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Mesenchymal Stem Cells from Rat Olfactory Bulbs can Differentiate into Cells with Cardiomyocyte Characteristics

Short title:

Rat olfactory bulb mesenchymal stem cells differentiate into cardiomyocytes

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ABSTRACT

Purpose: Mesenchymal stromal/stem cells (MSCs) are widely distributed in different tissues/organs including bone marrow, adipose tissues, peripheral blood, umbilical cord and the amnion. A prior study shown that MSCs isolated from rat olfactory bulb are a source of mesenchymal stem cells that can be induced to differentiate into adipo-, osteo-, and chondrocytes. However, it was not determined if these cells could differentiate into myocardial cells. In the present study, we examined whether olfactory bulb derived mesenchymal stromal cells could differentiate into myocardial cells *in vitro*.

Methods: fibroblast-like cells isolated from the olfactory bulb of neonatal rats were grown under four induced culturing: no treatment; in the presence of neuregulin-1, bFGF, and forskolin; coculture with cardiomyocytes; coculture with cardiomyocytes plus neuregulin-1, bFGF and forskolin. Cell differentiation into myocardial cells was monitored by RT-PCR, light microscopy, immunofluorescence, Western blot analysis, and changes in spontaneous contraction rates following pharmacological treatments.

Results: The isolated olfactory bulb-derived fibroblast-like cells expressed CD29, CD44, CD90, CD105, CD166 but not CD34 and CD45, consistent with them having molecular characteristics of MSCs. But only in the co-culture with rat cardiomyocytes plus neuregulin-1, bFGF and forskolin, long cylindical cells that spontaneously contracted were observed following 7 days of culture. RT-PCR and Western blot analysis indicated the cells expressed myocardial markers, such as Nkx2.5, GATA4, sarcomeric α-actinin, cardiac troponin I, cardiac myosin heavy chain, atrial natriuretic peptide and connexin 43. The differentiated cells contained sarcomeres and gap junction organization and were sensitive to pharmacologic treatments (adrenal and cholinergic agonists and antagonists).

Conclusions: These findings indicate that fibroblast-like cells with MSC characteristics from rat olfactory bulbs can differentiate into myocardial-like cells.

Key words: olfactory bulb, mesenchymal stem cells, cardiomyocytes, myocardial cells, induction, differentiation

1. INTRODUCTION

Chronic heart disease often results in heart damage and defects in cardiac function that are not reversible with current drug therapy. The use of stem cells therapy to repair this damage is of great interest and is actively being studied (Hida et al., 2008; Tomita et al., 2005; Tamura et al., 2011; Dowell et al., 2003).

Mesenchymal stem cells (MSCs) are adherent multi-potent stem cells that have similar to fibroblastic morphology (spindle shaped) and can differentiate into osteocytes, chondrocytes, adipocytes, and myocardial cells (Augello et al 2010). MSCs have been isolated from a variety of tissues including the bone marrow, adipose tissue, peripheral blood, umbilical cord, blood from the umbilical cord, the amnion fluid, compact bone, periosteum, menstrual blood, synovial fluid, synovial membrane, articular cartilage, and foetal tissues (Augello et al., 2010; Hida et al., 2008; Choi et al., 2010; Benard et al., 2004; Jumbay et al., 2009; Bin et al., 2006; Kadivar et al., 2005).

The olfactory bulb has a multi-layered cellular architecture and is one of the few places in the adult central nervous system where neurogenesis can occur and is a potential source of stem cells (Delorme et al., 2010). On the outer surface of the olfactory bulb is the axon fiber layer that includes fibroblasts, astrocytes, and olfactory ensheathing cells. Fibroblasts in the axon fiber layer are heterogeneous. To date, the types and functions of stromal cells in the olfactory bulb are still poorly understood. A prior study showed the stromal cells from olfactory bulb had characteristics of MSCs and could differentiate into osteocytes, chondrocytes, and adipocytes (Soleimani et al., 2008). Whether the stem cells from olfactory bulb have the potentials to differentiate into myocardial cells is not known.

In the present study, the rat olfactory bulb-derived fibroblast-like stromal cells were co-cultured with rat cardiomyocytes in a medium containing the growth factors neuregulin-1, bFGF and forskolin. We found that MSC-like cells from the central nervous system can differentiate into myocardial cells.

2. MATERIAL AND METHODS

2.1. Cell Culture

The isolation of fibroblast-like cells from olfactory bulb was performed according to previously reported with some modifications (Nash et al., 2001). The neonatal rats aged 5 to 6 days were sterilely sacrificed by decapitation and the skull and brain tissue were removed. The olfactory bulb was collected and washed several times in calcium and magnesium-free Hank's balanced saline solution (HBSS, Invitrogen, Carlsbad, CA). The frontal olfactory bulb was separated and cut into tissue pieces which were then digested for 10 min at 37°C in 0.1% trypsin (Invitrogen) and 0.05% DNase (Sigma, St. Louis MO). The enzymes were subsequently inactivated by DMEM with 10% fetal bovine serum (FBS). The homogenate was centrifuged at 1000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in growth medium (DMEM/F12 containing 10% FBS) and transferred into 25T flasks followed by incubation at 37°C in an atmosphere with 5% CO_2. Cells that adhered within 18 hours were collected and incubated in growth medium. The adherent cells had fibroblast-like morphology. The medium was refreshed every three days and cells were collected when confluence was observed. All in vitro differentiation experiments were performed on cells from passage 3-4.

The culture of rat cardiomyocytes was performed according to previous reports (Huang et al., 2007). The heart was obtained from neonatal rats aged 1-2 days under aseptic condition and the atrium and large vessels were removed. The remaining ventricle was washed in HBSS several times to remove blood, cut into pieces and incubated with 0.5 mg/ml collagenase (Sigma) and 0.6 mg/ml pancreatin (Sigma) at 37 ^{o}C for 15 min. The cell suspension was transferred into plating medium (MEM containing 10% FBS, 100 IU/ml penicillin, 100 g/ml streptomycin and 2 mM glutamine) to stop digestion. This procedure was repeated 3 to 5 times until the tissue was completely digested. The collected cells were then centrifuged at 1000 rpm for 10 min and the supernatant was removed and the pelleted cells were suspended in plating medium. The cell suspension was transferred into 10 cm dishes followed by

incubation at $37^{o}C$ for 1 hour in a CO_2 incubator. After fibroblasts attachment, the cardiomyocytes in the supernatant were collected and plated at a density of 10^5-10^6 cells/ml. These cells were subsequently grown on collagen coated transwell inserts for 1 day and then the medium was replaced with growth medium. The percentage of cardiomyocytes was greater than 90%, as determined by the proportion of cells showing spontaneous contraction or staining positive for the muscle-specific protein, titin.

2.2. Cardiogenic and other induction experiments

Cardiogenic differentiation: the cultured fibroblast-like cells were divided into 4 groups for induced culturing: Group 1, in standard medium as untreated control; Group2, in the presence of a cocktail that contained 50 ng/ml neuregulin-1 (R&D Systems, Minneapolis, MN), 20 ng/ml bFGF (Peprotech, Rochy Hill, NJ), and 10 uM forskolin (Calbiochem, La Jolla, CA); Group 3, in the presence of cardiomyocytes (transwell induction assay); Group 4, in the presence of cardiomyocytes (transwell induction assay) plus neuregulin-1, bFGF and forskolin. The medium was changed twice a week. The cells were monitored for cell differentiation 1-4 weeks later.

To investigate the multi-directional differentiation of the fibroblast-like cells from olfactory bulb of neonatal rat, we also performed the inductions of osteogenic differentiation and adipogenic differentiation.

Osteogenic differentiation: cell were stimulated every 3-4 days in growth medium supplemented with 0.1 uM dexamethasone , 50 mg/ml ascorbate, 10 mM β-glycerophosphate (all from Sigma). After 21 days, cells were rinsed twice with PBS, fixed with 10% formalin for 10 min, and washed with distilled water. To stain calcium deposits, cells were covered with 2% aqueous solution of alizarin red S (Sigma), pH 4.2 for 30 min. Stained cells were analyzed using an invert microscope (Olympus, Tokyo, Japan).

Adipogenic differentiation: cells were stimulated with growth medium supplemented with 0.1 uM dexamethasone, 200 uM indomethacin, 10 ug/ml insulin (all from Sigma) for 21 days with a medium change twice a week. Differentiated cells were analyzed using an inverted microscope (Olympus).

2.2.1. Transwell induction assay

For the transwell-induction assay, cardiomyocytes were grown on polycarbonate inserts (pore size 0.4 μm, Corning Incorporated, Corning, NY) that were placed into wells containing the fibroblast-like cells such that the different cell types were on either side of the polycarbonate filter and not in contact with one another. One to 2 weeks later, differentiation of the fibroblast-like cells into myocardial cells was examined.

2.2.2. Reverse Transcipatase-PCR (RT-PCR) analysis

RT-PCR was performed to prove whether the cells expressed the surface markers , including CD34, CD45, CD29, CD44, CD90, CD105 and CD116, and cardiac specific markers containing Nkx2.5, GATA-4, and cardiac α-myosin heavy chain (α-MHC). Total RNA was extracted from cells using TRIzol Reagent (Invitrogen). and cDNA was synthesized using SuperScriptTM Ⅲ First-Strand Synthesis System kit (Invitrogen). The sequences of PCR primers are shown in Table 1. The PCR conditions were: 94℃ for 2 min; 94℃ for 20 sec, 55℃ for 30 sec, 68℃ for 45sec, with a total 35 cycles; followed by 68℃ for 10 min. The PCR products were run on 1.6% agarose gel with ethidium bromide.

2.2.3. Flow cytometry analysis

The cultured cells (passage 3) were detached with Trypsin/ EDTA and counted. About $2x10^5$ cells were divided into 5 mL aliquots in centrifuge tubes and washed with PBS containing 2% BSA and 0.1% sodium azid (Sigma). Subsequently, the cells were stained with fluorescent isothiocyanate (FITC)-conjugated mouse anti–rat CD29 (Biolegend, San Diego, CA), mouse anti-rat CD90 (BD Biosciences, San Jose, CA),

mouse anti-rat CD34 (Novus Biologicals, Littleton, CO), mouse anti-rat CD45 (Biolegend), a concentration of 2 ug/ml at 4 ℃ for 45 minutes. The stained cells were analyzed using a FACS Callibur cytometry (BD Biosciences). CELLQuest software was used to create the histograms.

2.3. Detection of the rate of myocardial contraction

Spontaneous contraction were observed using an inverted microscope with heating board (Nikon, Tokyo, Japan) at 36 ± 1°C. Contractions were recorded with a video monitor, and the number of contraction of 4-8 aggregated cells (1 microscope field) was counted in 1 min. Five separate fields were selected from each dish for every experiment, and the number of contractions across the 5 fields were averaged and expressed as mean contraction per minute. Each experiment was repeated 3 times.

2.4. Western blot and immunofluorescence analysis

For immunofluorescence and Western blot analysis, primary antibodies for characterizing cardiomyocyte differentiation included anti-rabbit Nkx2.5, anti-rabbit GATA4 (both from Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit atrial natriuretic peptide (ANP, Millipore, Temecula, CA), anti-mouse sarcomeric α-actinin (Sigma), anti-mouse cardiac troponin I, cardiac MHC (both from Abcam, Cambridge UK), and anti-mouse titin and anti-rabbit Connexin 43 (Invitrogene), and anti-mouse β-tubulin (Abcam). Secondary antibodies included FITC-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG (both from Sigma), and Texas Red-conjugated horse anti-mouse IgG (Vector Labs, Burlingame, CA, US), alkaline phosphatase-conjugated goat anti-rabbit IgG, and alkaline phosphatase-conjugated goat anti-mouse IgG (both from Promega, Madison, WI).

2.4.1. Western blot

Cells were lysed in 0.15% Triton X-100, 10 mM EGTA, 2 mM $MgCl_2$, 60 mM PIPES, and 25 mM HEPES, pH 6.9 and the protein concentration determined using the protein assay kit (Bio-Rad, Hercules, CA, US). The cellular homogen (50 μg per

lane) was separated by 10% SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Schleicher & Schuell BioSciences Inc., Boston, MA). The nitrocellulose membrane was incubated in blocking solution [5% non-fat milk in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris, pH 8.2)] at room temperature for 1 hr and then incubated overnight with primary antibody at 4°C. After washing with TBS-0.1% Tween, the membrane was treated with alkaline phosphatase-conjugated secondary antibodies (diluted 1:7500) at room temperature for 1 hr followed by washing in TBS. The bands were visualized with 3.3 mg/ml nitroblue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris (pH 9.5). Each experiment was repeated at least 3 times.

2.4.2. Immunofluorescence

Cells were fixed in 10% formaldehyde for 10 min and then treated with blocking buffer (5% non-fat milk in 0.1% Triton X-100) for 15 min. The fixed cells were incubated with primary antibody at 37 ^{o}C for 1 hour and then washed 3X in PBS (5 min for each) and subsequently incubated with FITC-conjugated goat anti-mouse IgG at room temperature for 1 hour. For double immunofluorescence, cells were simultaneously incubated with mouse anti-MHC antibody and rabbit anti-Connexin 43 antibody for 1 hour. Then cells were treated with FITC-conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-rabbit IgG at room temperature for 1 h. After washing with PBS, cells were mounted with 3% n-propyl gallate and 50% glycerol in PBS and analyzed using a fluorescence microscope (Carl Zeiss, Oberkocheu, Germany).

2.5. Pharmacological Analysis

Pharmacological studies were performed on 14-day-old beating cells where the cardiomyocyte-like morphology predominated, because the characteristics of the beating cells at this time were suitable for this kind of analysis.

Chronotropic responses were assessed in DMEM-F12, 10% FBS by extracellular

recording of the beating rate in the presence of the appropriate drug. Dose-response experiments were performed with 0.5 to 2.5×10^{-6} mol/L isoproterenol (Calbiochem), 5 to 20×10^{-6} mol/L propranolol (Calbiochem), 0.25 to 1×10^{-6} mol/L carbamylcholine (Calbiochem), and 1 to 5×10^{-6} mol/L atropin (Calbiochem). Antagonist was added after the maximal dose of agonist.

2.6. Statistical analyses

Normally distributed continuous variables were compared by one-way analysis of variance (ANOVA). When a significant difference between groups was apparent, multiple comparisons of means were performed using the Bonferroni procedure with type-1 error adjustment. Data are presented as means ± SD. All statistical assessments were two-sided and evaluated at the 0.05 level of significant difference. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS Inc, Chicago, IL).

3. RESULTS

Cells with MCS-like characteristics were isolated from the neonatal rat olfactory bulb. These cells had fibroblast-like morphology after 3 and 7 days of being cultured (Fig. 1A and B). When the cells were cultured in osteogenic medium for 21 days, the morphology changed and was positive for alizarin red S staining (Fig. 1C). These cells were also able to differentiate into adipocytes, as they accumulated different amounts of lipid vacuoles (Fig. 1D, arrows) after cultivation in adipogenic medium. The fibroblast-like cells expressed mRNA for the MCS surface markers including CD29, CD44, CD90, CD105, and CD166, but not hematopoietic stem cell markers such as CD34 and CD45 (Fig.2A). Flow cytometry (Fig. 2B) and immunofluorescence (Fig. 2C) results showed that the isolated cells highly expressed CD29 (97.5 ± 1.8 %), whereas 29.2 ± 2.2 % of cells was positive for CD90. Simultaneously, cells were negative for hematopoietic markers CD34 and CD45 (Fig. 2B). Our results indicate that olfactory bulb derived fibroblast-like cells have

MSC-like molecular characteristics and multipotention similar to those of bone marrow derived MSC.

To determine if the fibroblast-like cells had the potential to differentiate into myocardial cells, they were divided into four groups for myocardial induction: Group1, cultured in standard medium; Group 2, cultured in medium with neuregulin-1, bFGF and forskolin; Group 3, co-cultured with cardiomyocytes; Group 4, co-cultured with cardiomyocytes plus neuregulin-1, bFGF and forskolin. After 7-days of culture, myogenic-like cells from group 4 formed myotube-like structures (Fig. 3D, arrows) and spontaneous contraction of 8.1% of the cells was observed (see video 1 in supplemental material), whereas there was no visible spontaneous beating of cells in the other groups (Fig.3A-C). After 4 weeks of culture, there were no signs of myotube-like structures of morphology in cells from group 1 (Fig.3A), group 2 (Fig.3B) and group 3 (Fig.3C). The cells in group 4 expressed mRNA for cardiac-specific marker, including Nkx2.5, GATA4, α-cardiac myosin heavy chain (α-MHC) by RT-PCR analysis (Fig.3E).

Western blot analysis further examined if the fibroblast-like cells had differentiated into cells with myocardial cell characteristics. Cells from group 4 expressed cardiac specific proteins including Nkx2.5, GATA4, cardiac MHC, sarcomeric α-actinin, cardiac Troponin I (TnI) and atrial natriuretic peptide (ANP) (Fig. 4: lanes 5). Olfactory bulb tissue extracts (Fig.4; lane 1) and the fibroblast-like cells from groups 1, 2, and 3 (Fig.4: lanes 2-4), did not express these cardiomyocyte specific markers except for α-actinin and ANP.

Immunofluorescence experiments also indicated that the olfactory bulb fibroblast-like cells had differentiated into cells with myocardial cell characteristics. The cells expressed α-actinin (Fig.5A), cardiac MHC (Fig.5B), titin (Fig. 5C). In addition, double label staining revealed connexin 43 (Cx43) expression in 2 neighboring MHC-positive cells, suggesting gap junction organization (Fig.5D, arrows). Periodic sarcomeric structures were visible with the α-actinin and titin staining (Figure 5A and 5C). Cells from groups 1-3 did not express these myocardial

cell markers or contain sarcomeric-like structures (data not shown).

Pharmacological agents that stimulate or inhibit cardiac muscle contraction were used to further examine the characteristics of these cells. Addition of the ß-agonist isoproterenol induced a dose dependent increase in the contraction rate of the cells ($P < 0.001$) (Fig. 6A). Subsequent addition of propranolol, a non-selective ß-adrenergic antagonist, reversed the increased contraction rate ($P < 0.001$) (Fig. 6A). Carbamylcholine, a non-selective cholinergic agonist, decreased the spontaneous contraction rate in a dose dependent manner ($P < 0.001$), subsequent addition of atropin, a muscarinic antagonist, reversed the decreased contraction rate ($P < 0.001$) (Fig. 6B). The pharmacologic response of these cells further indicates that they have myocardial-like cell properties.

4. DISCUSSION

Previously, fibroblast-like cells isolated from adult rat olfactory bulb were shown to have MSCs characteristics as they were capable of differentiate into cells with osteoblastic, adipogenic, and chondrogenic properties (Soleimani et al., 2008). In this study, we found neonatal rat olfactory bulb fibroblast-like cells were able to differentiate into myocardial-like cells. These findings further support the idea that these fibroblast-like cells are MSCs and indicate that these central nervous system derived cells have similar multi-potent characteristics as ectodermal derived MSCs.

The cell biological properties of the microenvironment of the stem cell niche are central in determining what cell lineage cells will follow upon differentiation (Augello et al., 2010). Cell-cell signal transduction pathways have been identified that control whether a multi-potent stem cell remains undifferentiated or is induced to follow a particular cell lineage. In this study, co-culture of the olfactory bulb MSCs with cardiomyocytes plus neuregulin-1, bFGF and forskolin was necessary to induced differentiation of these cells into myocardial-like cells. This suggests that cell receptors and intracellular signaling pathways are involved in inducing expression of genes required for differentiation along the myocardial cell lineage. Nkx2.5 and GATA4 are two transcription factors critical for myocardial cell differentiation. It is possible that induction of Nkx2.5 and GATA4 expression resulted in the transcriptional activation of key myocardial structural genes including sarcomeric α-actinin, cardiac MHC, and troponin I required for sarcomere formation.

Neuregulin-1, bFGF and foskolin are involved in the differentiation of myocardial cells. Neuregulin-1 acts via the ErbB/PI3 K/Akt signaling pathway to up-regulates the expression of the cardiac specific transcription factors Nkx2.5 and GATA4 and cardiac muscle structural proteins such as α- and ß-MHC and α-actinin (Wang et al., 2009) and induces proliferation of adult mononuclear cardiomyocytes (Bersell et al., 2009). bFGF influences the early maturation of embryonic stem cell-derived cardiomyocytes, their contraction properties, and expression of cardiac α- and ß-MHC (Khezri et al., 2007). bFGF is known to regulate myocardial infarct repair (Virag et al., 2007). Forskolin acts via cAMP to induced mouse embryonic stem cells

to differentiate into cardiomyocytes (Chen et al., 2006). Neuregulin-1 and cAMP also are able to induce a mixed population of fetal cardiomyocytes to be transformed into cardiac pacemaker-like cells (Ruhparwar et al., 2007). Our findings are consistent with these factors playing a role in inducing cells to become myocardial cells. Since all three factors were used simultaneously, it is not clear if 1, 2, or all 3 factors are required for the myocardial fate. Further analysis is required to characterize the mechanism of cell signaling pathway in this developmental myocardial experimental model system.

The development of olfactory bulb originated from a group of migrating cells which are derived from the 3 different regions: olfactory placode, the forebrain subventricular zone and neural crest. Pluri-potent neural crest cells mainly develop into frontonasal mesenchyme which then generates the olfactory ensheathing cells and stromal cells in the olfactory bulb and olfactory mucosa. The olfactory mucosa has been shown to contain multipotent stem cells (Delmore et al., 2010). It is possible that the olfactory bulb derived multi-potent MSCs in this study originated from neural crest cells.

This study found MSCs isolated from the olfactory bulb could differentiate into cardiomyocytes. Cardiomyocytes have also been generated from embryonic stem cells and induced pluripotent stem cells (So et al., 2010; Gai et al., 2009; Mauritz et al., 2008). Our findings identify a novel stem cell model system for studying early cardiac development and may provide a new source of cells for the investigation and treatment of heart disease.

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Table 1. PCR primers used in the study

Gene	Primer sequence (5'-3')	PCR product (bp)
CD34	F: TGTTGCCTCGGAGAATTCTAC R: GCACTCCTCGGATTCCTGAAC	305
CD45	F: AAAGAGGAAATGGCTCCTCAG R: CTATTTCTGTGCTTGTGGTGG	381
CD29	F: AATGTTTCAGTGCAGAGC R:TTGGGATGATGTCGGGAC	261
CD44	F: AAGACATCGATGCCTCAAAC R: CTCCAGTA GGC TGTGAAGTG	120
CD90	F: CCTGCCTGGTGAACCAGAACCTT R: GCAGGCTTATGCCACCACACTTG	327
CD105	F: CAGGCATCCAACACCATAGAG R: AAGTTCATGGCCGATGGTTCC	675
CD166	F: CTCGGATGGTACACTGTCAAC R: TGGACACCTCTCCATCAACAG	481
Nkx2.5	F: TTATCCGCGAGCCTACGGTGA R: CTGCCGCTGTCGCTTACACTT	319
GATA4	F: CTGTCATCTCACTATGGGCA R: CCAAGTCCGAGCAGGAATTT	257
α-MHC	F: AGTCAGAGAAGGAGCGCCTA R: TAGATCATCCAGGCCGCATA	292
GAPDH	F: CAAGGTCATCCATGACAACTTTG R: GTCCACCACCCTGTTGCTGTAG	496

FIGURES LEGENDS

Figure 1: Morphological characteristics and in vitro differentiation of rat olfactory bulb derived fibroblast-like cells. Cells were cultured in standard medium for three passages and then differentiation was started. Phase contrast of cells after (A) 3 days and (B) 7 days of culture displayed a spindle-shaped and fibroblastic appearance. (C) Cells after 21 days of osteogenic differentiation (alizarin red staining). (D) Cells after 21 days of adipogenic differentiation (note adipocytes containing lipid droplets, arrows). Bar = 50 um.

Figure 2: Molecular characterization of rat olfactory bulb derived fibroblast-like cells. (A) RT-PCR analysis of the expression of mRNA for specific cell markers, including CD34, CD45, CD29, CD44, CD90, CD105, and CD166. GAPDH was used as internal RT-PCR control. (B) Flow cytometry analysis of olfactory bulb derived fibroblast-like cells. Cells were labeled with FITC-conjugated antibodies against CD29, CD90, CD34 and CD45. (C) Immunofluorescence analysis of olfactory bulb derived fibroblast-like cells. Cells were labeled with FITC-conjugated antibodies against CD29 and CD90. Bar = 50 um.

Figure 3: Induction of olfactory bulb-derived fibroblast-like cells to differentiate into cells with myocardial cell characteristics. (A) Group1: olfactory bulb-derived fibroblast-like cells (control group), (B) Group 2: olfactory bulb derived fibroblast-like cells cultured in the presence of neuregulin-1, bFGF, and forskolin, (C) Group 3: olfactory bulb derived fibroblast-like cells co-cultured with cardiomyocytes, and (D) Group 4: olfactory bulb derived fibroblast-like cells co-cultured with cardiomyocytes plus neuregulin-1, bFGF and forskolin. After 7 days of culture, morphology of cultured cells was observed under an inverted microscope. Only the cells in group 4 developed a myocardial-like cell morphology (long and cylindrical; arrows) and contracted spontaneously. Bar = 30 um. (E) RT-PCR analysis for mRNA expression of Nkx2.5, GATA4, and α-cardiac myosin heavy chain in the

differentiated cells in group 4. M is DNA molecular weight standard and GAPDH was internal RT-PCR control.

Figure 4: Western blot analysis of the expressions of cardiac-specific proteins (Nkx2.5, GATA4, cardiac myosin heavy chain, sarcomeric α-actinin, cardiac Troponin I and ANP) in the olfactory bulb-derived fibroblast-like cells after different induction conditions. Myocardial proteins were not expressed in (Lane 1) olfactory bulb tissue extracts, (Lane 2) cells incubated in standard medium, (Lane 3) cells incubated with neuregulin-1, bFGF, and forskolin, and (Lane 4) cells co-cultured with cardiomyocytes. However, the myocardial proteins were present in cells that had been (Lane 5) co-cultured with cardiomyocytes plus neuregulin-1, bFGF and forskolin and (Lane 6) cultured rat cardiomyocyte extracts as a positive control.

Figure 5: Immunofluorescence of olfactory bulb derived fibroblast-like cells following co-culture with rat cardiomyocytes plus neuregulin-1, bFGF, and forskolin. After seven days induction, (A) sarcomeric α-actinin, (B) cardiac myosin heavy chain (MHC), (C) titin, and (D) double stained with cardiac MHC (green) and connexin 43 (Cx43, red) were detected by immunofluorescence. The periodic staining with α-actinin and titin antibodies indicates the presence of sarcomeric structures. Punctate Cx43 staining (arrows in D) expressed in neighboring MHC-positive cells. Bar = 30 μm.

Figure 6: Chronotropic response of contracting cells to adrenergic and cholinergic stimulation. In the co-culture of olfactory bulb-derived fibroblast-like cells with rat cardiomyocytes plus neuregulin-1, bFGF, and forskolin, after 7 days induction, spontaneous contracting cells were observed and their contracting rate was measured after treatment with increasing doses of (A) ß-adrenergic agonist isoproterenol and antagonist propranolol, and with (B) the cholinergic agonist carbamylcholine and

muscarinic antagonist atropine. The results are expressed as the mean ± SD of two separate experiments.

[*] Indicates a statistically significance difference between the given group and the control group (i.e., basal group).

[†] Indicates a statistically significance difference between the given group and the isoproterenol $2.5x10^{-6}$ mol/L for (A), and between the given group and carbamylcholine1 x 10^{-6} mol/L for (B)

Pair-wise multiple comparisons between groups were determined using Bonferroni's test with $\alpha = 0.001$ adjustment.

FIGURES

Figure 1

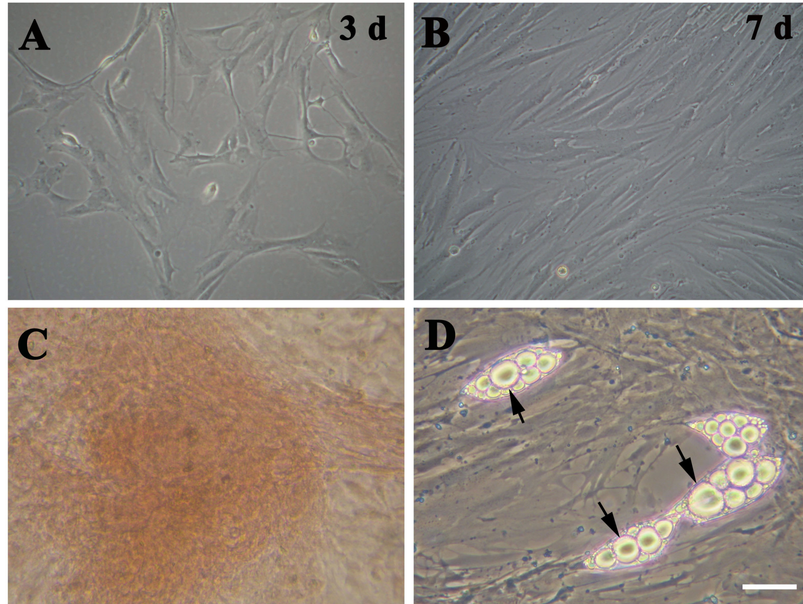

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Figure 2

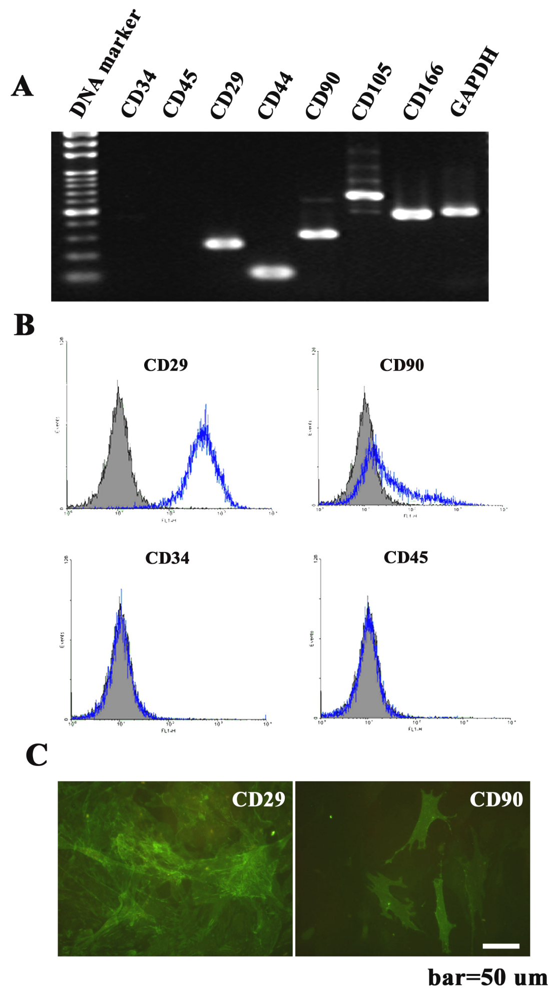

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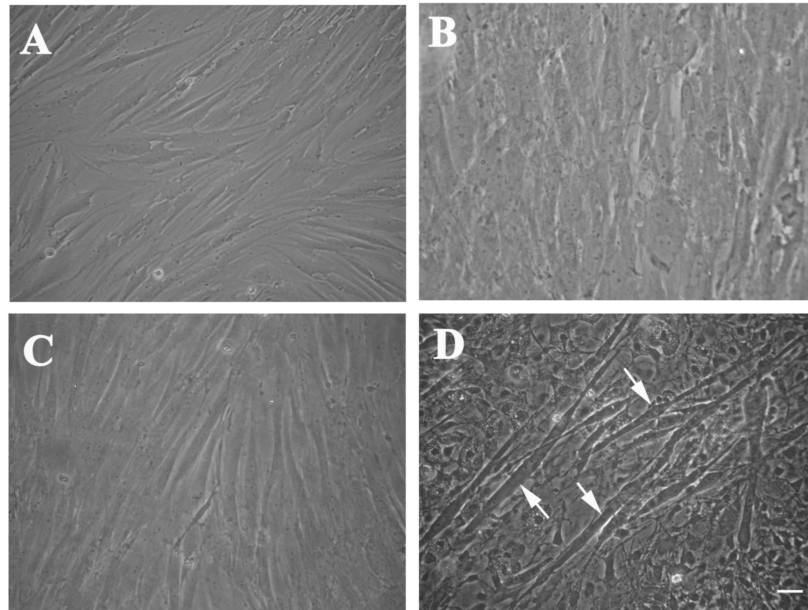
Figure 3



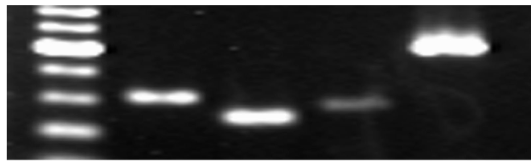


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Figure 4

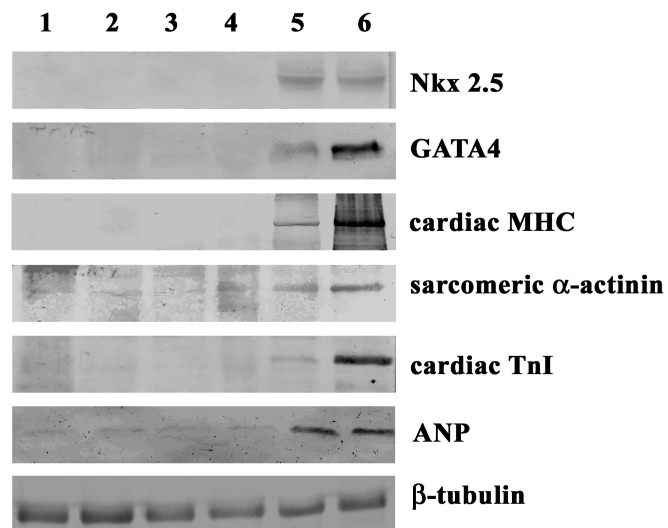

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Figure 5

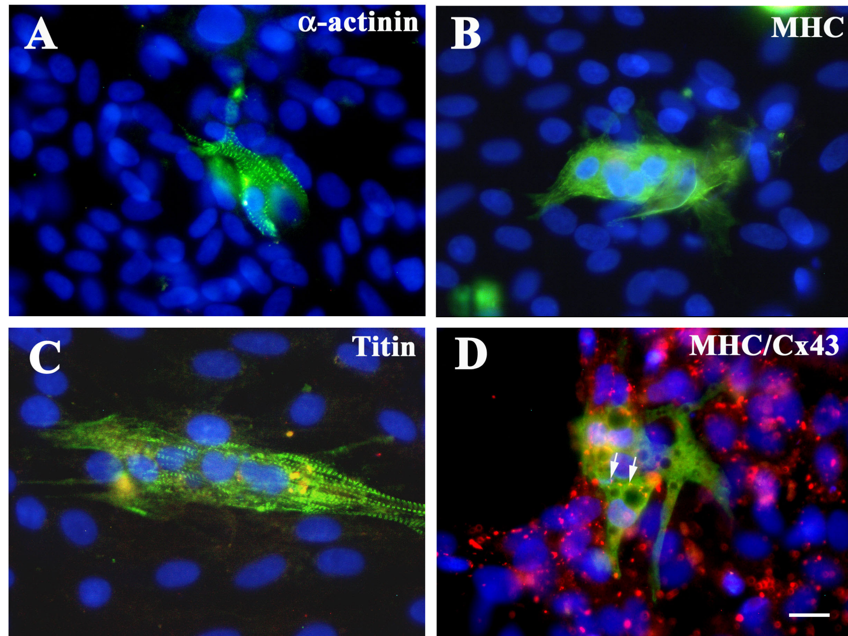

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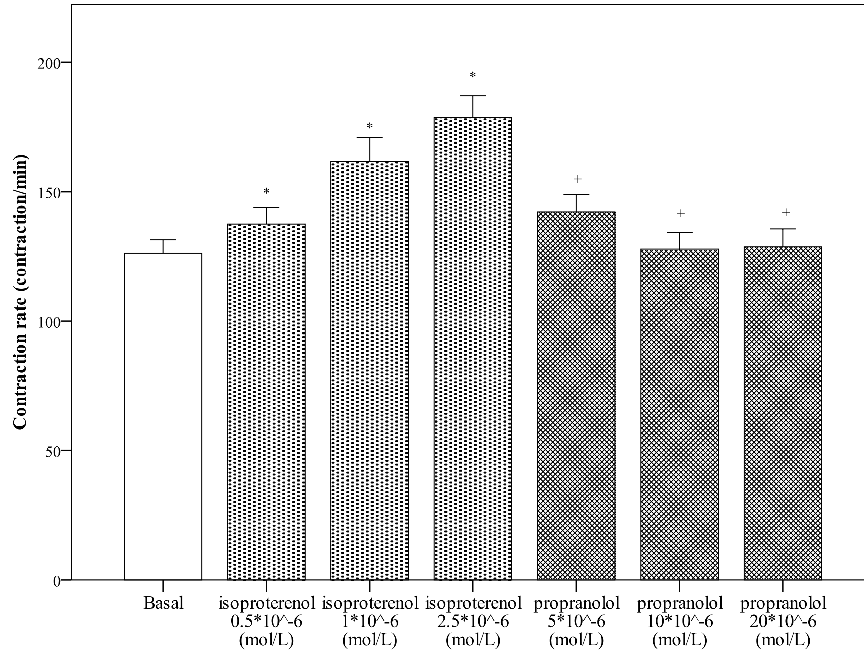
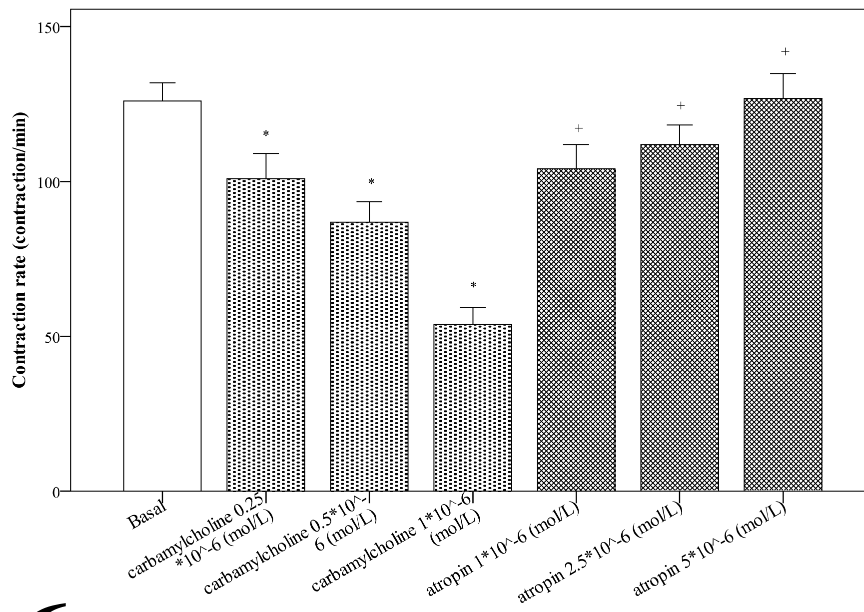
Figure 6

A.



B.



6

國科會補助計畫衍生研發成果推廣資料表

日期:2011/10/30

<table>
<tr><td rowspan="3">國科會補助計畫</td><td>計畫名稱: 由老鼠嗅球及嗅黏膜的多潛能幹細胞分化心肌細胞及修補老鼠心肌組織的潛能探討</td></tr>
<tr><td>計畫主持人: 黃雍協</td></tr>
<tr><td>計畫編號: 99-2314-B-040-016- 學門領域: 幹細胞/再生生物醫學</td></tr>
<tr><td colspan="2">無研發成果推廣資料</td></tr>
</table>

99年度專題研究計畫研究成果彙整表

計畫主持人：黃雍協	計畫編號：99-2314-B-040-016-
計畫名稱：由老鼠嗅球及嗅黏膜的多潛能幹細胞分化心肌細胞及修補老鼠心肌組織的潛能探討	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	1	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	對心臟再生醫學及幹細胞分化

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

　　□實驗失敗

　　□因故實驗中斷

　　□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 ■未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

對於心臟再生醫學的研究貢獻