Expression and functional role of GTP binding proteins in bone marrow derived mesenchymal stem cells

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Expression and functional role of GTP binding proteins in bone marrow derived mesenchymal stem cells

Directed by Professor Namsik Chung

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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June/2009

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June/2009

ACKNOWLEDGEMENTS

This paper would not have been possible without the support of many people. I would like to begin by expressing my sincere appreciation to my supervisor, Professor Namsik Chung for his tremendous guidance, encouragement and support. I would also like to express my deep gratitude to the committee members, Professor Ki-Chul Hwang, Professor Seok-Min Kang, Professor Kuk-In Park, and Professor Jaesang Kim for their enthusiasm, critical review, and valuable comments for the manuscript. I also sincerely appreciate the cooperation and support of all members of the Yonsei Cardiovascular Research Institute, especially Woo Chul Chang and Byung Wook Song.

I wish to thank professors of Cardiology Division, especially Professor Sung Soon Kim, Professor Seung-Yun Cho, Professor Won-Heum Shim, and Professor Yangsoo Jang. They guided me in every step and introduced me to what the cardiologist should do. I also wish to thank professors of Department of Internal Medicine at Yonsei University College of Medicine, Professor Chae Yoon Chon, Professor Shin-Wook Kang, Professor Seung Tae Lee, Professor Young Sam Kim, Professor Yong Chan Lee, and Professor Yumie Rhee. They have always shown me how the doctor of Internal Medicine should live through words and deeds.

I am very much indebted to my family, including my parents-in-law. They've always encouraged and understood me with love and patience. Lastly - and by no means the least - I want to give my love to my wife and two daughters who have always been beside me, understanding and encouraging me; without them I would not have been able to come this far.

Even though I feel very much ashamed of my work, I would like to dedicate this dissertation to the living and true God, who is a Spirit, infinite, eternal, and unchangeable, in his being, wisdom, power, holiness, justice, goodness, and truth.

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ABSTRACT

Expression and functional role of GTP binding proteins in bone marrow-derived mesenchymal stem cells

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(Directed by Professor Namsik Chung)

Mesenchymal stem cells (MSCs) are shown to differentiate into cardiomyocyte under proper situation. However the molecular mechanisms underlying cardiomyogenic differentiation from MSCs remain poorly understood. Previous study reported that GTP binding proteins (G proteins) might play a critical role in general differentiation of MSCs and also an important role in cardiac differentiation of embryonic stem cells. Thus, our study aimed to investigate the expression and functional role of heterotrimeric and monomeric G proteins in MSCs. To evaluate the cardiac differentiation potential in cardiogenic cells (CGCs), three different cell groups, thus MSCs, cardiomyocytes (CMs), and CGCs from MSCs were analyzed. First of all, we investigated the expression level of heterotrimeric and monomeric G proteins in these three groups. As for the heterotrimeric G protein, expression level of G_q and G_s were similar in CMs and CGCs. In monomeric G proteins, RhoA and Rab1b were similarly expressed in CGCs compared with CMs. We also investigated intracellular signaling pathways related to G proteins, especially in α-, β-adrenergic receptor related signaling. α-adrenergic receptor and G_q

related Rho signaling and subsequently ERK 1/2 signaling were confirmed in CGCs as well as in CMs. β-adrenergic receptor and G_s related Rab signaling and further downstream ERK 1/2 signaling were also confirmed in CGCs and CMs. In conclusion, CGCs and CMs showed similar G protein expression and function in α-, β-adrenergic receptor related signaling. Our results showed that G proteins may play important roles in cardiac differentiation of MSCs.

Key words: mesenchymal stem cell, cardiogenic cell, GTP binding protein

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I. INTRODUCTION

Mesenchymal stem cells (MSCs) are non-hematopoietic multi-potent stem cells that are capable of differentiating into both mesenchymal and non-mesenchymal lineages. In fact in addition to bone, cartilage, fat, and myoblasts, it has been demonstrated that MSCs are capable of differentiating neurons and astrocytes in vitro and in vivo[1-3]. MSCs are of much interest in cell therapy for several reasons. These cells have a high expansion potential, genetic stability, can be easily collected and shipped from the laboratory to the bedside and are compatible with different delivery methods and formulations. In addition, MSCs have two other extraordinary characteristics. They are able to migrate to sites of tissue injury and have strong immunosuppressive properties that can be exploited for successful autologous as well as heterologous transplantations[4]. For these reasons and also with the

extensive clinical experience already in place for marrow as a cell-based therapy, the rapid progress of clinical trial using bone marrow in cardiovascular medicine was facilitated. In general, these studies demonstrated that cell therapy with bone marrow derived stem cells is feasible, safe and showed modest improvements in physiologic and anatomic parameters in patients with both acute myocardial infarction and chronic ischemic heart disease[5, 6]. However the overall effects of MSCs were not satisfactory as anticipated. The initial enthusiasm which fueled initial clinical studies has largely faded. Instead further investigations on each cellular mechanism - differentiation, proliferation, migration, adhesion - are largely required. Among various topics for MSCs biology, understanding the mechanisms for differentiation into cardiomyocytes is very important for future cell based therapy in cardiovascular medicine.

MSCs are shown to differentiate into cardiomyocyte under proper situation[7-10]. However the molecular mechanisms underlying cardiomyogenic differentiation from MSCs remain poorly understood. Previous study reported that GTP binding proteins (G proteins) might play a critical role in general differentiation of MSCs[11-16] and also an important role in cardiac differentiation of embryonic stem cells[17]. GTP-binding proteins are involved in the regulation of cellular functions in virtually all living organisms and play key roles in signal transduction in cells as well as in the regulation of gene expression[18-22]. These specialized proteins are separated into two distinct classes consisting of (i) the heterotrimeric GTP binding proteins and (ii) the monomeric GTP binding proteins. Thus, our study aimed to investigate the expression and functional role of heterotrimeric and monomeric G proteins in MSCs. To evaluate the cardiac differentiation potential in cardiogenic cells (CGCs), three different cell groups, thus MSCs, cardiomyocytes (CMs), and CGCs from MSCs were analyzed.

II. Material and Methods

1. Materials

A. 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 neonatal rats. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care Committee.

2. Methods

A. 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, USA) and 1% antibiotic-penicillin and streptomycin solution (Gibco). Mononuclear cells recovered from the interface of Percoll-separated bone marrow were washed twice and resuspended in 10% FBS-DMEM, and plated at 1×10^6 cells/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) 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) for 5 minutes at 37℃, replated in 1 × 10^5/100-cm^2 plates, and again grown for approximately 10 days.

B. Isolation and primary culture of rat cardiomyocytes

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 mm^3 sized pieces and treated with 4 ml collagenase Ⅱ (1.4 mg/ml, 270 units/mg; 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°C humidified atmosphere chamber containing 5% CO_2 for five minutes, gained supernatant was mixed with alpha minimum essential medium (α-MEM; Gibco BRL) 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, most of fibroblasts became adherent cells, while NRVCMs turned into non-adherent cells. 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, USA).

C. Characterization of MSCs

Immunocytochemical characterization of MSCs was demonstrated below. Cells were cultured in 4-well chamber slides, washed with PBS and incubated 1% paraformaldehyde solution (Sigma, USA) for 10 minutes. After PBS

washing twice, cells have been permeated in 0.1% Triton X-100 for 7 minutes. Then they have been blocked for 1 hour (blocking solution: 2% bovine serum albumin, 10% horse serum in PBS) and adhered with the following antibodies: CD34 and CD71. FITC-conjugated mouse, rabbit and goat (Jackson Immunoresearch Laboratories, USA) were used as secondary antibodies. Then, they were detected by confocal microscopy (Carl Zeiss, Germany).

MSCs were characterized according to the protocol for fluorescence-activated cell sorting (FACS) staining methods 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℃ 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, CD105, and CD106; In the case of CD14 and CD34, 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 Calibur system (Becton Dickinson, USA) using CellQuest™ software (Becton Dickinson, USA) with 10,000 events recorded for each sample.

D. 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 (Phorbol 12-myristate 13-acetate, St Louis, MO, USA) at an interval of three days and changed fresh 10% FBS contained DMEM.

E. Sandwich ELISA

The capture antibody was bound to the bottom of each well and then the

plate was incubated overnight at 4℃. The plate was washed twice with PBS (Gibco) and treated with 100 μl of 3% BSA (Sigma) /PBS for 2~3 hours at 37℃ 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). After 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 antibody for 1 hour at 37℃. Finally, the plate was treated with 100 μl of TMB (tetramethylbenzidine, Sigma) 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.

F. 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, USA) and they were detected using confocal microscopy (Carl Zeiss).

G. Immunoblot analysis

Cells were washed once in PBS and lysed in a lysis buffer (Cell signaling, 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, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co., 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℃. 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, USA). The band intensities were quantified using Photo-Image System (Molecular Dynamics, Sweden).

H. RT-PCR analysis

(A) Isolation of total RNA

Total RNA was extracted by 500 μl/ 60 mm plate Tri-reagent (Sigma, USA). Chloroform was poured about 100 μl above Tri-reagent, vortexing a sample about 10 seconds. Then, sample was centrifuged at 12000 g, 4°C and 15 minutes. Three layers were appeared in the tube, transparency upper layer collected in new tubes. And, 2-propanol was poured about 250 μl over the sample, again vortexing a sample about 30 seconds. Centrifuge was accomplished about 12000 g, 4°C and 10 minutes. Left the pellet, supernatant was discarded and washed by 75% ethanol (Duksan, Korea) –mixed diethylpyrocarbonate (DEPC; Sigma) water. Centrifugation was also operated about 7500 g, 4°C and 5 minutes. The supernatant was dismissed, and pellet was dried on room temperature about 7 minutes. Finally, 30 μl nuclease free water (NFW) was poured onto pellet. The quality and quantity of the RNA was detected by OD_{260}/OD_{280} with DU 640 spectrophotometer (Effendorf, Germany).

(B) cDNA synthesis

Complementary DNA (cDNA) was synthesized with RT-&GO™.

Quantitative 1 μg total RNA was added to 1 μl anchored primer $(dT)_{25}V$, 2 μl dithiothreitol (DTT) and NFW, totally 9 μl. To prevent secondary structures, mixture was incubated for 5 minutes at 70°C, and added 8 μl of RT-&GO™ mastermix. The sample was incubated at 42°C for 1 hour and then reverse transcriptase was inactivated at 70°C for 15 minutes. Alike isolation of total RNA, sample was detected by OD_{260}/OD_{280} with DU 640 spectrophotometer.

(C) PCR analysis

Quantitative 1 μg cDNA, each 10 pmol primer (forward and backward; Table 1), 0.1 mM dNTP mixture, 1.25 U of Taq polymerase and 10 X reaction buffer were mixed with NFW, lastly total volume of 25 μl. PCR condition was fixed as follows. A cycle of denatureing at 94°C for 3 minutes followed by number of 35 cycles with denaturation at 94°C for 30 seconds, annealing at 48°C to 60°C for 30 seconds, and elongation at 72°C for 30 seconds. Then sample was kept up 72°C for 10 minutes. Specific primers were shown in Table 1. When PCR assay have finished, PCR product was separated by electrophoresis in a 1.2% agarose gel (Biorad) and Gel-Doc (Biorad) visualized after staining with ethidium bromide (EtBr; Sigma).

Table 1. Sequences of PCR primers

primer	sequence	primer	sequence
G_h	ATCTACCAGGGCTCTGTCAA ACTCCACCCAGCAGTGGAAA	G_i	CTCTAAGATGATCGACAAGA CATGCGATTCATCTCCTCAT
G_q	TCATTAAGCAGATGAGGATC CTCCACAAGAACTTGATCGT	G_s	AACAGTAAGACCGAGGACCA AGATGATGGCAGTCACATCA
RhoA	ACCAGTTCCCAGAGGTTTATGT TTTGGTCTTTGCTGAACACT	N-RAS	AAACTGGTGGTGGTTGGAGCAGG CCATCGTCACTGCTGTTGAG
R-RAS	ACACAGAAGACCACGTCTTCTC ATGGCTGCCTCTTGCTTATCTC	RAN	TCGTCTTCCATACCAACAG ACAGGTCGTCATCCTCATC
Rab1b	TACAGATTTGGGACACAGCTGGTC ACAGGAGTGCTGTCGATCTTCAG	Rab3b	TGCTCATCATTGGCAACAGCAGC TAGCAAGAGCAGTTCTGCTGGAG
Rab6b	GGTTCAGGAGCTTGATTCCTAGC GAACAGCCACCTTCATTGACTGG	Rab11	TACTATCGTGGAGCAGTAGGTGC AGATGTTCTGACAGCACTGCACC

Arf	TATCTGAGACGGTTTGGGCA ATCAGTGAGTTCCAAGGGGG	Arf1	GCGCCACTACTTCCAGAACA TGTACCAGTTCCTGTGGCGT
Arf4	GTTTGGGATGTTGGTGGTCA TCCTGCAGCTCATCTTCCAG	Arf5	CAGAAGATGCTGCAGGAGGA CGCTTTGATAGCTCATGGGA
Arf6	TCTGTGACCACCATTCCCAC GGTCATTGATAATGCGGTGC	18s rRNA	GTCCCCCAACTTCTTAGAG CACCTACGGAAACCTTGTTAC

I. 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, CD90, CD105, and CD106. They expressed neither CD34 nor CD14, which are hematopoietic markers (Figure 1).

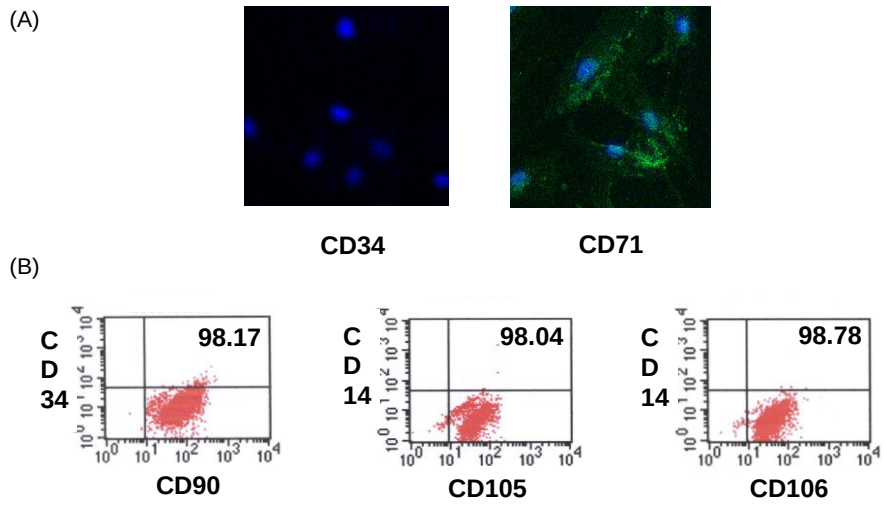

Figure 1. Characterization of rat MSCs. MSCs were primarily cultured in a PVC plate for 10 days. MSCs was transferred to a 4-well slide were executed before immunocytochemistry through a confocal microscopy was conducted. These cells were positive for CD71 but negative for CD34 (blue: DAPI stain, green: CD71; magnification: X400). (B) To confirm MSCs, cells were

analyzed by CD14, CD34, CD90, CD105, and CD106 by the FACS Calibur system.

2. Culture of MSCs differentiated into CGCs

Primary-cultured MSCs were treated with 1 mM PMA (final concentration was 1 μM) for 9 days. Treatment of PMA was every 3 days. Because dilution base of PMA was dimethylsulfoxide (DMSO, Sigma), counterpart was also treated DMSO as same volume. Normal MSCs was spindle-shaped until 9 days, but MSCs treated with PMA were changed into stick-like or branch-out appearance at 9 days (Figure 2A). To examine where cTnT and MLC-expressing cells are located in differentiated MSCs, immunostaining was performed (Figure 2B). Clearly, cardiogenic cells had significant morphological changes with positive immunocytochemical analysis for anti-cTnT and MLC, but normal MSCs showed neither significant morphological changes nor cardiac-specific protein expression of cTnT and MLC during the 9 days period of observation with the same treatment.

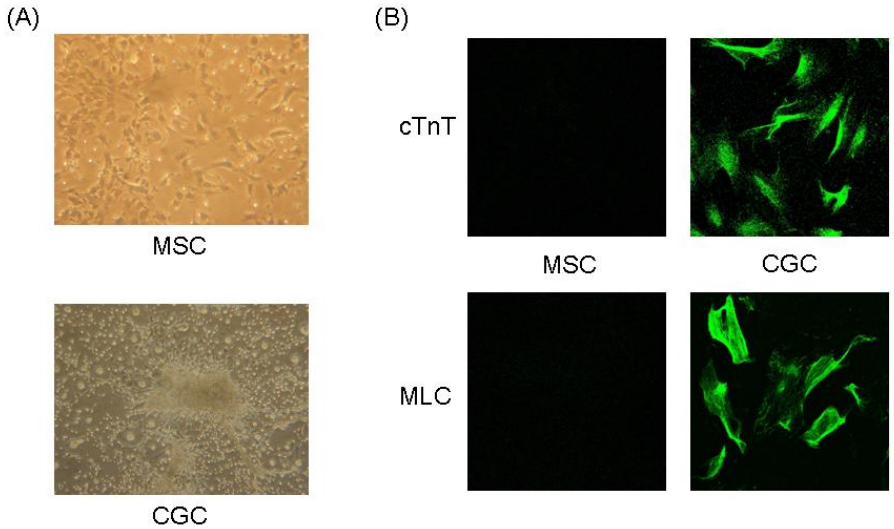

Figure 2. Comparative analysis for morphological and phenotypical changes of CGCs. (A) Light micrographs of CGC were taken after 9 days compared to normal MSC. CGC changed morphology to cardiomyocyte-like and fibroblast-like aggregated branches (magnification: X100). (B) Immunocytochemistry revealed cardiac specific TnT and MLC in CGC. Representative photos of normal MSC as positive control (Magnification: X400). Cardiac TnT and MLC visualized by FITC (green) is located on the stress fibers.

3. Cardiac-specific marker expression of CGCs

To confirm cardiogenic differentiation of MSCs, changes of cardiac specific markers expression were analyzed with sandwich ELISA. Cell lysates were used to antigen, and capture and detect antibody were treated with HRP-conjugated second antibody. These substrates were excited by TMB, then stop solution was stopped their reactions. 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 in time-dependent manner. Expression of cardiac specific markers was peaked around 9 days. Cardiogenic cells express multiple sarcomeric proteins associated with neonatal rat cardiomyocytes (Figure 3).

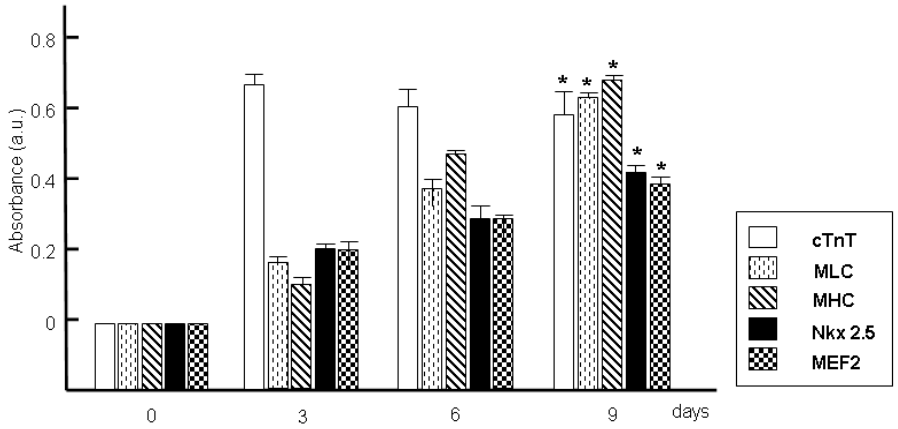

Figure 3. Changes of cardiac specific markers in CGCs. This change was detected by sandwich ELISA. Samples of protein were harvested and lysed each day (3, 6, and 9 days). This experiment was repeated three times. Cardiac-specific markers (cTnT, MLC, MHC, Nkx 2.5 and MEF2) were elevated from day 3 to day 9, especially at day 9. *$p<0.001$ vs. 0 day.

4. Expression of heterotrimeric G proteins in CGCs

To determine the involvement of heterotrimeric GTP-binding protein in CGCs, mRNA levels of heterotrimeric GTP-binding proteins were primarily examined. Experimental group was neonatal rat ventricular cardiomyocytes (CMs), MSCs and CGCs. Expression of G_s in CGCs was much alike in CMs, and G_q was increased in CGCs about 80% compared to CMs. However, G_h and G_i were not similar between CMs and CGCs (Figure 4). These data indicate that cardiogenic differentiation was related to the expression of G_q and G_s.

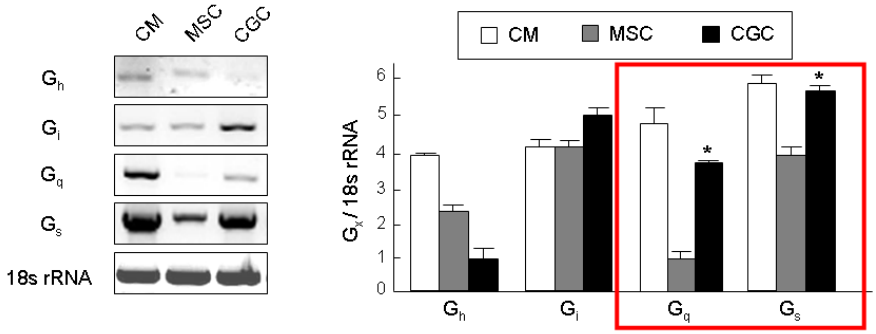

Figure 4. Alteration of expression levels for heterotrimeric GTP-binding proteins in CGCs. RNA was extracted from CMs, MSCs, and CGCs. The mRNA of heterotrimeric GTP-binding proteins (G_h, G_i, G_q, and G_s) was amplified by RT-PCR. The experiment was repeated 3 times with similar results. Results were normalized to 18s rRNS expression. *$p<0.05$ vs. CMs

5. Expression of monomeric G proteins in CGCs

To find whether the cardiogenic differentiation was involved with changes of monomeric GTP-binding proteins, expression levels of monomeric G proteins were examined as well. Primer sequences of small G protein were shown in materials and methods. These expression levels were analyzed in 5 monomeric G proteins, i.e. isotypes of Ras, Rho, Rab, Arf and Ran (Figure 5A). Among these results, mRNA levels of RhoA and Rab1b were similarly increased in CGCs compared to CMs (Figure 5B). These data indicate that cardiogenic differentiation is related to the expression of RhoA and Rab 1b.

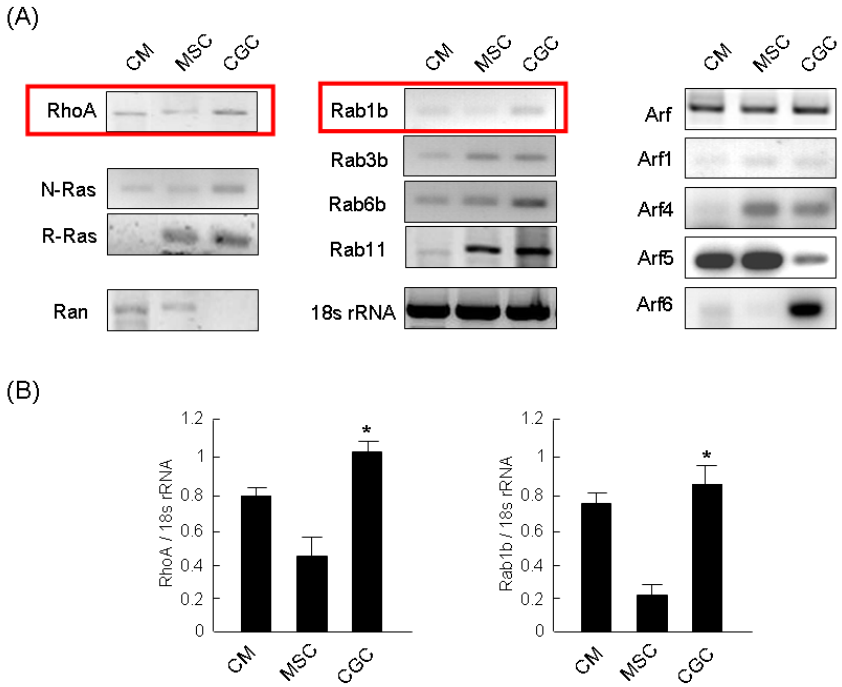

Figure 5. Alteration of expression levels for monomeric GTP-binding proteins in CGC. (A) RNA was extracted from CMs, MSCs, and CGCs. The mRNA of small GTP-binding proteins (Ras, Rho, Rab, Arf, and Ran) was amplified by RT-PCR. (B) The ratio of intensity of RhoA and Rab 1b were calculated for each samples and their mean ±S.E are plotted. The experiment was repeated 3 times with similar results. Results were normalized to 18s rRNS expression. *$p<0.05$ vs. CM.

6. Expression of α-adrenergic receptor-related Rho signaling in CGCs

To determine the influence of the α-adrenergic receptor-related Rho signaling in CGCs, agonist (NE; norepinephrine) of α-adrenergic receptor was treated in CGCs. It was tested the hypothesis that CGCs could mediate the activation of RhoA, which is a downstream molecule of α_1-adrenergic receptors. Ten μM of NE treated CGCs showed the formation of active GTP-bound RhoA as in CMs. But, NE treated MSCs did not reveal any significant

changes of Rho expression level (Figure 6).

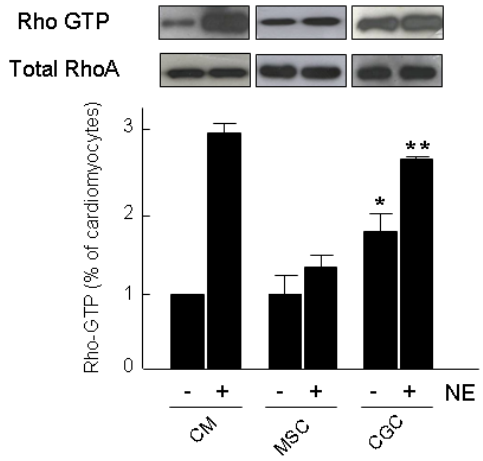

Figure 6. Effect of α-adrenergic stimulation on Rho signaling in CGCs. CMs and MSCs were selected and these cells were divided into positive and normal control group. NE (10 μM) was used as the agonist and samples were exposed to the agonist for 10 minutes. Representative immnoblots for Rho GTP and total RhoA were demonstrated and its result was compared to CM. *$p<0.01$ vs. CM (NE-), **$p<0.05$ vs. CM (NE+).

7. Expression of β-adrenergic receptor-related Rab1 signaling in CGCs

To verify the influence of β-adrenergic receptor related Rab1 signaling in CGCs, agonist of β adrenergic receptor (ISO: Isoproterenol) was treated in CGCs. It was tested the hypothesis that CGCs could mediate the activation of Rab1, which is a downstream molecule of β-adrenergic receptors. 0.1 μM ISO-treated CGCs showed the formation of active Rab1 GTPases as in CMs. However, ISO-treated MSCs did not reveal any significant changes of Rab1 expression level (Figure 7).

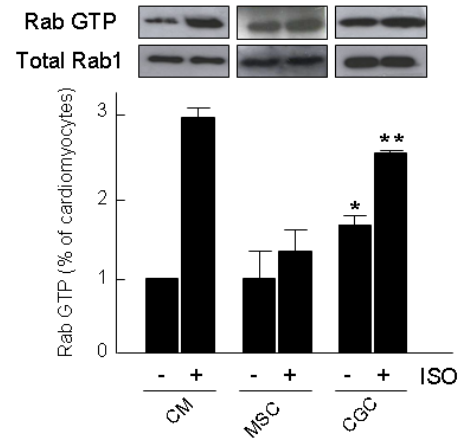

Figure 7. Effect of β-adrenergic stimulation on Rab signaling in CGCs. CMs and MSCs were selected and these cells were divided into positive and normal control group. Isoproterenol (0.1 μM) was used as the agonist and samples were exposed to the agonist for 10 minutes. Representative immnoblots for Rab GTP and total Rab1 were demonstrated and its result was compared to CM. *$p<0.01$ vs. CM (ISO-), **$p<0.05$ vs. CM (ISO+).

8. The α-adrenergic receptor-stimulated ERK1/2 signaling in CGCs

To assess the function of MAP kinases which is regulated by Rho GTPase, ERK1/2 in MAP kinase sub-signal was investigated with NE of 10 μM and PZ (prazosin) of 0.1 μM. CGCs showed about 3-fold upregulated ERK1/2 signal in NE-treatment group. Both PZ and NE-treated CGCs revealed about 35% downregulated ERK1/2 signal compared to NE only (Figure 8). These data indicated that α-adrenergic receptor-stimulated CGCs were able to activate sub-signal of MAP kinases.

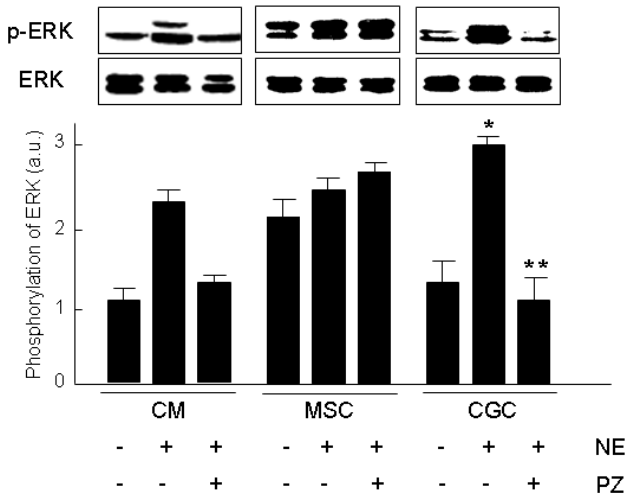

Figure 8. Comparison of phosphorylation of ERK1/2 on NE and PZ-stimulated CGCs. CMs and MSCs were selected and these cells were divided into positive and normal control group. NE (10 μM) was used as an agonist and PZ (0.1 μM) was used as an antagonist. Samples were exposed to the agonist for 10 minutes and antagonist for 30 minutes. Representative immnoblots for phosphorylated ERK1/2 were demonstrated and its result was compared to CM. *$p<0.01$ vs. CM (NE+), **$p<0.05$ vs. CM (NE+, PZ+).

9. Alteration of gene expression in CGCs treated with siRNA for G_q

To further confirm the involvement of G_q in CGCs, we investigated gene expression after treatment with siRNA for G_q. siRNA transfection for G_q effectively inhibited endogenous G_q and RhoA in CGCs and in CMs as well. Expression of G_q in CGCs was much alike in CMs, and G_q was decreased in G_q blocked CGCs about 60% compared to normal CGCs. However, MSCs did not show any similarities to CMs or CGCs (Figure 9). To assess the function of MAP kinases regulated by G_q, ERK1/2 in MAP kinase sub-signal was investigated with NE of 10 μM. CGCs showed about 1.4-fold increase in ERK1/2 signal in NE-treatment. However, the levels of ERK1/2 signal in G_q

blocked CGCs were downregulated about 35% compared with NE only treated group (Figure 10).

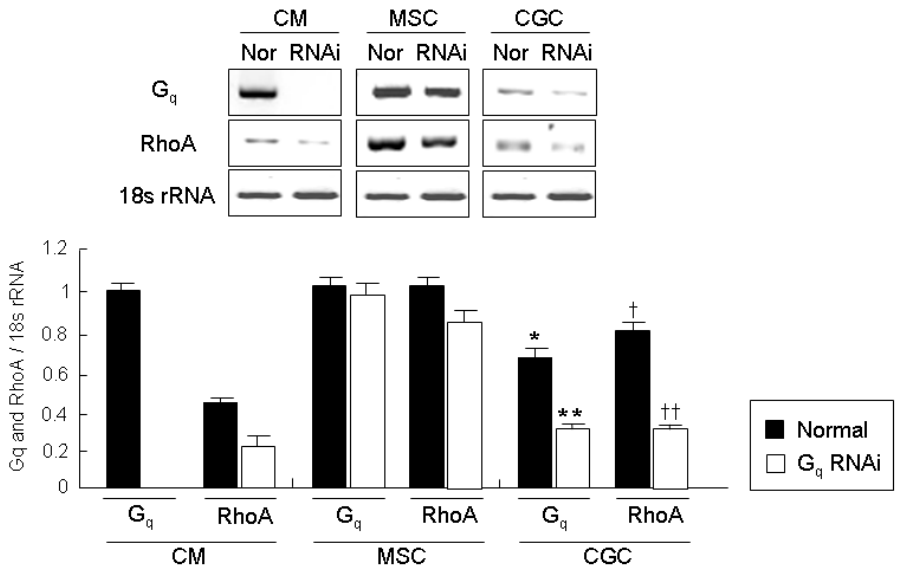

Figure 9. Inhibition of G_q expression by siRNA for G_q in CGC. CM, MSC, and CGC were transfected with siRNA to a final concentration of 10 nM. The mRNA of G_q and RhoA was amplified by RT-PCR. The experiment was repeated 3 times with similar results. Results were normalized to 18s rRNS expression. *p<0.01 vs. CM (Gq-normal), **p<0.001 vs. CM (Gq-Gq RNAi), †p<0.001 vs. CM (RhoA-normal), ††p<0.001 vs. CM (RhoA-Gq RNAi).

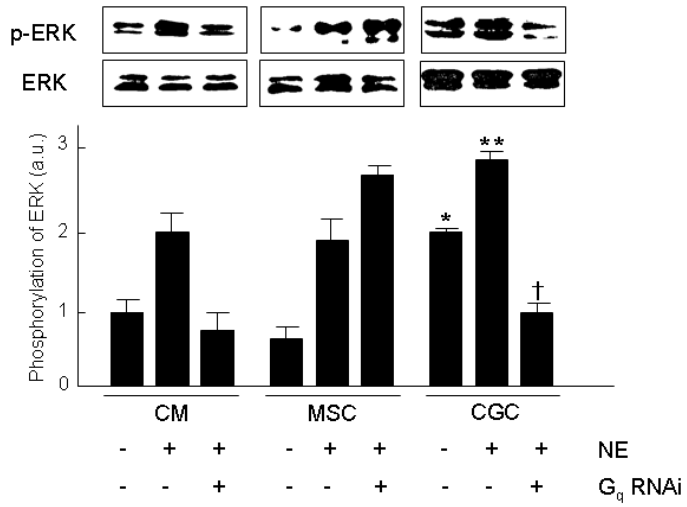

Figure 10. Alteration of ERK1/2 by siRNA for G_q in CGCs. CMs, MSCs, and CGCs were transfected with siRNA to a final concentration of 10 nM. The agonist were used NE (10 μM) and samples were exposed to the agonist for 10 minutes. Representative immnoblots for phosphorylated ERK1/2 were demonstrated and its result was compared to CM. *$p<0.001$ vs. CM (NE-, Gq RNAi -), **$p<0.01$ vs. CM (NE+, Gq RNAi -), †$p<0.05$ vs. CM (NE+, Gq RNAi +).

10. The β-adrenergic receptor-stimulated ERK1/2 signaling in CGCs

To determine whether β-adrenergic receptor stimulation is capable of regulating MAP kinases signaling in CGCs, agonist (ISO) and antagonist (PRO; propranolol) of β-adrenergic receptor were treated in CGCs. ERK1/2 activation by ISO of 0.1 μM was confirmed in CGCs and addition of PRO of 2 μM in CGCs with ISO of 0.1 μM resulted in decreased ERK1/2 activation (Figure 11). These data indicated ERK1/2, which is a sub-signal of MAP kinases could be regulated by β-adrenergic receptor agonist and antagonist.

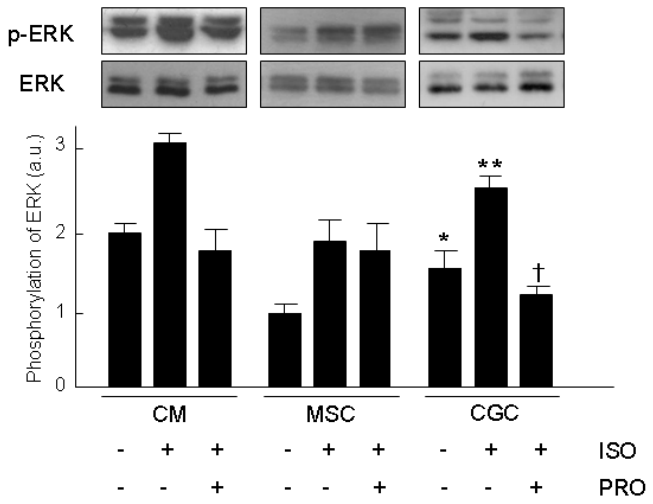

Figure 11. Comparison of phosphorylation of ERK1/2 on ISO and PRO-stimulated CGCs. CMs and MSCs were selected and these cells were divided into positive and normal control group. ISO (0.1 μM) was used as an agonist and PRO (2 μM) was used as an antagonist. Samples were exposed to the agonist for 10 minutes and antagonist for 30 minutes. Representative immnoblots for phosphorylated ERK1/2 were demonstrated and its result was compared to CM. *$p<0.05$ vs. CM (ISO-, PRO -), **$p<0.05$ vs. CM (ISO+, PRO -), †$p<0.01$ vs. CM (ISO+, PRO +).

IV. Discussion

The results of this study demonstrate the following: (1) MSCs, cultured in the presence of PKC activator can spontaneously generate cardiomyocyte like cells in vitro, that is CGCs; (2) G_q and G_s of heterotrimeric G protein showed similar expression pattern in CMs and CGCs; (3) RhoA and Rab1b of monomeric G protein were similarly expressed in CGCs compared to CMs; (4) α- and β-adrenergic receptor related intracellular MAP kinase signaling pathways were confirmed in both CGCs and CMs.

To confirm the cardiogenic differentiation of MSCs, we performed cardiac specific marker related sandwich ELISA. The highest expression of cardiac specific markers (cTnT, MLC, MHC, Nkx 2.5, and MEF2) was verified at day 9 (Figure 3). Moreover CGCs showed cardiomyocyte like cell morphology and immunocytochemistry revealed cardiac specific TnT and MLC only in CGCs (Figure 2).

The cardiovascular system is richly endowed with G protein-coupled receptors (GPCRs), members of the largest family of plasma membrane-localized receptors. Heterotrimeric G proteins are composed of non-identical α, β and γ subunits. The α subunit is the largest and is the Ras-like guanine-nucleotide-binding entity of the heterotrimer. In most cases, when it is GDP-bound, a Gα subunit forms a complex with one Gβ and one Gγ subunit, and functionally dissociates from the Gβγ complex when it binds GTP. In mammals there are 20 G proteins, each comprising a unique α subunit combined with a complex of one of 5 β subunits and one of 12 Gγ subunits. Agonists stimulate their cognate G-protein-coupled receptors, which are embedded in the cell membrane. GPCR activates the $G\alpha_s$ protein, which leads to at least two downstream responses: first, the generation of cyclic AMP (cAMP) by adenylyl cyclase (AC) and the subsequent activation of protein kinase A (PKA), which, through its catalytic subunits (C), can phosphorylate nearby substrates; and second, the Gβγ-complex-mediated activation of the

mitogen-activated protein kinase (MAPK) cascade, which results in the activation of extracellular signal-regulated kinase-1/2 (ERK1,2), which mediates transcriptional responses. GPCR2 activates $G\alpha_q$ and therefore phospholipase Cβ (PLCβ), which generates water-soluble inositol-1,4,5-triphosphate (InsP3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2). InsP3 regulates the mobilization of intracellular Ca2+ ions ($[Ca^{2+}]_i$) and the activation of Ca^{2+}/calmodulin-activated protein kinase II (CaMKII), whereas DAG activates protein kinase C (PKC)[18, 20, 21]. In this study, to determine the involvement of heterotrimeric GTP-binding proteins in CGCs, mRNA levels of heterotrimeric GTP-binding proteins were examined and G_q and G_s showed similar expression pattern in CGCs and CMs (Figure 4).

Monomeric GTP-binding proteins exist in eukaryotes from yeast to human and constitute a superfamily consisting of more than 100 members. This superfamily is structurally classified into at least five families: the Ras, Rho, Rab, Sar1/Arf, and Ran families. They regulate a wide variety of cell functions as biological timers that initiate and terminate specific cell functions and determine the periods of time for the continuation of the specific cell functions. They furthermore play key roles in not only temporal but also spatial determination of specific cell functions. The Ras family regulates gene expression, the Rho family regulates cytoskeletal reorganization and gene expression, the Rab and Sar1/Arf families regulate vesicle trafficking, and the Ran family regulates nucleocytoplasmic transport and microtubule organization[19, 22]. Previous study reported that Rho is related to $G\alpha_q$ and α_1-adrenergic receptor signaling in cardiomyocytes, and regulated myofibril formation and organization in neonatal rat ventricular myocytes[23-25]. Especially, RhoA-mediated actin cytoskeletal rearrangement appears to be necessary for the translocation of activated ERK to the nucleus, providing a mechanism for synergistic effects between the Ras/ERK and RhoA pathways

for cardiac hypertrophy. Specific mechanisms by which RhoA contributes to cardiomyocyte hypertrophic gene expression are suggested by the plethora of transcriptional mediators downstream of RhoA. For example, RhoA can regulate muscle specific gene expression through activation of serum response factor (SRF). In this study, RhoA was activated in NE-stimulated CGCs (Figure 6) and phosphorylation of ERK1/2 was regulated by α_1-adrenergic receptor agonist (NE) and antagonist (PZ) (Figure 8). Other previous studies demonstrated that increased expression of Rab1 in myocardium can cause cardiac hypertrophy and heart failure in dilated cardiomyopathy model which overexpress β-adrenergic receptors[26]. Transient expression of Rab4 markedly facilitated recycling of internalized β-adrenergic receptor to the cell surface and enhanced β-adrenergic receptor signaling as measured by ISO-stimulated cAMP production[27]. Thus Rab4 is a very important molecule for the recycling of endogenous β-adrenergic receptors. In this study, Rab was activated in ISO-stimulated CGCs (Figure 7) and phosphorylation of ERK1/2 was regulated by β-adrenergic receptor agonist (ISO) and antagonist (PRO) (Figure 11). α-adrenergic receptor - G_q - Rho - ERK 1/2 signaling and β-adrenergic receptor - G_s - Rab - ERK 1/2 signaling pathways were confirmed in CGCs and as well as in CMs. These results demonstrated that CGCs showed similar hypertrophy related signal, which is a unique characteristics of CMs. Thus GTP binding proteins may play an important role in cardiac differentiation of MSCs.

V. Conclusion

GTP binding proteins have shown to play a critical role in general differentiation of MSCs. However the molecular mechanisms underlying cardiomyogenic differentiation from MSCs remain poorly understood. This study investigated the expression and functional role of heterotrimeric and monomeric G proteins in CGCs, which is differentiated from MSCs under PKC activator.

Unlike MSCs, CGCs and CMs showed similarity not only in the expression level of heterotrimeric and monomeric G protein but also in the intracellular signaling pathway related to G proteins, especially in α-, β-adrenergic receptor related signaling. Our results showed that G proteins may play important roles in cardiac differentiation from MSCs. These results have important implications for understanding MSCs biology and for the MSCs-based cell therapy in cardiovascular medicine.

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ABSTRACT (IN KOREAN)

골수 간엽줄기세포에서 G 단백질의 발현과 기능적인 역할

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골수 간엽줄기세포는 적절한 자극 하에서 심근세포로 분화할 수 있지만 어떤 분자 생물학적 기전으로 심근세포 분화가 이루어지는지에 대해서는 잘 알려져 있지 않다. 최근에 간엽줄기세포의 전반적인 분화에 있어서 G 단백질이 중요한 역할을 하는 것이 증명되었고 배아줄기세포의 심근세포 분화에 있어서도 G 단백질이 중요한 역할을 하는 것이 확인되었다. 이에 본 연구에서는 간엽줄기세포와 심근세포 그리고 간엽줄기세포로부터 유도된 심근유사세포의 세 군에서 G 단백질의 발현 양상과 기능적인 역할을 비교 분석하였다. 삼중체 형태의 G 단백질에 있어서는 G_q와 G_s가, 단일체 형태의 G 단백질에서는 RhoA와 Rab1b가 심근유사세포와 심근세포에서 비슷한 양상으로 발현되었다. 또한 심근세포에서와 마찬가지로 심근유사세포에서도 알파 및 베타 아드레날린 수용체와 관련된 G_q-RhoA-ERK1/2 및 G_s-Rab-ERK1/2 신호전달체계가 모두 관여함을 확인하였다. 요약하면 간엽줄기세포로부터 유도된 심근유사세포와 심근세포가 비슷한 양상의 G 단백 발현 양상을 보였고 알파 및 베타 아드레날린 수용체에 관련된 세포 내 신호전달체계도 유사함을 확인하였다.

이를 통해 G 단백질이 간엽줄기세포로부터 심근세포로 분화하는 데에 있어서 중요한 역할을 하는 것임을 알 수 있다.

핵심 되는 말: 간엽줄기세포, 심근분화세포, G 단백질