Induction of cardiomyocyte-like cells from hair follicle cells in mice

YONG-HEE KIM[1,5], BANG-JIN KIM[2], SEOK-MAN KIM[1], SUN-UK KIM[3,4] and BUOM-YONG RYU[1]

[1]Department of Animal Science and Technology, College of Biotechnology and Natural Resources, Chung-Ang University, Anseong, Gyeonggi-do 17546, Republic of Korea; [2]Department of Cancer Biology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA; [3]National Primate Research Center and [4]Futuristic Animal Resource and Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju, Chungcheongbuk-do 28116, Republic of Korea

Received July 16, 2018; Accepted March 8, 2019

DOI: 10.3892/ijmm.2019.4133

Abstract. Hair follicles (HFs) are a well-characterized niche for adult stem cells (SCs), and include epithelial and melanocytic SCs. HF cells are an accessible source of multipotent adult SCs for the generation of the interfollicular epidermis, HF structures and sebaceous glands in addition to the reconstitution of novel HFs *in vivo*. In the present study, it was demonstrated that HF cells are able to be induced to differentiate into cardiomyocyte-like cells *in vitro* under specific conditions. It was determined that HF cells cultured on OP9 feeder cells in KnockOut-Dulbecco's modified Eagle's medium/B27 in the presence of vascular endothelial growth factors differentiated into cardiomyocyte-like cells that express markers specific to cardiac lineage, but do not express non-cardiac lineage markers including neural stem/progenitor cell, HF bulge cells or undifferentiated spermatogonia markers. These cardiomyocyte-like cells exhibited a spindle- and filament-shaped morphology similar to that presented by cardiac muscles and exhibited spontaneous beating that persisted for over 3 months. These results demonstrate that SC reprogramming and differentiation may be induced without resulting in any genetic modification, which is important for the clinical applications of SCs including tissue and organ regeneration.

Correspondence to: Professor Buom-Yong Ryu, Department of Animal Science and Technology, College of Biotechnology and Natural Resources, Chung-Ang University, 4726 Seodong-daero, Daedeok-myeon, Anseong, Gyeonggi-do 17546, Republic of Korea
E-mail: byryu@cau.ac.kr

Present address: [5]Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, CB2 1QN Cambridge, UK

Key words: hair follicle cell, reprogramming, cardiac differentiation, cardiomyocyte-like cell, mice

Introduction

Cardiovascular disease is the leading cause of mortality globally (1). Myocardial infarction is a common symptom of ischemic heart disease, which is associated with the irreversible necrosis of cardiomyocytes followed by ventricular dysfunction and progressive heart failure (2-4) due to an acute interruption of blood supply caused by the limited proliferative capacity of terminally differentiated cardiomyocytes or coronary artery thrombosis (2,5). Anywhere from 450,000 to 750,000 patients with heart failure are awaiting heart transplantation, with only 1,000 recipients per year receiving donor hearts (6). As such, there is an urgent requirement for alternative therapeutic options.

One promising strategy to replace lost cardiomyocytes and regenerate the myocardium is stem cell (SC)-based therapy. Various SC lines, including embryonic (E)SCs, induced pluripotent (iP)SCs, multipotent germline (mG)SCs and mesenchymal SCs, have been investigated for their therapeutic potential in a number of preclinical studies (7-13). Pluripotent cell lines are particularly useful cell sources for cardiac regeneration and cardiomyocyte differentiation; however, their clinical applicability is limited owing to teratoma formation following transplantation (14), despite various attempts to increase the efficiency of cardiac differentiation (15-17).

The hair follicle (HF) is characterized by cyclical periods of growth (anagen), regression (catagen) and rest (telogen) throughout the lifespan of mammals (18). HF SCs/progenitor cells permanently reside in the upper portion of the HF, known as the bulge area (19,20) which contains slow-cycling or label-retaining cells that define the SC population (20-22) that differentiate into various HF cell types in addition to epidermal cells and generate follicle structures during anagen (22). SCs in the bulge area may additionally differentiate into the hair matrix of the outer-root sheath, inner-root sheath and basal cells of the sebaceous gland (19,23). One previous study has reported that precursor cells derived from mammalian skin dermis may produce neurons, glia, smooth muscle cells and adipocytes (24). Pluripotent epidermal neural crest SCs are also present in the dermal papillae of HFs (25,26). The neural SC/progenitor cell marker nestin is selectively expressed in HF

bulge cells, which may be induced to differentiate into blood vessels, neurons, glial cells, smooth muscle cells, keratinocytes and melanocytes *in vitro* (27-31). Furthermore, all three sections (upper, middle, and lower) of the mouse vibrissa HF harbor HF-associated PSCs that may generate spontaneously beating cardiac muscle cells (32). However, a major drawback of the differentiation protocol is that other cell types including neurons, glial cells, keratinocytes and smooth muscle cells are simultaneously produced (32). It is a serious point to overcome for further practical application. Thus, a cardiac-specific differentiation protocol should be established.

The present study aimed to determine the conditions necessary for cardiac-specific *in vitro* differentiation of cells isolated from mouse dorsal skin HFs in order to identify defined culture conditions that may differentiate HF cells into cardiomyocyte-like cells expressing cardiac-specific markers and exhibiting spontaneous beating for over 3 months. Thus, HF cells may be a potential source for generating cells of cardiac lineage under defined culture conditions.

Materials and methods

Animals. Animal procedures were ethically approved by the Animal Care and Use Committee of Chung-Ang University (Gyeonggi-do, Korea; IACUC no. 2018-00073) and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (33). Mice had ad libitum access to a standard chow diet and water and were housed under controlled conditions at 21±2˚C and 55±10% humidity and subjected to 12:12-h light/dark cycle conditions. Male heterozygous OG2 [B6;CBA-Tg(Pou5f1-EGFP) 2Mnn/J] mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA; cat. no. 004654). A total of 8 mice (2-5 months old, 20-35 g) were used in the present study.

Isolation and collection of HF cells. Surgical procedures were performed in a sterile environment. The OG2 mice were sacrificed and their dorsal skin was removed and sliced into 1-2-cm pieces that were enzymatically digested as previously described (34) with some minor modifications. The piece of dorsal skin was treated overnight at 4˚C with 2.5 mg/ml dispase in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12; 1:1 mixture; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following day, whole HFs were gently plucked from the skin using fine forceps and were enzymatically digested at 37˚C for 5 min in a 4:1 solution of 0.25% trypsin-EDTA (Thermo Fisher Scientific, Inc.) and 7 mg/ml DNase I (Roche Diagnostics, Basel, Switzerland) in Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, Inc.). The digestion was terminated by adding 10% (final volume) fetal bovine serum (FBS; GE Healthcare Life Sciences, Little Chalfont, UK). The cell suspension was filtered through a nylon mesh having a 40-μm pore size (BD Biosciences, San Jose, CA, USA) and cell viability was determined by performing the Trypan Blue dye exclusion test. In brief, a cell suspension was simply mixed with the Trypan blue dye for a few second at room temperature and then visually examined immediately following staining to determine whether cells take up or exclude the dye under the light microscope (model no. TS100; Nikon Corporation, Tokyo, Japan) at a magnification of x100.

HF cell culture and differentiation. HF cells were initially cultured at a density of $1.0x10^6$ cells/well in 6-well culture plates (BD Biosciences) containing DMEM/F12 (3:1) supplemented with 10% FBS (GE Healthcare Life Sciences), 20 μl/ml B27 supplement (Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor (bFGF; BD Biosciences), 10 ng/ml glial cell line-derived neurotrophic factor (GDNF; R&D Systems, Inc., Minneapolis, MN, USA), 75 ng/ml GDNF family receptor α1 (R&D Systems, Inc.), and 1% penicillin/streptomycin (P/S; Thermo Fisher Scientific, Inc.) and were incubated at 37˚C in a humidified atmosphere of 5% CO_2 (34).

To induce their differentiation into cardiomyocyte-like cells, HF cells were cultured in α-Minimal Essential Medium (α-MEM; Thermo Fisher Scientific, Inc.)/FBS medium (supplemented with 10% FBS and 1% P/S), N2/B27 medium [DMEM-F12 supplemented with 1% B27, 0.5% N2 (Thermo Fisher Scientific, Inc.), 100 μM β-mercaptoethanol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 2 mM L-glutamine, and 1% P/S], KnockOut (KO)-DMEM (Thermo Fisher Scientific, Inc.)/B27 medium (supplemented with 2% B27 and 1% P/S), or mouse serum-free medium (MSFM) as previously described (35). Additional growth factors including 15 μM γ-secretase inhibitor (GSI; EMD Millipore, Billerica, MA, USA) and 25 ng/ml bone morphogenetic protein (BMP)-4, 2 ng/ml activin A, 150 ng/ml noggin and 5 ng/ml vascular endothelial growth factor (VEGF; all from R&D Systems, Inc.) were added to the media. HF cells were seeded on feeder cells, including mitotically inactivated OP9 cells (bone marrow stromal cells; this cell line was generously provided by Dr Toru Nakano), SIM mouse embryo-derived thioguanine and ouabain resistant (STO) cells (cat. no. CRL-1503), C166 cells (cat. no. CRL-2581; both from American Type Culture Collection, Manassas, VA, USA) or mouse embryonic fibroblasts (MEFs; this cell line was isolated from C57BL/6 mice at embryonic day 14.5). Two adult female C57BL/6 mice [weight, 28-30 g; age, 13-week-old; Korea Animal Tech (Koatech), Pyeongtaek, Korea] were housed in cages at 21±2˚C and 50±20% humidity with a 12-h light/dark cycle; the adult mice produced 10 mice (sex ratio, 1:1) weighing 232-289 mg. These feeder cells were maintained prior to mitotic inactivation. Briefly, OP9 cells, STO cells, C166 cells and MEFs were cultured at 37˚C in OP9 medium [α-MEM supplemented 20% FBS and 1% P/S], STO medium [DMEM supplemented 7% FBS, 115 μM β-mercaptoethanol, 2.4 mM L-glutamine and 1% P/S], C166 medium [DMEM supplemented 10% FBS, 1 mM sodium pyruvate (Sigma-Aldrich; Merck KGaA) and 1% P/S] and MEF medium [DMEM supplemented 10% FBS, 100 μM non-essential amino acids (cat. no. 11140050; Sigma-Aldrich; Merck KGaA) and 1% P/S], respectively. Medium was replaced every 2-3 days and cells were passaged every 3-5 days.

To inactivate cell lines, cells were incubated in a medium appropriate to each cell line, as described above, containing 10 μg/ml mitomycin C for 2-3 h at 37˚C. Following the incubation, they were extensively washed 3 times with Dulbecco's (D) PBS and collected by trypsinization. Mitomycin C-inactivated cells were plated onto tissue culture dishes at the following

cell densities: STO, MEF, and OP9 ($1x10^5$/2 cm^2) and C166 ($0.5x10^5$/2 cm^2).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was prepared from the HF cells cultured with OP9 cells, STO cells, C166 cells and MEFs using the PureLink RNA Mini kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Only RNAs with a 260/280 ratio of 1.8 or higher were reverse transcribed at 50˚C for 50 min and at 85˚C for 5 min using Superscript III Reverse Transcriptase (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-qPCR was performed using the TaqMan Gene Expression Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: Initial denaturation stage: 95˚C for 20 sec; PCR stage: 40 cycles of 95˚C for 1 sec and 60˚C for 20 sec; melt curve stage: 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. The level of gene expression was normalized to that of GAPDH (Assay ID, Mm99999915_g1). Relative transcript abundance was determined using the ΔΔCq method (36). The following TaqMan probes were used: Troponin T2, cardiac type 2 (Tnnt2; Assay ID, Mm01290256_m1), mesoderm posterior bHLH transcription factor 1 (Mespl; Assay ID, Mm00801883_g1), islet 1 (Isl1; Assay ID, Mm00517585_m1), myocyte enhancer factor 2c (Mef2c; Assay ID, Mm01340842_m1), NK2 homeobox 2.5 (Nkx2.5; Assay ID, Mm01309813_s1), and T-box 5 (Tbx5; Assay ID, Mm00803518_m1; all synthesized by Applied Biosystems; Thermo Fisher Scientific, Inc.).

Immunocytochemical analysis. To assess the expression of markers *in vitro*, HF cells were fixed in 4% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) in DPBS for 10 min at room temperature. They were subsequently blocked with DPBS containing 5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) for 30 min at room temperature and incubated overnight at 4˚C with primary antibodies against cardiac troponin T (cTnT; cat. no. MS-295-P0; Thermo Fisher Scientific, Inc.), pan-cadherin (cat. no. sc-1499) and nestin (cat. no. sc-21248; both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), cytokeratin 15 (CK15; cat. no. ab52816; Abcam, Cambridge, UK), and promyelocytic leukemia zinc finger (PLZF; cat. no. OP128; EMD Millipore) diluted 1:200 in 5% BSA solution. On the following day, the cells were washed twice with DPBS and incubated for 1 h at room temperature in the dark with Alexa Fluor 568-conjugated goat anti-mouse (cat. no. A11004) or donkey anti-goat (cat. no. A11057; both from Thermo Fisher Scientific, Inc.) immunoglobulin G (IgG) secondary antibodies, or with rhodamine (TRITC)-conjugated goat anti-rabbit IgG (cat. no. 11-025-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted 1:200 in 5% BSA solution. Following two washes with DPBS, the cells were mounted with Vectashield containing 4',6-diamidino-2-phenylindole for nuclear counterstaining (cat. no. H-1200; Vector Laboratories, Inc., Burlingame, CA, USA). The samples were observed using fluorescence microscopy (model no. TE2000) that employed NIS Elements imaging software (NIS-Elements BR 5.11.00; both from Nikon Corporation).

Statistical analysis. All results were presented as mean ± standard error of the mean. Statistical analyses were performed using SPSS software (v.23; IBM Corp., Armonk, NY, USA). Analysis of variance and Tukey's honest significant difference tests were used to assess the differences between the groups. $P<0.05$ was considered to indicate a statistically significant difference. Unless otherwise stated, all experiments were performed three times.

Results

Effect of culture medium on the cardiac differentiation of HF cells. To evaluate the effects of culture medium on cardiac differentiation, HF cells were cultured for 6 days in a range of different media, including HF medium, α-MEM/FBS, N2/B27, MSFM and KO-DMEM/B27, in the absence of growth factors for 6 days. Subsequent to the induction of cardiac differentiation, the mRNA levels of *Tnnt2*, a cardiomyocyte-specific gene, or *Isl1*, a cardiac transcription factor, were evaluated using RT-qPCR. The results of cells cultured in each of the aforementioned mediums were compared with those of the cells cultured in the HF medium. Only two groups (cells grown in MSFM and cells grown in KO-DMEM/B27) exhibited a significant difference ($P<0.01$) in *Tnnt2* expression levels compared with cells cultured in HF medium [mean fold difference ± standard error of the mean (SEM): HF medium, 1.00±0.36; α-MEM/FBS, 0.73±0.29; N2/B27, 1.77±0.07; MSFM, 3.17±0.35; and KO-DMEM/B27, 4.13±0.33; Fig. 1A], although the expression levels did not differ significantly between the two groups. *Isl1* also demonstrated an expression pattern similar to *Tnnt2*, in that its expression was significantly higher ($P<0.01$) in cells grown either in MSFM or KO-DMEM/B27 compared with in HF cells (mean fold difference ± SEM: HF medium, 1.05±0.13; α-MEM/FBS, 0.52±0.07; N2/B27, 1.08±0.1; MSFM, 2.22±0.43; and KO-DMEM/B27, 2.09±0.09; Fig. 1B). Therefore, MSFM and KO-DMEM/B27 were selected for use for subsequent experiments.

VEGF enhances the cardiac differentiation potential of HF cells. The effects of soluble growth factors, namely GSI, BMP-4, activin A, noggin and VEGF, on cultured HF cells were examined as these factors are known to enhance cardiac differentiation potential (13,15,17,37-42). HF cells were cultured in MSFM or KO-DMEM/B27 for 6 days in the presence of each growth factor, and *Tnnt2* or *Isl1* mRNA levels were evaluated using RT-qPCR. Cells cultured in MSFM containing VEGF exhibited a significant upregulation of *Tnnt2* and *Isl1* mRNA levels compared with the untreated cells in the control group ($P<0.05$; *Tnnt2*: 3.6±0.63; *Isl1*: 2.42±0.58 fold; Fig. 2A and B). In addition, the VEGF group demonstrated a higher fold change compared with GSI (0.11±0.02 fold), BMP-4 (0.11±0.03 fold), activin A (0.82±0.12 fold) and Noggin (0.48±0.1 fold) groups. Similar trends were observed in HF cells cultured in KO-DMEM/B27 in the presence of each growth factor; a significant difference was only observed in the cell population cultured with KO-DMEM/B27 containing

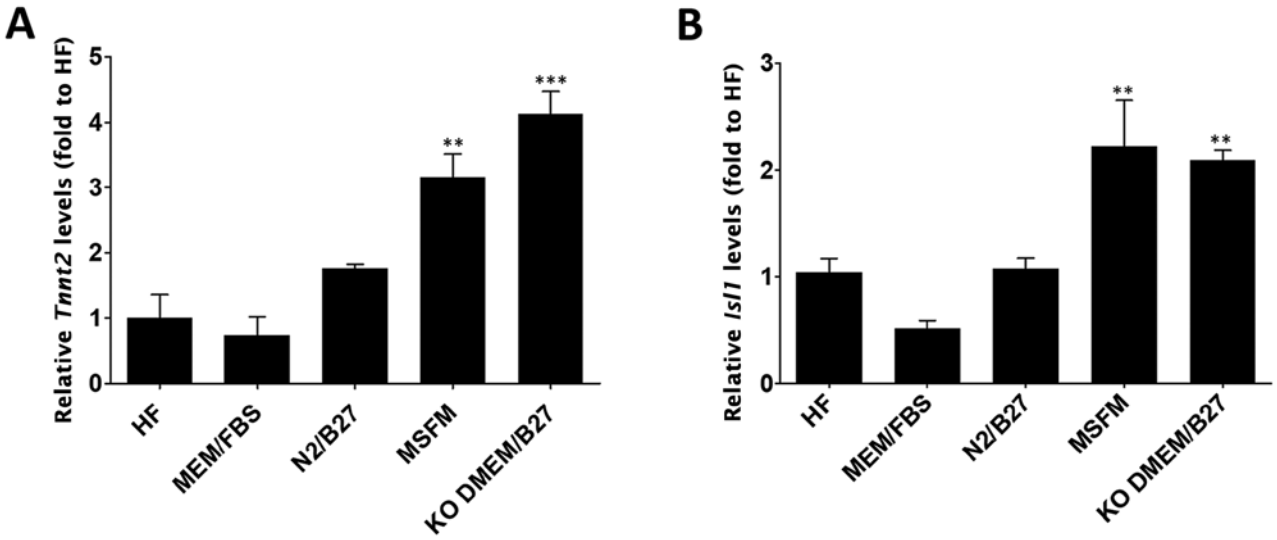

Figure 1. Cardiac differentiation of HF cells in culture media. mRNA expression levels of (A) the cardiomyocyte-specific gene *Tnnt2* or (B) the cardiac transcription factor *Isl1* in cells cultured in each type of medium were normalized to the level in cells cultured in the HF medium. Data was represented as the mean ± the standard error of the mean (n=3). **P<0.01 and ***P<0.001 vs. HF. HF, hair follicles; MEM, α-Minimal Essential Medium; FBS, foetal bovine serum; MSFM, mouse serum-free medium; KO-DMEM, KnockOut-Dulbecco's modified Eagle's medium; Tnnt2, troponin T2, cardiac type 2; Isl1, islet 1.

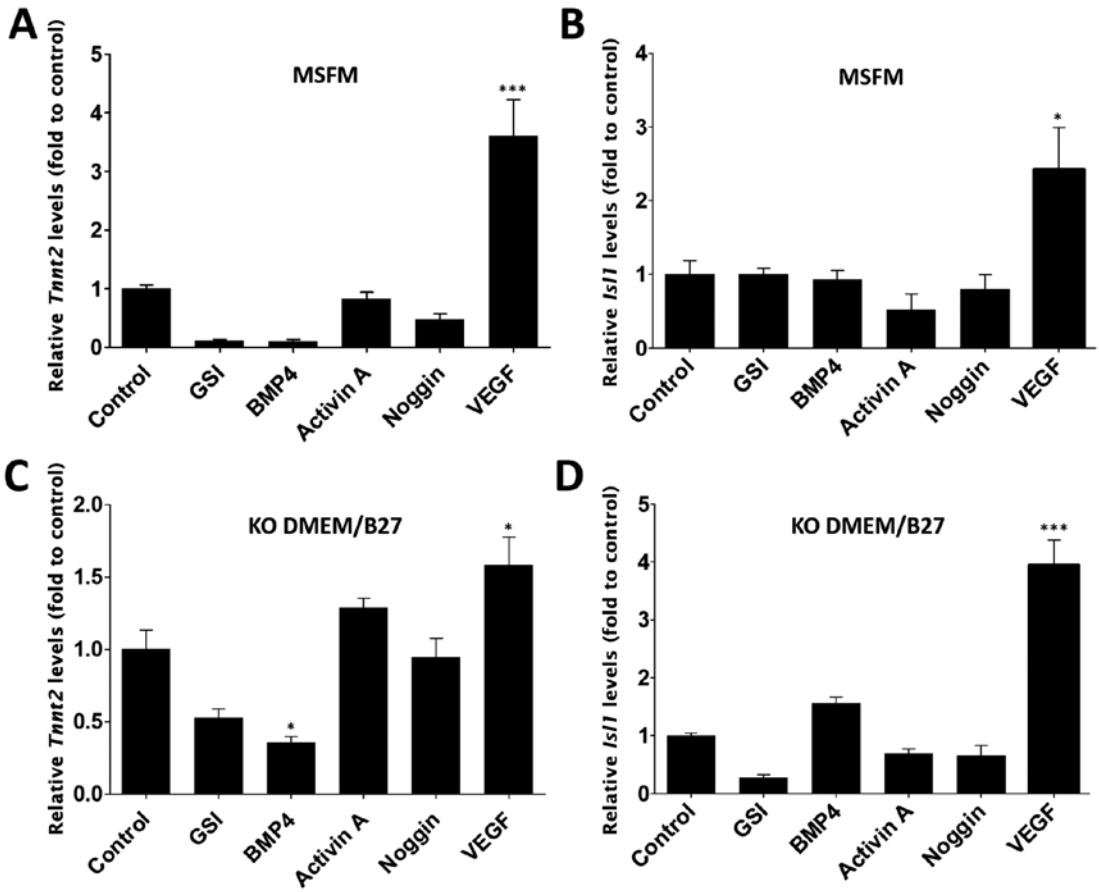

Figure 2. Effects of growth factors on the cardiac differentiation of HF cells. *Tnnt2* and *Isl1* mRNA expression levels were normalized compared with those in the control group. (A) *Tnnt2* or (B) *Isl1* mRNA expression levels in HF cells cultured in MSFM supplemented with various growth factors. Effect of various growth factors on (C) *Tnnt2* or (D) *Isl1* mRNA expression levels in the KO-DMEM/B27 group. Data represent the mean ± the standard error of the mean (n=3). *P<0.05 and ***P<0.001 vs. the control. HF, hair follicles; MSFM, mouse serum-free medium; KO-DMEM, KnockOut-Dulbecco's modified Eagle's medium; Tnnt2, troponin T2, cardiac type 2; Isl1, islet 1; GSI, γ-secretase inhibitor; BMP4, bone morphogenetic protein 4; VEGF, vascular endothelial growth factor.

VEGF compared with the control group (P<0.05; *Tnnt2*: 1.58±0.19; *Isl1*: 3.94±0.44 fold; Fig. 2C and D). These results demonstrate that VEGF is highly effective in inducing the cardiac differentiation of HF cells.

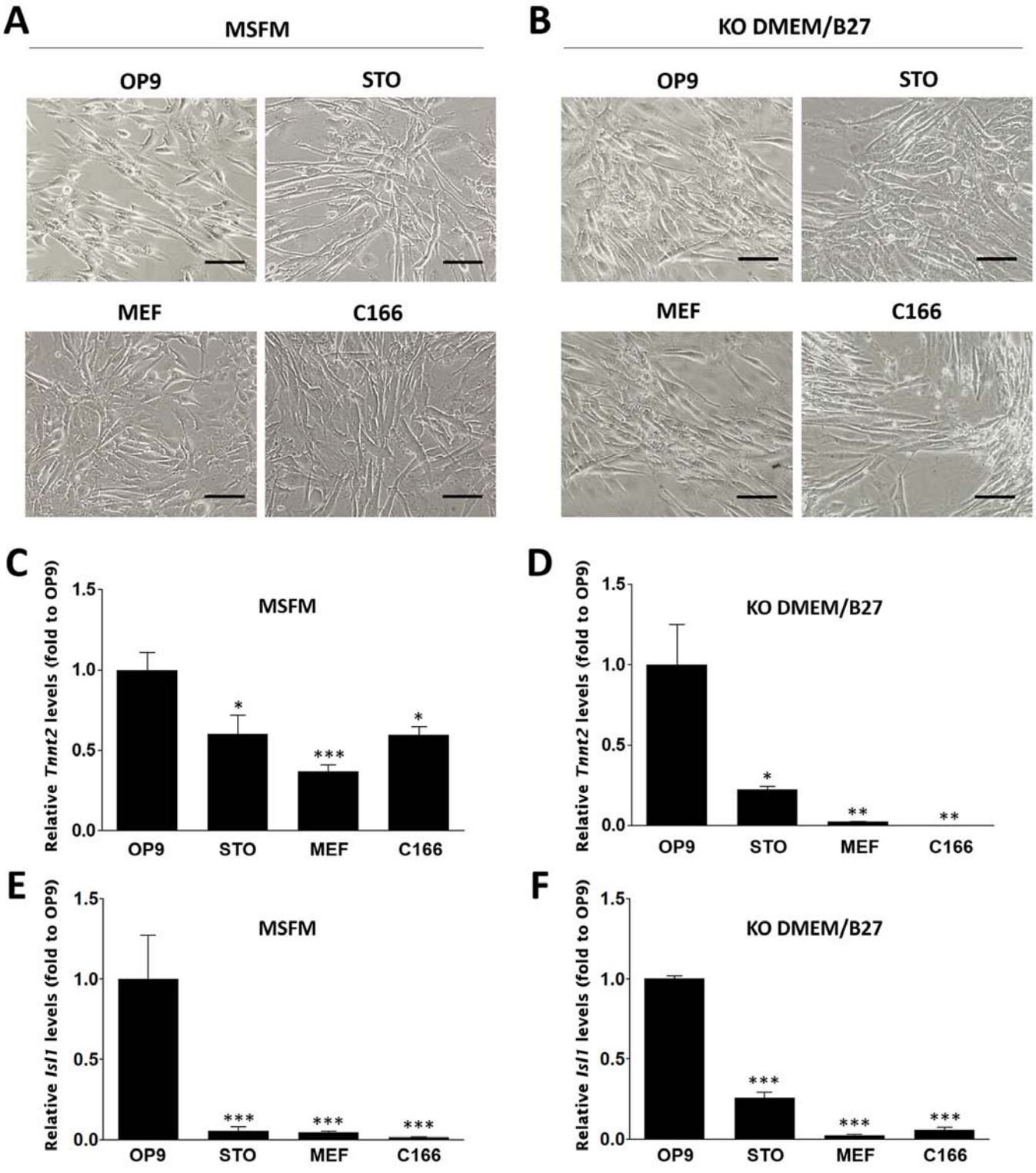

Figure 3. Cardiac differentiation of HF cells on feeder layers. Bright field micrographs of HF cells cultured on OP9, STO, MEF and C166 feeder cells in (A) MSFM or (B) KO-DMEM/B27 medium containing VEGF. Bar, 100 μm. Relative mRNA expression levels of *Tnnt2* in (C) MSFM and (D) KO-DMEM/B27 groups. Relative expression of *Isl1* in (E) MSFM and (F) KO-DMEM/B27 groups. Expression levels were normalized compared with those in the OP9 feeder cell group. Data is represented as the mean ± the standard error of the mean (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. the OP9 group. HF, hair follicles; MSFM, mouse serum-free medium; KO-DMEM, KnockOut-Dulbecco's modified Eagle's medium; Tnnt2, troponin T2, cardiac type 2; Isl1, islet 1; VEGF, vascular endothelial growth factor.

Effect of feeder layers on cardiac differentiation. To examine the influence of feeder cells on the induction of cardiac differentiation, HF cells were cultured on mitotically inactivated feeder cell lines (including OP9, STO, MEF and C166 feeder cells) in MSFM or KO-DMEM/B27 supplemented with VEGF. The morphology of the cells during the culture period was monitored (Fig. 3A and B). Interestingly, beating cardiomyocyte-like cells under each condition were observed. To evaluate the efficiency of cardiac induction *in vitro*, *Tnnt2* or *Isl1* expression levels were compared between different cell

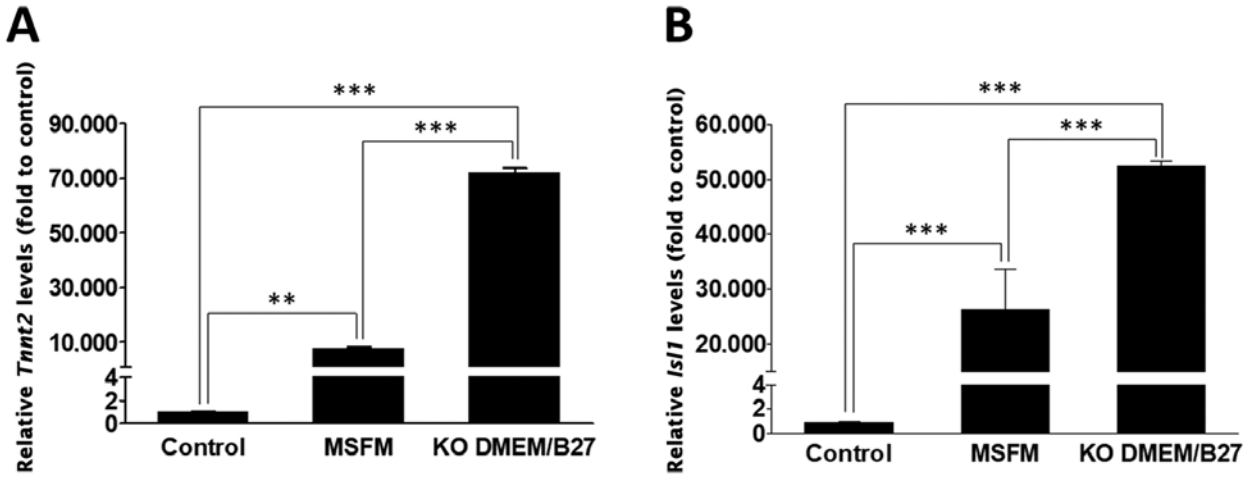

Figure 4. Cardiomyocyte-specific gene expression in HF cells cultured on OP9 feeder cells in the MSFM or KO-DMEM/B27 medium. mRNA expression levels of (A) *Tnnt2* or (B) *Isl1* in the two cell populations were normalized compared with those in OP9 feeder cells. Data is represented as the mean ± standard error of the mean (n=3). **P<0.01 and ***P<0.001 with comparisons shown by lines. HF, hair follicles; MSFM, mouse serum-free medium; KO-DMEM, KnockOut-Dulbecco's modified Eagle's medium; Tnnt2, troponin T2, cardiac type 2; Isl1, islet 1.

populations. The two genes had significantly higher expression levels in cells cultured on OP9 feeder cells with MSFM (*Tnnt2*: 1.00±0.11; *Isl1*: 1.00±0.27 fold) compared with those cultured with STO (*Tnnt2*: 0.6±0.12; *Isl1*: 0.6±0.03 fold), MEFs (*Tnnt2*: 0.37±0.04; *Isl1*: 0.05±0.01 fold) or C166 (*Tnnt2*: 0.6±0.05; *Isl1*: 0.02±0.01 fold) feeder cells (P<0.05; Fig. 3C and E). Additionally, HF cells cultured in KO-DMEM/B27 exhibited significantly higher mRNA levels of *Tnnt2* when OP9 cells were used as the feeder compared with any other group (OP9, 1.00±0.25; STO, 0.22±0.02; MEF, 0.03±0.0 and C166, 0.01±0.0 fold; P<0.001; Fig. 3D); similarly, *Isl1* expression was also significantly higher in cells cultured in KO-DMEM/B27 with OP9 feeder cells compared with any other group (OP9, 1.00±0.02; STO, 0.26±0.04; MEF, 0.02±0.01; and C166, 0.06±0.02 fold; P<0.001; Fig. 3F). These data suggest that OP9 feeder cells highly stimulated the cardiac differentiation of HF cells in the two types of media.

To determine which of the two media (MSFM or KO-DMEM/B27) is more effective in inducing cardiac differentiation, HF cells were cultured on OP9 feeder cells with MSFM or KO-DMEM/B27 in the presence of VEGF, and *Tnnt2* or *Isl1* expression levels between the two cultures were compared. OP9 feeder cells alone cultured in MSFM or KO-DMEM/B27 served as the control. RT-qPCR analysis revealed that *Tnnt2* and *Isl1* mRNA levels were significantly upregulated in HF cells cultured on OP9 in MSFM or KO-DMEM/B27 medium compared with the cells grown in the control medium (P<0.01; Fig. 4); additionally, significantly higher expression levels of *Tnnt2* and *Isl1* genes (*Tnnt2*: 9.43; *Isl1*: 1.99 fold) were observed in KO-DMEM/B27 compared with in the MSFM group (P<0.001). KO-DMEM/B27 was, therefore, used to analyze the phenotypic characteristics of cardiomyocyte-like cells.

Phenotype of cardiomyocyte-like cells derived from HF cells. Following 2 weeks of culturing on OP9 feeder cells in KO-DMEM/B27 containing VEGF, HF cells were analyzed using immunocytochemistry. To exclude the expression of non-specific markers, the present study initially confirmed that the various markers used were not expressed by OP9 cells (Fig. 5). Numerous cells strongly expressed the cardiomyocyte marker cTnT and the cardiac intercellular adherens junction marker pan-cadherin (Fig. 5). On the other hand, these cells were negative for the neural stem/progenitor cell marker nestin, the HF bulge marker CK15 and the undifferentiated spermatogonia marker PLZF (Fig. 5). These results indicate that the culturing of HF cells on OP9 feeder cells in KO-DMEM/B27 medium supplemented with VEGF promotes the expression of genes specific to the cardiac lineage but not to other lineages including neural, glial, HF and germline cells.

The present study additionally compared HF cells with cardiomyocyte-like cells in terms of the mRNA expression levels of cardiac lineage-specific genes including *Tnnt2, Mesp1, Isl1, Mef2c, Nkx2.5 and Tbx5* (9,43-46) and it was revealed that all these genes were significantly more highly expressed in the latter group of cells compared with the HF group (P<0.05; Fig. 6), demonstrating that these differentiated cells exhibited a phenotype that was distinguishable from that of the parent HF cells.

It was observed that cardiomyocyte-like cells had a spindle- and filament-shaped morphology similar to that of cardiac muscles and were capable of beating spontaneously for >3 months without any stimulation (Video S1). Thus, beating cardiomyocyte-like cells may be derived from HF cells by culturing on OP9 feeder cells in KO-DMEM/B27 medium supplemented with VEGF. These cardiomyocyte-like cells may potentially function normally as spontaneous beating is thought to be an *in vitro* indicator of functional cardiomyocytes (47,48).

Discussion

In the present study, it was postulated that the use of media, exogenous factors or feeders essential for cardiac differentiation may be an important first step in priming HF cells to be receptive to the conversion into the cells of cardiac lineages. In this regard, it is noteworthy that the differentiation and/or specification of pluripotent cells into specific cell types have

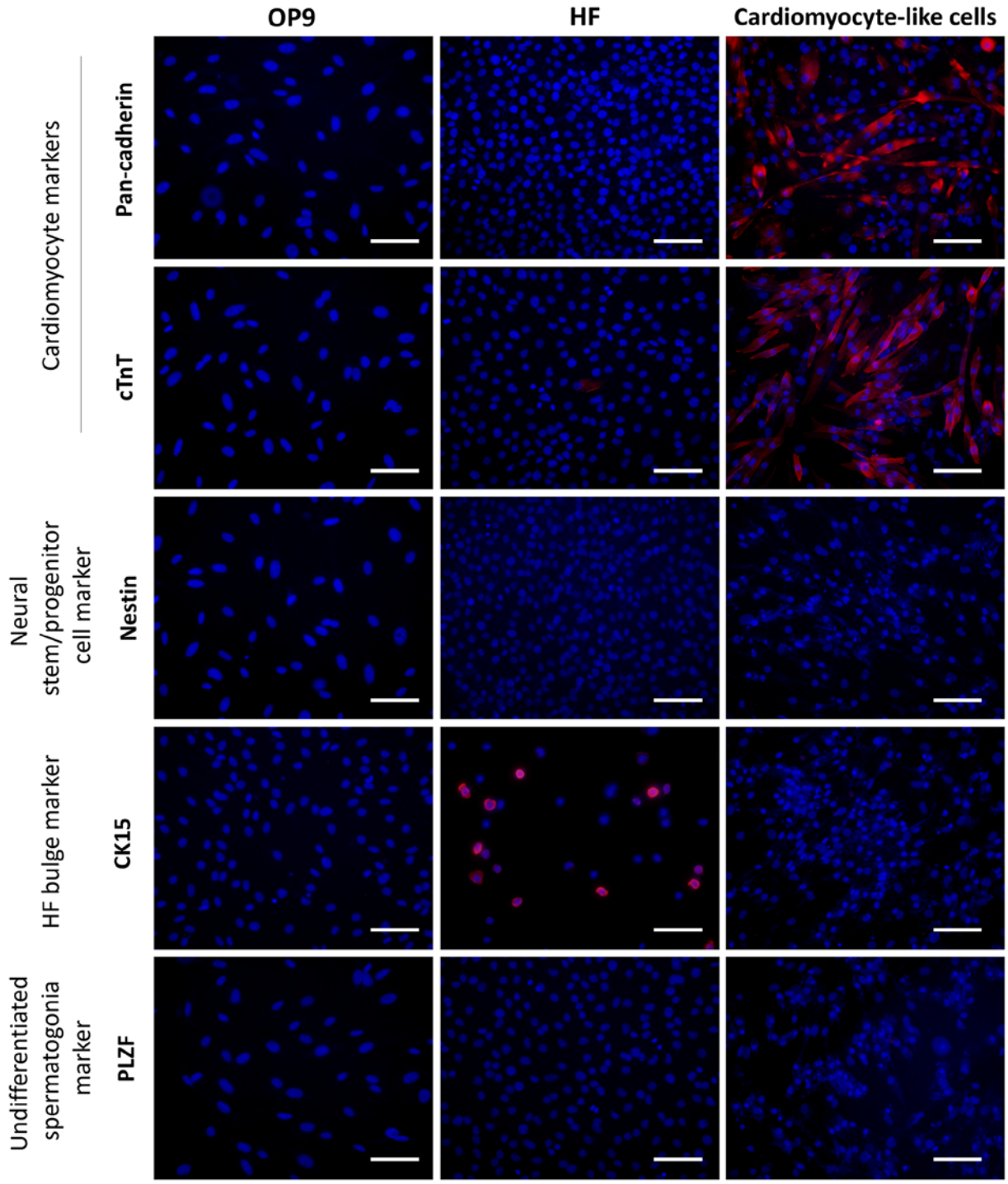

Figure 5. Immunocytochemistry analysis of lineage-specific marker expression in OP9 feeder cells, HF cells and cardiomyocyte-like cells. Cells were stained with cardiomyocyte marker, cTnT, cardiac intercellular adherens junction marker, pan-cadherin, neural stem/progenitor cell marker, nestin, HF bulge marker, CK15, or undifferentiated spermatogonia marker, PLZF (red). Cells were then counterstained with 4',6-diamidino-2-phenylindole (blue). Bar, 100 μm. HF, hair follicles; MSFM, mouse serum-free medium; KO-DMEM, KnockOut-Dulbecco's modified Eagle's medium; cTnT, cardiac troponin T; CK15, cytokeratin 15; PLZF, promyelocytic leukemia zinc finger.

been frequently demonstrated to require the addition of growth factors that are specific to the cell type (49,50). For example, ESCs and iPSCs are differentiated into cardiomyocytes by adding growth factors, which are implicated in normal cardiac development, into the media (41,43). In a similar manner, media, growth factors or feeders that have been implicated in cardiac development were added to the HF cultures to determine whether they were able to facilitate reprogramming. Finally, the culture conditions required for the cardiac-specific differentiation of HF cells isolated from the mouse dorsal skin were established. The HF cells were initially cultured in different media (α-MEM/FBS, N2/B27, MSFM and KO-DMEM/B27) with different growth factors (GSI, BMP-4, activin A, noggin and VEGF) and feeder cells (OP9, STO, C166 and MEF cells) to determine the optimal culture conditions for inducing cardiac differentiation; the

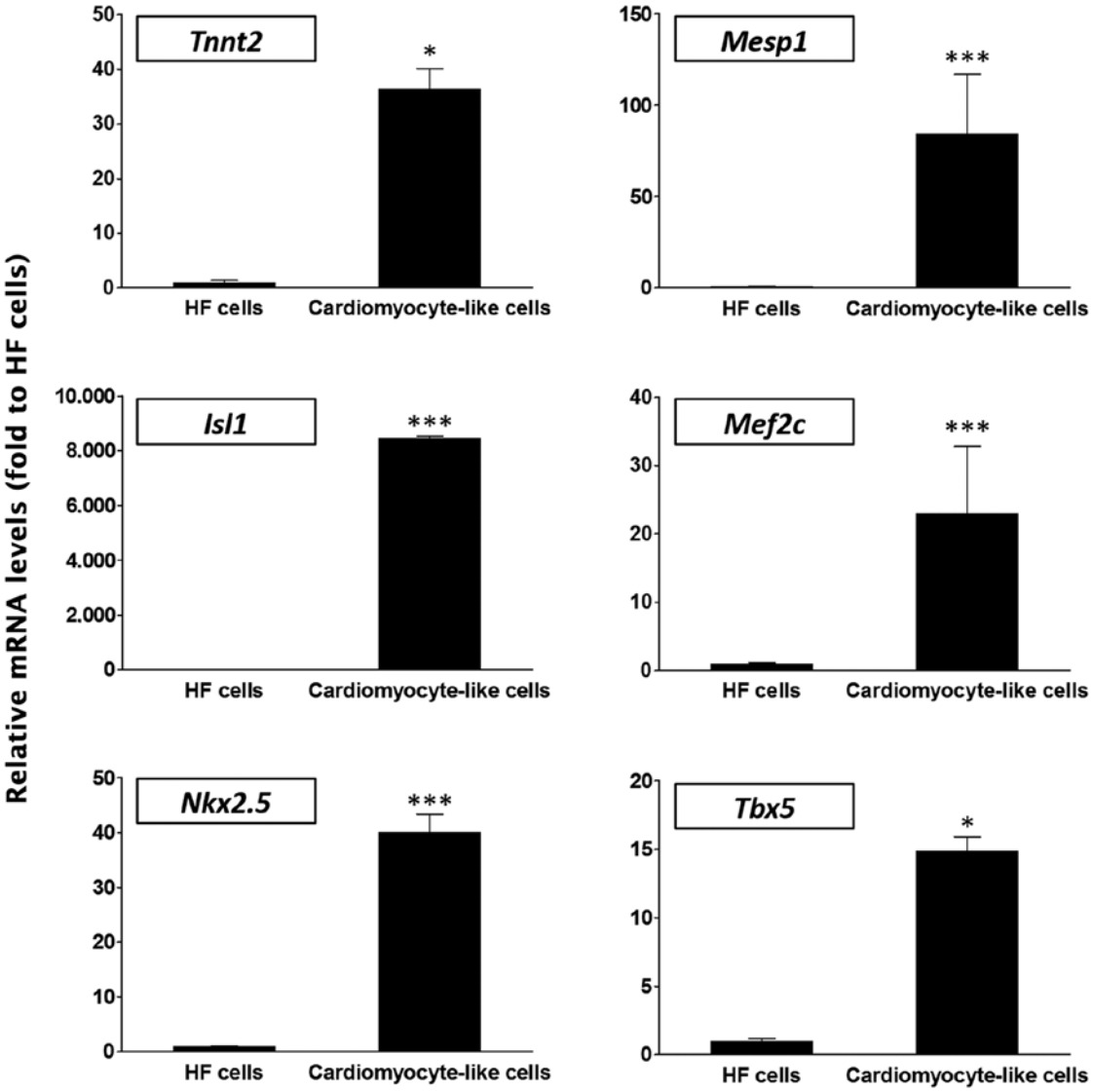

Figure 6. Relative expression levels of cardiac lineage-specific genes in HF and cardiomyocyte-like cells. Expression levels of the cardiac lineage-specific genes *Tnnt2*, *Mesp1*, *Isl1*, *Mef2c*, *Nkx2.5* and *Tbx5* were examined and normalized to the levels in the HF cell group. Data is represented as the mean ± standard error of the mean (n=3). *P<0.05 and ***P<0.001 vs. the HF group. HF, hair follicles; Tnnt2, troponin T2, cardiac type 2; Isl1, islet 1; Mesp1, mesoderm posterior bHLH transcription factor 1; Mef2c, myocyte enhancer factor 2c; Nkx2.5, NK2 homeobox 2.5; Tbx5, T-box 5.

cardiac differentiation ability of cultured cells was evaluated based on their expression of cardiac lineage-specific genes and beating capacity. It was revealed that HF cells cultured in KO-DMEM/B27 containing VEGF exhibited the upregulation of cardiac lineage-specific genes, which was further enhanced by culturing on OP9 feeder cells.

Numerous studies have been conducted to establish suitable conditions for inducing the differentiation of PSCs into specific cell types (13,39,51,52). mGSCs are similar to embryonic stem and germ cells in terms of phenotype and their capacity of differentiating into cardiomyocytes and endothelial cells; these cells were induced to differentiate into mesodermal cells by culturing in α-MEM containing 10% fetal calf serum and 2-mercaptoethanol on an OP9 stromal cell layer (51). The cardiac differentiation efficiency of mouse and human ESCs was enhanced by culturing in DMEM containing 20% FBS or KO-DMEM containing 20% FBS in the presence of apelin, BMP-4, activin A and bFGF (13). Although efficient differentiation into mature cardiomyocytes was achieved, the inclusion of FBS remains controversial as its precise components are unknown. For this reason, various chemically defined media have been used to induce the cardiac differentiation of PSCs, including one that promotes the self-renewal of human ESCs grown on a Matrigel-coated surface over multiple passages (52). This previous study additionally selectively induced the differentiation of a human ESC monolayer into cells of cardiac muscle lineage by transient treatment with activin A and BMP-4 in N2/B27, a chemically defined medium comprising DMEM/F12 with N2 and B27 supplements and BSA, amongst other ingredients. As a result of differentiation, hESCs maintained in N2/B27 proceeded from the mesendoderm lineage toward the mesoderm-cardiac muscle lineage. The differentiation of human ESCs into the cardiac mesoderm and simultaneous suppression of the neuroectodermal lineage was promoted by culturing in a reduced volume medium with GSI followed by KO-DMEM. The KO-DMEM/B27 culture conditions induced the differentiation and maturation of the cardiac lineage cells (39). MSFM is

another chemically well-defined medium that supports the proliferation and self-renewal of mouse spermatogonial SCs (35,53). In the present study, α-MEM containing FBS and a number of chemically defined media (N2/B27, KO-DMEM/B27 and MSFM) without FBS were assessed for their ability to promote cardiac lineage commitment in HF cells. The mRNA expression levels of the cardiac-specific genes *Tnnt2* and *Isl1* were higher in a chemically defined medium when compared with a serum-containing medium, suggesting that the presence of serum inhibits cardiac differentiation owing to reasons that have yet to be determined.

Various growth factors are known to promote cardiogenesis in PSCs (13,38,39,41,42). Although GSI alone does not affect the self-renewal and differentiation capacity of human ESCs by blocking Notch signaling, GSI in a reduced-volume culture medium may accelerate mesodermal differentiation (37,39,40). A number of other studies have demonstrated that activin/nodal/transforming growth factor-β, Wnt and BMP signaling pathways are critical for establishing the development of the cardiovascular system (15,17,41,54-61). The BMP antagonist noggin is transiently but notably expressed in the heart-forming region during gastrulation and functions at the level of mesendoderm induction to establish conditions conducive to cardiogenesis (42). Although GSI, BMP-4, activin A and noggin are known to influence cardiac differentiation at a number of different stages, the present study did not examine the positive effects of these growth factors on cardiac-specific differentiation of HF cells. However, HF cells cultured in the presence of VEGF demonstrated increased cardiac-specific gene expression levels. In a previous study, VEGF receptor 2 was demonstrated to be expressed in a cell population that had the potential to generate cell types of cardiac lineage (38). Thus, it is likely that VEGF is necessary for cardiac differentiation.

In the present study, spontaneously beating cardiomyocyte-like cells were generated using HF cells cultured on a feeder layer in KO-DMEM/B27 supplemented with VEGF. Specifically, OP9 feeder cells increased the expression levels of cardiac-specific genes to a greater extent compared with other feeder cells. OP9 cells have been used to induce the differentiation of PSCs into cardiac and endothelial cells, indicating that OP9 cells have a potential function in the differentiation of PSCs into contracting cardiac colonies (51,62-64). The results of the present study are consistent with these earlier reports. However, it may be difficult to determine the underlying mechanism and number of cells involved, but with an increase in the expression of specific genes owing to the implementation of the aforementioned method, the results of the present study may lay a necessary foundation for further study. Interestingly, HF cells were induced to differentiate into beating cardiomyocyte-like cells when cultured on each of the feeder cell lines, although the precise functions of the feeder cells in cardiac differentiation remains unclear. Thus, the precise mechanism and necessity of each growth factor requires further elucidation.

In conclusion, the results of the present study demonstrate that cardiomyocyte-like cells may be derived from HF cells under suitable culture conditions. These may provide a useful *in vitro* system for the standardization of the method for the derivation of cardiomyocytes, and offer a potential cell source for the development of cell-based therapeutics.

Acknowledgements

The authors would like to thank Dr Toru Nakano (Department of Pathology, Medical School, Osaka University, Osaka, Japan) for the OP9 cell line.

Funding

The present study was supported by the Bio and Medical Technology Development Program of the National Research Foundation of Korea (NRF) funded by the Korean government, MSIT (grant no. 2018M3A9H1023139), the Basic Science Research Program through the NRF funded by the Ministry of Education (grant no. 2018R1A6A1A03025159) and the Korea Research Institute of Bioscience and Biotechnology Research Initiative Programs (grant no. KGM4251824).

Availability of data and materials

All data generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YHK and BYR conceived and designed the project. YHK, BJK and SMK performed the experiments. YHK, BJK, SUK and BYR analyzed the data. YHK and BYR wrote the manuscript. BYR supervised the design of study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal procedures were approved by the Animal Care and Use Committee of Chung-Ang University (Gyeonggi-do, Republic of Korea; IACUC no. 2018-00073) and were performed in accordance with the Guideline for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Mortality GBD and Causes of Death C: Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet 388: 1459-1544, 2016.
2. Weir RA and McMurray JJ: Epidemiology of heart failure and left ventricular dysfunction after acute myocardial infarction. Curr Heart Fail Rep 3: 175-180, 2006.
3. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, *et al*; American Heart Association Statistics Committee and Stroke Statistics Subcommittee: Heart disease and stroke statistics--2012 update: A report from the American Heart Association. Circulation 125: e2-220, 2012.

4. Thygesen K, Alpert JS and White HD; Joint ESC/ACCF/AHA/WHF Task Force for the redefinition of myocardial infarction: Universal definition of myocardial infarction. J Am Coll Cardiol 50: 2173-2195, 2007.
5. Hunter JJ and Chien KR: Signaling pathways for cardiac hypertrophy and failure. N Engl J Med 341: 1276-1283, 1999.
6. Beaglehole R, Bonita R, Horton R, Adams O and McKee M: Public health in the new era: Improving health through collective action. Lancet 363: 2084-2086, 2004.
7. Dubois NC, Craft AM, Sharma P, Elliott DA, Stanley EG, Elefanty AG, Gramolini A and Keller G: SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. Nat Biotechnol 29: 1011-1018, 2011.
8. Fijnvandraat AC, van Ginneken AC, de Boer PA, Ruijter JM, Christoffels VM, Moorman AF and Lekanne Deprez RH: Cardiomyocytes derived from embryonic stem cells resemble cardiomyocytes of the embryonic heart tube. Cardiovasc Res 58: 399-409, 2003.
9. Hidaka K, Lee JK, Kim HS, Ihm CH, Iio A, Ogawa M, Nishikawa S, Kodama I and Morisaki T: Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. FASEB J 17: 740-742, 2003.
10. Hattori F, Chen H, Yamashita H, Tohyama S, Satoh YS, Yuasa S, Li W, Yamakawa H, Tanaka T, Onitsuka T, et al: Nongenetic method for purifying stem cell-derived cardiomyocytes. Nat Methods 7: 61-66, 2010.
11. Baba S, Heike T, Yoshimoto M, Umeda K, Doi H, Iwasa T, Lin X, Matsuoka S, Komeda M and Nakahata T: Flk1(+) cardiac stem/progenitor cells derived from embryonic stem cells improve cardiac function in a dilated cardiomyopathy mouse model. Cardiovasc Res 76: 119-131, 2007.
12. Guan K, Wagner S, Unsöld B, Maier LS, Kaiser D, Hemmerlein B, Nayernia K, Engel W and Hasenfuss G: Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. Circ Res 100: 1615-1625, 2007.
13. Wang IN, Wang X, Ge X, Anderson J, Ho M, Ashley E, Liu J, Butte MJ, Yazawa M, Dolmetsch RE, et al: Apelin enhances directed cardiac differentiation of mouse and human embryonic stem cells. PLoS One 7: e38328, 2012.
14. Zhu WZ, Hauch KD, Xu C and Laflamme MA: Human embryonic stem cells and cardiac repair. Transplant Rev (Orlando) 23: 53-68, 2009.
15. Klaus A, Saga Y, Taketo MM, Tzahor E and Birchmeier W: Distinct roles of Wnt/beta-catenin and Bmp signaling during early cardiogenesis. Proc Natl Acad Sci USA 104: 18531-18536, 2007.
16. Taha MF and Valojerdi MR: Effect of bone morphogenetic protein-4 on cardiac differentiation from mouse embryonic stem cells in serum-free and low-serum media. Int J Cardiol 127: 78-87, 2008.
17. Ladd AN, Yatskievych TA and Antin PB: Regulation of avian cardiac myogenesis by activin/TGFbeta and bone morphogenetic proteins. Dev Biol 204: 407-419, 1998.
18. Hoffman RM: The hair follicle as a gene therapy target. Nat Biotechnol 18: 20-21, 2000.
19. Oshima H, Rochat A, Kedzia C, Kobayashi K and Barrandon Y: Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell 104: 233-245, 2001.
20. Cotsarelis G, Sun TT and Lavker RM: Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61: 1329-1337, 1990.
21. Morris RJ and Potten CS: Highly persistent label-retaining cells in the hair follicles of mice and their fate following induction of anagen. J Invest Dermatol 112: 470-475, 1999.
22. Taylor G, Lehrer MS, Jensen PJ, Sun TT and Lavker RM: Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102: 451-461, 2000.
23. Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M and Fuchs E: Defining the epithelial stem cell niche in skin. Science 303: 359-363, 2004.
24. Toma JG, Akhavan M, Fernandes KJ, Barnabé-Heider F, Sadikot A, Kaplan DR and Miller FD: Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 3: 778-784, 2001.
25. Fernandes KJ, McKenzie IA, Mill P, Smith KM, Akhavan M, Barnabé-Heider F, Biernaskie J, Junek A, Kobayashi NR, Toma JG, *et al*: A dermal niche for multipotent adult skin-derived precursor cells. Nat Cell Biol 6: 1082-1093, 2004.
26. Sieber-Blum M, Grim M, Hu YF and Szeder V: Pluripotent neural crest stem cells in the adult hair follicle. Dev Dyn 231: 258-269, 2004.
27. Li L, Mignone J, Yang M, Matic M, Penman S, Enikolopov G and Hoffman RM: Nestin expression in hair follicle sheath progenitor cells. Proc Natl Acad Sci USA 100: 9958-9961, 2003.
28. Yano K, Brown LF and Detmar M: Control of hair growth and follicle size by VEGF-mediated angiogenesis. J Clin Invest 107: 409-417, 2001.
29. Mecklenburg L, Tobin DJ, Müller-Röver S, Handjiski B, Wendt G, Peters EM, Pohl S, Moll I and Paus R: Active hair growth (anagen) is associated with angiogenesis. J Invest Dermatol 114: 909-916, 2000.
30. Amoh Y, Li L, Yang M, Moossa AR, Katsuoka K, Penman S and Hoffman RM: Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. Proc Natl Acad Sci USA 101: 13291-13295, 2004.
31. Amoh Y, Li L, Katsuoka K, Penman S and Hoffman RM: Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. Proc Natl Acad Sci USA 102: 5530-5534, 2005.
32. Yashiro M, Mii S, Aki R, Hamada Y, Arakawa N, Kawahara K, Hoffman RM and Amoh Y: From hair to heart: Nestin-expressing hair-follicle-associated pluripotent (HAP) stem cells differentiate to beating cardiac muscle cells. Cell Cycle 14: 2362-2366, 2015.
33. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals, 8th edition. National Academies Press (US), Washington, DC, 2011.
34. Nath M, Offers M, Hummel M and Seissler J: Isolation and in vitro expansion of Lgr6-positive multipotent hair follicle stem cells. Cell Tissue Res 344: 435-444, 2011.
35. Kubota H, Avarbock MR and Brinster RL: Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci USA 101: 16489-16494, 2004.
36. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
37. Fox V, Gokhale PJ, Walsh JR, Matin M, Jones M and Andrews PW: Cell-cell signaling through NOTCH regulates human embryonic stem cell proliferation. Stem Cells 26: 715-723, 2008.
38. Kattman SJ, Huber TL and Keller GM: Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell 11: 723-732, 2006.
39. Jang J, Ku SY, Kim JE, Choi K, Kim YY, Kim HS, Oh SK, Lee EJ, Cho HJ, Song YH, *et al*: Notch inhibition promotes human embryonic stem cell-derived cardiac mesoderm differentiation. Stem Cells 26: 2782-2790, 2008.
40. Noggle SA, Weiler D and Condie BG: Notch signaling is inactive but inducible in human embryonic stem cells. Stem Cells 24: 1646-1653, 2006.
41. Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J and Keller G: Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 8: 228-240, 2011.
42. Yuasa S, Itabashi Y, Koshimizu U, Tanaka T, Sugimura K, Kinoshita M, Hattori F, Fukami S, Shimazaki T, Ogawa S, *et al*: Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. Nat Biotechnol 23: 607-611, 2005.
43. Kim BJ, Kim YH, Lee YA, Jung SE, Hong YH, Lee EJ, Kim BG, Hwang S, Do JT, Pang MG, *et al*: Platelet-derived growth factor receptor-alpha positive cardiac progenitor cells derived from multipotent germline stem cells are capable of cardiomyogenesis in vitro and in vivo. Oncotarget 8: 29643-29656, 2017.
44. Org T, Duan D, Ferrari R, Montel-Hagen A, Van Handel B, Kerényi MA, Sasidharan R, Rubbi L, Fujiwara Y, Pellegrini M, *et al*: Scl binds to primed enhancers in mesoderm to regulate hematopoietic and cardiac fate divergence. EMBO J 34: 759-777, 2015.
45. Bai F, Ho Lim C, Jia J, Santostefano K, Simmons C, Kasahara H, Wu W, Terada N and Jin S: Directed differentiation of embryonic stem cells into cardiomyocytes by bacterial injection of defined transcription factors. Sci Rep 5: 15014, 2015.
46. Li G, Plonowska K, Kuppusamy R, Sturzu A and Wu SM: Identification of cardiovascular lineage descendants at single-cell resolution. Development 142: 846-857, 2015.

47. Eng G, Lee BW, Protas L, Gagliardi M, Brown K, Kass RS, Keller G, Robinson RB and Vunjak-Novakovic G: Autonomous beating rate adaptation in human stem cell-derived cardiomyocytes. Nat Commun 7: 10312, 2016.
48. Radaszkiewicz KA, Sýkorová D, Karas P, Kudová J, Kohút L, Binó L, Večeřa J, Víteček J, Kubala L and Pacherník J: Simple non-invasive analysis of embryonic stem cell-derived cardiomyocytes beating in vitro. Rev Sci Instrum 87: 024301, 2016.
49. Hayashi K, Ohta H, Kurimoto K, Aramaki S and Saitou M: Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell 146: 519-532, 2011.
50. Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H and Saitou M: Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. Science 338: 971-975, 2012.
51. Baba S, Heike T, Umeda K, Iwasa T, Kaichi S, Hiraumi Y, Doi H, Yoshimoto M, Kanatsu-Shinohara M, Shinohara T, *et al*: Generation of cardiac and endothelial cells from neonatal mouse testis-derived multipotent germline stem cells. Stem Cells 25: 1375-1383, 2007.
52. Yao S, Chen S, Clark J, Hao E, Beattie GM, Hayek A and Ding S: Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc Natl Acad Sci USA 103: 6907-6912, 2006.
53. Ryu BY, Kubota H, Avarbock MR and Brinster RL: Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc Natl Acad Sci USA 102: 14302-14307, 2005.
54. Conlon FL, Lyons KM, Takaesu N, Barth KS, Kispert A, Herrmann B and Robertson EJ: A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development 120: 1919-1928, 1994.
55. Gadue P, Huber TL, Paddison PJ and Keller GM: Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc Natl Acad Sci USA 103: 16806-16811, 2006.
56. Liu Y, Asakura M, Inoue H, Nakamura T, Sano M, Niu Z, Chen M, Schwartz RJ and Schneider MD: Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. Proc Natl Acad Sci USA 104: 3859-3864, 2007.
57. Marvin MJ, Di Rocco G, Gardiner A, Bush SM and Lassar AB: Inhibition of Wnt activity induces heart formation from posterior mesoderm. Genes Dev 15: 316-327, 2001.
58. Schneider VA and Mercola M: Wnt antagonism initiates cardiogenesis in Xenopus laevis. Genes Dev 15: 304-315, 2001.
59. Schultheiss TM, Burch JB and Lassar AB: A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev 11: 451-462, 1997.
60. Tzahor E and Lassar AB: Wnt signals from the neural tube block ectopic cardiogenesis. Genes Dev 15: 255-260, 2001.
61. Yatskievych TA, Ladd AN and Antin PB: Induction of cardiac myogenesis in avian pregastrula epiblast: The role of the hypoblast and activin. Development 124: 2561-2570, 1997.
62. Iida M, Heike T, Yoshimoto M, Baba S, Doi H and Nakahata T: Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation. FASEB J 19: 371-378, 2005.
63. Murakami Y, Hirata H, Miyamoto Y, Nagahashi A, Sawa Y, Jakt M, Asahara T and Kawamata S: Isolation of cardiac cells from E8.5 yolk sac by ALCAM (CD166) expression. Mech Dev 124: 830-839, 2007.
64. Misfeldt AM, Boyle SC, Tompkins KL, Bautch VL, Labosky PA and Baldwin HS: Endocardial cells are a distinct endothelial lineage derived from Flk1^{+} multipotent cardiovascular progenitors. Dev Biol 333: 78-89, 2009.