Signaling pathways mediating cardiac myocyte gene expression in cardiogenic cells derived from mesenchymal stem cells

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Directed by Professor Kyung-Jong Yoo

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Abstract

Signaling pathways mediating cardiac myocyte gene expression in cardiogenic cells derived from mesenchymal stem cells

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Despite treatment of mesenchymal stem cells (MSCs) for cardiac repair, specific stimuli and signal pathways that may control cardiogenic differentiation remains to be completely defined. In cardiogenic cells (CGCs) derived from MSCs using PKC activator (PMA, phorbol 12-myristate 13-acetate), cardiac-specific signal pathways were investigated by various stimuli; i) MAPK pathway by platelet-derived growth factor (PDGF) and U0126 (MEK inhibitor), ii) PKB/Akt pathway by insulin growth factor (IGF), iii) Ca^{2+}-related pathway by endothelin-1 (ET-1). In the mitogen activated protein kinase (MAPK), phosphorylation level of MEK and ERK1/2 was elevated each 2.5-fold and 1.5-fold by PDGF stimulation, respectively. And, CGCs treated with U0126 were down-regulated in phosphorylation of ERK1/2 about 6-fold compared to PDGF only. Stress-responsive MAPK family, namely, the c-Jun N-terminal kinases (JNKs) and the p38-MAPKs were analogous to cardiomyocytes in the phosphorylation level of PDGF treated CGCs about 92% and 99%,

respectively. In PKB/Akt signaling pathway, phosphorylation level of Akt, mTOR (mammalian target of rapamycin) and 4E-BP (eIF4E binding protein) of CGCs by IGF were similar to those of cardiomyocytes. The lasting increase in Ca^{2+} of cytoplasm was reflected by fluo4-AM combined with intracellular Ca^{2+}. In ET-1 stimulation, phosphorylation and activation of CaMKⅡ and calcineurin (CaN) were detected in CGCs by immunoblot and immunocytochemistry. These result demonstrate that gene expression of CGCs are likely to cardiomyocytes and it is assumed that these pathways function to influence the change of intracellular signaling when cardiogenic differentiation are progress.

Key Words: mesenchymal stem cells, cardiogenic cells, cardiac gene expression

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I. INTRODUCTION

Chronic heart diseases such as ischemia and hypertensive heart failure are characterized by irreversible loss of cardiomyocytes. Generally accepted paradigms are that adult cardiomyocytes lack the ability to regenerate the myocardium in part because proliferation slows down at the time of birth. Although after birth cardiomyocytes do undergo some rounds of cell division (in human dividing cells are still found up to 3 weeks after birth), the last round of cell division in both human and mouse is actually incomplete. The cells undergo nuclear division only, and as a result the adult cardiomyocytes either have two diploid nuclei or one tetraploid nucleus. In addition, the heart was considered to lack stem cells. However, several recent reports have described resident stem cell

populations in human and rodent hearts that proliferate and may give rise to cardiomyocytes, vascular smooth muscle cells and endothelial cells.

Nevertheless, the regenerative capacity of the heart is still limited, and cell transplantation for the treatment of cardiac disease remains an attractive concept. Finding the most suitable source of cells for this purpose is, however, a major challenge of experimental cardiology at the present time. Stem cells may be derived either from adult human tissues (adult stem cells (ASCs)) or from human embryos (embryonic stem cells (ESCs)). First, ESCs are conceived as a highly promising therapeutic approach because they have the 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 from all three embryonic germ layers. But use of ESCs has been hampered due to the 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 the only suitable cells, as differentiated somatic cells neither possess ability to proliferate nor to differentiate into any other cell types. Second, ASCs is an undifferentiated cell found among differentiated cells in a tissue or organ, can renew itself, and can differentiate to yield the major specialized cell types of the tissue or organ. Its primary roles in a living organism are 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.[1-3]

Especially, MSCs, first characterized by Friedenstein and colleagues in 1987, are multipotent cells capable of differentiating into several lineages including; cartilage, bone, muscle, tendon, ligament and adipose tissue, both in vivo and in vitro, under appropriate culture conditions. Origin of MSCs is either the cambium layer of periosteum or bone marrow, although

other sources such as muscle, fat, and synovium provide a limited source. MSCs are also characterized by human leukocyte antigen (HLA) class I positive and HLA class II negative. Thus, these cells are hypoimmunogenic, that is, they do not elicit an immune response, and are suitable for allotransplantation, even between mismatched individuals.

Most strategies used involve inducing differentiation of stem cells in culture to a cardiac phenotype compatible with survival, electromechanical coupling and integration in the host heart tissue, without causing arrhythmias. Adult cardiomyocytes do not survive after transplantation into the adult heart, and skeletal muscle myoblasts are functionally isolated from their host cells, which may lead to arrhythmias. However, fetal cardiomyocytes from various species, including humans, do survive transplantation in the heart, so the goal may be to derive cells with fetal cardiomyocyte phenotype.[4-10]

As mentioned above, 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). These cells appeared spontaneous heart-like beating and expressed NK2 transcription factor related, locus 5 (Nkx 2.5), myocyte-specific enhancer factor 2 (MEF 2A), MEF 2C, which is cardiac specific marker and transcription factor. In that, cardiogenic differentiation is associated with changes in gene expression including increased expression of immediate early genes and re-expression of genes which are expressed early in development (the "fetal" pattern of gene expression; e.g. b-myosin heavy chain, atrial natriuretic factor). Although significant progress has been made in the molecular understanding of myocardiogenic differentiation, little is known about the mechanisms of genes expression involved in heart development. Therefore,

research efforts focused on identifying signaling pathway that regulate cardio-specific gene expression in cardiogenic cells derived from MSCs.[11-14]

An important question is how to mediating cardiac myocyte gene expression in cardiogenic cells derived from mesenchymal stem cells. To analyze signaling pathway related to gene expression in cardiogenic cells derived from mesenchymal stem cells, we will compare signaling mechanisms between two different cell types (MSC and cardiogenic cells, Figure 1)

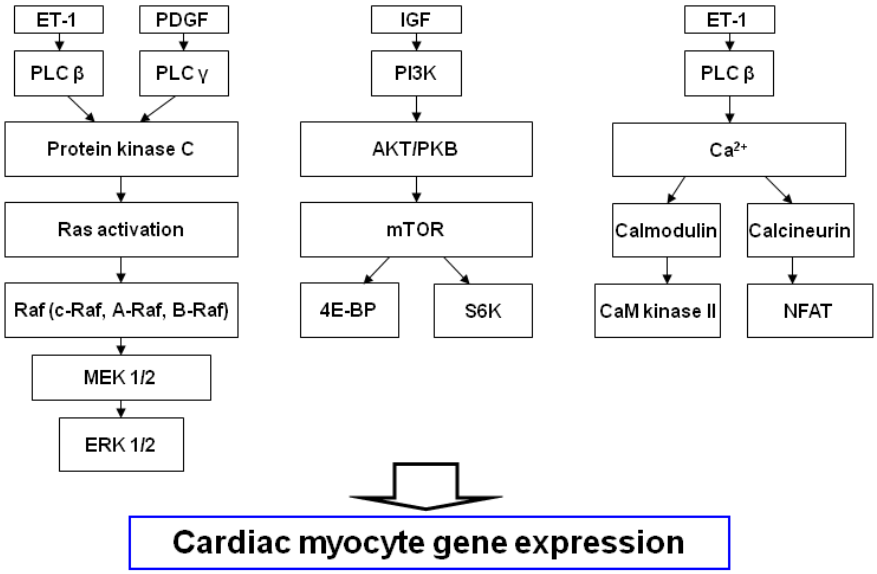

Figure 1. Signaling pathways mediating cardiac myocyte gene expression

II. MATERIALS AND METHODS

1. Materials

1) Animals

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

2. Methods

1) Isolation and primary culture of rat MSCs

Isolation and primary culture of MSCs from the femoral and tibial bones of donor rats were performed. Bone marrow-derived mesenchymal stem cells were collected from the aspirates of the femurs and tibias of 4-week-old Sprague-Dawley male rats (about 100 g) with 10 ml of MSC medium consisting of Dulbecco's modified Eagle's medium (DMEM)-low glucose supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and 1% antibiotic-penicillin and streptomycin solution (Gibco, Carlsbad, CA, USA). Mononuclear cells recovered from the interface of Percoll-separated bone marrow were washed twice and resuspended in 10% FBS-DMEM, and plated at 1×10^6cells/100 cm^2 in flasks. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 or 72 hours, nonadherent cells were discarded, and the adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). Fresh complete medium was added and replaced every 3 or 4 days for about 10 days. To further purify the MSCs, Isolex Magnetic Cell Selection System (Baxter Healthcare Corporation, Irvine, CA, USA) was used. Briefly, cells were incubated with Dynabeads M-450 coated with anti-CD34 monoclonal antibody. A magnetic field was applied to the chamber and the CD34+ cell-bead complexes were separated magnetically from the remaining cell suspension with the CD34− fraction being further cultured.

The cells were harvested after incubation with 0.25% trypsin and 1 mM EDTA (Gibco, Carlsbad, CA, USA) for 5 minutes at 37°C, replated in 1 × 10^5/100-cm^2 plates, and again grown for approximately 10 days.

2) Isolation and primary culture of rat cardiomyocytes

Neonatal rat ventricular cardiomyocytes 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, Carlsbad, CA, USA) in order to remove red blood cells. After depletion of the atria, the hearts were sliced up approximately 0.5 mm^3 sized pieces and treated with 4 ml collagenase Ⅱ (1.4 mg/ml, 270 units/mg; Gibco BRL, Carlsbad, CA, USA) solution for five minutes. The supernatant was removed, and the pellet was suspended in collagenase Ⅱ. After cells were incubated in a 37°C humidified atmosphere chamber containing 5% CO_2 for five minutes, gained supernatant was mixed with alpha minimum essential medium (α-MEM; Gibco BRL, Carlsbad, CA, USA) then centrifuged at 2000 rpm for 2 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°C 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 mm plates with α-MEM plus 10% FBS at 37°C in 5% CO_2 chamber. To eliminate fibroblast expansion, we used 0.1 mM 5-bromo-2'-deoxyuridine (Brd-U; Sigma-Aldrich, St. Louis, MO, USA).

3) Characterization of MSCs

Immunocytochemical characterization of MSCs was demonstrated below. Cells were cultured in 4-well side chamber, washed with PBS and incubated 1% paraformaldehyde solution (Sigma, St. Louis, MO, USA) for 10 minutes. PBS washing twice, then cells has permeated in 0.1% Triton X-100 for 7 minutes. After PBS washing twice, they have blocked for 1 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, West Grove, PA, USA) were used as secondary antibodies. Then, they were detected confocal microscopy (Carl Zeiss, Jena, Germany).

MSCs were performed according to the procedure of fluorescence-activated cell sorting (FACS) staining described below. Briefly, cells were detached from the plate with 10% trypsin-EDTA (Gibco BRL, Carlsbad, CA, USA), 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/ml in blocking buffer (1% BSA, 0.1% FBS) containing the following antibodies: CD14, CD34, 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 area. After two more washes, flow cytometric analysis was performed on a FACS Caliber system (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest™ software (Becton Dickinson, Franklin Lakes, NJ, USA) with 10,000 events recorded for each sample.

4) Cardiogenic differentiation of MSCs

MSCs were seeded on 60 mm plates at 2×10^5 cells/ml. To induce differentiation, MSCs were treated with 1 μM (total concentration) of PMA

at an interval of three days and changed fresh 10% FBS contained DMEM.

5) Sandwich ELISA

The capture antibody was bound to the bottom of each well and then the plate was incubated overnight at 4°C. The plate was washed twice with PBS (Gibco, Carlsbad, CA, USA) and treated with 100 μl of 3% BSA (Sigma, St. Louis, MO, USA) /PBS for 2~3 hours at 37°C at room temperature. After washing the plate twice with PBS, cell lysate was added to each well and the plate was incubated for at least 2 hours at room temperature in a humid atmosphere. The plate was washed four times with PBS containing 0.02% tween-20 (Sigma, St. Louis, MO, USA). Following adding the detector antibody, the plate was incubated for 2 hours at room temperature in a humid atmosphere. The plate was incubated again with addition of peroxidase conjugated secondary Ab for 1 hour at 37°C. Finally, the plate was treated with 100 μl of TMB (tetramethylbenzidine, Sigma, St. Louis, MO, USA) as substrate and 25 μl of 0.1 M H_2SO_4 as stop buffer, then detected immediately at 450 nm on an ELISA plate reader.

6) Immunocytochemistry

Immunocytochemistry methods were the same as those for 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, Jena, Germany).

7) Creatine kinase assay

Creatine kinase assay system was purchased by EnzyChrom^{TM} Creatine

Kinas Assay Kit (BioAssay Systems, Hayward, CA, USA). Samples should be assayed within cell lysate of 1mg if they remain at -20°C below. For each reaction well, buffer solution was prepared 10 μl substrate solution, 100 μl assay buffer and 1 μl enzyme. Ten μl calibrator and 100 μl water (calibrator) were mixed into wells of a clear bottom 96-well plate, and samples were transferred into calibrator. Reconstituted reagent (buffer solution) was added 100 μl and tap plate to mix. Samples were incubated at room temperature or 37°C. CK is fully activated within 10 minutes by glutathione provided in the substrate solution. They were read OD340 nm at 10 minutes and again at 40 minutes.

8) Immunoblot analysis

Cells were washed once in PBS and lysed in a lysis buffer (Cell signaling, Danvers, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF. Protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co., Ballerica, MA, USA). After blocking the membrane with Tris-buffered saline-tween 20 (TBS-T, 0.1% tween 20) containing 5% non-fat dried milk for 1 hour at room temperature, membrane was washed twice with TBS-T and incubated with primary antibodies for 1 hour at room temperature or for overnight at 4°C. The membrane was washed three times with TBS-T for 10 minutes, and then incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent

(Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using Photo-Image System (Molecular Dynamics, Sweden).

9) Measurement of intracellular Ca^{2+} overload in early ischemic cardiomyocytes

The measurement of cytosolic free Ca^{2+} was performed by confocal microscopy analysis. Neonatal rat cardiomyocytes were plated on glass coverslips coated with laminin (5 mg/cm^2) for 1 day in cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL, Carlsbad, CA, USA) and 0.1 μM BrdU. Cardiomyocytes were put in a hypoxic chamber for established ischemic time. After incubation, the cells were washed with modified Tyrode's solution containing 0.265 g/l $CaCl_2$, 0.214 g/l $MgCl_2$, 0.2 g/l KCl, 8.0 g/l NaCl, 1 g/l glucose, 0.05 g/l NaH_2PO_4 and 1.0 g/l $NaHCO_3$. Cells were then loaded with 5 mM of the acetoxymethyl ester of Fluo-4 (Fluo-4 AM, Invitrogen, Carlsbad, CA, USA) for 20 minutes, in the dark and at room temperature, by incubation in modified Tyrode's solution. Fluorescence images were obtained using an argon laser confocal microscope (Carl Zeiss Inc., Thornwood, NY, USA). This fluorochrome is excited by the 488 nm line of an argon laser and emitted light is collected through a 510-560 nm band pass filter. Relative changes of free intracellular Ca^{2+} were determined by measuring fluorescent intensity.

10) Statistical analysis

Data are expressed as means ±SE. Statistical comparisons between the two groups were performed using the Student´s t-test. In addition, a one-way ANOVA using a Bonferroni test was used when comparing more than

two groups. A p-value <0.05 was considered significant.

III. RESULTS

1. Primary culture and characterization of MSCs

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, 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, CD105, CD106, and ICAM. They expressed neither the hematopoietic marker CD34 nor CD14 (Figure 2).

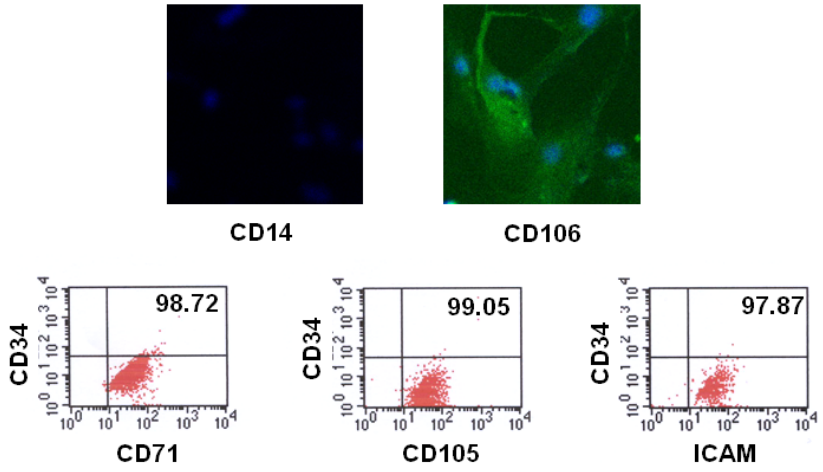

Figure 2. Characterization of MSCs purified from bone marrow. MSCs were primarily cultured in a PVC plate for 10 days. MSCs was transferred to a 4-well slide were executed before immunocytochemistry through a

confocal microscopy was conducted. These cells were positive for CD106 but negative for CD14 (blue: DAPI stain, green: CD106; magnification: X400). To confirm MSCs, cells were analyzed by CD34, CD71, CD105, and ICAM by the FACS Calibur system.

2. The change of cardiac specific marker in cardiogenic cells

To reconfirm myogenic differentiation of MSCs, changes of cardiac specific markers expression were analyzed with sandwich ELISA (Figure 3). 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 time-dependently. Expression of most of the specific markers was peaked around 9 days. Cardiogenic cells express multiple sarcomeric proteins associated with neonatal rat cardiomyocytes.

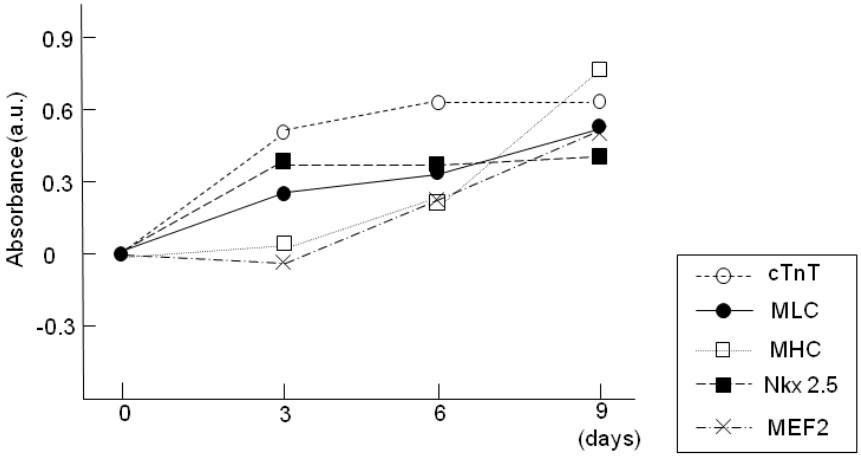

Figure 3. Alteration of cardiac specific marker in CGCs derived from MSCs. This change was detected by sandwich ELISA. Samples of protein were harvested and lysed each day (3, 6, and 9 days). This experiment was repeated three times, and each standard deviation was 0.05. Cardiac-specific markers (cTnT, MLC, MHC, Nkx 2.5 and MEF2) were elevated from two days to 9 days, especially day nine, which highly expressed in

five markers.

3. The formation of cardiogenic cells on morphology and expression

Primary culture MSCs were treated with 1 mM PMA (final concentration was 1 μM) according to the experimental group. This group was separated at 3 days, 6 days and 9 days. Treatment with PMA was every 3 days. Because the dilution base for PMA was dimethylsulfoxide, the control group was also treated with DMSO of the same volume. Normal MSCs were spindle-shaped until 9 days, but MSCs treated with PMA changed into a stick-like or branching-out appearance at 9 days (Figure 4A). To examine where MHC and cTnT-expressing cells are located in differentiated MSCs, immunostaining was performed. Clearly, cardiogenic cells had significant morphological changes with positive immunocytochemical analysis for anti-MHC and cTnT, but normal MSCs showed neither significant morphological changes nor cardiac-specific protein expression of MHC and cTnT during the 9 days period of observation with the same treatment (Figure 4B).

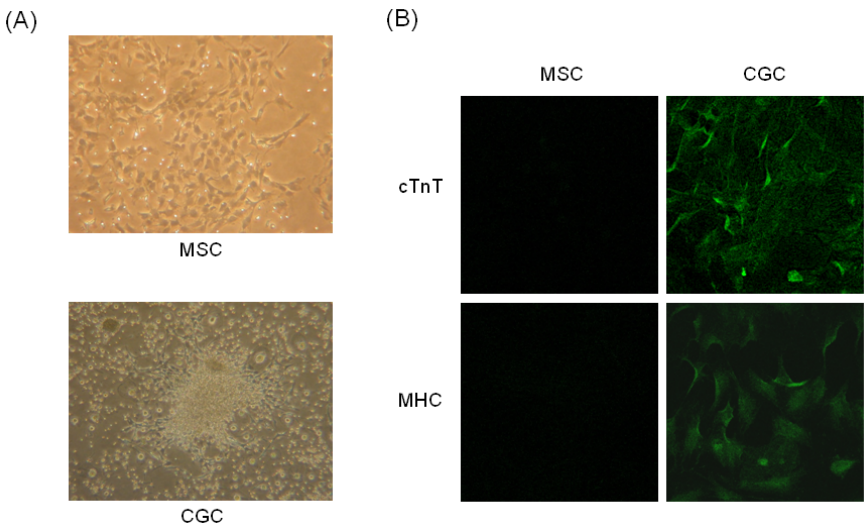

Figure 4. (A) Alteration of cardiogenic morphology. Electron micrographs of CGC were taken after 9 days compared to normal MSC. CGC changed morphology to cardiomyocyte-like and fibroblast-like aggregated branches (magnification: X100). (B) Expression of cardiac-specific factor in CGC. Immunocytochemistry was detected for cardiac TnT and MHC in CGC. Representative photos of normal MSC as positive control (Magnification: X400). Cardiac TnT and MHC visualized by FITC (green) is located on the stress fibers.

4. Measurement of cardiogenic differentiation by creatine kinase assay

Creatinine kinase (CK) is found in skeletal and cardiac muscle, as well as the gastrointestinal tract. To detect CK activity, MSCs and cardiogenic cell were exposed in 10 μM norepinephrine (hypertrophic response) for 10 minutes and hypoxic condition of 6 hours (ischemic condition). CK activity was up regulated about 1.36-fold in normal cardiogenic cells, 1.25-fold in norepinephrine for 10 mins and 1.66-fold in ischemic condition for 6 hours (Figure 5).

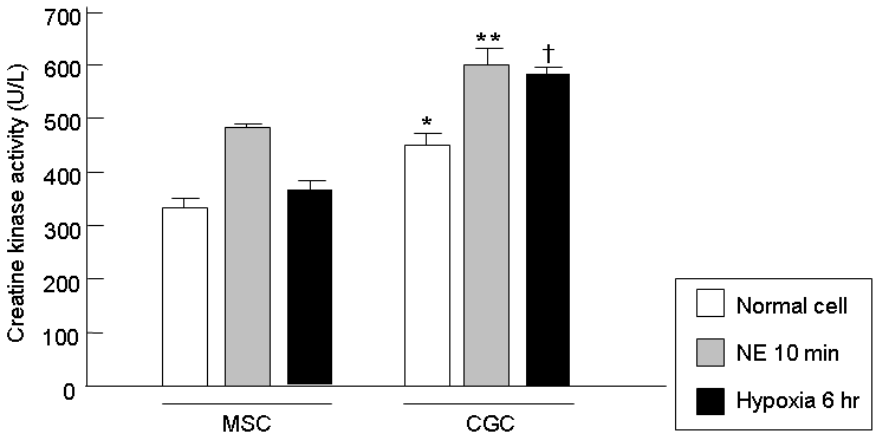

Figure 5. Cardiogenic differentiation on expression of creatine kinase. MSC and CGC was exposed norepinephrine (10 μM) for 10 minutes or hypoxic condition (less than 1% O_2) for 6 hours. Calibrator (negative control) and samples were added into reconstituted reagent for 10 and 40 minutes. OD_{40min} and OD_{10min} are OD_{340nm} values at 40 minutes and 10 minutes for the sample. $OD_{CALIBRATOR}$ and OD_{H2O} are OD340nm values of the calibrator and water blank at 40 minutes. The value 100 is the equivalent activity (U/L) of the Calibrator under the assay conditions. The mean±SE of three independent experiments is reported. *p<0.001 vs. MSC normal cell, **p<0.001 vs. MSC NE 10 minutes, †p<0.001 vs. MSC hypoxia 6 hours.

5. The change of MAPK cascades in cardiogenic cells

To assess the effect on ERK, JNK and p38 (MAPKs) activation, immunoblot analysis was performed by treating cardiogenic cells with PDGF of 2 ng/ml. Immunoblots of total cell lysates were performed with antibodies against phosphor-active MAPKs and total MAPKs. Analysis of MAPK activation shows, a high level of phospho-ERKs, -MEKs, -JNKs and -p38 similarly occurred in cardiogenic cells with PDGF compared to neonatal rat cardiomyocytes with PDGF (Figure 6).

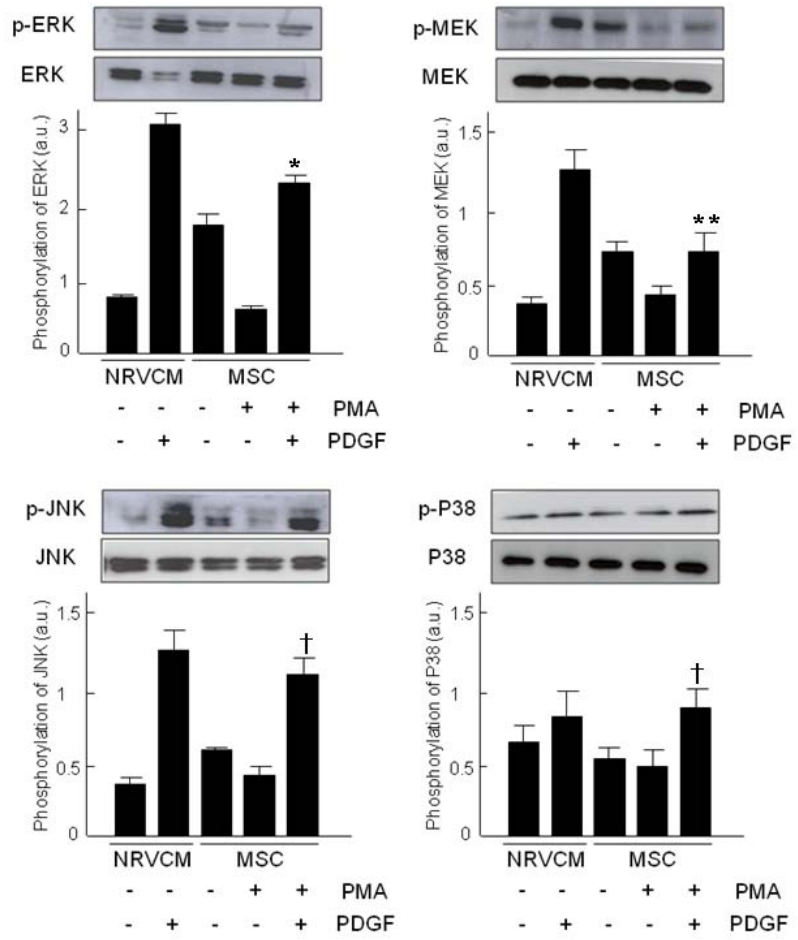

Figure 6. Effect of PDGF on MAPK cascades of CGC. The phosphorylated states or total expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the ERK1/2, MEK, JNK, and P38. Representative immunoblots for phosphorylation of ERK1/2, MEK, JNK, and P38 were demonstrated and its result was compared to NRVCM. *p<0.001 vs. p-ERK (NRVCM PDGF+), **p<0.01 vs. p-MEK (NRVCM PDGF+), †p<0.05 vs. p-JNK and p-P38 (NRVCM PDGF+).

6. Inhibition of MAPK cascade by MEK inhibitor in CGC

As shown in Figure 7, PDGF of 2 ng/ml was increased phosphorylation of PKC and ERK1/2 about 3.1-fold and 1.3-fold, respectively. To clarify

whether alteration of CGC on MEK inhibition is mediated through signaling pathway of MAPK cascade, we used U0126, MEK inhibitor, in CGC treated PDGF. In CGC, treatment with PDGF and U0126 was not affected phosphorylation of PKC. But, phosphorylation of ERK1/2 was increased in PDGF-treated CGC about 2-fold compared to normal CGC and co-treated with PDGF and U0126 was decreased about 6-fold compared to PDGF-treated CGC (Figure 7). This data indicates that there is a direct relationship between CGC and MAPK cascades.

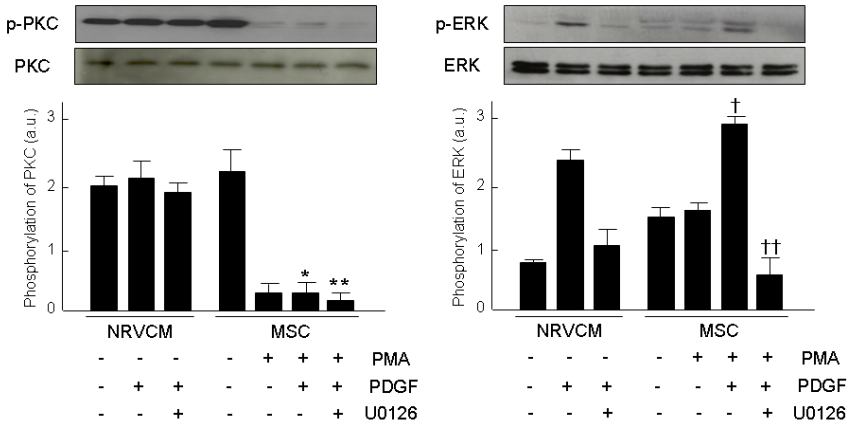

Figure 7. Effect of U0126 on PKC and ERK phosphorylation. The phosphorylated states or total expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the PKC and ERK1/2. Representative immunoblots for phosphorylation of PKC and ERK1/2 were demonstrated and its result was compared to NRVCM. *p<0.001 vs. p-PKC (NRVCM PDGF+), **p<0.001 vs. p-PKC (NRVCM PDGF+, U0126+), †p<0.05 vs. vs. p-ERK (NRVCM PDGF+), ††p<0.05 vs. vs. p-ERK (NRVCM PDGF+, U0126+).

7. The role of PKB/Akt in cardiogenic differentiation

To confirm that PKB/Akt signaling in cardiogenic cells was transduced through mTOR, cardiogenic cells were serum-starved and then challenged

with IGF in the presence PMA. Phosphorylation of Akt, mTOR and 4EBP-1 were up-regulated in IGF-treated cells from 2- to 2.5-fold (Figure 8). These observations suggest that mTOR-4EBP-1 signaling is downstream of Akt in gene expression of cardiogenic cells.

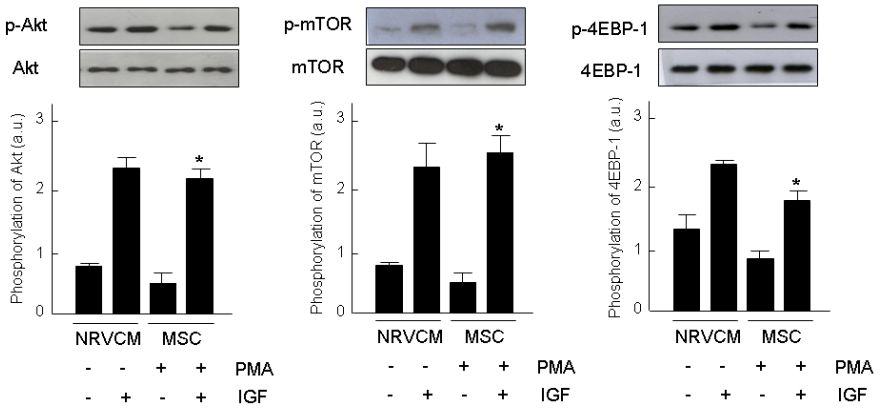

Figure 8. Alteration of PKB/Akt pathway in IGF-treated CGC. IGF1 (100 nM)-treated CGC was compared to NRVCM. The phosphorylated states or total expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the Akt, mTOR, and 4EBP-1. Representative immunoblots for phosphorylation of Akt, mTOR, and 4EBP-1 were demonstrated and its result was compared to NRVCM. *p<0.05 vs. NRVCM IGF+.

8. Activation of Ca^{2+}-related transcription factors in cardiogenic cells

The lasting increase in [Ca^{2+}] of cytoplasm was necessary for the activation of calcineurin (Ca^{2+}/CaM-dependent phosphatase) and CaMKⅡ (Figure 9). CaMKⅡ activated directly can be autophosphorylated, resulting in sustained CaMKⅡ activation. Exposure of resting myocytes to ET-1 induced CaMKⅡ autophosphorylation had the same effect in cardiogenic cells. And, ET-1-induced calcineurin expression was up regulated in cardiomyocytes and cardiogenic cells alike. NFAT was

constitutively detectable while ET-1 treated in cardiogenic cells (Figure 9A). The [Ca^{2+}] in plasmas and nuclear of cardiogenic cells were reflected by fluorescence intensity of fluo4-AM combined with intracellular Ca^{2+} (Figure 9B). These experiments mechanistically explain how calcium to CaMKⅡ and calcineurin/NFAT work in concert to activate transcription in cardiogenic cells.

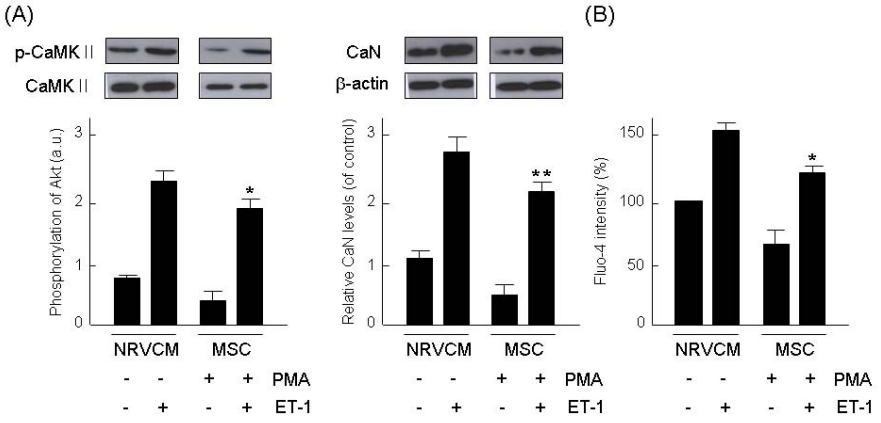

Figure 9. Effect of ET-1-treated CGC in calcium regulation. (A) ET-1 (100 nM)-treated CGC was compared to NRVCM. The phosphorylated states or total expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the CaMKⅡ and CaN. Representative immunoblots for phosphorylation of CaMKⅡ and CaN were demonstrated and its result was compared to NRVCM. *$p<0.05$ vs. NRVCM ET-1+, **$p<0.01$ vs. NRVCM ET-1+. (B) The 2 μM Fluo-4 was loaded into the cardiomyocytes and they were cultured for 20 minutes in a humidified chamber. Relative intracellular Ca^{2+} intensity was determined from the manufacturer's manual. *$p<0.05$ vs. NRVCM ET-1+.

IV. DISCUSSION

Although MSCs are being increasingly used for cell-based cardiac repair, little is known about whether these cells are capable of adopting a cardiac

phenotype.[14-16] This study was developed a new method for inducing cardiogenic differentiation of MSCs using the PMA as the only PKC activator.

Cardiomyocytes have been generated from murine and human adult stem cells as well as from embryonic stem cells. Approaches to increase the cardiac differentiation of adult bone marrow-derived in vivo may exploit the molecular and cellular signals mediating the in vitro induction of cardiac myocytes differentiation. Initial in vitro studies used 5'-azacytidine treatment of undifferentiated bone marrow cells to generate cardiac myocytes. The molecular events mediating this derivation, however, have yet to be identified. More recently, report indicates that non-canonical Wnt signaling enhances differentiation of circulating progenitor cells to cardiomyogenic cells. But, this study has also yet to be identified for molecular events of cardiogenic differentiation.[17-21]

The principal cascades which are particularly associated with cardiac myocyte responses are the MAPKs and the PKB/Akt pathway. A detailed description of the pathways is beyond the scope of this study. It was studied that cardiogenic cells related with cardiac myocyte biology, highlight novel or neglected aspects of the signaling, and focus on the influence on transcriptional regulation. In cultured cardiac myocytes, the extracellular signal-regulated kinase 1/2 [ERK1/2, a subfamily of the mitogen-activated protein kinases (MAPKs)] are potently activated by endothelin-1 (ET-1) and minimally activated by insulin.[35] In proliferating cells, signaling through ERK1 and ERK2 provides an important cue for cell cycle progression and ERK1/2 may thus be viewed as a "growth" signal. JNKs and p38-MAPKs were originally identified as "stress-responsive" MAPKs. [22-27] This study indicated that MAPKs cascades were operated to PDGF-treated condition in cardiogenic cells.

Activation of phosphatidylinositol 3' kinase (PI3K) and signaling through PKB/Akt were first delineated in response to insulin, and is almost universally associated with increased rates of protein synthesis and cytoprotection. Insulin or insulin-like growth factor Ⅰ potently activate PKB/Akt in cardiomyocytes. [28-32] Indeed, activation of cytoprotective signal may be required for apoptosis to proceed in a regulated manner. With IGF, cardiogenic cells were expressed in phosphorylation of mTOR and 4EBP.

Some DNA-binding transcription factors are regulated by their subcellular localization, and phosphorylation/dephosphorylation events modulate their migration into the nucleus where they can regulate gene expression. Probably the best known of these in relation to cardiac hypertrophy are the NFATs. Calcineurin is uniquely activated by sustained elevation of $[Ca^{2+}]_i$. Nuclear factor of activated T cells (NFAT), a family of transcriptional regulators and an established target of the calcineurin signaling pathway, is dephosphorylated by calcineurin and subsequently translocates to nucleus. CaMKⅡ is another local partner of nuclear $InsP_3R$. In ventricular myocytes, CaM is also known to interact with $InsP_3R$. The activated CaMKⅡ could then go on to phosphorylate other targets. In the heart, phosphorylation of these HDACs (Histone deacetylases) appears to be particularly mediated by CaM kinases in response to an increase in intracellular/nuclear Ca^{2+}. [33-35] This study indicated that CaM and calcineurin/NFAT signaling were associated with gene expression of cardiogenic cells.

Stem-cell-based therapies have improved cardiac function in recent clinical trials, and have become the most promising means of treatment in regenerative cardiovascular medicine. However, more information

including more appropriated cell type, cell fate, mechanism of effect, cell modifications, and feasible delivery methods need to be clarified. In this study we tried to clarify the signaling pathway mediating differentiation of cardiac myocyte which may help future genetic therapy in clinical bases.

V. CONCLUSION

This study has shown that MSCs are able to alter expression of cardiac specific factor in cardiogenic differentiation. We experimented about cardiogenic differentiation with PKC activator. PKC activating signaling is sufficient to elicit a cardiomyocytic phenotype in these cells via the induction of the cardiac fetal gene program. In CGC, signal pathway of MAPK cascades was expressed, using PDGF and U0126. PKB/Akt pathway was significantly elevated expression level in stimulus treated group (IGF). Ca^{2+}-related signaling was operated in ET-1 treated CGC. Cardiogenic differentiation has been made in identifying protein kinase signaling pathways which are likely to be involved in regulating cardiac myocyte response, and it is assumed that these pathways regulate gene/protein expression to influence the changes that are seen. Many of the genes which are modulated encode ‘regulators’ of gene/protein expression may be linked to the changes in gene and protein expression observed in cardiogenic differentiation of stem cells.

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ABSTRACT IN (KOREAN)

골수 간엽 줄기세포에서 분화된 심근 세포에서 관련 유전자 발현을 매개하는 신호기전

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이 삭

골수 간엽 줄기세포의 심근 재생 치료 효과가 잘 알려져 있음에도 불구하고 심근세포로의 분화를 유도하는 특이 자극이나 신호전달 체계에 대해서는 완전하게 알려져 있지 않다. 본 연구에서는 PKC 활성제를 이용하여 골수에서 유도된 중간엽 줄기세포에서 i)혈소판 유도 성장인자(PDGF)와 U0126에 의해 유도되는 MAPK 신호전달체계, ii)인슐린 성장인자(IGF)에 의해 유도되는 PKB/Akt 신호전달체계, iii) endothelin-1 (ET-1)에 의해 유도되는 칼슘관련 신호전달체계의 활성화 여부를 관찰하였다. MAPK 신호전달체계에서는 PDGF 자극에 의해 MEK와 ERK1/2의 인산화가 각각 2.5배와 1.5배 증가하였으며, U0126으로 처리된 심근세포에서는 PDGF 단독 처리한 심근에 비해 ERK1/2의 인산화가 약 6배 감소되었다. 압박-반응(stress-responsive) MAPK 군, 일명 c-Jun N-terminal kinases와 p38-MAPKs는 PDGF로 처리된 심근세포에서 각각 92%와 99%의 유사한 인산화율을 보였다.

PKB/Akt 신호전달 체계에서는 IGF 자극에 의해 중간엽세포에서 유도된 심장계통 세포와 심근세포가 유사한 정도로 mTOR와 4E-BP의 인산화가 이루어졌다. 세포질내의 지속적인 칼슘증가는 세포내 칼슘과 flou4-AM의 결합에 의해 반영되었다. ET-1 자극에 의해 CaMK II와 calcineurin의 활성과 인산화가 증가되는 것을 immunoblot과 면역세포화학 방법으로 측정하였다. 이러한 결과는 심근세포계통으로의 분화하는 과정에서 심근세포양 세포와 심근세포의 유전자 발현을 유도하는 세포내 신호전달체계가 비슷한 과정에 의해 매개된다는 것을 보여주는 것이라 하겠다.

핵심되는 말 : 골수간엽 줄기 세포, 심근계통 세포, 심장 유전자 발현