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RESEARCH ARTICLE

Cardiomyogenic differentiation of human sternal bone marrow mesenchymal stem cells using a combination of basic fibroblast growth factor and hydrocortisone

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Abstract

The alarming rate of increase in myocardial infarction and marginal success in efforts to regenerate the damaged myocardium through conventional treatments creates an exceptional avenue for cell-based therapy. Adult bone marrow mesenchymal stem cells (MSCs) can be differentiated into cardiomyocytes, by treatment with 5-azacytidine, thus, have been anticipated as a therapeutic tool for myocardial infarction treatment. In this study, we investigated the ability of basic fibroblastic growth factor (bFGF) and hydrocortisone as a combined treatment to stimulate the differentiation of MSCs into cardiomyocytes. MSCs were isolated from sternal marrow of patients undergoing heart surgery (CABG). The isolated cells were initially monitored for the growth pattern, followed by characterization using ISCT recommendations. Cells were then differentiated using a combination of bFGF and hydrocortisone and evaluated for the expression of characteristic cardiac markers such as CTnI, CTnC, and Cnx43 at protein level using immunocytochemistry and flow cytometry, and CTnC and CTnT at mRNA level. The expression levels and pattern of the cardiac markers upon analysis with ICC and qRT-PCR were similar to that of 5-azacytidine induced cells and cultured primary human cardiomyocytes. However, flow cytometric evaluation revealed that induction with bFGF and hydrocortisone drives MSC differentiation to cardiomyocytes with a marginally higher efficiency. These results indicate that combination treatment of bFGF and hydrocortisone can be used as an alternative induction method for cardiomyogenic differentiation of MSCs for future clinical applications.

Keywords: basic fibroblast growth factor; cardiomyocytes; human mesenchymal stem cells; hydrocortisone

Introduction

Myocardial infarction has been increasing at an alarming rate and is one of the leading causes of mortality around the globe. The pathogenesis involves death of cardiomyocytes at localized areas leading to dysfunction of tissue (Ma et al., 2013), increasing the work load burden on viable cardiomyocytes (Takala, 1981; Domingos et al., 2002), thus escalating the chances of subsequent infarctions and eventual cardiac failure. Heart muscles are terminally differentiated and, unlike skeletal muscles, lack the capability to regenerate. In addition, conventional treatments do not restore the functions of necrotic tissue, instead merely manages the symptoms and are far from satisfactory in improving the survival rate of the patient (Yoon et al., 2005). This drives the search for cells with the potential to differentiate, regenerate, and replace the injured heart muscle and augment cardiac function upon implantation (Toma et al., 2002).

Cells isolated from a wide variety of tissues, including embryonic, fetal, and adult tissues, have been used for the regeneration and management of infarcted myocardium (Dawn et al., 2005). Among those, human bone marrow mesenchymal stem cells (MSCs) have been particularly

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Abbreviations: bFGF, basic fibroblast growth factor; CTnC, cardiac troponin C; CTnI, cardiac troponin I; CTnT, cardiac troponin T; Cnx43, cardiac connexin 43; MSC, mesenchymal stem cells

exciting because of the relatively less ethical hurdles and their ability to differentiate into cardiomyocytes in vitro and in vivo setting. In addition to their potential to differentiate and replenish the damaged myocardium upon implantation, MSCs are also believed to exert cyto-protective effects on native cardiomyocytes through secretion of trophic factors, stimulation of angiogenesis, activation of resident cardiac stem cells, and modulation of invading immune cells (Orlic et al., 2001; Abarbanell et al., 2009; Rogers et al., 2011).

MSCs require induction with appropriate factors for in vitro cardiomyogenic differentiation. Over the past two decades researchers have presented different approaches to induce the differentiation of MSCs into cardiomyocytes, including use of chemical inducers (Banfi et al., 2000; Lin et al., 2004; Antonitsis et al., 2007; Hsu et al., 2010), growth factors (Khezri et al., 2007; Hahn et al., 2008; Franco et al., 2011), and co-culture with primary cardiomyocytes (Labovsky et al., 2010). 5-Azacytidine is a widely used chemical induction factor in differentiation of stem cells and has demonstrated significant success in deriving cardiomyocytes from stem cells of various sources. However, 5-azacytidine is a nucleoside analog, which gets incorporated into nucleic acids and its carcinogenicity has been widely reported both in vitro (Holliday, 1991) and in vivo (Carr et al., 1984; Cavaliere et al., 1987). In addition, a recent report from International Agency for Research on Cancer (IARC, WHO) classified 5-azacytidine as probably carcinogenic to humans (group 2A drugs; Alagesan and Griffin, 2014). Taken together, the aforementioned reports raise a cautionary note on the use of 5-azacytidine for generation of cardiomyocytes for therapeutic applications, and emphasize the need for alternative induction methods.

Recent rise in understanding of the factors involved in cardiac development resulted in the use of growth factors to induce in vitro differentiation of stem cells. Bone morphogenic protein (BMP-2/4), activin A (Han et al., 2011), insulin like growth factor (IGF-1), and fibroblast growth factor (FGF-2; Franco et al., 2011; Asumda and Chase, 2012; Asumda, 2013) are few examples that have been tested to induce cardiomyogenic differentiation of multiple stem cell sources, including embryonic stem cells, induced-pluripotent stem cells, and MSCs. Basic fibroblast growth factor (bFGF) is a member of heparin binding growth factor and is known to have an important role in driving MSCs to the cardiomyogenic lineage during embryogenesis (Sugi and Lough, 1995; Sugi et al., 1995; Rosenblatt-Velin et al., 2005). Hydrocortisone, a stress hormone produced by the adrenal gland, has been shown to stimulate the entry of cardiomyocytes into division cycle, and thus enhances their proliferation in vitro (Zuk et al., 2002; Giraud et al., 2006; Vindigni et al., 2013).

Present study characterized the sternal MSCs and tested the potential of these MSCs to differentiate into cardiomyogenic lineage. We demonstrated that sternal marrow is a good source for MSCs and possesses the potential to differentiate into cells of mesodermal tri-lineage. It was also found that combination of bFGF and hydrocortisone induced the differentiation of sternal marrow MSCs into cardiomyocytes with efficiency comparable to that induced by 5-azacytidine.

Materials and methods

Ethics statement

Samples were collected from patients suffering from coronary artery disease and scheduled for coronary artery bypass grafting (CABG) with informed consent. Collection and usage of samples were approved by the Universiti Kebangsaan Malaysia Medical Research and Ethics committee (FF-385-2011).

Isolation and culture of sternal marrow MSCs

Approximately 7 ± 1 mL of sternal marrow was collected using heparinized syringe from sternum and processed for MSC isolation within 45 min of sample collection as described elsewhere (Hsu et al., 2010). In brief, the bone marrow mononuclear cells were separated using Ficoll paque (GE, USA) density gradient method and plated in 75 cm^2 tissue culture treated flasks with 15 mL MSCs medium, consisting of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA), 10% fetal bovine serum (FBS, Invitrogen, USA), ascorbic acid (Sigma, USA), Glutamax (Invitrogen, USA), and antibiotic-antimycotic that contains 10,000 U/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of Fungizone® (Gibco, USA). Cells were incubated at 37°C in a 5% CO_2 humidified incubator for 3 days before the first medium change. MSCs were isolated based on their ability to attach to culture surface. Once adherent cells reached 80–90% confluence, the cells were detached with 0.05% trypsin ETDA (Gemini, USA) and subcultured. Cells in passage3 (P3) were used for downstream experiments.

Growth kinetics analysis of sternal marrow MSCs

To identify the initial density of sternal marrow MSCs in primary culture, non-attached cells were removed by washing with PBS at 72 h. Cells were harvested using 0.05% trypsin and total cell number and viability was estimated via trypan blue exclusion assay using a hemocytometer. Growth kinetics and doubling time were determined according to a previous protocol with modifications (Hsu et al., 2010). In brief, MSCs were seeded at a density of 1,000 cells/well in 12-well plate. Cells from triplicate wells

were harvested using 0.05% trypsin-EDTA and cell number was determined every 48 h until day 12. The growth kinetics of cultured MSCs in different passages (P1, P2, and P3) was determined by plotting the cell number against days in culture and cell doubling time was calculated using an online doubling time calculator following formula $X = h \times \ln(2)/\ln(c2/c1)$, where "c" is the number of cells at each time of collection and "ln" is a neperian logarithm (Roth, 2006, http://www.doubling-time.com/compute.php).

Immunophenotype analysis by flow cytometry

Human sternal marrow MSCs were tested at passage 1 by flow cytometry for surface marker expression to ensure that the cells meet the minimal criteria described by international society of cellular therapy (ISCT; Dominici et al., 2006). The cells were harvested with 0.05% trypsin-EDTA, washed with 0.2% bovine serum albumin (BSA) in PBS, and stained with the following list of antibodies; mouse anti-human CD14, CD29, CD44, CD45, CD73, CD90, CD105, anti-HLA-DR (BD Pharmingen, USA), CD13, and CD34 (Life Technology, USA). In brief, 2×10^5 cells were suspended in 100 μL of 0.2% BSA in PBS and stained with individual antibodies (at concentration as per manufacturer's recommendation) in separate tubes for 30 min. The cells were then washed with 0.2% BSA/PBS twice and fixed in 4% paraformaldehyde. Samples were washed twice in PBS, suspended in 0.2% BSA/PBS, and analyzed by FACS Calibur cytometer (BD Biosciences, USA) using CellQuest Pro software. Ten thousand gated events were recorded. Gating was determined based on unstained controls.

Differentiation potential analysis of sternal marrow MSCs

Multi-lineage differentiation potential of sternal marrow MSCs was assayed in passage 3 by inducing their differentiation into osteocytes, adipocytes, and chondrocytes. MSCs cultured in medium without induction factors were used as control.

Osteogenic differentiation

Osteogenic differentiation of human sternal marrow MSCs was induced in α-MEM (Sigma–Aldrich, USA) supplemented with 0.1 μM dexamethasone (Sigma–Aldrich), 10 mM β-glycerol phosphate (Sigma–Aldrich), and 0.2 mM ascorbic acid-2-phosphate (Sigma–Aldrich). Cultures were replenished with fresh medium twice/week and changes in cell morphology and growth pattern were closely monitored using a phase contrast microscope until day 21. Differentiation was assessed by staining with Alizarin red for calcium deposition. Briefly, cells were fixed with cold ethanol for 1 h, rinsed with PBS, and stained with Alizarin red for 1 h. Excess stain was washed off using PBS, followed by incubation with boric acid buffer and counterstaining with hematoxylin. Controls and differentiated cultures were then evaluated using a bright field microscope (Olympus-CK40, Japan).

Adipogenic differentiation

Adipogenic differentiation of human sternal bone marrow MSCs was induced in DMEM/F12 supplemented with 0.25 mmol/L 3-isobutyl-1-methylxanthine (Sigma–Aldrich), 100 nmol/mL dexamethasone, and 100 nmol/L human recombinant insulin (Sigma–Aldrich). Culture medium was changed every 3 days and was closely monitored using phase contrast microscopy for lipid deposition. On day 21, cells were stained with Oil Red O to identify lipid deposition. Briefly, the adipogenic cultures were rinsed once with PBS and fixed with 10% formalin for 60 min at room temperature. Formalin was discarded and the cells were stained with 0.36% Oil Red O stain for 50 min. The cultures were then examined using a bright field microscope

Chondrogenic differentiation

Chondrogenic differentiation of human sternal bone marrow MSCs was induced in DMEM/F12 supplemented with transforming growth factor-beta-3 (TGFβ; 10 ng/mL). Briefly, 2×10^6 human sternal bone marrow MSCs were pelleted in a 15 mL polypropylene tube and cultured in chondrogenic medium for 21 days. Resulting tissue was fixed, sectioned, and stained with Safranin O to identify the presence of proteoglycans and glycosaminoglycan within the tissue matrix. In brief, slides were stained by Weigert's iron hematoxylin (Sigma–Aldrich) working solution and washed with tap water and acid alcohol. The sections were then stained with fast green solution (Dako, Denmark), counter stained with Safranin O solution (Stain pur^{TM}), and examined using a bright field microscope.

In vitro cardiomyogenic induction of sternal marrow MSCs

To induce cardiomyogenic differentiation, 70% confluence human sternal marrow MSCs cultured in 25 cm^2 flasks were treated with a combination of 10 nM bFGF (Invitrogen) and 50 μM hydrocortisone (Sigma–Aldrich) for 24 h. Concurrently, MSCs were induced by 10 μM 5-azacytidine (Sigma–Aldrich) as test control for the differentiation assay. Cells were then washed twice with PBS and replenished with DMEM without induction factors. Medium was replenished every 3 days until the assay was terminated at day 14. Primary human cardiomyocytes (ScienCell, USA) were used as positive control for the differentiation assay analysis.

Immunocytochemistry staining

Immunocytochemistry staining of control cultures, induced cultures, and primary cardiomyocytes was performed to

examine the expression of cardiac troponin I (CTnI), cardiac troponin C (CTnC), and cardiac connexin43 (Cnx43; 1:300 dilution; Abcam, USA). Briefly, cells were fixed with 4% paraformaldehyde (Sigma–Aldrich) for 30 min at room temperature followed by permeabilization with 0.1% Triton X-100 (Invitrogen) in PBS for 5 min. Cells were then incubated with 10% goat serum for 1 h at 37°C to block non-specific binding of antibodies. Subsequently, cells were incubated with primary antibodies at 4°C overnight, followed by washing twice with PBS and incubation with appropriate secondary antibodies (Invitrogen) at room temperature for 2 h. Nuclei were counterstained with 0.1 μg/mL of DAPI (Invitrogen) for 5 min and imaged with a fluorescence microscope (Nikon, Japan).

Flow cytometry

Percentage of cells expressing cardiac markers after differentiation was estimated by flow cytometry. In brief, cells were fixed with 4% paraformaldehyde (Sigma–Aldrich) for 30 min at room temperature followed by permeabilization with 0.1% Triton X-100 (Invitrogen) in PBS for 5 min. Cells were then incubated with 10% goat serum for 1 h at 37°C to block non-specific binding of antibodies. Subsequently, cells were incubated with primary antibodies for CTnI, CTnC, and Cnx43 (1:200 dilution; Abcam), at room temperature for 1 h, followed by washing twice with PBS, and incubation with appropriate secondary antibodies (Invitrogen) at room temperature for 1 h. The cells were subsequently washed once with PBS and suspended in 300 μL of 0.2% BSA in PBS. Flow cytometry analysis was performed on BD FACS Calibur™ flow cytometer (BD, USA) equipped with BD CellQuestPro™ software (BD). Controls stained with secondary antibodies alone were used to negate the non-specific binding and define the gating.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with Trizol Reagent (Gibco, USA) from untreated MSCs, cardiomyogenic induced MSCs, and primary cardiomyocytes. Two-step qRT-PCR for cardiomyocyte specific genes; cardiac troponin C and cardiac troponin T were performed using iScript™ cDNA Synthesis Kit followed by amplification using iQ™ SYBR® Green Supermix (BioRad, USA) on MyiQ™ iCycler Real-Time PCR Detection System (BioRad) as per kit instructions. Relative quantification of the gene expression was performed using the ΔC_t method. Housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used for internal normalization. Primer sequences are shown in Supplementary Table S1.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and statistical significance was analyzed by ANOVA using SPSS version 20.

Results

Morphology and growth properties of sternal marrow cultures

The morphological, phenotypic, and growth kinetic characteristics of sternal marrow MSCs were studied to understand their physiological and growth properties that were important for the design of downstream experiments. MSCs cultures of passages 1–3 were used in this study and the cells were routinely sub-cultured before reaching 90% confluence to maintain ideal growth environment. As a general evaluation of culture quality, cell viability and growth kinetics were determined using trypan blue exclusion assay, and cell morphology was monitored using phase contrast microscopy. At passage 0, the cells were short and spindle in shape, which then grew into distinct small colonies. The cells were trypsinized and re-plated into new flask when they reached ~90% confluence. In passage 1, the cells were in quiescent state (lag phase) till day 4 and then divided quickly until day 10 (Figure 1a). By day 10, the cells entered stationary phase as indicated by the slowdown in cell proliferation rate. The growth pattern of MSCs at passages 2 and 3 was similar to those of passage 1, except having a shorter lag phase (2 days). Doubling time of MSCs at different passages was also determined and shown in Supplementary Table S2.

Multi-lineage differentiation of human sternal marrow MSCs

Sternal marrow MSCs were tested for differentiation into the three main cell lineages: osteocyte, adipocyte, and chondrocyte. In adipogenic differentiation assay, lipid droplets appeared inside the cells by day 7. At day 21, these droplets increased in numbers and formed clusters of shiny oily appearances and stained positive with Oil red O confirming the lipid deposition (Figure 1b). Cells cultured in osteogenic medium displayed small stratified-like cluster of cells starting from day 10. At day 21, osteogenic differentiation was confirmed by Alizarin Red staining to detect calcium deposits (Figure 1c). Chondrogenic differentiation was achieved by culturing the cells in 15 mL tube in optimized chondrogenic medium. At day 21, staining with safranin O detected the presence of proteoglycans and glycosaminoglycan deposition (Figure 1d).

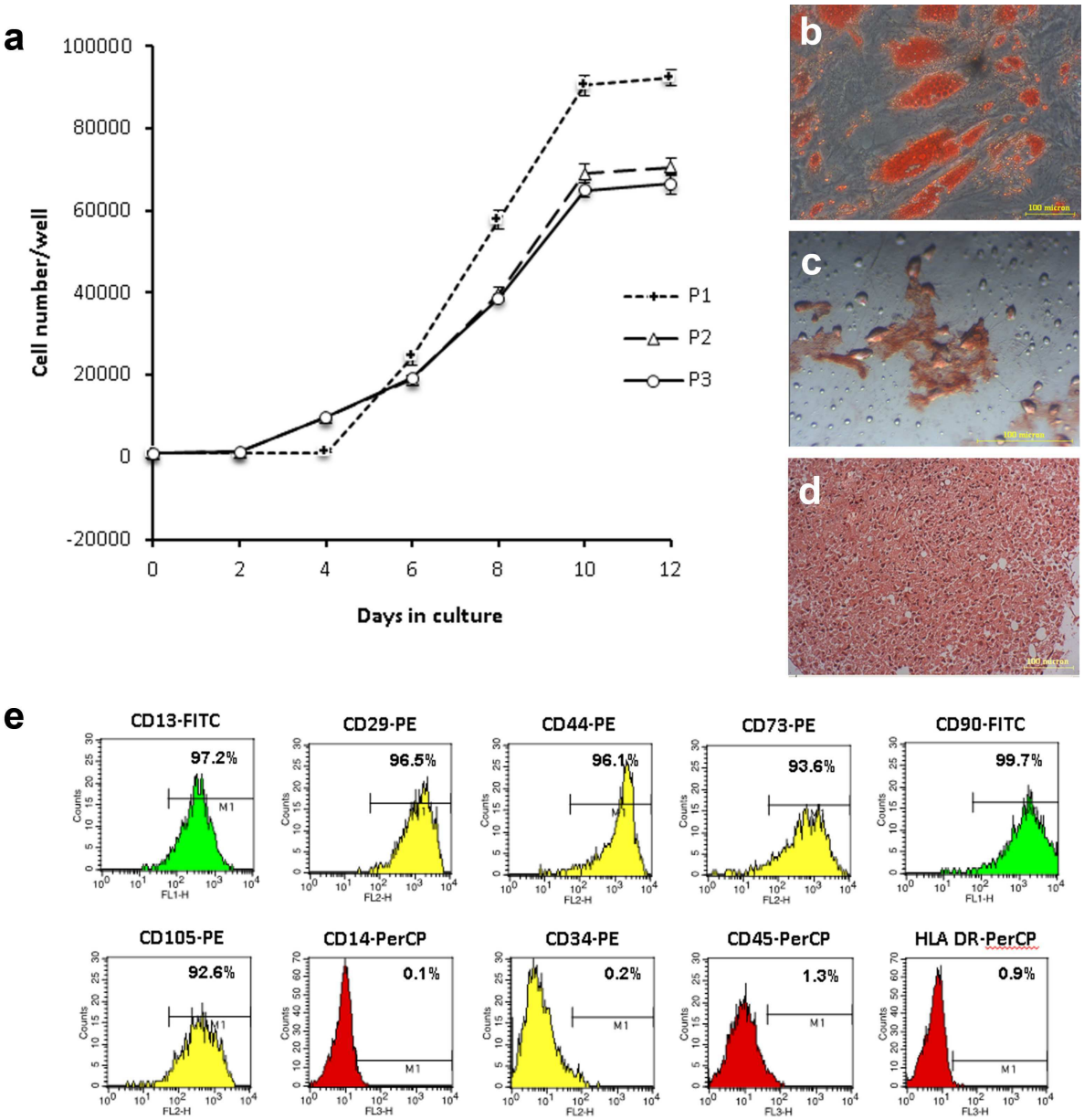

Figure 1 Characterization of sternal marrow MSC. (a) Growth curve of human sternal marrow MSCs in culture in the initial three passages. Results are presented as mean ± SD from six biological replicates. (b–d) Tri-lineage differentiation of human sternal marrow MSCs. Oil red O (b), alizarin red (c), and safranin O (d) staining demonstrating the tri-lineage differentiation potential of sternal marrow MSC. (e) Flow cytometry histograms show the expression levels CD13, CD29, CD44, CD73, CD90, CD105, CD14, CD34, CD45, and HLA-DR. The numbers on the top right corner indicate percentage of positive cells. (b–e) Results are from a representative of three independent experiments.

Immunophenotypic characterization of human sternal marrow MSCs

Immunophenotyping of passage 1 MSCs were performed using a panel of markers based on the ISCT guidelines. Flow cytometric analysis confirmed the presence of MSCs in sternal marrow cultures as more than 95% of cells expressed CD13, CD29, CD44, CD73, CD90, and CD105, and less than 5% of cells expressed CD14, CD34, CD45, and the immune activation marker HLA-DR (Figure 1e; Supplementary Table S3).

In vitro cardiomyogenic differentiation of human sternal marrow MSCs

Sternal marrow MSCs were differentiated with 5-azacytidine or with combination of bFGF and hydrocortisone. MSCs expressed cardiac specific markers in both treatment

conditions. Immunofluorescence analysis detected the expression of CTnI and CTnC in cells induced with bFGF and hydrocortisone (Figures 2a–h). Expression pattern and intensity of these cardiac markers were similar to that of 5-azacytidine induced MSCs and cultured primary human cardiomyocytes. Similarly, 5-azacytidine and bFGF and hydrocortisone induced the MSCs to express cardiac intracellular gap junction protein Cnx43 (Figures 2i–l) and the expression pattern was similar to that of in primary cardiomyocytes. Expression of all three markers was not detected in non-induced MSCs.

Flow cytometric evaluation of the differentiated cells was performed to compare the efficiency of the two different differentiation methods tested here. Staining for CTnC, CTnI, and Cnx43 revealed that stimulation with bFGF and hydrocortisone induce cardiomyogenic differentiation of MSC (Figure 3) with a marginal non-significant superior efficiency compared to induction using 5-azacytidine. bFGF plus hydrocortisone stimulation induced expression of CTnC and CTnI in approximately 50% of MSC compared to 40% in those induced by 5-azacytidine (Supplementary Figure S1). It is also noteworthy that bFGF + hydrocortisone stimulation successfully induced Cnx43 expression in $13.96 \pm 0.45\%$ ($^{*}P < 0.05$, versus non-induced controls) of cells, whereas 5-azacytidine induced Cnx43 only in $7.89 \pm 1.23\%$ cells.

Cardiomyogenic differentiation of MSCs was also evaluated in mRNA level by determining the expression of CTnC and CTnT using qRT-PCR. Expressions of CTnC and CTnT were significantly up-regulated in both induced human sternal marrow MSCs groups compared to the non-induced controls (Figure 4). However, no significant differences in the expression of these cardiac markers were detected between the induced groups, indicating that bFGF and hydrocortisone induction can be used as an alternative induction cocktail in place of the widely used 5-azacytidine method.

Discussion

Enumeration of bone marrow is normally performed from either iliac bones or femur due to the availability of relatively large volume of marrow in these sites. Though bound with the constraint of small volume, sternum is considered as an equally good source of bone marrow MSCs, as their cell division cycle and proliferative features are comparable to the cells from iliac bones (Harker et al., 1983). In this study, we have shown that human mesenchymal stem cells can be isolated from sternal marrow aspiration of patients undergoing CABG. Instead of discarding during the sternotomy procedure, marrow can be aspirated using a small bone marrow aspiration needle. This approach has additional benefits of avoiding a second round of anesthesia and associated discomfort. In addition, the isolation of bone marrow through the same incision line of CABG reduces the risk of contamination.

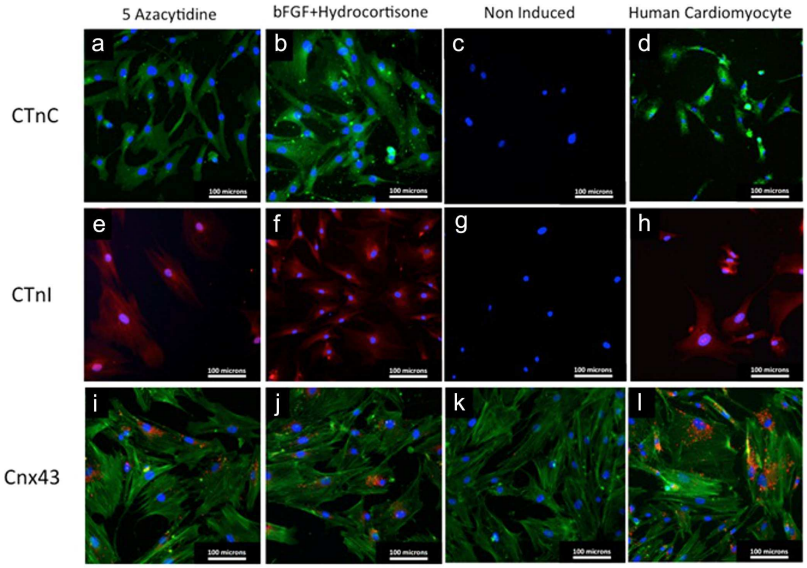

Figure 2 **Immunocytochemical staining of structural cardiac markers.** Induction with bFGF, hydrocortisone combination induced the expression of CTnC (b), CTnI (f), and Cnx43 (j) in MSCs. MSCs induced with 5-azacytidine (a, e, i) also expressed all three markers. Primary human cardiomyocytes and non-induced MSCs were used as positive and negative controls, respectively. Cells were double stained with cytoplasmic actin (green, AF 488) and connexin 43 (red, AF594) in i–l. DAPI was used as a counter stain in all samples. Results are from a representative of three independent experiments.

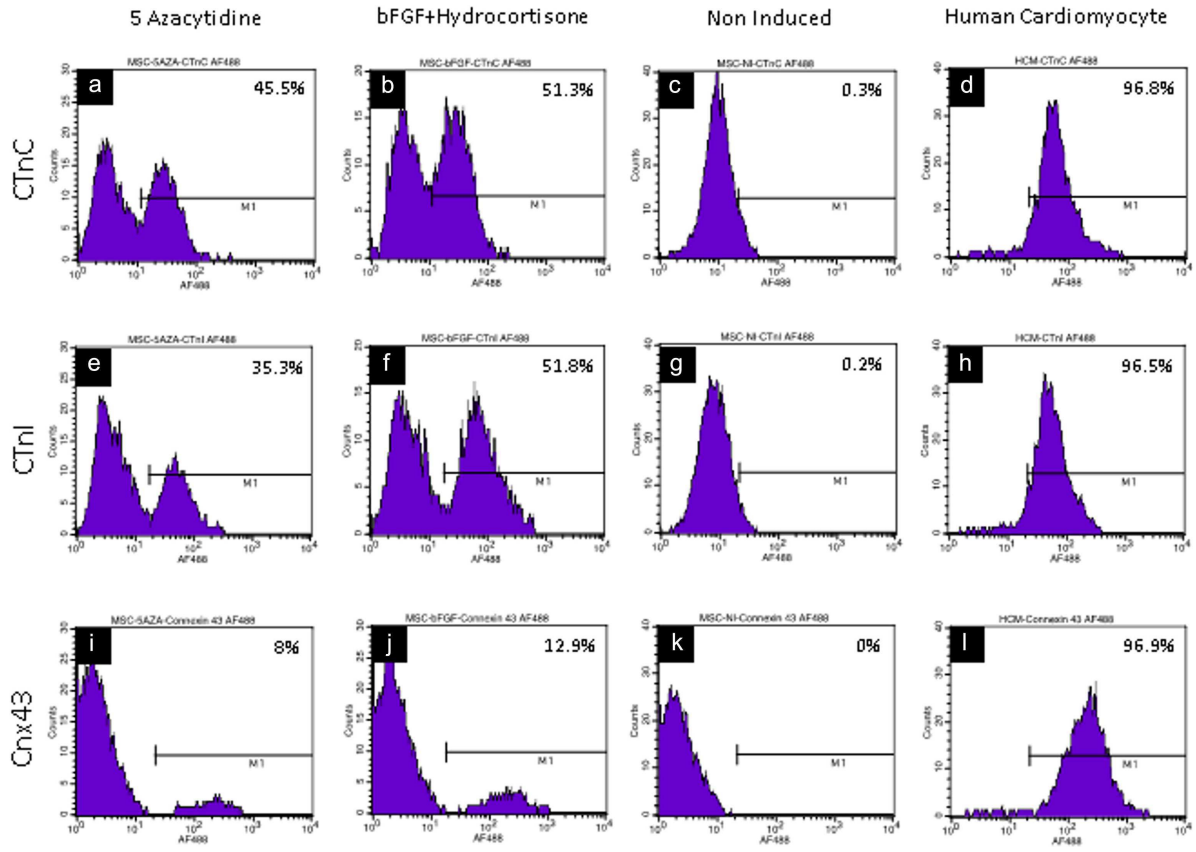

Figure 3 Flow cytometric evaluation of cardiac markers. Stimulation with bFGF, hydrocortisone combination induced the expression of CTnC (b), CTnI (f), and Cnx43 (j) in MSCs. MSCs induced with 5-azacytidine (a, e, i) also expressed all three markers. Primary human cardiomyocytes and non-induced MSCs were used as positive and negative controls, respectively. The numbers on the top right corner indicate percentage of positive cells. Results are from a representative of three independent experiments.

We used the conventional ficoll paque density gradient approach to separate the mononuclear cells, followed by selection of MSCs based on their plastic adherence. Consistent with previous reports, MSCs isolated from sternal marrow expressed classic MSCs markers (CD13, CD24, CD44, CD73, CD90, and CD105) and were devoid of CD14, CD34, CD45, and immune activation marker (HLA-DR). The isolated MSCs also exhibited potential to differentiate into the three main mesodermal lineages, osteocyte, adipocyte, and chondrocyte. These results clearly showed that the cells isolated from sternal marrow met the minimal criteria for defining mesenchymal stem cells, which included plastic adherence, high expression ($\geq$95%) of CD105, CD73, CD90, lack of ($\leq$2%) CD45, CD34, CD14, and HLA class II, and potential to differentiate to osteoblast, adipocytes, and chondroblasts (Pittenger et al., 1999; Gronthos et al., 2003; Dominici et al., 2006; Hsu et al., 2010).

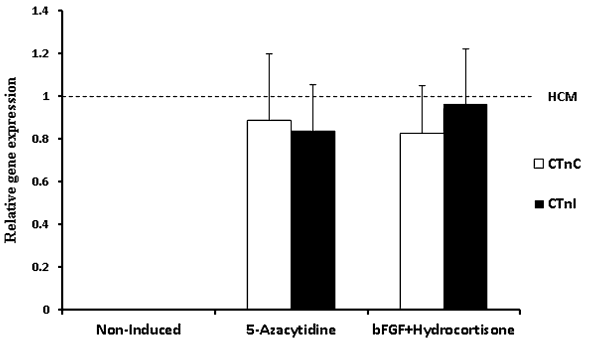

Figure 4 qRT-PCR evaluation of cardiac genes. Cardiac troponin C and T gene expressions were only detected in the induced groups, with no significant differences compared to human cardiomyocytes. Results are presented as mean $\pm$ SD from three biological replicates.

Another important question we addressed in the present study is, whether the isolated MSCs have the potential to differentiate toward cardiomyocyte phenotype in vitro. Previous studies have reported the ability of human marrow MSCs to differentiate into the cardiomyogenic lineage (Antonitsis et al., 2007, 2008). Several investigators have demonstrated the ability of 5-azacytidine to induce animal and human MSCs in vitro to cardiogenic lineage (Wakitani et al., 1995; Antonitsis et al., 2007, 2008; Hsu et al., 2010;

HassanHeidari et al., 2013). Consistent with their findings, MSCs were successfully differentiated into cardiomyocytes as characterized by the expression of cardiac markers in both protein and mRNA levels. Considering the challenges in moving 5-azacytidine-induced cardiomyocytes into clinic due to the potential carcinogenic hazard, we sought to elucidate a safer method for induction of MSCs into cardiomyocytes. Among the numerous growth factors used in differentiation of stem cells, bFGF and hydrocortisone were selected due to their role in maintenance and differentiation of mesodermal cells. For example, bFGF has been reported to play a significant role in driving mesodermal cells to cardiomyogenic lineage during embryogenesis (Solloway and Harvey, 2003; Mummery et al., 2012; D'Amario et al., 2014). However, Khezri et al. (2007) evaluated the possible biological role of bFGF in the differentiation of mouse ESCs into cardiomyocytes and narrated that bFGF alone is incapable of inducing the development of cardiomyocytes, indicating that combination with other factors is necessary for cardiomyocyte differentiation. Hydrocortisone has been found to play a critical role in the regulation of cardiomyocyte proliferation (Vindigni et al., 2013) and cell differentiation toward the myogenic lineages (Zuk et al., 2002). In addition, our own unpublished results showed that in combination, bFGF and hydrocortisone successfully induce differentiation of sheep marrow MSCs into cardiomyocytes.

In the present study, we compared the differentiation of MSCs into cardiomyocytes upon induction with a combination of bFGF and hydrocortisone to that of 5-azacytidine. Immunocytochemistry staining of cardiac specific markers, CTnC, CTnI, and Cnx43 revealed that MSCs treated with both differentiation protocols demonstrated the presence of these markers in a pattern identical to primary human cardiomyocytes (positive control). Flow cytometric evaluation revealed that induction with bFGF and hydrocortisone consistently induces differentiation of approximately 50% of MSC to $CTnC^{+}/CTnI^{+}$ cardiomyocytes. It is also noteworthy that our protocol stands superior in induction of the expression of Cnx43; a gap junctional protein shown to play critical role in cardiac Ca^{2+} uptake and cardiac development (Eckardt et al., 2004). An earlier report using lower concentration of 5-azacytidine (5 μM) to induce cardiomyocytes differentiation failed to stimulate the expression of Cnx43 (Hsu et al., 2010). In addition, the differentiated cells expressed cardiac specific genes in levels comparable to human cardiomyocytes indicating that combination of bFGF and hydrocortisone can be used as an alternative induction cocktail for the successful differentiation of MSCs into cardiomyocytes. However, none of the differentiation protocols used in the present study induced contractile functions in the differentiated cells. This is inconsistent with earlier reports (Makino et al., 1999; Liu et al., 2003; Antonitsis et al., 2007). This discrepancy may be due to the differences in the age of MSC donors, concentration of induction factor, duration of induction, and total duration in culture before end point analysis. Although the present study did not address functionality of differentiated cells, we postulate that the cells might be functional because of the expression of gap junction protein Cnx43. It is possible that prolonging the duration in culture might facilitate the complete differentiation of MSC and might lead to acquisition of functional properties of cardiomyocytes including contractile function.

In summary, we proved that sternal marrow is an ideal source of MSCs, whereby highly pure MSCs with excellent growth kinetics can be isolated and expanded in vitro. Futhermore, the MSCs demonstrated the plasticity to differentiate into adipogenic, chondrogenic, and osteogenic lineages. We also demonstrated the efficacy of combined treatment of bFGF and hydrocortisone in inducing MSCs differentiation into cardiomyogenic lineage is comparable to the conventional 5-azacytidine mediated differentiation.

Conclusion

Sternal marrow is an ideal source of MSCs, whereby highly pure MSCs with excellent growth kinetics can be isolated and expanded in vitro, sternal marrow MSC can be differentiated to cardiomyocytes with combinatory stimuli of bFGF and hydrocortisone. This novel differentiation method can be used as an alternative induction method for cardiomyogenic differentiation of MSCs for future clinical applications.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Author contribution

PH collected the samples, performed the experiments, analyzed the data, and wrote the manuscript. SJ optimized the flow cytometry analysis, analyzed the data, and wrote the manuscript. SRC, MHN, and RBHI designed the experiments and made critical comments on the manuscript. RBHI and RARM conceived the study, obtained funding for the work, and analyzed the data. All authors read and approved the final draft.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. bFGF + hydrocortisone combinatory stimuli induces differentiation of MSC to cardiomyocytes.

Figure S2. Flow cytometric histograms of unstained and secondary alone controls.

Table S1. Cardiac troponin C, T, and GADPH primer sequences.

Table S2. Growth properties of human sternal marrow MSCs in P1, P2, and P3.

Table S3. Data set for immunophenotyping of human sternal marrow MSCs in P1.