Adult cardiac Sca-1 positive cells differentiate into beating cardiomyocytes

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Running title: Cardiac stem cells in the adult murine heart

Summary

Although somatic stem cells have been reported to exist in various adult organs, there have been few reports on stem cells in the heart. We here demonstrate that Sca-1 positive (Sca-1+) cells in adult hearts have some features of stem cells. Sca-1+ cells were isolated from adult murine hearts by the Magnetic Cell Sorting system and cultured on gelatin-coated dishes. A part of Sca-1+ cells stuck to the culture dish and proliferated slowly. When treated with oxytocin, Sca-1+ cells expressed genes of cardiac transcription factors and contractile proteins, and showed sarcomeric structure and spontaneous beating. The isoproterenol treatment increased the beating rate, which was accompanied with the intracellular Ca^{2+} transients. The cardiac Sca-1+ cells expressed oxytocin receptor mRNA and the expression was upregulated after the oxytocin treatment. Some of the Sca-1+ cells expressed alkaline phosphatase after osteogenic induction and were stained with Oil-Red O after adipogenic induction. These results suggest that the Sca-1+ cells in the adult murine heart have a potential as stem cells and may contribute to the regeneration of injured hearts.

Introduction

The heart has long been thought to adapt to increased work and loss of cardiomyocytes by the cellular hypertrophy of residual cardiomyocytes, but not by the proliferation of mature cardiomyocytes or the differentiation of undifferentiated cells. However, recent reports have suggested that adult cardiomyocytes can proliferate under certain pathologic conditions and that there are the cells expressing stem cell markers in the adult heart (1-4). It has been reported that Sca-1 and c-kit positive (+) cells exist in the adult heart (5) and that adult murine hearts contain potential stem cells, side population (SP) cells (6, 7). However, it remains to be clarified whether these cells have the characteristics of stem cells such as abilities of self-renewal and differentiating into various types of cells including mature cardiomyocytes.

Sca-1 is a member of Ly-6 family and has first reported as one of cell surface markers of hematopoietic stem cells (8). Recently many reports have demonstrated that multipotential stem cells derived from bone marrow and skeletal muscle express Sca-1. Okumoto et al (9) have reported that Sca-1+ cells from bone marrow differentiate into hepatocyte when treated with hepatic growth factor. Gojo et al (10) have reported that adult mesenchymal stem cells from bone marrow abundantly express Sca-1 and differentiate into cardiomyocyte in vivo. Qu-Petersen et al (11) have shown that skeletal muscle-derived stem cells which highly express Sca-1 contribute to the regeneration of the skeletal muscle in a mouse model of Duchenne muscle dystrophy. They also demonstrated that the skeletal muscle-derived stem cells were able to differentiate into neural cells and endothelial cells. Asakura et al (12) have reported that ~90 % of SP cells in skeletal muscle express Sca-1. It has been reported that skeletal muscle-derived Sca-1+ and CD34+ cells restore dystrophin in mdx mice (13) and that CD34+ and CD45- cells in the interstitial spaces of skeletal muscle, which highly express Sca-1, differentiate into adipocytes, endothelial and myogenic cells (14). These findings suggest that Sca-1 might be one of the important clues of somatic stem

cells.

Currently little is known about the humoral or growth factors, which induce cardiomyogenic differentiation. It has been shown that ectopic application of bone morphological protein (BMP) -2 and -4 elicits cardiogenic responses in chick in vivo system (15) and fibroblast growth factor (FGF) -2 and -4, combined with BMP-2 or BMP-4 can induce cardiogenesis in chick non-precardiac mesoderm (16). The non-canonical Wnt / c-Jun-N-terminal kinase pathways have been reported to be essential for cardiac induction in frog and chick embryo systems (17, 18). However, these factors are prerequisites for proceeding early cardiac differentiation but not sufficient for accomplishing the differentiation into mature beating cardiomyocytes. Recently Paquin et al (19) have reported that oxytocin induces differentiation of P19 embryonic carcinoma cells to beating cardiomyocytes. In support of a potential of oxytocin on cardiac development, oxytocin receptor is increased at protein levels in the murine heart from day 7 of gestation, when cardiac differentiation starts (20). Although the precise mechanism of the effect of oxytocin is not clear, oxytocin may play an important role in the differentiation into cardiomyocytes from primitive cells including adult somatic stem cells.

Here, we first report that a novel population from Sca-1+ cells derived from the adult murine heart proliferates and differentiates into beating cardiomyocytes with the oxytocin treatment.

Experimental procedures

Animals and reagents

Wild mice (B57Bl/6) were purchased from Takasugi Experimental Animals Supply, Co, LTD, Japan. All protocols were approved by the Institutional Animal Care and Use Committee of Chiba University. Phycoerythrin (PE) conjugated anti-Sca-1 (anti-Ly6A/E), anti-c-kit (anti-CD117) antibodies were purchased from eBioscience

(San Diego, CA). PE conjugated anti-CD34, anti-CD45 and biotin conjugated anti-Sca-1 antibodies were purchased from BD Pharmingen (San Diego, CA). Following antibodies were used for immunostaining: mouse monoclonal anti-cardiac troponin T (RV-C2, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany), rabbit polyclonal anti-atrial natriuretic factor-1 (ANF) (Peninsula laboratories, San Carlos. CA), goat polyclonal anti-GATA4 (Santa Cruz Biotechnology, Santa Cruz. CA), mouse monoclonal anti-myosin light chain-2v (MLC-2v) (BioCytex. France), mouse monoclonal anti-tropomyosin (Sigma Aldrich, St. Louis. MO), mouse monoclonal anti-sarcomeric myosin heavy chain (MF-20) (American Type Culture Collection, Rockville. Maryland), rabbit polyclonal anti-connexin 43 (Zymed Laboratories, South San Francisco. CA). Fluorescent secondary antibodies were purchased from Jackson Immuno Research Laboratory (Bar Harbor. MO). Other reagents which are not specified were obtained from Sigma-Aldrich.

Isolation and culture of Sca-1+ cells from the adult murine heart

A heart of adult B57Bl/6 mouse (10-12 weeks old) was enzymatically dissociated into a single cell suspension as described previously (21). Enrichment of Sca-1+ cells was achieved by sorting using the Magnetic Cell Sorting (MACS) system (Miltenyl Biotec, Sunnyvale, CA). Whole primary cell suspension was incubated with PE conjugated anti-Sca-1 antibody for 10 minutes on ice, washed in PBS supplemented with 3 % FBS, incubated with anti-PE micro beads for 15 minutes at 4 °C and washed with PBS supplemented with 3 % FBS. The samples were passed through a MACS column set up in a Miltenyl magnet and the Sca-1+ cells were eluted from the column by washing with PBS supplemented with 3 % FBS. To increase the purity of the Sca-1+ cells, magnetic sorting was performed one more time. The Sca-1+ cells were cultured on 1 % gelatin-coated dishes with Iscove's Modified Dulbecco's Medium (IMDM)

supplemented with 10 % FBS, 100 μg/ml of penicillin and 250 μg/ml of streptomycin at 37 °C in humid air with 5 % CO_2. Twenty-four hours after seeding, the cells were treated with 10 μM of 5'-azacytizine for initial 72 hours or 100 nM of oxytocin (WAKO, Japan). After treatment, the medium was changed every 3 days.

Characterization of cardiac muscle-derived stem cells for flow cytometric analysis

Sca-1+ cells were isolated by the MACS system with biotin conjugated anti-Sca-1 antibody and anti-biotin micro beads. Magnetic sorting was repeated twice and the cells were incubated with PE conjugated anti-CD45 antibody, PE conjugated anti-CD34 antibody, PE conjugated anti-c-kit antibody respectively for 10 minutes on ice and washed with PBS supplemented with 3 % FBS. The percentages of CD45+, CD34+ and c-kit+ cells were analyzed by the EPICS ALTRA flow cytometer using EXPO32 software (BECKMAN COULTER, Miami, FL).

RNA extraction and reverse transcriptase-PCR

Total RNA was extracted from the adult murine heart, liver and Sca-1+ cells by RNA-bee reagent (TEL-TEST, Friendswood, TX). Reverse transcriptase (RT)-PCR of genes of cardiac transcription factors, including Csx/Nkx-2.5 (22), GATA4 (23), muscle enhancer factor-2C (MEF-2C) (24), and cardiac structural proteins, including α- and β-myosin heavy chain (MHC) (25), myosin light chain-2a (MLC-2a), MLC-2v (26), cardiac α-actin (27), and oxytocin receptor (19), alkaline phosphatase (28), osteocalcin (29) were performed using 0.1 μg of total RNA. β-actin (30) was used as an internal control. The primers used in this study and PCR conditions are described in table 1. The PCR products were size-fractionated by 2 % agarose gel electrophoresis.

Immunocytochemistry

Cells were fixed with 4 % paraformaldehyde for 15 minutes at room temperature.

After preblocking with PBS containing 2 % donkey serum, 2 % BSA and 0.2 % NP40 for 30 minutes, primary antibodies in PBS containing 2 % donkey serum, 2 % BSA and 0.1 % NP40 were applied over night in 4 °C. Subsequently cells were washed three times in PBS and then FITC- or Cy5-conjugated secondary antibodies were applied to visualize expression of specific proteins. Nuclear staining was performed with TO-PRO-3 (Molecular Probes, Eugene, OR). Images of cells were taken by laser confocal microscopy (Radiance2000, BioRad, Hercules, CA).

Phase contrast live imaging

Live images were taken by a Zeiss inverted microscope (Carl Zeiss, Jena, Germany) equipped with phase-contrast objectives and an AxioCam camera. Live image of beating cells were obtained by a chilled CCD camera (Hamamatsu) using I-O DATA Videorecorder software.

Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in beating cells derived from cardiac Sca-1+ cells was measured with a previously described (31). The beating cells on gelatin-coated glass coverslips were incubated in HEPES (loading) solution containing 1 μM fluo 3-acetoxymethyl ester (fluo 3-AM; Molecular Probes) at 36 °C in the dark for 30 minutes. The loading solution was prepared by diluting a 100 μM fluo 3 stock solution, which contained 0.45 % Pluronic F127 (Molecular Probes), 10 % dimethyl sulfoxide, and 90 % FBS. HEPES solution consisted of (in mM) 126 NaCl, 4.4 KCl, 1.0 $MgCl_2$, 1.08 $CaCl_2$, 24 HEPES, 13 NaOH, 11 glucose, and 0.5 probenecid (pH 7.4). The coverslips were washed twice with dye-free HEPES solution and placed in a flow-through chamber on the microscope. Fluo 3-loaded beating cells were excited by 480-nm light and emitted fluorescence was recorded at 530 nm by a photomultiplier tube (AIM-10, InterMedical, Co, Japan) and digitized (PowerLab 2/20, ADinstruments,

Castle Hill, Australia). Curve fits were performed with Origin 7J software (MicroCal Software, Northampton, MA). The intensity of the fluorescence at 530 nm increased with an increase in $[Ca^{2+}]_i$.

Estimation of pluripotency

Osteogenic differentiation of Sca-1+ cells from the adult murine heart was induced in IMDM supplemented with 10 % FBS, 50 μM ascorbic acid 2-phosphate, 0.1 μM dexamethasone and 10 mM β-glycerophosphate as described previously (32). For detection of osteocytes, alkaline phosphatase staining (Leukocyte Alkaline Phosphatase Assay Kit, Sigma-Aldrich) was used. Adipogenic differentiation was induced as described previously (28). Briefly the cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5 % horse serum with MDI-I cocktail; 0.5 mM methyl-isobutylxanthine, 1 μM dexamethasone, 100 mM indomethacin and 10 μg/ml insulin for 2 days and then cultured with DMEM supplemented with 5 % horse serum and 10 μg/ml of insulin for 1 day. The treatment with MDI-I followed by insulin was repeated 4 times. For detection of accumulated oil droplets, Oil-Red O staining was performed followed by nuclear hematoxylin counter staining.

Statistical analysis

Values are presented as mean ± SEM. The significance of differences among mean values was determined by ANOVA. The accepted level of significance was $P<0.05$.

Results

Cell surface antigens of Sca-1+ cells derived from the adult murine heart

Flow cytometric analysis revealed that Sca-1+ cells were enriched to over 90 % when adult murine cardiac cells were sorted twice with the MACS system using PE conjugated anti-Sca-1 antibody and anti-PE micro beads (Fig. 1A). The number of the

purified Sca-1+ cells was ~1 X 10^4 cells. Limana et al have estimated the number of cardiomyocytes in an adult murine heart as approximately 3 X 10^6 (33). Therefore the percentage of cardiac Sca-1+ cells was ~0.3 % of total number of cardiomyocytes. Next we examined other cell surface antigens such as CD45, CD34 and c-kit in Sca-1+ cells. After repeated magnetic sorting with biotin conjugated anti-Sca-1 antibody and anti-biotin micro beads, enriched Sca-1+ cells were incubated with PE-conjugated anti-CD45, CD34 and c-kit antibodies and analyzed by flow cytometry. In enriched Sca-1+ cells, ~40 % of the cells expressed CD45 (Fig. 1B), and ~10 % of the cells expressed CD34 (Fig. 1C) and c-kit (Fig. 1D).

Sca-1+ cells from the adult murine heart differentiate into beating cardiomyocytes

In order to induce differentiation into cardiomyocytes, Sca-1+ cells were treated with either 5'-azacytizine or oxytocin. When Sca-1+ cells were cultured with medium containing FBS, they showed various cell shapes and spindle-like and elongated shapes were predominant (Fig. 2A). Two weeks after the treatment with oxytocin, small round cells with prominent nucleus and little cytoplasm have appeared (arrow heads in Fig. 2B). These round cells rapidly proliferated, formed clusters and detached from the culture dish so that spindle-shaped cells were left. The cells were re-plated when reached to confluent. Four weeks after starting the treatment with oxytocin, some spontaneously beating cells were recognized among spindle-shaped cells (arrow in Fig. 2C, D and Supplemental data). Spontaneous beating was observed at ~1 % cells. On the other hand, cells after the treatment with 5'-azacytizine or vehicles showed fibroblast like morphology, and never showed round or spindle-shaped morphology, or spontaneous beating.

Next we examined the gene expression of cardiac transcription factors and cardiac structural proteins in the Sca-1+ cells by RT-PCR. Before the treatment with 5'-azacytizine or oxytocin, only Csx/Nkx-2.5 and GATA4 were slightly expressed (Fig.

3, lane P). Four weeks after the treatment with 5'-azacytizine or oxytocin, all of genes of cardiac transcription factors including Csx/Nkx-2.5, GATA4 and MEF-2C and structural proteins such as α- and β-MHC, MLC-2a, MLC-2v and cardiac α-actin were expressed (Fig. 3, lane A for 5'-azacytizine and lane OT for oxytocin). Treatment with 100 nM of oxytocin antagonist (OTA, [d$(CH_2)_5^1$,Tyr(Me)^2,Thr-4,Orn-8,Tyr-NH_2^9] vasotocin, Wako, Japan) completely inhibited oxytocin-induced expression of cardiac genes (Fig. 3, lane OT+OTA). Total RNA obtained from the adult murine heart and liver was used as positive and negative controls (Fig. 3, lane H for heart and lane L for liver). Loading of equal amount of RNA was confirmed by expression of β-actin gene. Cardiac gene expression was not observed in cells cultured with vehicles (Fig. 3, lane V).

To examine the expression and localization of cardiac proteins, the Sca-1+ cells treated with oxytocin and 5'-azacytizine were stained with specific antibodies against cardiac proteins. The cells treated with oxytocin expressed GATA4 (Fig. 4A), ANF (Fig. 4B) and cardiac troponin T (Fig. 4A, B). MLC-2v (Fig. 4C), sarcomeric myosin heavy chain (Fig. 4D) and tropomyosin (data not shown) were also expressed. Notably, staining of each contractile protein showed a fine striated pattern. Connexin 43 was expressed at the junction between two cardiac troponin T expressing cells (Fig. 4E). These findings indicate that the treatment with oxytocin induced differentiation of Sca-1+ cells derived from the adult murine heart into mature cardiomyocytes, which had well-organized structures and electrical junctions. After the treatment with 5'-azacytizine, a part of cells expressed sarcomeric myosin heavy chain in fibrillar pattern (Fig. 4F), but not cardiac troponin T (data not shown). Next we sorted cardiac Sca-1+ cells on the basis of CD45 expression and cultured with oxytocin. Some of Sca-1+/CD45- cells expressed sarcomeric myosin after the oxytocin treatment, but none of Sca-1+/CD45+ cells expressed myosin (data not shown), suggesting that Sca-1+ cells that can differentiate into cardiomyocytes are in CD45- population.

Cardiac contraction is regulated by beat to beat change in $[Ca^{2+}]_i$. To ascertain that the spontaneous beating of differentiated cardiac Sca-1+ cells depends on intracellular level of Ca^{2+}, we analyzed $[Ca^{2+}]_i$ transients of the beating cells. As shown in upper panel of Fig. 5A, the spontaneous beating of differentiated Sca-1+ cells was accompanied with the $[Ca^{2+}]_i$ transients. After the treatment with 10^{-7} M of isoproterenol for 5 minutes, the frequency of $[Ca^{2+}]_i$ transients were increased in comparison with control (Fig. 5A upper panel vs. lower panel). Next we examined the predominant subtype of β receptors, which mediate changes in beating rate. Differentiated cardiac Sca-1+ cells were treated with vehicle (PBS), propranolol, CGP20712A (β_1-selective blocker), or ICI118551 (β_2-selective blocker) for 30 minutes and then stimulated with isoproterenol alone for 5 minutes. Isoproterenol significantly increased the beating rate of the control cells (control: 131.9 ± 5.6, n=10 vs. isoproterenol: 228.9 ± 7.3, n=10, $P<0.01$, Fig. 5B). The pretreatment with propranolol (average 196.4 ± 5.6, n=10, $P<0.05$ vs isoproterenol) and CGP20712A (average 188.9 ± 7.5, n=10, $P<0.05$ vs isoproterenol) reduced the increase in beating rate in response to isoproterenol significantly (Fig. 5B). ICI118551 had no effect on isoproterenol-induced increase in beating rate.

Sca-1+ cells from the adult murine heart express oxytocin receptor mRNA

To elucidate the role of oxytocin receptor in cardiomyogenesis of Sca-1+ cells, we examined the expression of oxytocin receptor in Sca-1+ cells. Oxytocin receptor mRNA was present at low levels in Sca-1+ cells before the oxytocin treatment (Fig. 6, lane P). Expression levels of oxytocin receptor remained low in cells cultured with vehicles (Fig. 6, lane V). After the treatment with oxytocin, expression levels of oxytocin receptor were upregulated (Fig. 6, lane OT). In accordance with inhibitory effect of oxytocin antagonist on oxytocin-induced cardiac differentiation, oxytocin antagonist inhibited oxytocin-induced upregulation of oxytocin receptor (Fig. 6, lane

OT+OTA). These results suggest that the positive feedback mechanism, namely oxytocin-induced upregulation of oxytocin receptor, plays an important role in oxytocin-induced cardiomyocyte differentiation of cardiac Sca-1+ cells.

Sca-1+ cells can differentiate into osteocytes and adipocytes

It has been reported that Sca-1+ cells from skeletal muscle and bone marrow differentiate into various type of cells such as adipocytes, endothelial cells, muscle, neural and hepatic cells (9, 11, 14). To determine whether the Sca-1+ cells from the adult murine heart have pluripotency, we examined whether these cells could differentiate into cells other than cardiomyocytes. When treated with osteogenic inducers, some of Sca-1+ cells were stained with alkaline phosphatase, one of the early markers of osteogenesis (Fig. 7A). RT-PCR clearly revealed that osteogenic marker mRNAs such as alkaline phosphatase and osteocalcin were induced in Sca-1+ cells after the treatment with osteogenic inducers (Fig. 7B). On the other hand, Sca-1+ cells treated with oxytocin never expressed alkaline phosphatase and osteocalcin. When Sca-1+ cells were cultured with MDI-I cocktail for twelve days, some of Sca-1+ cells showed cytoplasmic accumulation of oil droplets stained with Oil-Red O, indicating that Sca-1+ cells differentiated into adipocytes (Fig. 7C).

Discussion

In this report, we have first demonstrated that adult cardiac Sca-1+ cells can differentiate into beating cardiomyocytes in vitro by the treatment with oxytocin. When treated with oxytocin, the Sca-1+ cells expressed cardiac genes including Csx/Nkx-2.5, GATA4, MEF-2C, α-MHC, β-MHC, MLC-2a, MLC-2v, and cardiac α-actin, and cardiac proteins including GATA4, cardiac troponin T, tropomyosin, MLC-2v, sarcomeric myosin heavy chain, ANF and connexin 43. Furthermore, some of Sca-1+ cells showed well organized sarcomere and spontaneous beating. Although

transient treatment with 5'-azacytizine also induced expression of cardiac genes in Sca-1+ cells, it did not induce expression of cardiac troponin T, assembly of sarcomere or spontaneous beating. These results suggest that the treatment with 5'-azacytizine induces differentiation of Sca-1+ cells into cardiomyocytes incompletely and that oxytocin is a more potent inducer of cardiac differentiation than 5'-azacytizine.

P19 teratocarcinoma cells differentiate into beating cardiomyocytes after the treatment with DMSO and have been considered as a good model of in vitro cardiogenesis (34, 35). Several essential transcription factors in cardiomyogenesis such as GATA4 (34), Csx/Nkx-2.5 (34), and MEF-2C (36) are upregulated in P19 cells treated with DMSO. Recently Paquin et al (19) have reported that oxytocin induces P19 embryonic carcinoma cells to differentiate into cardiomyocytes. Treatment with oxytocin as well as with DMSO induced colony formation of beating cardiomyocytes, expression of cardiac proteins and oxytocin receptor proteins. In this study, cardiac Sca-1+ cells expressed low levels of oxytocin receptor mRNA which were positively regulated by oxytocin itself and pretreatment with oxytocin antagonist completely inhibited oxytocin-induced expression of cardiac genes. These results suggest that oxytocin induces cardiomyocytes differentiation of cardiac Sca-1+ cells through oxytocin receptors. Furthermore Sca-1+ cells treated with oxytocin did not express osteogenic marker mRNAs, suggesting that oxytocin is not a non-specific inducer like 5'-azacytizine but has some specificity for cardiac lineage.

Oxytocin receptors are coupled to $G_{q/11}$ class GTP binding proteins and stimulate the generation of inositol triphosphate and diacylglycerol, leading to Ca^{2+} release and activation of protein kinase C (37). Oxytocin stimulates cell proliferation through calcium (38, 39) and protein kinase C pathways (38). Cassoni et al (40) have reported that oxytocin stimulates cell proliferation through oxytocin receptors that lead to an increase in intracellular Ca^{2+} and tyrosine phosphorylation. Tyrosine phosphorylation in oxytocin signaling has been reported to activate both p38

mitogen-activated protein kinase and extracellular signal-regulated kinase 2 (41, 42). The mechanism by which oxytocin stimulates tyrosine phosphorylation has not been elucidated, but may be mediated by $G_{\beta\gamma}$ subunit dissociating from G_{α} subunit. Oxytocin inhibits the proliferation of human brain tumors (43), breast-cancer cells (44) and adenocarcinoma of endometrium (45) via the cyclic adenosine monophosphate - protein kinase A pathway. Tahara et al (46) have reported that the RhoA/Rho-kinase cascade is involved in oxytocin-induced rat uterine contraction. Among the considerable diversity of oxytocin-mediated signaling pathways, the specific pathway that activates cardiogenesis is currently unknown. Recently posttranslational modification of cardiac transcription factors has been reported to be important for their transcriptional activities. Rho-like GTPases can phosphorylate GATA4 via activation of the p38 mitogen-activated protein kinase pathway, which enhances the potency of GATA4 (47). MEF2 is stimulated by calmodulin kinase activation in the heart (48). It remains to be determined which oxytocin signaling pathways are important for differentiation of cardiomyocytes.

It has been reported that c-kit+, Sca-1+, lineage- and CD34-/low fraction of bone marrow cells contain hematopoietic stem cells, which contribute to long-term multi-lineage reconstitution of blood system in mice (49). Orlic et al (50) and Gojo et al (10) have reported that c-kit+ bone marrow cells and c-kit+ bone marrow derived-mesenchymal cells transdifferentiate into cardiomyocytes in vivo, suggesting that c-kit is one of cell surface markers of multipotent stem cells in bone marrow. The multipotential stem cells also reside in skeletal muscle although the origin of the stem cells is still controversial (51). Skeletal muscle-derived stem cells reported by Qu-petersen et al (11) and Torrente et al (13) highly express CD34 and Sca-1 but not c-kit and CD45 and differentiate into neural and endothelial cells. In our study, cardiac Sca-1+ cells expressed low levels of c-kit, suggesting that the feature of stem cell markers on cardiac stem cells is distinct from bone marrow-derived stem cells and

rather similar to skeletal muscle-derived stem cells.

Tamaki et al (14) isolated CD34+ and CD45- cells from the interstitial space of skeletal muscle, which highly expressed Sca-1 but not other endothelial progenitor cell markers. The CD34+/CD45- cells differentiated into adipocytes, endothelial and myogenic cells and expressed Bcrp1/ABCG2 gene mRNA, which is an important determinant of the SP phenotype. Recently Polesskaya et al (52) have reported that CD45+/Sca-1+ cells from injured skeletal muscle differentiate into myoblasts much more than CD45-/Sca-1- cells. Because of the hematopoietic restricted expression of CD45 antigen, skeletal myogenic CD45+/Sca-1+ cells might be a hematopoietic origin. In our study, cardiac Sca-1+ cells expressed low levels of CD34 and approximately 40% of the cardiac Sca-1+ cells expressed CD45, one of hematopoietic cell markers. We sorted cardiac Sca-1+ cells on the basis of CD45 expression and cultured with oxytocin. Some of Sca-1+/CD45- cells expressed sarcomeric myosin after the oxytocin treatment, but none of Sca-1+/CD45+ cells expressed myosin (data not shown), suggesting that Sca-1+ cells that can differentiate into cardiomyocytes are in CD45- population. Therefore, in terms of the expression of CD34 and CD45, the cardiac muscle stem cells are distinct from the previously reported skeletal muscle-derived stem cells.

Sca-1+ cells from the adult heart expressed GATA4 and Csx/Nkx-2.5, but not Oct-3/4 before the treatment with oxytocin (data not shown), suggesting that the Sca-1+ cells are committed to cardiomyocytes in some degree. Makino et al (24) have reported that mouse bone marrow-derived mesenchymal stem cells (CMG cells) differentiate into cardiomyocyte after the 5'-azacytizine treatment. Although the cell surface antigens of CMG cells were not analyzed, the bone marrow-derived mesenchymal stem cells, which differentiated into cardiomyocytes after the 5'-azacytizine treatment in vivo, expressed Sca-1, c-kit and CD34 (10), suggesting that the cardiac Sca-1+ cells are different from bone marrow-derived mesenchymal stem cells. Cardiac Sca-1+ cells differentiated into osteocytes and adipocytes in appropriate

conditions, suggesting that cardiac Sca-1+ cells have the intra-germ layer multipotency. It remains to be determined whether cardiac Sca-1+ cell population contains stem cells capable of differentiating to extra-germ layer lineage.

The spontaneously beating differentiated cardiac Sca-1+ cells showed $[Ca^{2+}]_i$ transients and treatment with isoproterenol increased the frequency of $[Ca^{2+}]_i$ transients and beating rate. The similar response to isoproterenol has been reported in adult murine cardiomyocytes (53), embryonic stem cells-derived cardiomyocytes (54), and CMG cells (55). β_1-selective blocker, CGP20712A, significantly reduced isoproterenol-induced increase in beating rate to the same extent as the nonselective β-blocker, propranolol, but β_2-selective blocker, ICI118551, did not. These results suggest that the β_1 receptor is the predominant subtype which mediates the changes in beating rate of cardiomyocytes derived from Sca-1+ cells.

During the preparation of our manuscript, two studies on cardiac stem cells have been reported (56, 57). They have shown that c-kit+ or Sca-1+ cells derived from the adult murine heart express cardiac genes and proteins after the cardiogenic induction. We showed for the first time that there are potential adult cardiac stem cells which have an ability to proliferate and differentiate into various types of cells including beating cardiomyocytes in vitro. Although the role of cardiac stem cells is uncertain, our results suggest their possible role in cardiac repair. In addition, understanding of precise molecular mechanisms of differentiating process of cultured cardiac stem cells may provide us a new insight into the cardiac development and regeneration.

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Figure Legends

Figure 1 Flow cytometric analysis of Sca-1+ cells. Sca-1+ cells were enriched by the MACS system with PE conjugated anti-Sca-1 antibody and anti-PE micro beads, and after sorting twice, ~90 % of the cells expressed Sca-1 (A). Sca-1+ cells were stained with PE conjugated anti-CD45 antibody, anti-CD34 antibody, and anti-c-kit antibody. In enriched Sca-1+ cells, ~40 % of the cells expressed CD45 (B), ~10 % of the cells expressed CD34 (C), and c-kit (D).

Figure 2 Phase contrast images of Sca-1+ cells before and after the oxytocin treatment. (A) Sca-1+ cells before the oxytocin treatment show small spindle-shaped morphology (0 weeks). (B) Two weeks after the treatment with oxytocin, small round cells (arrow heads) have appeared. These round cells rapidly proliferated, formed clusters and detached from the culture dish so that spindle-shaped cells were left. The cells were re-plated when reached to confluent. Four weeks after starting the treatment with oxytocin, some spontaneously beating cells (arrow in C, D) were recognized among spindle-shaped cells (C). (D) A large scale of the Figure C. Bars, 100 μM

Figure 3 RT-PCR analysis of cardiac genes. Sca-1+ cells after the treatment with oxytocin (lane OT) or 5'-azacytizine (lane A) expressed Csx/Nkx-2.5, GATA4, MEF-2C, α-MHC, β-MHC, MLC-2a, MLC-2v, and cardiac α-actin. Although Sca-1+ cells before the treatment (lane P) expressed Csx/Nkx-2.5 and GATA4 slightly, none of cells after the treatment with vehicles (lane V) or oxytocin antagonist combined with oxytocin (lane OT+OTA) expressed any cardiac transcription factors. Heart (lane H) and liver (lane L) were used as positive and negative controls, respectively.

Figure 4 Immunocytochemical analysis of cardiac proteins. (A-E) Cardiac

differentiation of Sca-1+ cells after the oxytocin treatment. Cells were double stained using anti-GATA4 antibody (A green), anti-ANF antibody (B green), and anti-cardiac troponin T antibody (A, B blue). Cells were stained with anti-MLC-2v (C green) and anti-sarcomeric myosin heavy chain antibodies (D green) and nuclei were stained with TO-PRO-3 (C, D blue). Cells were double stained using anti-cardiac troponin T antibody (E green), and anti-connexin 43 antibody (E blue). (F) Differentiation of Sca-1+ cells after the 5'-azacytizine treatment. Cells were stained with anti-sarcomeric myosin heavy chain (F green) and TO-PRO-3 (F blue). Bars, 50 μm

Figure 5 Physiological analysis of differentiated cardiac Sca-1+ cells. (A) $[Ca^{2+}]_i$ transients of beating cells derived from cardiac Sca-1+ cells before (upper panel) and after (lower panel) the treatment with isoproterenol. (B) The effects of subtype specific β receptor blockers on isoproterenol-induced increase in beating rate of differentiated cardiac Sca-1+ cells. Preincubation with 10^{-7} M of propranolol (nonselective β blocker) and 10^{-7} M of CGP20712A (β_1-selective blocker) reduced isoproterenol-induced increase in beating rate significantly but 10^{-7} M of ICI118551 (β_2-selective blocker) did not. *P<0.01 vs control, **P<0.05 vs isoproterenol only, n.s., not significant

Figure 6 RT-PCR analysis of oxytocin receptor expression in Sca-1+ cells. Oxytocin receptor mRNA was present at low levels in Sca-1+ cells before treatment (lane P). After the oxytocin treatment, oxytocin receptor mRNA expression was upregulated (lane OT), but oxytocin antagonist inhibited oxytocin-induced oxytocin receptor upregulation (lane OT+OTA).

Figure 7 Osteogenic and adipogenic differentiation potential of Sca-1+ cells derived from the adult murine heart. (A) Osteogenic differentiation of Sca-1+ cells was

induced by the treatment with ascorbic acid 2-phosphate, dexamethasone and β-glycerophosphate for 3 weeks. Alkaline phosphatase staining (blue) was used for detection of osteocytes. (B) RT-PCR experiment clearly revealed that osteogenic marker mRNAs such as alkaline phosphatase (ALP) and osteocalcin were induced in Sca-1+ cells by the treatment with osteogenic inducers (lane P for pre-treatment, lane V for vehicle treatment, lane O for osteogenic induction). Sca-1+ cells treated with oxytocin never expressed osteogenic marker mRNAs (lane OT for oxytocin treatment). β-actin was used as an internal control. (C) Adipogenic differentiation of Sca-1+ cells was induced by the treatment with adipogenic cocktail (MDI-I) for twelve days. Oil-Red O staining showed adipogenic differentiation of Sca-1+ cells. Hematoxylin was used for counter staining of nuclei. Bars, 50 μm

Video legends

Video Cardiac Sca-1+ cells differentiate into beating cardiomyocytes. Four weeks after starting the treatment with oxytocin, some of cardiac Sca-1+ cells showed spontaneous beating.

Table 1 PCR primers and PCR conditions

Primer	Product Size, bp	Annealing Temperature, °C
α-MHC		
5'-GGAAGAGTGAGCGGCCATCAAGG-3'	302	65
5'-CTGCTGGAGAGGTTATTCCTCG-3'		
β-MHC		
5'-GCCAACACCAACCTGTCCAAGTTC-3'	205	66
5'-TGCAAAGGCTCCAGGTCTGAGGGC-3'		
MLC-2a		
5'-CAGACCTGAAGGAGACCT-3'	286	54
5'-GTCAGCGTAAACAGTTGC-3'		
MLC-2v		
5'-GCCAAGAAGCGGATAGAAGG-3'	499	55
5'-CTGTGGTTCAGGGCTCAGTC-3'		
Cardiac α-actin		
5'-CTGAGATGTCTCTCTCTCTCTTAG-3'	99	60
5'-ACAATGACTGATGAGAGATG-3'		
Csx/Nkx-2.5		
5'-CAGTGGAGCTGGACAAAGCC-3'	216	55
5'-TAGCGACGGTTCTGGAATTT-3'		
GATA4		
5'-CTGTCATCTCACTATGGGCA-3'	275	60
5'-CCAAGTCCGAGCAGGAATTT-3'		
MEF-2C		
5'-GGCCATGGTACACCGAGTACAACGAGC-3'	401	62
5'-GGGGATCCCTGTGTTACCTGCACTTGG-3'		

Table 1 (continued) PCR primers and PCR conditions

Primer	Product Size, bp	Annealing Temperature, °C
Oxytocin receptor		
5'-AAGATGACCTTCATCATTGTTC-3'	303	61
5'-CGACTCAGGACGAAGGTGGAGGA-3'		
ALP		
5'-TTGAAACTCCAAAAGCTCAACACCA-3'	450	62
5'-TCTCGTTATCCGAGTACCAGTCCC-3'		
Osteocalcin		
5'-CCGGGAGCAGTGTGAGCTTA-3'	92	62
5'-TAGATGCGTTTGTAGGCGGTC-3'		
β-actin		
5'-GGACCTGGCTGGCCGGGACC -3'	583	60
5'-GCGGTGCACGATGGAGGGGC -3'		

MHC, myosin heavy chain; MLC, myosin light chain; MEF, muscle enhancer factor

ALP, alkaline phosphatase

Fig. 1

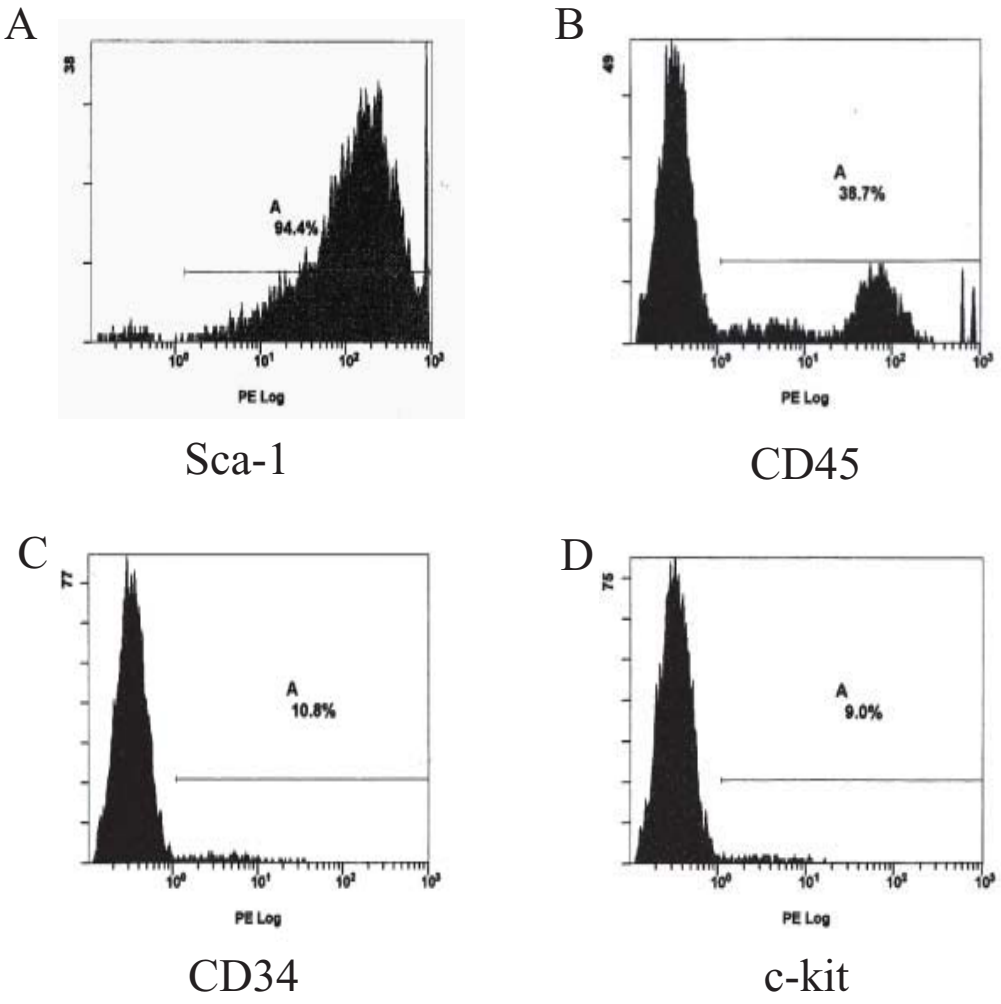


Fig. 2

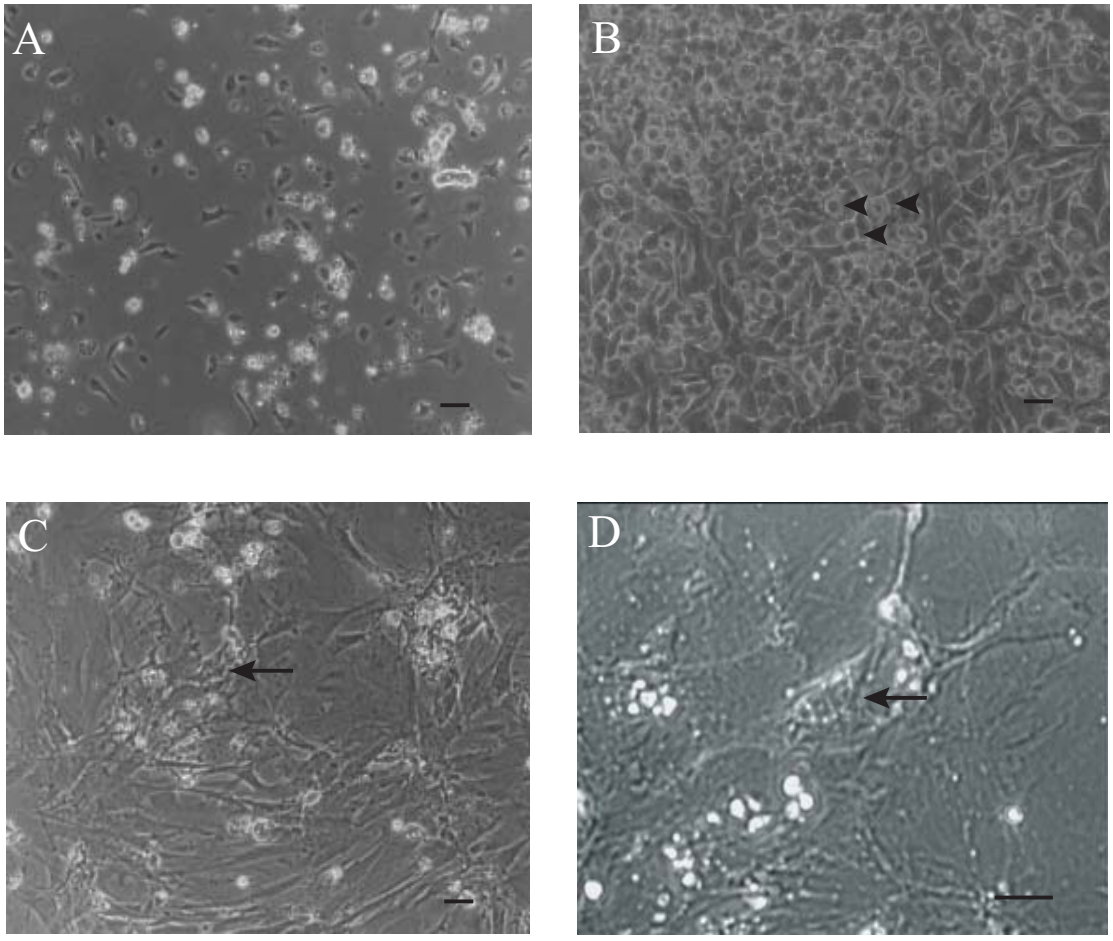


Fig. 3

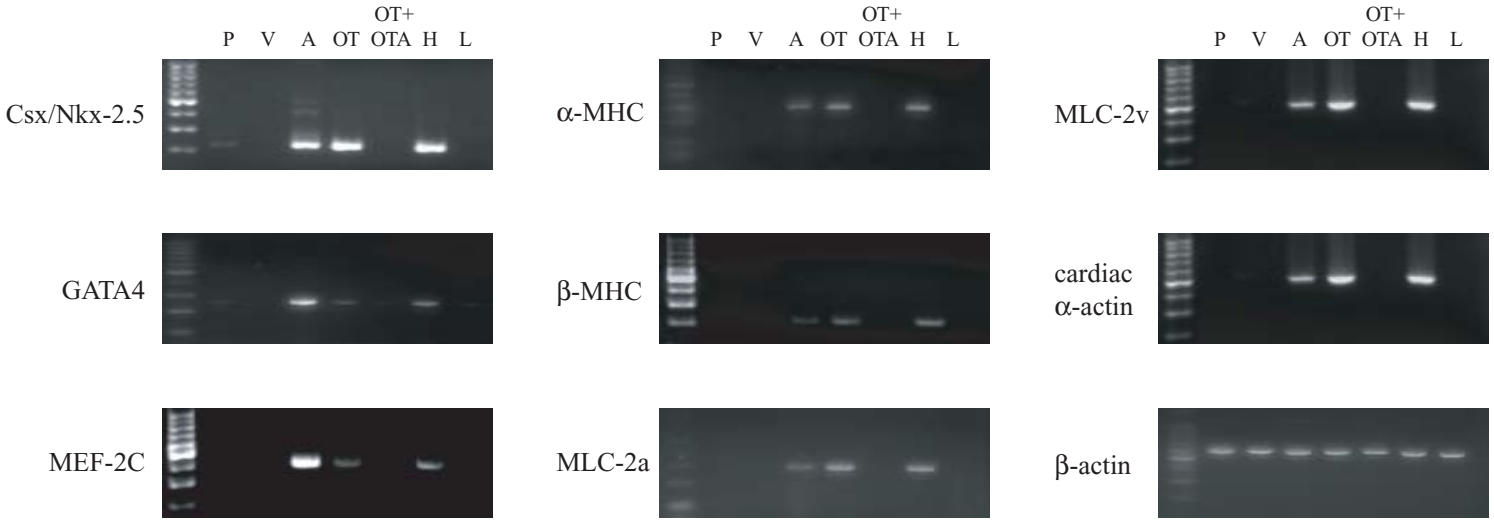


Fig. 4

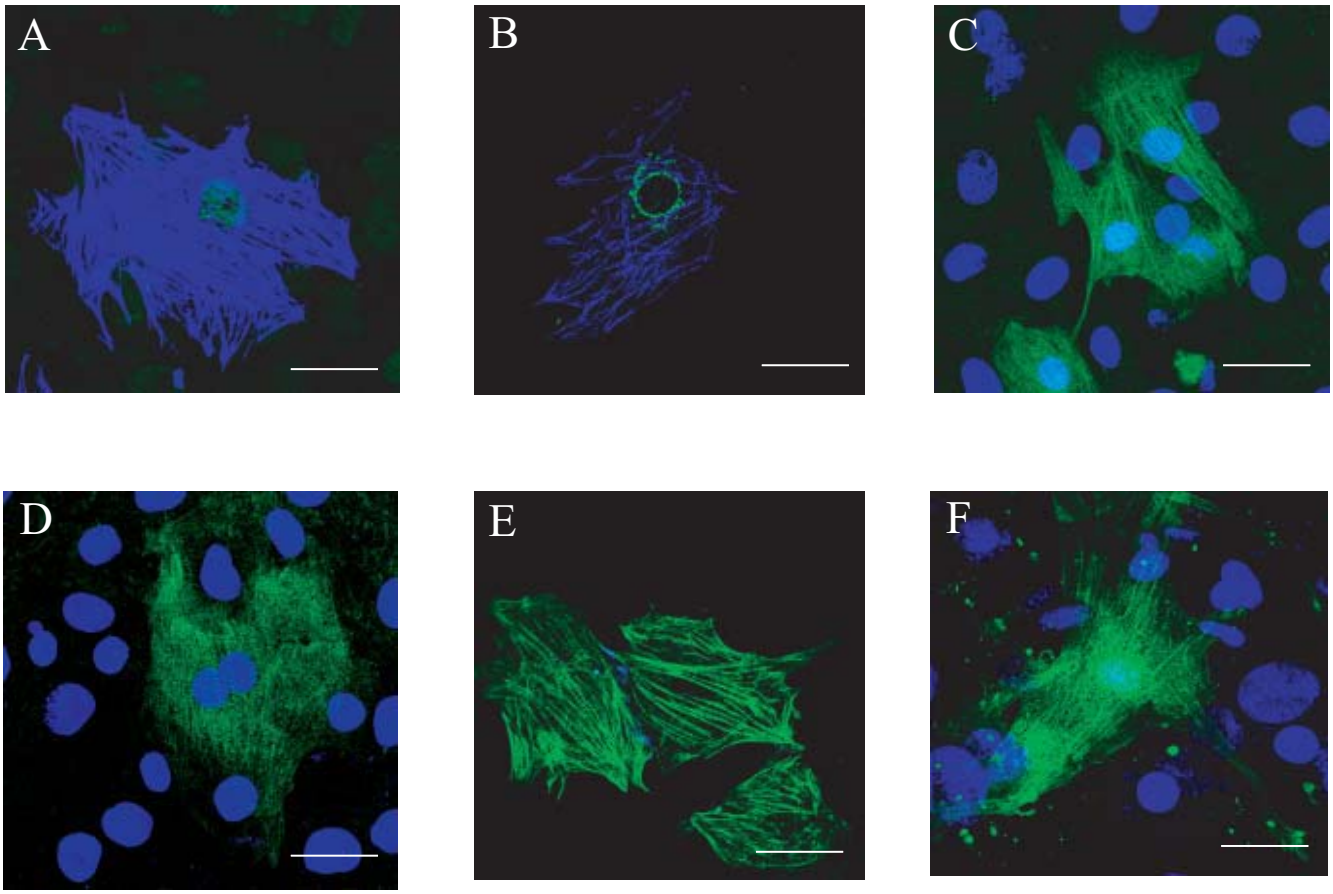
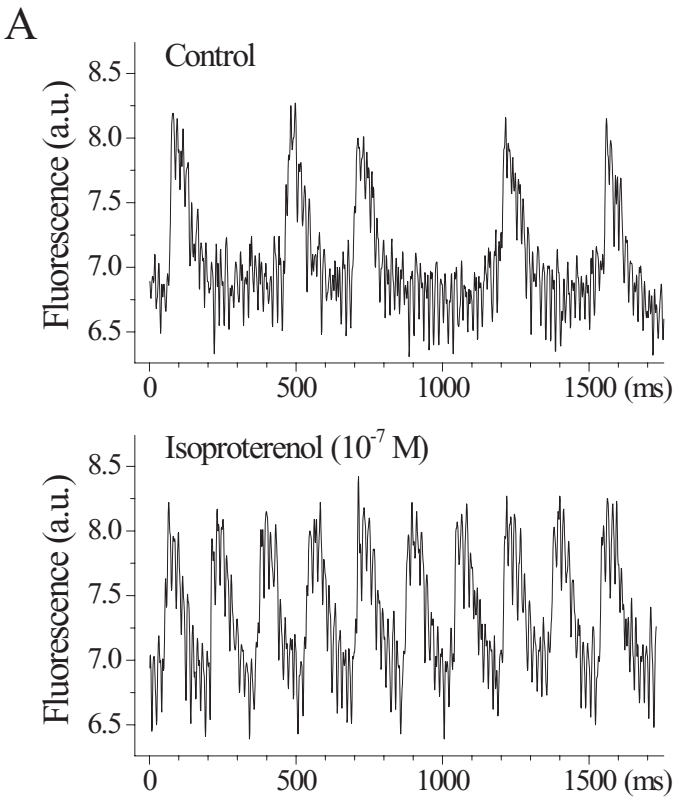


Fig. 5



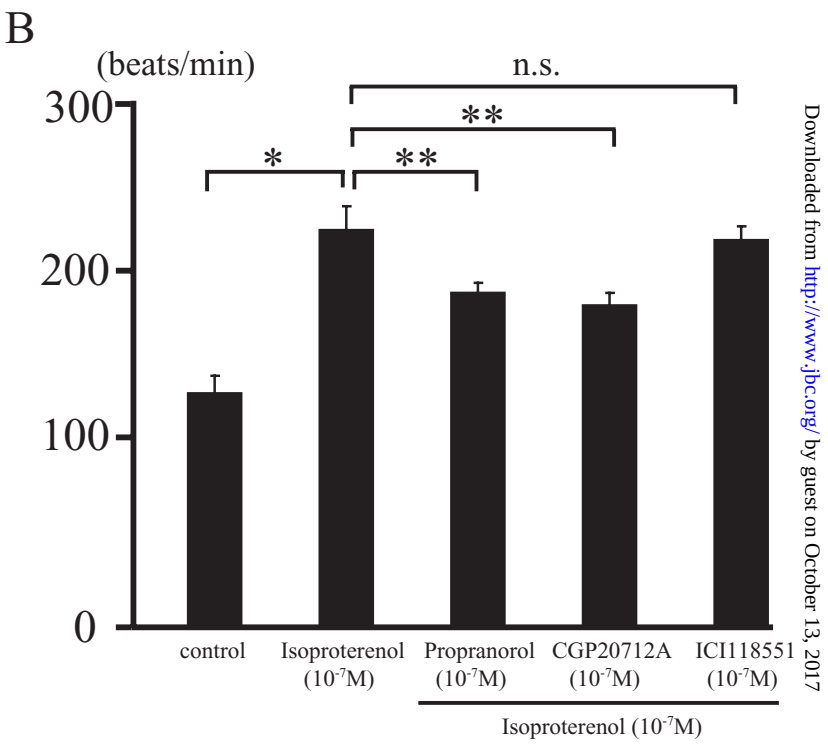


Fig. 6

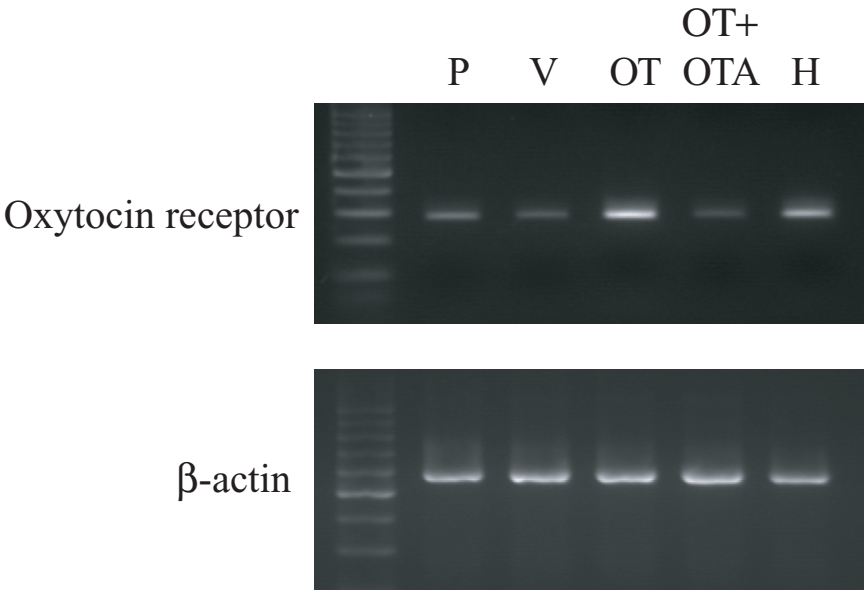
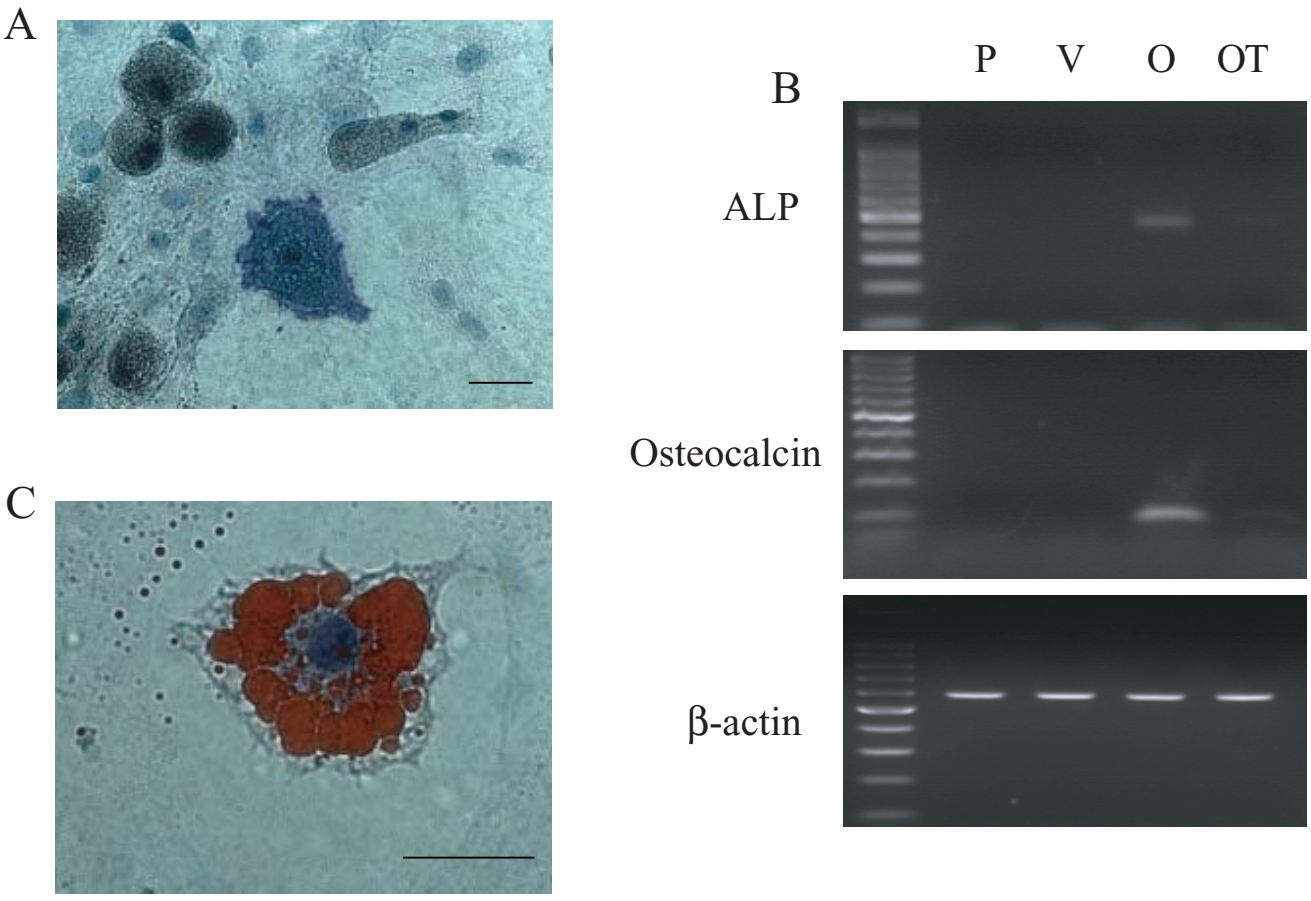


Fig. 7



ADDITIONS AND CORRECTIONS

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Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes.

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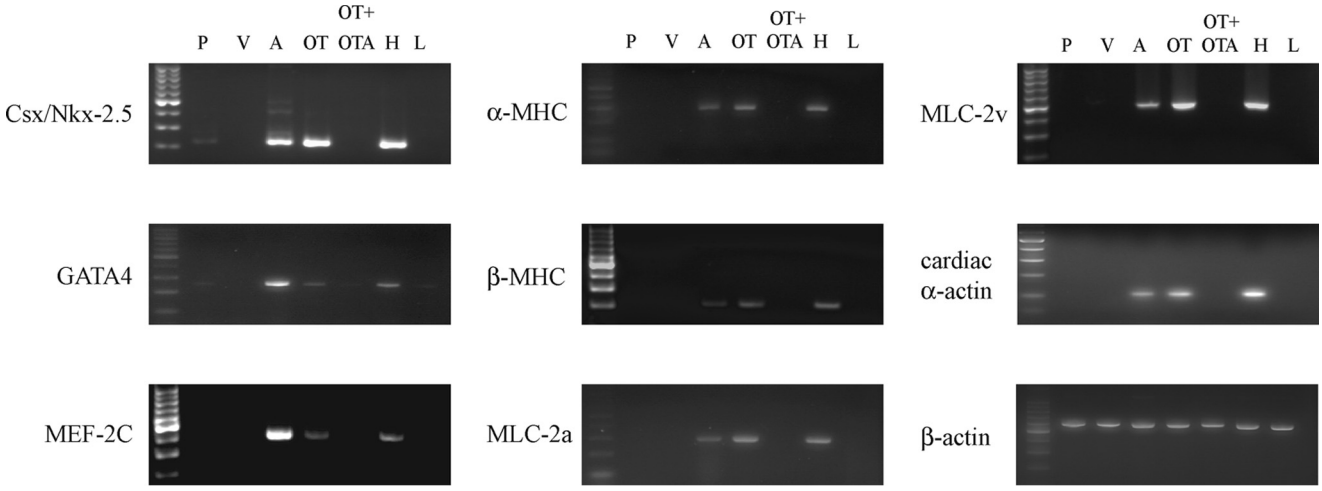
PAGE 11388:

The MLC-2v panel was duplicated in the cardiac α-actin panel of the original Fig. 3. We have now replaced the cardiac α-actin panel with the correct panel. This correction does not change the interpretation of the results or the conclusions.



Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

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