Specific differentiation of bone marrow-derived mesenchymal stem cells by regulation of protein kinase system

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Specific differentiation of bone marrow-derived mesenchymal stem cells by regulation of protein kinase system

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Abstract

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Protein kinases, the members of a large family of proteins involved in modulating many known signaling pathways, are likely to play important roles in regulating stem cell differentiation programs because important bio-processes such as cell fate are likely to be determined by an elaborate orchestration of multiple signaling pathways. In addition, cell permeable small molecules to modulate activation of protein kinase have several merits that include the ability for temporal, tunable and modular control of specific protein function.

Therefore, research efforts focused on search for chemical reagent and mechanism that regulates systematic differentiation of MSCs. It was found that some of protein kinase cause recognizable changes in differentiation rates of MSCs based on screening inhibitors of major protein kinase subfamilies to alter the orchestration of multiple signaling pathways. At first, chondrogenesis of MSCs *in vitro* was significantly improved in cells treated with H-89, a PKA inhibitor. After treatment of H-89, the data of alcian blue staining assay for the quantitation of chondrogenesis was increased. Aggrecan, one of the extracellular matrix genes in chondrocytes, was induced in MSCs treated with various concentrations of H-89 (0.1–1 μM). During chondrogenesis of MSC, activation of ERKs was increased in MSCs treated with H-89 but cotreatment with H-89 and U0126 did not lead to a change in ERK activation. Semiquantitative RT-PCR showed a significantly changed level of cell adhesion molecule during chondrogenesis. MSCs gave rise to cardiomyocytes expressing of cardiac-specific markers (cardiac troponin T, myosin light chain, myosin heavy chain, NK2 transcription factor-related, locus 5, and Myocyte-specific enhancer factor 2) by addition of phorbol myristate acetate (PMA), a PKC activator. Differentiated cardiomyocytes expressed functional adrenergic and muscarinic receptors and norepinephrine induced phosphorylation of ERK via

α_1-adrenoceptors. The mRNA level of Ca^{2+}-related factor (sarcoplasmic reticulum Ca2+-ATPase, L-type Ca2+ channel) in differentiated MSCs was similar to that in cardiomyocytes. Differentiation into endothelial-like cells was induced by cultivation of cells in the presence of GSK-3β inhibitor. The differentiated cells showed a strong increase of expression of endothelial-specific markers (CD31, CD34, eNOS, VE-cadherin, VCAM-1, and VEGF-R2) and functional behavior of endothelial cells such as a formation of capillary-like structure. Treatment of proliferating MSCs with GSK-3β inhibitor leads to depletion of S-phase cells and over all decreased proliferation. The differentiation of MSCs into endothelial-like cells by the treatment of GSK-3β inhibitor was associated with GSK-3β/β-catenin signaling. The current study suggests that the regulation of protein kinase may emerge as a remarkable tool for systematic differentiation of MSCs. This system would greatly contribute to a novel understanding of MSC biology and the development of novel regenerative medicine.

Key words: Mesenchymal stem cell, Differentiation, Protein kinase, Signaling pathway

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I. INTRODUCTION

A stem cell is an extraordinary type of cell that has the ability to self-renew for long periods of time and to differentiate into specialized cells under appropriate physiological or experimental conditions [1]. Traditionally, stem cells are classified as either embryonic or adult (tissue-specific) stem cells. Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst stage embryo. They possess an unlimited capacity for self-renewal and have the potential to develop into any cell type found in the three primary germ layers of

the embryo (endoderm, mesoderm and ectoderm), as well as germ cells and extraembryonic cells [2-4]. In contrast, adult stem cells are found in differentiated tissues, have limited self-renewal capability, and generally can only differentiate into specialized (mature) cell types of the tissue in which they reside.

It may be that adult stem cells in adult tissues are reservoirs of reparative cells, ready to mobilize and differentiate in response to wound signals or disease conditions. Little information is currently available about the biology of endogenous stem cell populations in adults and their precise role in tissue repair or regeneration. This may be due in part to the lack of useful cell-specific markers. What is clear, however, is the ease with which these cells can be isolated and expanded in culture through many generations while retaining the capacity to differentiate. Recent progress in the isolation and characterization of these cells has led to the development and testing of therapeutic strategies in a variety of clinical applications.

Mesenchymal stem cells (MSCs), which reside within the stromal compartment of bone marrow were first identified in the pioneering studies of Friedenstein and Petrakova, who isolated bone-forming progenitor cells from rat marrow [5].

They play a role in providing the stromal support system for haematopoietic stem cells in the marrow. In addition, they have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage and muscle. The multilineage differentiation potential of MSC populations derived from a variety of different species has been extensively studied *in vitro* since their first discovery in 1960s [5]. These studies demonstrate that populations of bone marrow derived MSCs from human, canine, rabbit, rat, and mouse have the capacity to develop into terminally differentiated mesenchymal phenotypes both *in vitro* and *in vivo*, including bone, cartilage, tendon, muscle, adipose tissue, and hematopoietic-supporting stroma [6-13]. In that, MSCs are considered a readily accepted source of stem cells because such cells have already demonstrated efficacy in multiple types of cellular therapeutic strategies, including applications in treating children with osteogenesis imperfecta, hematopoietic recovery, and bone tissue regeneration strategies [14-17]. MSCs represent a very small fraction, 0.001–0.01% of the total population of nucleated cells in marrow. However, they can be isolated and expanded with high efficiency. More importantly, these cells may be directly obtained from individual patients, thereby eliminating the complications associated with immune rejection of allogenic tissue.

As state above, an important feature about MSCs is their multilineage differentiation potential. Under defined inductive conditions, MSCs are able to acquire characteristics of cells derived from embryonic mesoderm, such as osteoblasts, chondrocytes, adipocytes, tendon cells, as well as cells possessing ectodermal and neuronal properties. Based on the genetic and genomic information provided by various studies, MSCs undergo transcriptional modification, generating precursor cells without apparent changes in phenotype and self-renewal capacity. Similar to MSCs residing in adult bone marrow, the majority of MSCs cultured *in vitro* remain quiescent and growth arrested in G0/G1, until stimulated, for example, by the supplementation of growth factors. Upon stimulation, multipotent, uncommitted MSCs undergo asymmetric division, giving rise to two daughter cells, one being the exact replica of the mother cell and maintaining multilineage potential, and the other daughter cell becoming a precursor cell, with a more restricted developmental program. In this, the precursor cell continues to divide symmetrically, generating more tripotent and bipotent precursor cells. These tripotent and bipotent precursor cells are morphologically similar to the multipotent MSCs, but differ in their gene transcription repertoire, and therefore, still reside in the stem cell compartment. The progression of MSCs to precursor cells is considered the first

step in stem cell commitment. The transition or exit from the ‘stem cell compartment’ to the ‘commitment compartment’ occurs when precursor cells continue to divide symmetrically to generate unipotent progenitor cells, simultaneous with the acquisition of lineage specific properties, rendering them fully committed mature cells with distinguishable phenotypes.

Despite diverse and growing information concerning MSCs and their use in cell-based strategies, the mechanisms that govern MSC self-renewal and multilineage differentiation are not well understood and remain an active area of investigation. Especially, what is not fully understood is the mechanism that governs the transit of uncommitted stem cells to partially committed precursor or progenitor cells, and then to fully differentiated cells. Therefore, research efforts focused on identifying factors that regulate and control MSC cell fate decisions are crucial to promote a greater understanding of the molecular, biological and physiological characteristics of this potentially highly useful stem cell type.

Stem cell fate is determined by both intrinsic regulators and the extra-cellular environment (niche), and their expansion and differentiation ex vivo are generally controlled by growing them in a specific configuration (monolayer or

three dimensional culture) with ‘‘cocktails’’ of growth factors and signaling molecules, as well as genetic manipulations [18-22]. However, most of these conditions are either incompletely defined, or non-specific in regulating the desired cellular process. As a consequence, more efficient and selective methods to control the fate of stem cells to produce homogenous populations of particular cell types will be essential to the therapeutic use of stem cells and will facilitate studies of the molecular mechanisms of development. In this study, we hypothesize that pathway specific screens of synthetic small molecules to effect protein kinases system will provide useful chemical tools to modulate and study complex cellular processes because protein kinases play a critical role in many signaling pathways such as development, differentiation, proliferation or death not also undefined media often result in inconsistency in cell culture and/or heterogeneous populations of cells which would not be useful for cell-based therapy and would complicate the biological study of a particular cellular process.

II. MATERIALS AND METHODS

1. Isolation and culture of MSCs

MSCs were isolated from the femoral and tibial bones of rats. Bone marrow-derived MSCs were collected from the aspirates of the femurs and tibias of 4-week-old male Sprague-Dawley rats (approximately 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 and 1% antibiotic-penicillin and streptomycin solution. Mononuclear cells recovered from the interface after centrifugation in Percoll were washed twice, resuspended in 10% fetal bovine serum (FBS)-DMEM, and plated in flasks at 1×10^6 cells per 100 cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 or 72 hrs, 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 approximately 10 days. To further purify the MSCs, the 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-negative fraction being further cultured. The cells were harvested after incubation with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, replated in 1×10^5/100-cm^2 plates, and again grown for approximately 10 days.

2. MSCs characterization

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 hours (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, Thornwood, NY, USA). 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, Grand Island, NY, 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, CD 105, ICAM-1 ; 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 Calibur system (Becton Dickinson, Franklin Lakes NJ, USA) using CellQuest™ software (Becton Dickinson, Franklin Lakes NJ, USA) with 10,000 events recorded for each sample.

3. Screening of protein kinase inhibitors for differentiation of MSCs

We assembled a small library of commercially available chemicals. Of these, a subset of compounds was selected based on their low cytotoxicity. These compounds were screened for their activity to differentiate MSCs.

4. Alcian Blue staining

Cells were first rinsed with PBS (Gibco BRL, Grand Island, NY, USA) three times then fixed with 100% methanol (Sigma, St. Louis, MO, USA) for 10 min at -20°C. Staining was accomplished by applying a solution of 1% Alcian blue 8GX (Bio Basic, Ontario, Canada) in 0.1 M HCl (pH 1.0) (Sigma, St. Louis, MO, USA) to the cells for 2hr at room temperature. To quantify the intensity of the staining, the stained culture plates were rinsed with PBS three times and each well was extracted with 1 ml of 6M guanidine-HCl (Sigma, St. Louis, MO, USA) overnight at room temperature. The optical density of extracted dye was measured at 650nm.

5. 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 BRL, Grand Island, NY, USA) and treated with 100 μl of 3% BSA (Sigma, St. Louis, MO, USA) /PBS for 2~3 hrs 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 hrs 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 hrs at room temperature in a humid atmosphere. The plate was incubated again with addition of peroxidase conjugated secondary Ab for 1 hr 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 450nm on an ELISA plate reader.

6. Immunocytochemistry

Cells were grown on 4-well plastic dishes (SonicSeal Slide, Nalge Nunc, Rochester, NY, USA). Following incubation, cells were washed twice with PBS and then fixed with 4% paraformaldehyde in 0.5 ml PBS for 30 min at room temperature. The cells were washed again with PBS and then permeabilized for 30 min in PBS containing 0.2% triton. The cells were then blocked in PBS containing 10% goat serum and incubated for 1 hr with primary antibody. The cells were rewashed three times for 10 min with PBS and incubated with FITC-conjugated secondary antibody for 1 hr. Photographs of cells were taken under fluorescence by immunofluorescence microscopy (Olympus, Melville,

NY, USA). All images were made by using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software ver. 4.6 (Universal Imaging Corporation Ltd., Marlow, UK).

7. Immunoblot analysis

Cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 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 phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Proteins were separated in a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% nonfat dried milk for 1 hr at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody for 1 hr at room temperature or

overnight at 4°C. The membrane was washed three times with TBS-T for 10 min and then incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using NIH ImageJ version 1.34e software.

8. RT-PCR analysis

8.1. Isolation of total RNA

Total RNA was extracted by 500 μl/ 60 ㎜ plate Tri-reagent (Sigma, St. Louis, MO, USA). Poured 100 μl chloroform above Tri-reagent, voltexing a sample about 10 seconds. Then, sample was centrifuged at 12000 g, 4 ℃ and 15 minutes. 3 layers were appeared in the tube, transparency upper layer collected in new tubes. And poured 250 μl 2-propanol over the sample, again voltex a sample about 30 seconds. Centrifuge was accomplished about 12000 g, 4 ℃ and 10 minutes. Left the pellet, supernatant was discarded and washed by 75 % ethanol (Sigma, St. Louis, MO, USA) –mixed diethylpyrocarbonate (DEPC; Sigma, St. Louis, MO, USA) water. Centrifugation was also operated

about 7500 g, 4 ℃ and 5 minutes. Dismissed the supernatant, pellet was dried on room temperature about 7 minutes. Finally, poured the 30 µl nuclease free water (NFW). The quality and quantity of the RNA was detected by OD_{260}/OD_{280} with DU 640 spectrophotometer (Effendorf, Hambug, Germany)

8.2. 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 volume. To prevent secondary structures, incubated the mixture for 5 minutes at 70 ℃ and added 8 µl of RT-&GO™ mastermix. Sample was incubated the assay at 42 ℃ for 1 hour. At the conclusion, sample was inactivated the reverse transcriptase at 70 ℃ for 15 minutes. Alike isolation of total RNA, sample was detected by OD_{260}/OD_{280} with DU 640 spectrophotometer.

8.3. PCR analysis

Quantitative 1 µg cDNA, each 10 pmol primer (forward and backward), 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 fix as fellow. A cycle of denatureing at 94 ℃ for 3 minutes follwed by number of 35 cycles with denaturation at 94 ℃ for 30 seconds, annealing at 48 ℃ to 60 ℃ for 30 seconds, and elongation at 72 ℃ for 30 seconds[26]. Then keep up 72 ℃ for 10 minutes. Primers were appeared Table 1. When PCR assay have finished, PCR product was separated by electrophoresis in a 1.2 % agarose gel (Bio-Rad, Hercules, CA, USA) and Gel-Doc (Bio-Rad, Hercules, CA, USA) visualized after staining with ethidium bromide (EtBr; Sigma, St. Louis, MO, USA).

Table 1. PCR primers used in this study

Gene	Primer	Size (bp)	Temp.
Fibronectin	5’-cct taa gcc ttc tgc tct gg-3’ 5’-cgg caa aag aaa gca gaa ct-3’	300	48
α5 integrin	5’-ctt cgg ttc act gtt cct c-3’ 5’-tgg ctt cag ggc att t	283	43
β1 integrin	5’-gcc agt gtc acc tgg aaa at 5’-tcg tcc att ttc tcc tgt cc	344	48
N-cadherin	5’-gcc acc ata tga ctc cct ttt agt 5’-cag aaa act aat tcc aat ctg aaa	454	48
α_{1A}-adrenergic receptor	5’-gag aat tcc gag gcc tca agt ccg gcc t 5’-ttg aat tct cgg gaa aac ttg agc ag	169	59
α_{1B}-adrenergic receptor	5’-ctg ggg aga gtt gaa aga tgc c 5’-ccg aca gga tga cca aga tgt t	158	58
α_{1D}-adrenergic receptor	5’-ttg aat tcc tac aga gac cca cga ccc ag 5’-cgg aat tct taa atg tca gtc tcc cgg ag	229	58
β_1-adrenergic receptor	5’-acg ctc acc aac ctc ttc at 5’-agg ggc acg tag aag gag ac	440	53
β_2-adrenergic receptor	5’-cct cat gtc ggt tat cgt cc 5’-ggc acg tag aaa gac aca atc	519	53
M1-muscarinic receptor	5’-ctg gtt tcc ttc gtt ctc tg 5’-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
CD34	cac ctc aga ggc tgt tct tg ctg gtg acc aaa aag tcc caa caa agt	437	60
eNOS	tcc agt aac aca gac agt gca cag gaa gta agt gag agc	693	40
VE-cadherin	aag aca tca atg aca act tcc cct cca cag tca ggt tat acc	590	54
VCAM-1	cct aag gat cca gag att caa ttc a ggg taa aca tca gga gcc aac	85	58
VEGF-R2	aga ctt tga gca tgg aag cca ttc cac caa aag atg	310	51
GAPDH	acc aca gtc cat gcc atc ac tcc acc acc ctg ttg ctg ta	450	49

9. In Vitro Angiogenesis

Analysis of capillary formation was performed using the in vitro angiogenesis kit (Chemicon International Inc, Temecula, CA, USA) according to the manufacturer's instructions. Fifty microliters of gel matrix solution were applied into one well of a 96-well plate and incubated for 1 hour at 37 ℃. Cells were then trypsinized and 5 x 10^3 cells were suspended in 50 μl of the DMEM containing VEGF and plated onto the gel matrix and incubated for 4 hours. Cells were counted by eye for the formation of capillary structures. The percentages of formed capillaries were calculated from three independent experiments.

10. Cell counting by trypan blue stain

The cells were washed by phosphate-buffered saline (PBS) (Gibco BRL, Grand Island, NY, USA). 0.5 ml of Trypan blue solution was transferred to test tube and added to 0.3 ml of PBS, 0.2 ml of the cell suspension was mixed thoroughly. Allow to stand for 5 to 15 minutes. A small amount of trypan blue-cell suspension was transferred to both chambers of the hematocytometer by carefully touching the edge of the cover-slip with the pipette tip and allowing

each chamber to fill by capillary action. Chamber of the hematocytometer was counted all the cells and dyed cell in the 1mm center square and four 1mm corner squares.

11. Cell cycle analysis

For cell cycle analysis, cells were harvested with trypsin, collected by centrifugation at 200 *g* for 5 min and re-suspended in PBS. Cells were again collected by centrifugation, monodispersed in 0.5 mL PBS and transferred to tubes containing 4.5 mL of cold 70% ethanol; tubes were stored at –20 °C until analysis. Before analysis, ethanol-suspended cells were collected by centrifugation at 200 *g* for 5 min, rinsed with PBS and re-suspended in PBS containing propidium iodide (50 μg/mL) and RNase A (0.2 mg/mL). A FACS Calibur cytometer operated with CellQuest software (Becton Dickinson, Franklin Lakes NJ, USA) was used for data collection. Histogram analysis and calculation of G1, S and G2/M percentages was performed with ModFit LT (Verity Software House, Topsham, ME, USA).

12. Statistical analysis

Data are expressed as means ±SE. Statistical analysis of two groups were estimated by Student`s t-test. And examining from more than two groups was done by one-way ANOVA, using bonferroni test. $p < 0.05$ was considered significant.

III. RESULTS

1. Isolation and characterization of phynotypic markers of MSCs

MSCs were first isolated from the mixed culture with hematopoietic cells based on their preferential attachment on polystyrene surfaces and the isolated MSCs were further purified using bead targeting hematopoietic marker CD34, yielding $3x10^6$ cells by 2 weeks culture and 95% purity. The MSCs retained a fibroblastic morphology through repeated passages and their identity was confirmed by immunocytochemistry and FACS analysis. The cultured MSCs expressed CD71, CD90, CD105, CD106, and ICAM. They expressed neither the hematopoietic marker CD34, nor CD14 (Fig. 1).

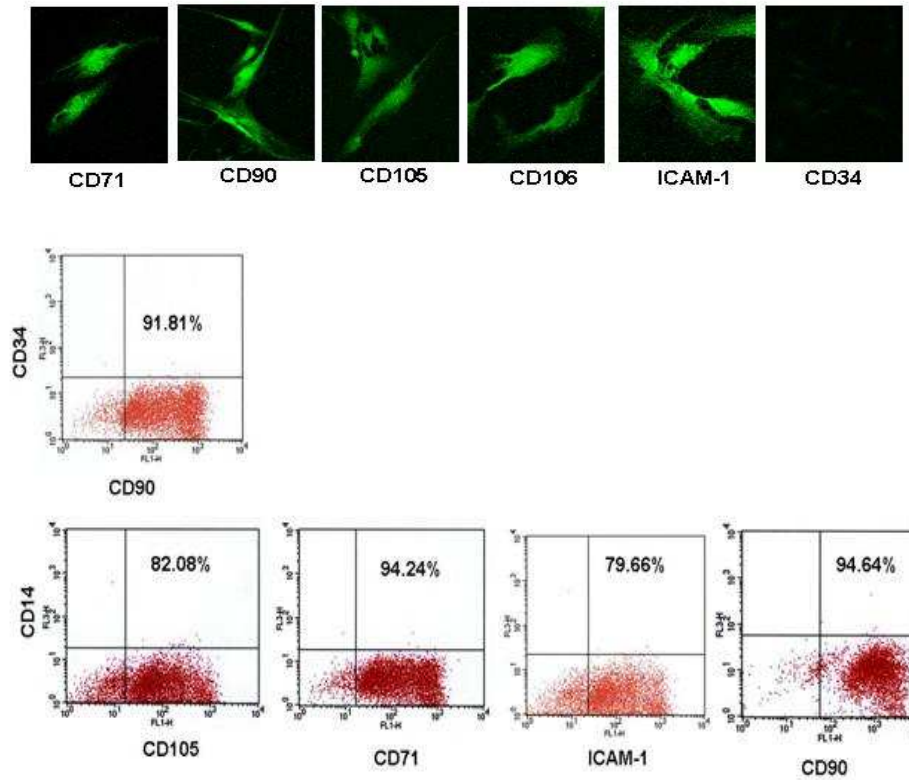


Figure 1. Characteristics of MSCs. Most adherent MSCs are practically fibroblastic in morphology and some polygonal cells were contained after a 6-week culture. Magnification, 100x. Cells were cultured from bone marrow after density fractionation and are shown at 10 days after plating. At 14 days, the MSCs were positive for CD71, CD90, CD105, CD106, and ICAM-1 and were negative for CD14 and CD34 by flow cytometry.

2. Expression profile of target cell markers in MSCs treated with various kinase inhibitors

Under proper stimulation, MSCs can be induced to differentiate into chondrocytes, myocytes, adipocytes, osteoblasts, tenocytes, and hematopoietic-supporting stroma. Protein kinase is the most common protein family that play critical role as signal switch in many signaling pathways presumably including cell differentiation. Since important cellular processes such as differentiation is likely to be controlled by a complicated orchestration of many signaling pathways. To examine the effect of these kinase inhibitors on the differentiation of rat MSCs into various cell types, the MSCs were seeded and treated every 3 days with the kinase inhibitors for up to 11 days. Among the 41 inhibitors tested, 14 showed recognizable indications of inducing various cell types at different degrees. To assess the effectiveness of these 14 inhibitors for directed target cell development, we constructed a profile matrix, where the rows and columns represent target cell markers and kinase inhibitors, respectively. Using the principal component analysis (PCA) method on the profile matrix, we examined the relative strength of the induction and the cross-relationship among them. The size of the profile matrix used in PCA was 7 x 15 in which we obtained the coordinates of inhibitors and target cell types

by using the first three principal components from PCA and scaled the coordinates to plot them together in the map for visualization. From the examination of Figure 2, we found several good candidates that drive rat MSCs into specific cell types.

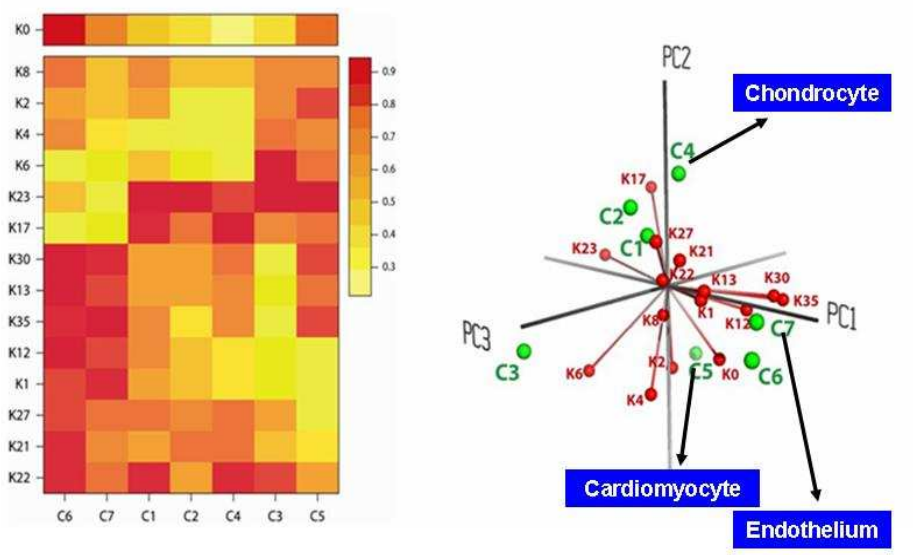


Figure 2. Use of protein kinase inhibitors for the differentiation of MSCs. (*A*) The relative expression level of seven target cell markers (C1–C7) in the presence of various kinase inhibitors are detected by sandwich ELISA, and normalized in the range of 0 to 1 for standardization. Kinase inhibitors used (at 1 μM) are from Calbiochem and are distinguished by the numerals after K. The kinases they preferentially inhibit are in parentheses: K0, no inhibitor; K1 (AKT 1,2),1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl) methyl)-4-piperidinyl)-2H-benzimidazol-2-one; K2 (AKT), 1L6-hydroxymethyl-chiroinositol-2-(R)-2-*O*-methyl-3-*O*-octadecyl-sn-glycerocarbonate; K4 (CaMK II), Lavendustin (5-(*N*-2',5'-dihydroxybenzyl) aminosalicylic acid); K6 (calcium channel), HA 1077 (Fasudil, 5-isoquinolinesulfonyl)homopiperazine, 2HCl); K8 (CaseinK I), D4476(4-(4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide; K12 (CDK 1,2), NU6102

[6-cyclohexylmethoxy-2-(4'-sulfamoylanilino)purine]; K13 (TGF-βR I kinase), [3-(pyridin-2-yl)-4-(4-quinonyl)]-1Hpyrazole; K17 (PKA), H-89 [*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl]; K21 (PKC), Go¨ 6983 (2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)) maleimide; K22 (PKG-1α, β), guanosine 3',5'-cyclic monophosphorothioate, β-phenyl-1, N2-etheno-8-bromo-, Rp-isomer, sodium salt; K23 (EGFR PTK), compound 56 (4-[(3-bromophenyl)amino]-6,7-diethoxyquinazoline); K27 (ROCK), *N*- (4-pyridyl)-*N*'-(2,4,6-trichlorophenyl) urea; K30 (DNA-PK), 4,5-dimethoxy-2-nitrobenzaldehyde; K35 (p38 MAPK), SB 202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole]. The target cells tested for each cell type were: C1, MSC positive; C2, MSC negative; C3, osteocyte; C4, chondrocyte; C5, cardiomycyte; C6, hepatocyte; C7, endothelium. The target cell markers assayed for each cell type were: C1, CD105; C2, CD34; C3, osteopontin (OPN); C4, aggrecan (AGG); C5, cardiac troponin T (CTnT); C6, CK-18, C7: CD31. (*B*) The effectiveness of inhibitors relative to the directed target cell development was analyzed by the principal component analysis (PCA) method using numerical values used to generate *A*. We obtained the coordinates of inhibitors and target cell types by using the first three principal components (for visualization) from PCA and scaled the two sets of the coordinates to plot them together in the map. The three largest principal components of PCA analysis are represented as PC1, PC2, and PC3. The inhibitors are indicated by red balls and the cell types are shown as green balls. The balls attached to the longer vectors are more efficient differentiation inducers for the cell types nearest to the balls.

3. Effect of PKA inhibitor on the differentiation of MSCs

3.1. Effect of H-89 on the differentiation of MSCs into chondrocyte-like cells

Among them, H-89, an inhibitor of protein kinase A, was found to be potentially implicated in chondrogenesis of the MSCs. H-89 is a derivative of isoquinolinesulfonamide, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl (Calbiochem/EMD Biosciences). The compound is cell-permeable and known to be selective and a potent inhibitor of protein kinase A (K_i = 48 nM). Among the kinases tested, it inhibited other kinases only at much higher concentrations: CaM kinase II (K_i = 29.7 μM), casein kinase I (K_i = 38.3 μM), myosin light chain kinase (K_i = 28.3 μM), protein kinase C (K_i = 31.7 μM), and Rho-associated kinase (ROCK)-II (IC_{50} = 270 nM). To examine the capability of H-89 in inducing chondrogenesis and mechanisms underlying the process, we carried out several experiments with cell culture assays. Alcian blue bound to sulfated glucosaminoglycans was extracted with 6M guanidine-HCl and analyzed by measuring the absorbance at 650nm. Quantification of chondrogenesis by measuring an absorbance of Alcian blue extract indicated that H-89 enhanced chondrogenesis to over 2-fold of

control. MSCs treated with various concentrations of H-89 (0.1–1 μM) were differentiated into chondrocytes in a dose-dependent manner as judged by the up-regulated expression of aggrecan, a chondrocyte marker. We used H-89 at 1 μM concentration for the rest of our studies (Fig. 3).

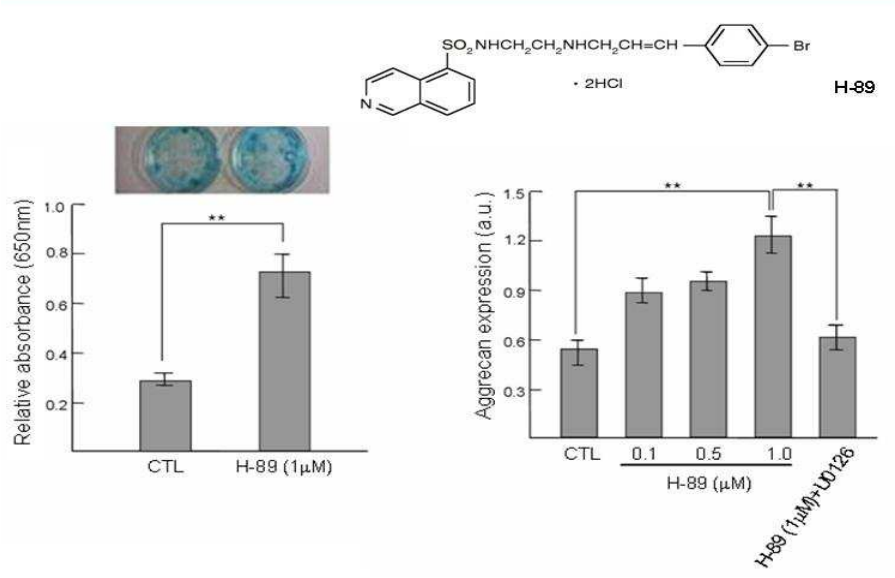


Figure 3. Effect of H-89 on the differentiation of MSCs into chondrocyte-like cells. (A) Chondrogenesis of MSCs *in vitro* was significantly improved in cells treated with 1 μM H-89 for 11 days. The data of Alcian blue staining for sulfated proteoglycan indicate the quantitation of chondrogenesis. (B) Aggrecan, one of the extracellular matrix genes in chondrocytes, was induced in MSCs treated with various concentrations of H-89 for 11 days (0.1–1 μM). The effect of U0126, a selective inhibitor of MEK, was examined on the expression of aggrecan. Cotreatment with 1 μM H-89 and 10 μM U0126 did not lead to a change of expression level. During chondrogenesis of MSC, the change of expression level in aggrecan was examined by sandwich ELISA. (**p<0.01).

3.2. Effect of H-89 on the phosphorylation of ERK

The induction of chondrogenesis by H-89 is thought to be mediated by the (ERK) MAP kinase signaling pathway. H-89 treatment increased the phosphorylation of ERK through unknown pathways. ERK was expressed at the same level during chondrogenesis of MSCs treated with H-89. In contrast, ERK phosphorylation was enhanced significantly more and more during chondrogenesis of MSC treated with H-89. Furthermore, the treatment with U0126, a selective inhibitor of MEK, was shown to block the phosphorylation of ERK even in the presence of H-89 and nullified the chondrogenic inducibility of H-89 (Fig. 4).

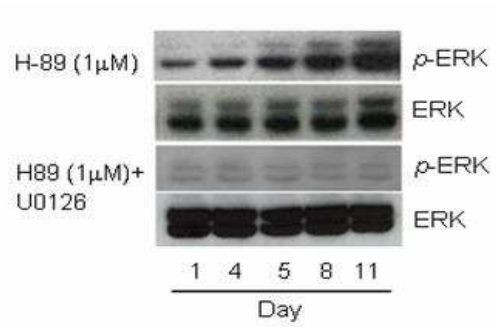


Figure 4. Effect of H-89 on the phosphorylation of ERK. Activation of ERKs was increased in MSCs treated with 1 μM H-89 but cotreatment with 1 μM H-89 and 10 μM U0126 did not lead to a change in ERK activation for 11 days.

3.3. Effect of H-89 on the expression of cell adhesion molecules

The effect of H-89 on differentiation of MSCs into chondrocye-like cells was investigated by expression level of cell adhesion molecules and extracellular matrix (ECM) components. Expression of N-cadherin, which mediates cell-cell interaction, was highest on the first day of H-89 treatment, but decreased as differentiation of MSC to chondrocytes proceeded. Because the interaction of cell with ECM in addition to cell-cell interaction is involved in the regulation of chondrogensis, potential involvement of H-89 in regulation of integrin α5β1 and its ligand fibronectin was also examined. During chondrogenic differentiation of MSCs treated with H-89, the fibornectin-receptor (integrin α5β1) and fibronectin were down-regulated (Fig. 5).

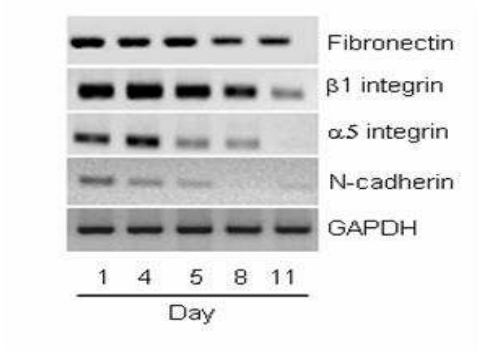


Figure 5. Effect of H-89 on the expression of cell adhesion molecules. Semiquantitative RT-PCR showed a significantly changed level of cell adhesion molecule during chondrogenesis, using primers to the RNAs indicated. MSCs were cultured for the indicated time periods in the presence of 1 μM H-89.

4. Effect of PKC activator on the differentiation of MSCs

4.1. Characteristics of differentiated cardiomyocyte-like cells

After 9 days in culture, MSCs adhered to the plastic surface and were spindle-shaped with one nucleus. MSCs were treated with 1 μM PMA according to the experimental group. Treatment with PMA was every 3 days. The morphological differentiation from MSCs to myogenic-like cells evolved gradually after PMA induction. 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. To examine where MHC or cTnT-expressing cells are located in differentiated MSCs, immunostaining was performed. Clearly, cardiomyocyte-like cells (CLCs) had significant morphological changes with positive immunocytochemical analysis for anti-MHC or -cTnT, but normal MSCs showed neither significant morphological changes nor cardiac-specific protein expression of MHC or cTnT during the 9 day period of observation with the same treatment (Fig. 6).

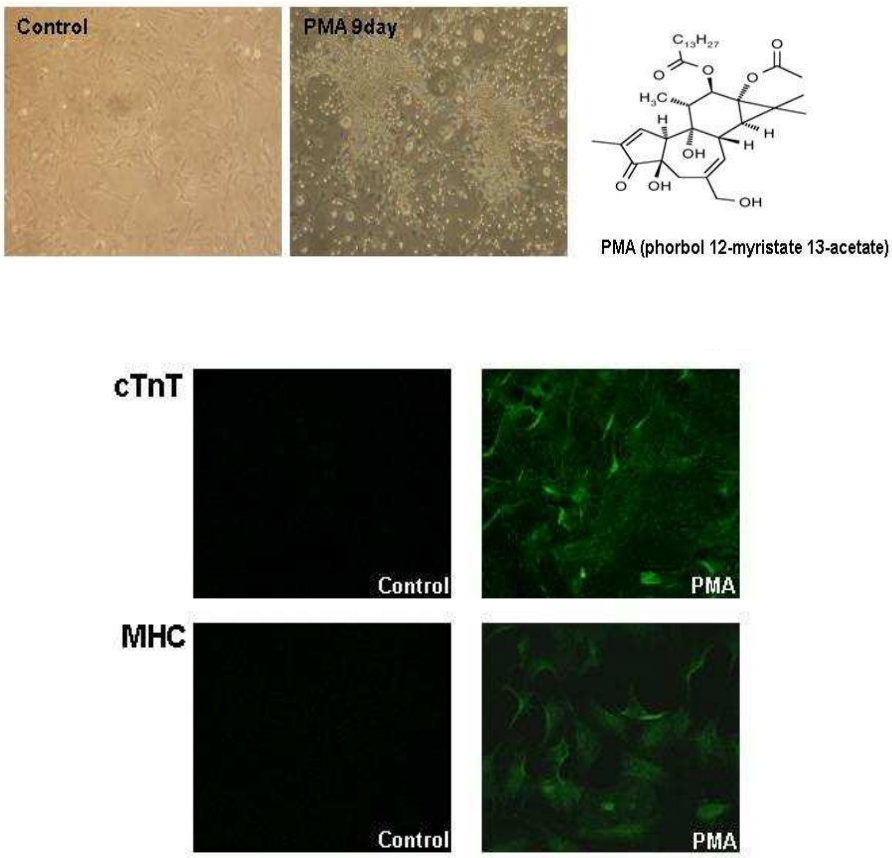


Figure 6. Effect of PMA on morphology of MSCs. (A) Micrographs of MSCs were taken after nine days. MSCs treated with PMA changed morphology to cardiomyocyte-like and fibroblast-like aggregated long-branches compared to their counterparts. (B) Immunocytochemistry was detected for cardiac MHC and cTnT in MSCs treated with PMA. Representative photos of normal MSCs as positive control and MSCs treated with 1 μM PMA are shown (Magnification: X400). Cardiac MHC or cTnT visualized by FITC is located on the stress fibers.

4.2. Myogenic differentiation of MSCs by upregulating expression of cardiac specific markers after PMA treatment

After 9 days of culture with the supplement of PMA, MSCs had a myocyte-like morphology. To reconfirm the effect of PMA on myogenic differentiation of MSCs, changes of cardiac specific markers expression were analyzed with Sandwich ELISA. 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 time-dependently. Expression of most of the specific markers was peaked around 9 days. MSCs treated with PMA express multiple sarcomeric proteins associated with neonatal rat ventricular cardiomyocytes (NRVCM) (Fig. 7).

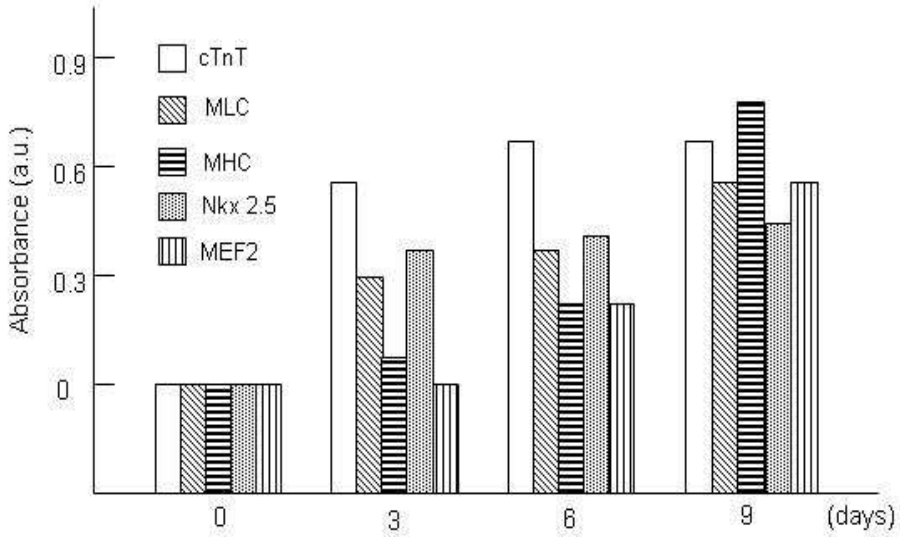
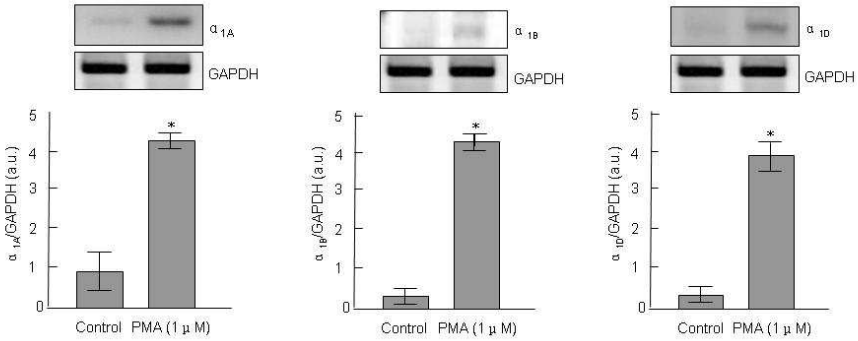


Figure 7. Changes of cardiac specific markers in MSCs treated with PMA. This change was detected by sandwich ELISA. Samples of protein were lysed to MSCs treated with PMA each harvest day (3, 6, and 9 days). This experiment was repeated three times, and each standard deviation was 0.05. Cardiac-specific 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.

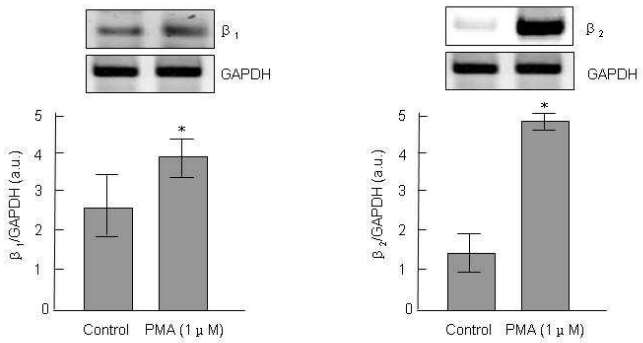
4.3. Changes of adrenergic and muscarinic receptor mRNA after PMA treatment

To begin to address whether α 1-adrenergic receptors were involved in modulating the function of CLCs, we first detected α 1 receptor expression. CLCs did not express their transcripts before PMA treatment. They expressed α_{1A}, α_{1B}, and α_{1D} receptor mRNA after treatment by PCR level. β_1- and β_2-adrenergic receptors play a crucial role in catecholamine-induced increases in heart rate, conduction velocity, and contractility. To explore whether β_1 and β_2 receptors are functional, we examined their expression. CLCs expressed all the β_1 and β_2 before PMA treatment. A low level of expression of β_1 and β_2 was observed before PMA treatment, but their expression was markedly augmented after the treatment. To date, 5 subtypes (M1 through M5) of muscarinic receptors have been cloned. M1 and M2 receptor subtypes are expressed in murine neonatal and adult cardiomyocytes. As shown in Figure 8, a low level of expression of both receptors was observed before PMA treatment, but their expression was markedly augmented after the treatment. These findings showed that adrenergic and muscarinic receptors are expressed when they obtain the cardiomyocyte phenotype (Fig. 8).

A


α 1A
GAPDH
α 1B
GAPDH
α 1D
GAPDH
α 1A/GAPDH (a.u.)
α 1B/GAPDH (a.u.)
α 1D/GAPDH (a.u.)
0
1
2
3
4
5
*
Control
PMA (1 μ M)

B


β 1
GAPDH
β 2
GAPDH
β 1/GAPDH (a.u.)
β 2/GAPDH (a.u.)
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Control
PMA (1 μ M)

C

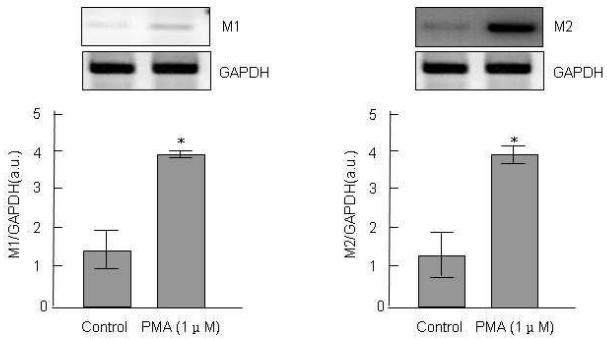


Figure 8. Changes of adrenergic and muscarinic receptor subtype in MSCs treated with PMA. Each panel shows RT-PCR products for expression of α- (A) and β- (B) adrenergic and muscarinic (C) receptor subtypes. Bar-type densitometry showed that all subtypes were more expressive than normal control. The 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.

4.4. Effect of norepinephrine on phosphorylation of ERK in MSCs treated with PMA

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. To examine whether α_1-adrenergic receptors are expressed at the protein level and transduce hypertrophic signals, we stimulated MSCs treated with PMA by NE and detected phosphorylation of ERK. Phosphorylation of ERK was induced by NE in a time-dependent manner. Activation of ERK by NE was detected in both MSCs treated with PMA and cardiomyocytes. These aspects indicated that MSCs treated with PMA expressed functionally active α_1-adrenergic receptors (Fig. 9).

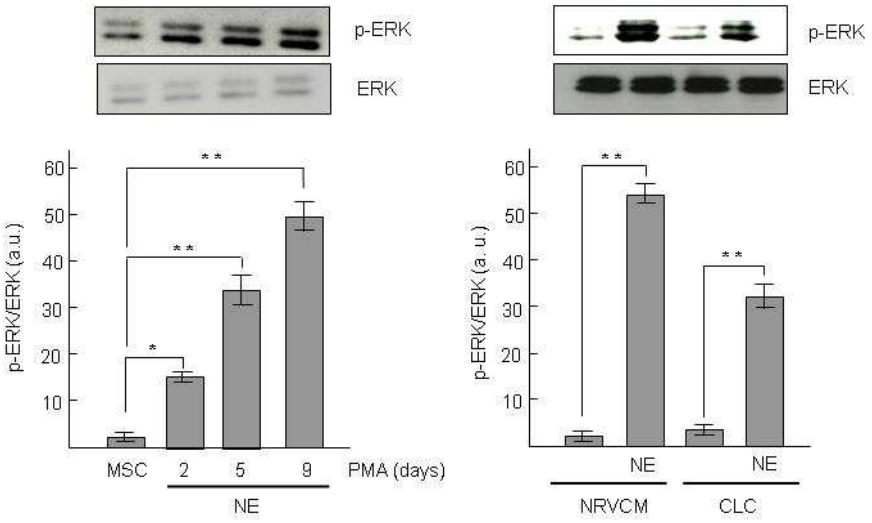


Figure 9. Effect of NE on activation of ERK in MSCs treated with PMA. Cells at two, five, and nine days (time-dependently) after PMA treatment were stimulated with 1 μM norepinephrine, and western blot analysis was performed to detect phosphorylation of ERK1/2. When MSCs treated with PMA were compared with cardiomyocytes under norepinephrine stimulation, the phosphorylation of ERK1/2 was elevated in both. *P<0.01, **P<0.001.

4.5. Changes in Ca^{2+} handling-related protein expression with 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 and LTCC. So PCR was enforced for the expression of SERCA 2a and LTCC. Neonatal cardiomyocytes were highly expressed, but even MSCs treated with PMA were time-dependently expressed. These findings suggest that MSCs treated with PMA might influence Ca^{2+} handling-related protein expression (Fig. 10).

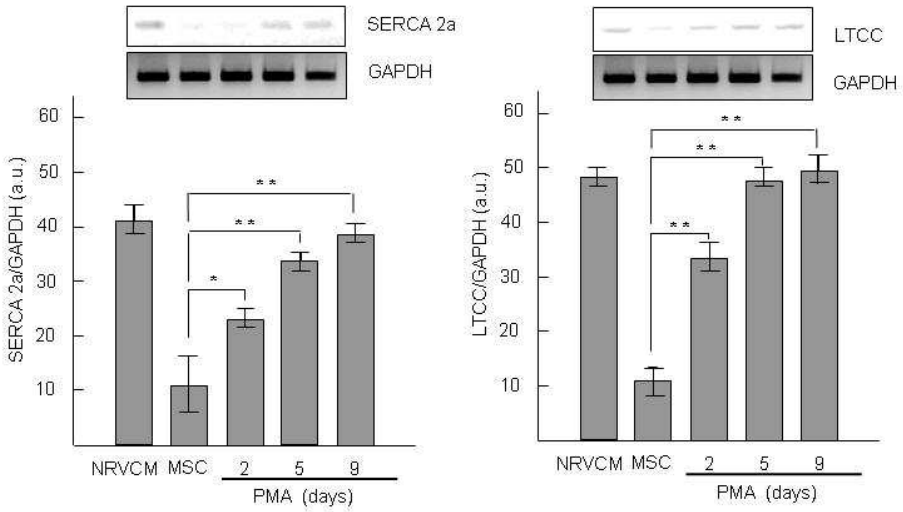


Figure 10. Changes of Ca^{2+}-related proteins in MSCs treated with PMA. MSCs treated with PMA compared to control and NRVCM in time-dependent measure. Each panel shows RT-PCR products for the expression of Ca^{2+}-related proteins (SERCA 2a, LTCC). Densitometric analysis was performed, and the ratio 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, **P<0.001.

5. Effect of GSK-3β inhibitor on the differentiation of MSCs

5.1. Effect of GSK-3β inhibitor on the differentiation of MSCs into endothelial-like cells

Stem cells were seeded and treated every 3days with GSK-3β inhibitors up to a 16-day culture. Because related specific markers were expressed during differentiation of stem cells into specific cell type, expression level of such markers were detected by sandwitch ELISA methods. An inhibitor of GSK-3β, was found to be potentially implicated in endogenesis of the MSCs. The compound is cell-permeable and known to be selective and a potent inhibitor of GSK-3β. Among the kinases tested, it inhibited only GSK-3β. MSCs treated with various concentrations of GSK inhibitor (0.1–1 μM) were differentiated into endothelial-like cells in a dose-dependent manner as judged by the up-regulated expression of CD31, a endothelial cell marker. We used GSK inhibitor at 1 μM concentration for the rest of our studies (Fig. 11).

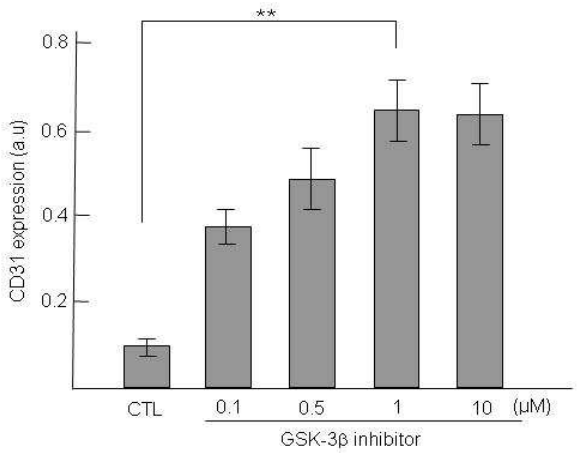


Figure 11. Effect of GSK-3β inhibitor on the endothlium differentiation of MSCs. CD31, one of specific endothelial cell markers, was induced in MSCs treated with various concentrations of GSK-3β inhibitor for 16 days (0.1–1 μM). During differentiation of MSC, the change of expression level in CD31 was examined by sandwich ELISA. (**p<0.01).

5.2. Differentiation of MSCs into endothelial-like cells

During differentiation into endothelial-like cells by cultivating confluent MSCs in the presence of GSK-3β inhibitor for 16 days, cell morphology showed no difference compared with undifferentiated MSCs. Immunohistochemical staining for vWF was chosen for the basal characterization of endothelial-like cells. Undifferentiated MSCs showed almost no specific staining for vWF, but after 16 days of cultivation the overall fluorescence intensity of the differentiated MSCs was markedly enhanced. To further establish whether these MSC could differentiate in response to GSK-3β inhibitor, changes of endothelial specific markers was analyzed with RT-PCR. After 16 days culture in the presence of GSK-3β inhibitor, expression of CD34, eNOS, VE-cadherin, VCAM-1, and VEGF-R2 was increased as compared with normal MSC (Fig. 12).

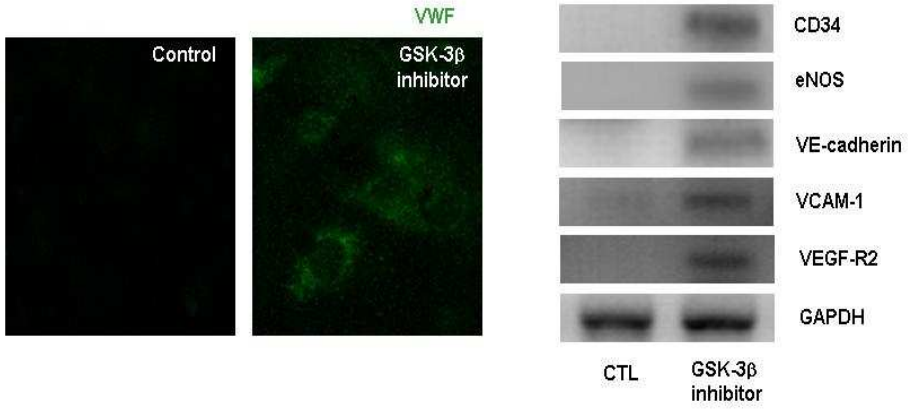
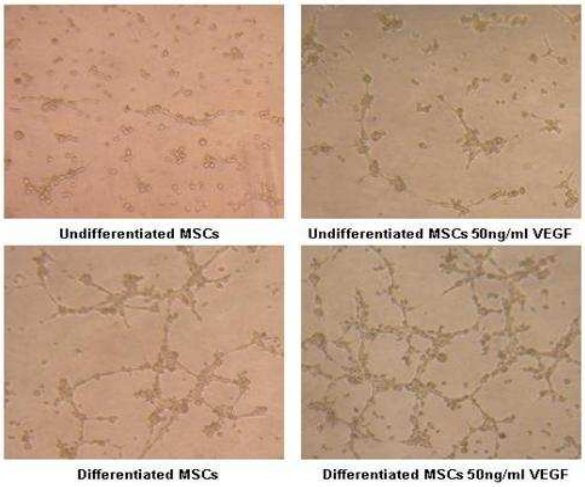
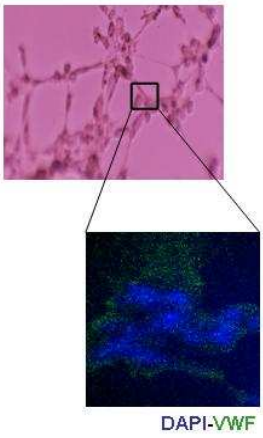


Figure 12. Changes induced by differentiation of MSC into endothelial-like cells. (A) Immunocytochemistry was detected for VWF in MSCs treated with GSK-3β inhibitor. Representative photos of normal MSCs as positive control and MSCs treated with 1 μM GSK-3β inhibitor are shown (Magnification: X400). (B) This change was detected by RT-PCR. Endothelial markers (CD34, eNOS, VE-cadherin, VCAM-1, and VEGF-R2) were expressed in MSCs treated with GSK-3β inhibitor.

5.3. Effect of GSK-3β inhibitor on the in vitro formation of capillary structures

The ability to form capillaries in semisolid medium was tested with an in vitro angiogenesis kit. Undifferentiated and differentiated MSCs were trypsinized and seeded on top of the ECmatrix gel solution. Cells were cultivated in the presence of VEGF and once without VEGF. The undifferentiated MSCs showed very few capillaries after 4 hours and most of the cells stayed round in the medium. When cultivated for 4 hours in the presence of VEGF, more tube-like structures were visible. Undifferentiated MSCs showed a substantial formation of capillary structures when cultivated for 4 hours in the presence of VEGF. After differentiation more than 70% of MSCs form capillary structures both in the presence and in the absence of VEGF. The cells in capillary structures distinctly expressed endothelial specific vWF (Fig. 13).





Percentage of capillar-like cells after cultivation on semisolid medium

	Undifferentiated MSCs	Differentiated MSCs
-VEGF	25±1	75±3
+VEGF	60±5	85±1

Figure 13. Endothelial-specific functional characterization of differentiated MSCs. (A) Light microscopic analysis of differentiated and undifferentiated MSC on semisolid medium in presence and absence of VEGF. Vascular tubelike networks were formed by differentiated MSCs on semisolid medium after plating. (B) Immunocytochemical staining of the vascular networks formed on semisolid medium for VWF (green) and DAPI (blue). Stable lumen structures were observed in the vascular networks during in vitro culture.

5.4. Effect of GSK-3β inhibitor on change of stem cell fate

During endothelium differentiation, GSK-3β inhibitor treatment reduced the proliferation of MSCs compared with non-treated cells, indicating that GSK-3β inhibitor might modulate the balance between differentiation and proliferation. Cell cycle analysis showed that GSK-3β inhibitor addition led to a decrease in the percentage of cells in S phase. These results taken together indicate that treatment of proliferating MSCs with GSK-3β inhibitor leads to depletion of S-phase cells and over all decreased proliferation (Fig. 14).

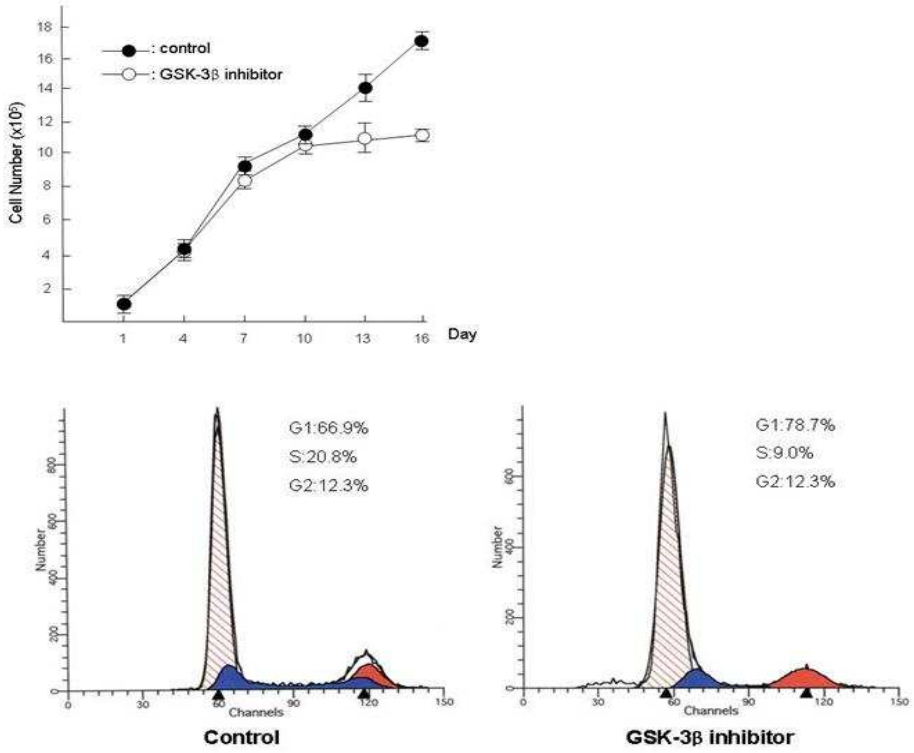


Figure 14. Changes of MSC fate by GSK-3β inhibitor. (A) For cell number (upper panel), MSCs were seeded in plates at 1 x 10^5 cells/well. GSK-3β inhibitor was added at the indicated concentrations. The experiment was performed three times. Results are shown as mean ± SD. (B) Cell cycle analysis of cells treated with GSK-3β inhibitor or not. Cells were stained with propidium iodide and analysed by means of a FACS Calibur cytometer. A representative set of data from one of three independent experiments is shown.

5.5. Mechanism of GSK-3β inhibitor-induced differentiation of MSCs

In order to study the mechanism by which GSK-3β inhibitor may induce differentiation of MSCs into endothelial cells, we evaluated the GSK-3β/β-catenin axis. GSK-3β inhibitor treatment was associated with a significantly increased phosphorylation of Akt, and GSK-3β. Consequently, this was associated with decreased phosphorylation of β-catenin, a well-known downstream molecule of the Akt/GSK-3β axis. In addition, the expression level of β-catenin in MSCs was decreased consecutively with the extension of differentiation time. In controls, β-catenin was predominantly immunolocalized at the cell periphery. However, nuclear accumulation of β-catenin immunostaining was markedly increased in MSCs treated with GSK-3β inhibitor (Fig. 15).

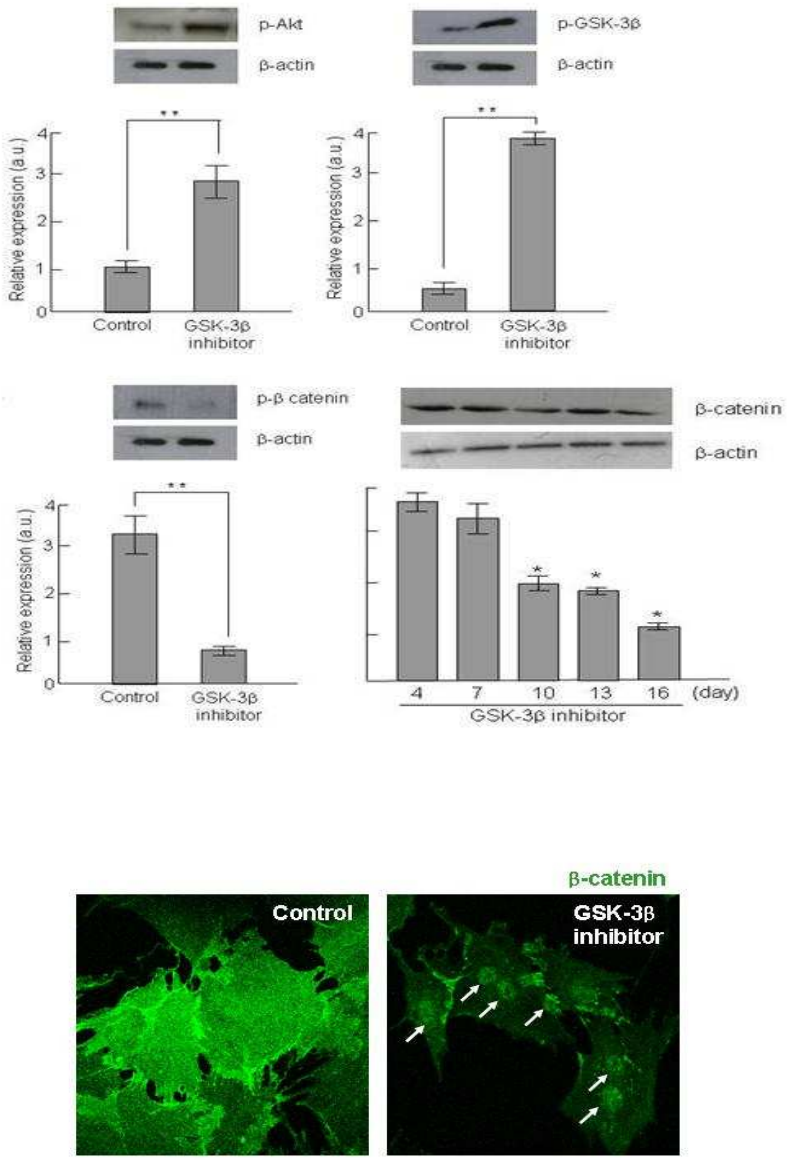


Figure 15. Effect of GSK-3β inhibitor on intracellular signaling pathway in endothelium differentiation of MSCs. (A) Western blot of MSCs after

GSK-3β inhibitor treatment. GSK-3β inhibitor treatment was associated with activation of Akt, increased phosphorylation (thus inactivation) of GSK-3β, and downregulation of phospho-β-catenin. Protein levels of β-catenin decreased significantly during differentiation process. (B) Confocal micrographs of MSCs treated with GSK-3β inhibitor. Cells were stained for β-catenin (green) after addition of GSK-3β inhibitor.

IV. DISCUSSION

Stem cells have induced increasing interest and enthusiasm because of their biological properties and potential medical importance. Recognition of these properties will need a better understanding of the signaling pathways that control stem cell fate and an improved ability to manipulate stem cell proliferation and differentiation. Specifically, the fate of stem cells is controlled both by intrinsic mechanism and the niche interaction. In general, stem cells are classified as either embryonic or adult stem cells. Embryonic stem (ES) cells are derived from the inner cell mass of mammalian blastocysts and are able to proliferate indefinitely *in vitro* while maintaining an undifferentiated state and differentiate into any possible cell type under certain conditions. By contrast, adult stem cells, which reside in differentiated tissues, have less potential of tumorigenesis but possess restricted self-renewal capacity, and can only differentiate into specialized cell types of the tissue in which they reside. These properties of stem cells make them a good source for the treatment of a number of diseases as well as tissue/organ injuries, including cardiovascular disease, neurodegenerative disease, musculoskeletal disease, diabetes, and spinal-cord injuries.

Above all, the regeneration therapy using stem cells has to meet at least two prerequisites: (1) the directed differentiation of stem cell to specific cell types and (2) prevention of undifferentiated cells becoming cancer cells. Recently, an increased understanding of the molecular and developmental processes for stem cell fate will improve the ability to manipulate stem cells for therapeutic applications. It has been known that expansion and differentiation of stem cell fate is controlled by both intrinsic regulators and the niche, such as a specific configuration (monolayer or three dimensional matrix), growth factors, as well as genetic manipulations. However, most of these conditions have some difficulties such as incompletely definition, or non-specificity in regulating the desired cellular process. Undefined condition often results in inconsistency in cell culture and/or heterogeneous populations of cells which would not be useful for cell therapy. More efficient and selective methods to control the fate of stem cells to produce homogenous populations of particular cell types will be essential to the therapeutic use of stem cells. The application of a chemical approach to the field of stem cell biology facilitates control over and understanding of stem cell fate.

Small molecules offer several advantages that include the ability for temporal, tunable and modular control of specific protein function. Small molecules are

powerful tools in the manipulation of stem cells and complement other currently utilized techniques to arrive at insights into the complex molecular mechanisms that modulate stem cell fate. Chemical approaches hold the distinct advantages of precisely controlling stem cell fate in vitro for research and therapy, probing for new biological mechanisms, and facilitating small molecule therapeutic developments for modulating stem cell function in vivo for regenerative medicine.

Since such important cellular processes are likely to be controlled by a complicated orchestration of many signaling pathways, including many as yet undiscovered pathways, signal modulators, such as protein kinases, are likely to play important role in balancing multiple signals to induce one or both of the prerequisites. Protein kinases belong to one of the largest protein families in human genome, and they play a critical role in many signaling pathways such as development, differentiation, proliferation or death. Several signaling pathways have been known to induce or suppress the differentiation of stem cells including those involving mitogen-activated kinases, glycogen synthase kinase-3, PI3 kinase, and others [23-27].

The screening of protein kinases inhibitors described above was based on the

assumption that the complex orchestration of signaling pathways is responsible for triggering the differentiation of MSCs, and that protein kinase members play key role in many of the signaling pathways (Fig. 2). To test chodrogenic characteristics of the MSCs treated with H-89, we carried out several experiments using cell culture assays. Quantification of chondrogenesis by measuring an absorbance of Alcian blue extract indicated that H-89 enhanced chondrogenesis to over 2-fold of control. MSCs treated with various concentration of H-89 (0.1-1 μM) were differentiated dose-dependently into chondrocyte-like cells and chondrogenesis of MSCs upregulated expression of extracellular matrix gene, aggrecan. The treatment of MSCs with 1 μM H-89 induced maximal increase of aggrecan expression to over 2 folds of the control (Fig. 3).

Previous studies showed that activation of MAPK was related to induction of specific gene during chondrogenesis of MSCs [28]. To ask whether regulation of chondrogenesis was mediated by MAP kinase signaling, the effect of H-89, a selective inhibitor of PKA, was examined on the phosphorylation and activation of ERK, a downstream target of PKA. Chondrogenesis of MSCs was mediated by ERK activation following PKA inhibition. In addition, co-treatment with U0126, a selective inhibitor of MEK, and H-89 did not induce the expression of

aggrecan and the activation of ERK during chondrogenesis of MSCs due to the inhibition of MEK, an upstream signaling molecule of ERK (Fig. 4).

Extracellular molecules known to regulate chondrogenesis exert their effects at the level of cell proliferation and/or precartilage condensation [29-31]. Precartilage condensation is a process that reduces intercellular spaces and formation of extensive cell-cell contacts between prechondrogenic mesenchymes [32-34]. The biochemical events leading to cell condensation are not yet understood clearly. However, both cell-cell and cell-ECM interactions have been considered to play a role in precartilage condensation. Therefore, the possibility of whether H-89, a PKA inhibitor, regulates chondrogenesis by modulating the expression of cell adhesion molecules and ECM components such as N-cadherin, integrin α5β1, and fibronectin was investigated.

N-Cadherin has been known to be expressed in prechondrogenic mesenchymes and during cell condensation [35, 36], and blockage of its function inhibited precartilage condensation [37]. However, it was not expressed in chondrocytes that were completely surrounded by cartilage-specific ECM molecules [35]. Expression of N-cadherin, which mediates cell-cell interaction, was high in first day but was reduced as differentiation of MSC treated with H-89 advanced to

chondrocyte-like cells (Fig. 5).

Because the interaction of cell with extracellular matrix (ECM) in addition to cell-cell interaction is involved in the regulation of chondrogensis, a potential of H-89 regulation of integrin α5β1 and its ligand fibronectin was also examined. During chondrogenic differentiation of MSCs treated with H-89, the fibronectin-receptor (integrin α5β1) and fibronectin were down-regulated (Fig. 5). The above results are consistent with the facts that an interaction of cells with fibronectin via integrin α5β1 is necessary for cell condensation to occur and that a reduction of fibronectin and its integrin α5β1 receptor after cell aggregation is necessary for the progression of cartilage differentiation [34, 38, 39]. Indeed, it has been known that enhanced expression of fibronectin exerts negative effects on chondrogenesis [40-42].

Treatment MSCs with H-89 retarded the proliferation activities of MSCs during chondrogenesis compared with control but H-89 did not have the cytotoxicity effect during chondrogenesis of MSCs for 11days. Namely, H-89 might modulate the relationship between proliferation and differentiation (Data not shown). In principle, one may be able to extract information about the identities of other protein kinases that may also negatively regulate the differentiation of

MSCs if one has the inhibition profile of H-89 for all known protein kinases. This approach can be applied to any stem cells to find chemical molecules that can trigger initiation of differeniation

Results seem to validate these assumptions. Furthermore, it is also assumed that, since the structure of the kinase domain of all protein kinases are very similar, especially among the members of the same subfamily, a given kinase inhibitor will inhibit multiple kinases at different levels. Thus, we posit that H-89 inhibits, in addition to PKA, multiple signaling pathways at different levels, and the orchestration of these signaling pathways triggered the differentiation. Our result suggests that one or more signaling pathways that can be regulated by PKA and other unidentified kinases negatively regulate differentiation of MSCs.

Mesenchymal stem cells (MSCs) have the potential for myocardial regeneration due to their self-renewal and myocardiogenic differentiation. Previously, researchers studying 5'-azacytidine reported that MSCs were used to differentiate into myocytes [43]. This led to believe that MSCs are multi-differential cell types, and differentiation is possible without the genotoxic effects of 5'-azacytidine [43-45]. Despite these promising results, the molecular mechanisms underlying myogenic differentiation from MSCs remain

poorly understood. The major finding of this study is a new molecular biological method of inducing differentiation of MSCs into cardiomyocyte-like cells by PMA, a protein kinase C activator, treatment.

Protein kinases belong to one of the largest protein families in human genome, and they play a critical role in many signaling pathways such as development, differentiation, proliferation or death. 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 [46, 47]. Recent study demonstrated that protein kinase C (PKC) have the critical role of the cardiac differentiation of embryonic stem cells [48, 49]. In addition, above data for the effect of various kinase inhibitors on differentiation of MSCs showed treatment of MSCs with a protein kinase C

inhibitor suppressed the expression of cardiomyocetic marker gene, indicating that activation of protein kinase C might be critical to differentiating MSCs into cardiomyocytes. Therefore, we hypothesized that MSCs might differentiate into cardiomyocyte-like cells (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 markers (cTnT, MHC, MLC, Nkx 2.5 and MEF-2) was nine days (Fig. 7). Moreover, the cell resembled a cardiac myocyte in morphology by nine days (Fig. 6). Especially, MHC and cTnT 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 and cTnT (Fig. 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 [50]. We explored α_{1A}, α_{1B}, α_{1D}, β_1, and β_2-adrenergic receptors and M_1 and M_2 muscarinic receptors. The characteristics of receptors include the following: critical implications in modulating cardiac function, α_{1A}-, α_{1B}-, α_{1D}-, catecholamine-induced increases in heart rate, conduction velocity and contractility, β_1- and β_2-adrenergic receptors, expression in murine neonatal and adult cardiomyocytes, and M1 and M2 muscarinic receptors [51-54] (Fig. 8). Surprisingly, when MSCs treated with PMA in nine days were compared with normal MSCs, differentiated MSCs had a higher expression of these receptors.

PKC is concerned about hypertrophy-related signaling. Other researchers showed the translocation of PKC-α in response to the α_1-adrenergic and $G\alpha_q$-coupled receptor agonist phenylephrine [55]. Likewise, norepinephrine (NE) influences the contractile properties of the heart and induces a series of changes characteristic of the hypertrophic phenotype through α_1-adrenergic receptors in cardiac myocytes [56]. If MSCs differentiated into CLCs, α_1-adrenergic receptors are expressed at the protein level and transduce hypertrophic signals. Because of this, the change of ERK1/2 phosphorylation was detected. When differentiated MSCs were treated with NE, their activation of ERK1/2 increased

in a time-dependent manner (Fig. 9).

Cardiomyocytes are linked by excitation-contraction 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 [57, 58]. 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, their expression level was analyzed. As seen in Fig. 10, even though NRVCMs had a slightly higher expression level, the MSCs' expression level increased in a time-dependent manner.

Finally, the prevailing evidence suggests that MSCs might differentiate into cardiomyocyte-like cells via PKC activation. This simple new model for differentiated MSCs may help clarify the cascade of transcriptional activation that regulates differentiation into CLCs.

Activation of PKC is considered to be the key point in the myogenic

differentiation of MSCs because protein kinases play a critical role in many intracellular signaling pathways and differentiation of embryonic stem cell. Treatment of MSCs with PMA induced cardiac-specific marker expression, and 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 conclusion, PMA, a PKC activator, might induce the myogenic differentiation of MSCs.

The relative ease of isolating MSCs from bone marrow and the great plasticity of the cells make them ideal tools for an autologous or allogeneic cell therapy [59, 60]. The use of autologous vascular endothelial progenitor cells seems attractive for the development of engineered vessels as well as for the vascularization of engineered tissues, and may also be useful to augment vessel growth in ischemic tissue [61, 62].

In previous studies, serial analysis of gene expression (SAGE) revealed that single cell-derived colonies of MSCs expressed mRNAs of multiple cell lineages, including characteristic epithelial and endothelial molecules like Epican and Keratins 8 and 10 [63]. These data suggest that the in vitro differentiation potential of MSCs is not restricted to mesodermal lineages but

also transdifferentiation of MSCs into other lineages like endothelial could be realized in vitro and in vivo.

The formation of endothelial tissue (vasculogenesis) is a process in which the embryo angioblasts are differentiated from mesodermal cells and organized to form a primitive vascular network [64]. Angiogenesis, the formation of new blood vessels by sprouting from pre-existing vessels, occurs in many situations such as embryonic development and pathological conditions like tissue ischemia. Although the molecular mechanisms responsible for vasculogenesis and angiogenesis are currently not fully understood, the pivotal role of glycogen synthase kinase-3β (GSK-3β) for both processes is evident.

GSK-3β is a serine/threonine kinase that is under the control of Wnt [65, 66] signaling pathways. Recently, it has been shown that a pharmacological inhibitor of GSK-3β promotes the self-renewal of embryonic stem cells *in vitro* [67]. GSK-3β controls several downstream transcription factors that are crucial in cell survival and function including β-catenin [68], heat shock factor-1 [69], cAMP-response element-binding protein [70], AP-1 [71], Myc [72], NFAT [73], CCAAT/enhancer-binding protein α [74], and cyclin D1 [75]. GSK-3β has also been shown to be involved in the regulation of angiogenesis through its ability to

modulate vascular endothelial cell migration and survival [76]. Previous studies indicated a pharmacological inhibitor of GSK-3β lead to the differentiation of endothelial progenitor cells and monocytes into endothelial cells [77, 78]. Hence, our study shows that GSK-3β is one of factors for the in vitro differentiation of mesenchymal stem cells into endothelial cells in vitro.

Several populations of bone marrow-derived cells have the potential to differentiate into endothelial-like cells. $CD133^+$ HSCs cultivated at high cellular density and in the presence of endothelial growth factors like VEGF were shown to acquire endothelial features [79, 80], and $CD34^+$ HSCs isolated from peripheral blood can differentiate into endothelial cells in vitro [81] and contribute to vascularization in animal models [82]. Regardless of this fact, the major advantage of MSCs is the vast number of cells that can be achieved from one bone marrow aspirate. MSCs were shown to be genetically stable over many passages [83].

The treatment of MSCs with GSK-3β inhibitor triggered endothelium differentiation in a dose-dependent (0.1–1 μM) manner as judged by the up-regulated expression of CD31, a endothelial cell marker (Fig. 11). In our differentiation system MSCs acquire major characteristics of mature

endothelial-like expression of CD 34, eNOS, VCAM-1, VEGF-R2, VE-cadherin, and vWF (Fig. 12).

After differentiation, the formation of capillary-like structures in semisolid medium was markedly enhanced when cells were cultivated without VEGF. Recent studies have shown that murine stroma cells can also be differentiated into vasculature-forming cells under hypoxic conditions or genetic modification [84, 85]. It was also found that MSCs form tube-like structures when cultivated in semisolid medium; the presence of VEGF markedly enhanced this behavior. Interestingly, the numbers of capillary-like cells in this assay were strongly enhanced in predifferentiated MSCs (Fig. 13). Hypoxia upregulates several genes involved in angiogenesis like basic fibroblast growth factor, VEGF, the VEGF receptors KDR and FLT-1, and components of the plasminogen system [86]. Differentiation of MSCs with GSK-3β inhibitor also upregulates the expression of the VEGF receptors KDR and FLT-1, which play a major role in angiogenesis in vivo and contribute together with matrix-metalloproteases to the formation of capillary-like structures in vitro.

In the figure 14, a pharmacological inhibitor of GSK-3β retarded the proliferation activities of MSCs during endothelium differentiation of MSCs.

Namely, GSK-3β might modulate the balance between proliferation and differentiation. In cell cycle analysis, a decrease in the percentage of cells in S phase ascertained differentiation induction of GSK-3β. In addition, GSK-3β has been known as a regulator of angiogenesis through modulation of downstream transcription factors including β-catenin and so on. Our present study showed that the treatment of GSK-3β inhibitor is associated with a significant increase in GSK-3β phosphorylation, resulting in its inactivation, and thus downregulation of phospho-β-catenin and nuclear accumulation of β-catenin (Fig. 15).

Observations described above, the enhanced differentiation of MSCs to chondrocytes, cardiomyocytes, and endothelial cells, support the notion that, although cell differentiation may be the result of a complex orchestration of many signals from multiple signaling pathways, even a single chemical reagent, a kinase inhibitor in this case, can alter the relative balance of many signals enough to enhance differentiation of stem cells to particular cell types. They also suggest that the process may be optimized by a ‘‘mixture’’ of various multiple kinase inhibitors.

Because these compounds may interact with ‘‘off-target’’ kinases as well as

other unknown proteins, our observation should be considered as a practical approach for finding chemical reagents for inducing stem cell differentiation to a specific cell type even when most of the signaling pathways are unknown. Thus, the approach described or some variation of it may be applied to other stem cells, including human stem cells, to find chemical molecules that can trigger initiation, inhibition, or even reversion of the differentiation process of stem cells or progenitor cells.

V. CONCLUSION

Although cell differentiation may be the result of a complex orchestration of many signals from multiple signaling pathways, even a single chemical reagent, a kinase inhibitor/activator in this case, can alter the relative balance of many signals enough to enhance differentiation of stem cells to particular cell types. They also suggest that the process may be optimized by a ‘‘mixture’’ of various multiple kinase inhibitors/activators. Because these compounds may interact with ‘‘off-target’’ kinases as well as other unknown proteins, the observation should be considered as a practical approach for finding chemical reagents for inducing stem cell differentiation to a specific cell type even when most of the signaling pathways are unknown. The approach described or some variation of it may be applied to other stem cells, including human stem cells, to find chemical molecules that can trigger initiation, inhibition, or even reversion of the differentiation process of stem cells or progenitor cells.

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Abstract (in Korean)

단백질 키나아제 신호 체계 조절에 의한 골수 유래 간엽줄기세포의 특이적 분화 유도

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장 우 철

현재까지 알려진 많은 신호 기전을 조절하는 단백질들 중 하나인 단백질 키나아제는 줄기세포의 분화 조절에서 중요한 역할을 할 수 있으며 이는 세포의 운명과 같은 중요한 생물학적 과정이 다양한 신호기전의 섬세한 조합에 의해 결정되어지기 때문이다. 게다가, 단백질 키나아제의 활성을 조절하는 세포 투과성 저분자화합물은 특정 단백질의 기능을 시간적, 양적 그리고 모듈단위로 조절할 수 있는 장점을 가지고 있다. 따라서 본 연구에서는 간엽줄기세포의 분화를 체계적으로 조절하는 화학물질과 기전에 대해 알아보고자 하였으며 다양한 신호기전의 조합을 조절할 수

있는 주요한 단백질 키나아제 군의 억제제 스크리닝을 통해 몇몇 단백질 키나아제가 간엽줄기세포의 분화에 상당한 영향을 미침을 발견하였다. 우선, PKA 억제제인 H-89를 줄기세포에 처리한 결과, 생체외에서 연골세포로의 분화과정이 증진되었다. H-89를 처리한 뒤 연골세포로의 분화 정도를 측정하는 alcian blue 염색 정도가 증가하였다. 다양한 농도(0.1-1 μM)의 H-89를 간엽줄기세포에 처리할 경우, 연골세포의 세포외 기질에 존재하는 단백질들 중 하나인 aggrecan의 발현이 유도되었다. 간엽줄기세포가 연골세포로 분화되는 과정에서 ERK의 활성은 증가하였으나 H-89과 U0126을 동시에 처리할 경우 ERK의 활성은 증가하지 않았다. 연골세포로의 분화과정에서 세포 생착과 관련된 분자의 발현에서 상당한 변화가 유발됨을 RT-PCR을 통해 확인하였다. PKC 활성제인 phorbol myristate acetate (PMA)의 처리는 간엽줄기세포에서 심근세포의 특이적 표현인자 (cardiac troponin T, myosin light chain, myosin heavy chain, NK2 transcription factor-related, locus 5, 그리고 Myocyte-specific enhancer factor 2)의 발현을 유발시켰다. 분화 유도된 심근세포는 기능인자인 adrenergic과 muscarinic 수용체가 발현되고 norepinephrine을 처리하면 발현된 α_1-adrenergic 수용체를 통해 ERK의 인산화가 유발되었다. 칼슘 관련 인자(sarcoplasmic reticulum Ca2+-ATPase 그리고 L-type Ca2+ channel)의 mRNA 발현 수준은 분화 유도된

간엽줄기세포와 심근세포에서 유사성을 보였다. GSK-3β inhibitor 처리에 의해 내피유사세포로의 분화가 유도되었으며 분화 유도된 세포는 내피세포의 특이적 표현인자(CD31, CD34, eNOS, VE-cadherin, VCAM-1, 그리고 VEGF-R2) 발현의 상당히 증가하였으며, 또 모세혈관과 유사한 구조의 형성과 같은 기능적 특성을 보였다. 간엽줄기세포에 GSK-3β inhibitor를 처리하면 세포주기 중 S기가 감소하고 전반적으로 생육이 저하되었다. 신호 물질의 분석을 통해 GSK-3β inhibitor 처리에 의한 내피세포로의 분화에는 GSK-3β/β-catenin 하위신호기전이 관련되어 있음을 알 수 있었다. 본 연구는 단백질 키나아제의 조절이 간엽줄기세포의 체계적인 분화 조절 방법이 될 수 있으며 이 체계는 간엽줄기세포에 대한 학문적 이해와 새로운 재생의학의 발달에 기여할 것으로 기대된다.

핵심되는 말: 간엽줄기세포, 분화, 단백질 키나아제, 신호기전

PUBLICATION LIST

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