-related protein expression with BM MSCs treated with PMA

Cardiac myocytes are progressed in excitation-contraction coupling. Molecular mechanisms responsible for Ca^{2+}-handling are closely related to SERCA 2a[30] and LTCC[31]. So PCR was enforced the expression of SERCA 2a and LTCC. NRVCMs were highly expressed, but even BM MSCs treated with PMA were time-dependently expressed (Figure 10). These findings suggest that BM MSCs treated with PMA can influence Ca^{2+} handling-related protein expression.

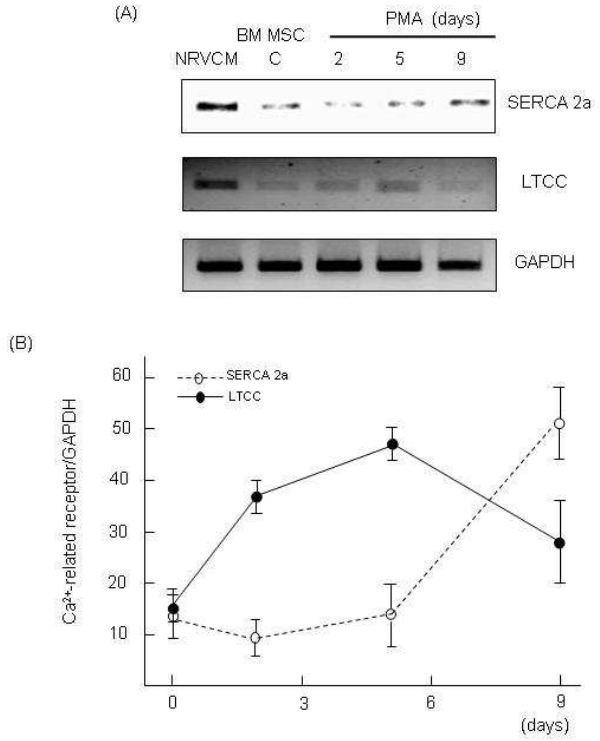

Figure 10. The change of Ca2+-related proteins in BM MSCs treated with PMA. BM MSCs treated with PMA compared to control and NRVCM in time-dependent measure. (A) Each panel shows RT-PCR products for the expression of Ca^{2+}-related proteins (SERCA 2a, LTCC) (B) Densitometric analysis was performed, and the ratio of RT-PCR product of SERCA 2a and LTCC to that of GAPDH is shown. The mRNA expression was established by DNA electrophoresis and visualized by EtBr. Data are presented as arbitrary units over controls. Values are mean±SEM. *P<0.01 vs. controls.

7. Effects of norepinephrine (NE) on phosphorylation of ERK1/2 in BM MSCs treated with PMA and its hypertrophy

Through α_1-adrenergic receptors in cardiac myocytes, norepinephrine (NE) influences the contractile properties of the heart and induces a series of changes characteristic of the hypertrophic phenotype[32]. To examine whether α_1-adrenergic receptors are expressed at the protein level and transduce hypertrophic signals, we stimulated BM MSCs treated with PMA by NE and detected phosphorylation of EKR1/2. Phosphorylation of ERK1/2 was induced by NE in a time-dependent manner (Figure 11-A). BM MSCs treated with PMA were compared to controls (not treated with NE). As well, BM MSCs treated with PMA and NE were more expressive than their counterparts (Figure 11-B). These aspects indicated that BM MSCs treated with PMA expressed functionally active α_1-adrenergic receptors.

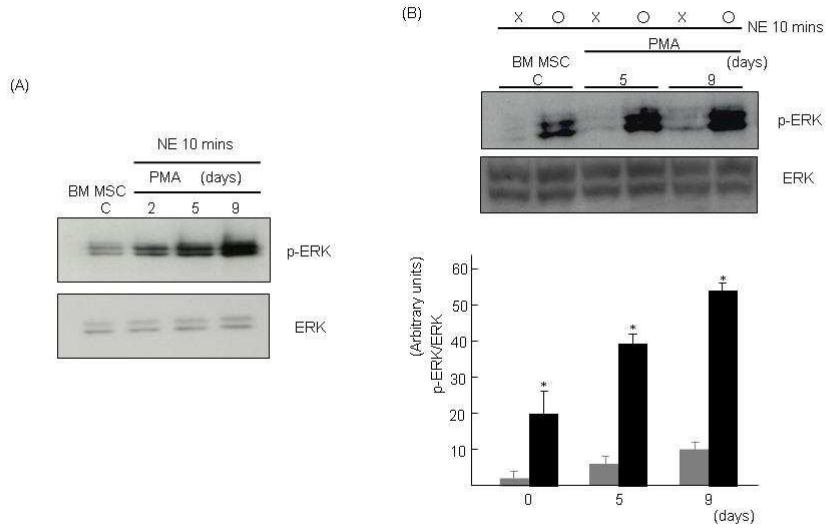

Figure 11. The effect of NE on activation of ERK1/2 in BM MSCs treated with PMA. (A) Cells at two, five, and nine days (time-dependently) after PMA treatment were stimulated with 1 μM norepinephrine, and Western blot analysis was performed to detect phosphorylation of ERK1/2. (B) Panels show that BM MSCs treated with PMA were compared with normal BM MSCs on the same day. In Both (A) and (B), the phosphorylation of ERK1/2 was elevated in PMA-treated cells. **P*<0.01 vs. controls.

IV. DISCUSSION

The major finding of this study is that MSCs can differentiate into cardiomyocyte-like cells with PMA treatment. Previously, researchers studying 5'-azacytidine reported that MSCs were used to differentiate into myocytes[16]. This led us to believe that MSCs are multi-differential cell types, and differentiation is possible without the genotoxic effects of 5'-azacytidine [13, 16, 33].

PMA is a protein kinase C activator. The PKC family of calcium or lipid-activated serine-threonine kinases is regulated downstream of nearly all membrane-associated signal transduction pathways. About 12 different isozymes comprise the PKC family. The conventional PKC isozymes (PKC-α, -βⅠ, -βⅡ and -γ) are calcium and lipid-activated, whereas other isozymes are calcium independent but activated by distinct lipids (PKC-ε, -θ, -η, -δ, -ξ, -ι, -ν and -μ). PKC is influenced by calcium or diacylglycerol (DAG): their isoenzymes play a critical role in cardiomyocyte hypertrophy. Especially, PKC-α is reportedly capable of inducing a hypertrophic response characterized by enhanced sarcomeric organization and increased atrial natriuretic factor (ANF) expression[34, 35]. We hypothesized that MSCs might differentiate into CLCs after PMA treatment.

To determine whether MSCs treated with PMA have a heart-like cell phenotype, we performed cardiac-specific marker-related sandwich ELISA. The highest expression time of all antibodies (cTnT, MHC, MLC, Nkx 2.5 and MEF-2) was nine days (Figure 5). Moreover, the cell resembled a cardiac myocyte in morphology by nine days (Figure 4). Especially, MHC expression was monitored by immunocytochemistry and was found in the cytoplasm of the cells induced with PMA. Compared to normal MSCs, differentiated MSCs had a higher expression of MHC (Figure 6).

To confirm whether differentiated MSCs became CLCs through a molecular biological approach, neurohumoral factor was used to distinguish their expression levels. Neurohumoral factors control heart rate, myocardial contractility, and cardiac hypertrophy. The most powerful system controlling cardiac function is the autonomous nervous system, including the sympathetic and parasympathetic nerves acting through adrenergic and muscarinic receptors. The adrenergic receptors are divided into α-adrenergic and β-adrenergic receptor subtypes, and muscarinic receptors have five subtypes, M_1 through M_5[36]. We explored α_{1A}, α_{1B}, α_{1D}, β_1, and β_2-adrenergic receptors and M1 and M2 muscarinic receptors, which are reported in another paper[37]. The characteristics of receptors include the following: critical implications in modulating cardiac function, α_{1A}-, α_{1B}-, α_{1D}-[38] (Figure

6), catecholamine-induced increases in heart rate, conduction velocity and contractility, β_1- and β_2-adrenergic receptors[38, 39, 40] (Figure 7), expression in murine neonatal and adult cardiomyocytes, and M1 and M2 muscarinic receptors[41] (Figure 8). Surprisingly, when MSCs treated with PMA in nine days were compared with normal MSCs, differentiated MSCs had a higher expression.

Cardiomyocytes are linked by EC coupling, which operates by Ca^{2+} influx. LTCC and SERCA 2a are especially meaningful factors in this process. The mRNA of SERCA 2a is also a myocardial marker similar to the heart muscle-specific marker gene cTnT[22, 23]. Moreover, a 1000-fold Ca^{2+}-gradient is maintained across the cardiac sarcoplasmic reticulum membrane by these factors, and LTCC is physiologically important in many excitable cells but particularly important in the heart because Ca^{2+} entry through these channels not only contributes to impulse generation and conduction but also serves as a second messenger to modulate regulatory protein kinases and the activation of contractile proteins. Thus, we watched their expression level. As seen in Figure 10, even though NRVCMs had a slightly higher expression level, the MSCs' expression level increased in a time-dependent manner.

Likewise, PKC is concerned about hypertrophy-related signaling and cardiomyocyte is also related with hypertrophy. Other researchers showed

the translocation of PKC-α in response to the α_1-adrenergic and $G\alpha_q$-coupled receptor agonist phenylephrine[42]. If MSCs differentiated into CLCs, then they might have similar characteristics to cardiomyocytes. Because of this, we experimented with the phosphorylation of ERK1/2 when differentiated MSCs were treated with NE, and noted that their expression increased in a time-dependent manner (Figure 11). In conclusion, we found that MSCs treated with PMA might differentiate into CLCs through various methods.

The prevailing evidence suggests that BM MSCs can regenerate myogenic or cardiomyocyte-like cells in cardiac tissue. These advances raise the prospect that damaged cardiac tissues might be repaired by administering adult human BM MSCs[43, 44]. This simple new model for differentiated BM MSCs may help clarify the cascade of transcriptional activation that regulates differentiation into CLCs[15, 37].

Ⅴ. CONCLUSION

Our study suggests that BM MSCs treated with PMA differentiated into CLCs. BM MSCs were isolated from rat tibias and femurs, and characterized with MSC positive and negative markers. We found that cardiac-specific marker expression was the highest score at nine days by sandwich ELISA. The MSCs treated with PMA displayed different morphological patterns, a high expression of neurohumoral factor and Ca^{2+}-related protein, and a time-dependent hypertrophic expression compared with normal MSCs.

In summary, the present study demonstrated that BM MSCs might be extensively expanded *in vitro* and can be induced to differentiate into CLCs by PMA treatment if the cells are not immortalized. This implies that there is still a long way to go before the cells can be used for a cell/gene therapy for heart diseases.

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ABSTRACT (in Korean)

Protein Kinase C 활성체에 의한 중간엽 줄기세포로부터 심근유사세포로의 분화

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송 병 욱

적절한 자극 하에 중간엽 줄기세포는 *in vivo* 와 *in vitro* 에서 근육세포, 연골세포, 지방세포, 조골세포, 건 세포 그리고 조혈 유지 기질로의 분화가 가능한 다기능 세포이다. 5-azacytidine 에 의해 유도된 심근세포 분화가 가능한 결과임에도 불구하고, 중간엽 줄기세포에서 심근세포 분화를 기초로 하는 분자 기작들은 잘 알려져 있지 않다. 손상된 심근의 효과적인

재생을 위하여, MSCs 의 *in situ* 심근세포 분화를 개선시키는 생리학적 방법을 찾는 것이 중요하다. 단백질 키나아제 C (PKC)는 배아 줄기세포의 심근세포 분화를 수반하는 신호변환 요소 중 하나이다. 본 연구에서는, 중간엽 줄기세포의 심근세포로 분화에서 PKC 활성체인 phorbol myristate acetate (PMA)의 역할을 조사하였다. PMA 효과를 증명하기 위해, 1 μM PMA 를 처리한 중간엽 줄기세포는 각각 2, 5, 그리고 9 일간 배양되었다. 효소 면역 측정법에서, 심장 특정 표지인자 (cardiac troponin T; cTnT, myosin light chain; MLC, myosin heavy chain; MHC, Nkx2.5; NK2 transcription factor related, locus 5, MEF2; Myocyte-specific enhancer factor 2)의 발현은 배양 9 일째에 모든 부분에서 높아졌다. PMA 를 처리한 중간엽 줄기세포에서 MHC 의 발현은 immunocytochemistry 를 통해 확인되었다. 알파 1A-아드레날린 수용체는 중간엽 줄기세포에 거의 존재하지 않는다고 알려져 있지만, 이를 포함한 알파 1-아드레날린 수용체 하위유형은 PMA 를 처리한 중간엽 줄기세포에서 우위적으로 발현되었다. 게다가, 베타-아드레날린 수용체와 무스카린 수용체의 발현도 처리하지 않은 중간엽 줄기세포와 비교하여 2 배 이상 증가하였다. 처리한 중간엽 줄기세포에서 칼슘관련 인자 (SERCA 2a;

sarcoplasmic reticulum Ca2+-ATPase, LTCC; L-type Ca^{2+} channel)의 mRNA 수준은 심근세포와 유사한 양상을 보였다. 노르에피네프린 (NE)에 의한 비대성 반응에서, 비대성 인자인 ERK1/2 의 활성은 PMA 를 처리한 중간엽 줄기세포에서 시간 의존적으로 증가되었다. 결론적으로, PKC 활성체인 PMA 는 중간엽 줄기세포의 심근세포 분화를 유도했다. 이는 단백질 키나아제 조절이 줄기세포의 분화의 선택적 방법이 될 수 있음을 암시한다.

핵심 되는 말: 중간엽 줄기세포, PKC 활성체, 심근세포 분화