Fetal heart extract facilitates the differentiation of human umbilical cord blood-derived mesenchymal stem cells into heart muscle precursor cells

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Abstract Human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) are a promising stem cell source with the potential to modulate the immune system as well as the capacity to differentiate into osteoblasts, chondrocytes, and adipocytes. In previous publications, UCB-MSCs have been successfully differentiated into cardiomyocytes. This study aimed to improve the efficacy of differentiation of UCB-MSCs into cardiomyocytes by combining 5-azacytidine (Aza) with mouse fetal heart extract (HE) in the induction medium. UCB-MSCs were isolated from umbilical cord blood according to a published protocol. Murine fetal hearts were used to produce fetal HE using a rapid freeze–thaw procedure. MSCs at the 3rd to 5th passage were differentiated into cardiomyocytes in two kinds of induction medium: complete culture medium plus Aza (Aza group) and complete culture medium plus Aza and fetal HE (Aza + HE group). The results showed that the cells in both kinds of induction medium exhibited the phenotype of cardiomyocytes. At the transcriptional

level, the cells expressed a number of cardiac muscle-specific genes such as *Nkx2.5*, *Gata 4*, *Mef2c*, *HCN2*, *hBNP*, α -Ca, *cTnT*, *Desmin*, and β -MHC on day 27 in the Aza group and on day 18 in the Aza + HE group. At the translational level, sarcomeric α -actin was expressed on day 27 in the Aza group and day 18 in the Aza + HE group. Although they expressed specific genes and proteins of cardiac muscle cells, the induced cells in both groups did not contract and beat spontaneously. These properties are similar to properties of heart muscle precursor cells in vivo. These results demonstrated that the fetal HE facilitates the differentiation process of human UCB-MSCs into heart muscle precursor cells.

Keywords 5-Azacytidine · Fetal heart extract · Heart muscle precursor cells · Mesenchymal stem cells · Umbilical cord blood

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Introduction

Cell therapy is one of the new approaches for the treatment of cardiovascular diseases. The regenerative potential of damaged heart muscle is very low (Ellison et al. 2007; Laflamme and Murry 2011; Nadal-Ginard et al. 2003), and the differentiation potential of stem cells is high (Gonzalez and Bernad 2012). Hence, stem cells have become the main source of cells for this therapy. Several attempts have been made to

differentiate stem cells into cardiomyocytes using bone marrow-derived mesenchymal stem cells (BM-MSCs) (Hakuno et al. 2002; Siegel et al. 2012; Supokawej et al. 2013), adipose-derived mesenchymal stem cells (Choi et al. 2010; Zhu et al. 2009), amniotic fluid-derived stem cells (Connell et al. 2013), embryonic stem cells (ESCs) (Cao et al. 2011; Caspi et al. 2007; Yamashita et al. 2000), and induced pluripotent stem cells (iPSCs) (Gherghiceanu et al. 2011; Yu et al. 2013; Zhang et al. 2009).

Cardiomyocytes derived from ESCs or iPSCs have been shown its ability to beat spontaneously after induced differentiation. However, previous published results about the ability of mesenchymal stem cells (MSCs) to achieve spontaneous beating are not consistent. Kehat and Zhang reported that induced cells were able to beat after differentiation (Kehat et al. 2002; Zhang et al. 2009), but other authors have claimed the opposite (Koninckx et al. 2011; Rangappa et al. 2003). However, these types of stem cells also have their limitations. For example, ESCs are often used for heart development studies rather than for cardiovascular disease treatment due to moral barriers (De Wert 2003). Although iPSCs reduce the risk of cell rejection by the immune system, their propensity to develop into tumors in transplant patients is a concern (Ben-David and Benvenisty 2011; Guha et al. 2013; Zhao et al. 2011). BM-MSCs do not tend to be tumor-producing when transplanted but are difficult to obtain from older patients (Sethe et al. 2006). Amniotic fluid contains a multi-potential stem cell population, but these cells were rejected when transplanted into a myocardial infarction rat model (Chiavegato et al. 2007). Thus, human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) which are young, healthy, easy to acquire, and potential immune modulators (Iafolla et al. 2014; Pranke et al. 2005; Wagner et al. 2005) should be considered for differentiation into cardiomyocytes and treatment of cardiovascular disease.

There are various established methods for differentiating stem cells into cardiomyocytes, such as using 5-azacytidine (Aza) (Supokawej et al. 2013), Aza combined with other substances (Jumabay et al. 2010), and co-culture with rat cardiomyocytes (Connell et al. 2013). It has been shown that Aza is potentially toxic to differentiated cells (Li et al. 2007; Stresemann et al. 2006), but it is able to induce the expression of specific genes in a short time (Bel et al. 2003). Therefore, it

should be supplemented with external factors (Bhang et al. 2010). Mouse fetal extract contains several unknown cardiac stimulating factors necessary for the differentiation of stem cells into heart cells (Gaustad et al. 2004; Hakelien et al. 2004). However, the extract itself is not enough to induce stem cells to differentiate into cardiac muscle cells in vitro because stem cells induced with fetal extract were shown to lack Gata4 expression (Connell et al. 2013).

Therefore, this study aimed to determine whether UCB-MSCs could be differentiated into heart muscle cells using Aza, to determine the effect of using mouse fetal extract in combination with Aza on stem cell differentiation, and to ascertain whether combination treatment would be more effective than treatment with Aza alone.

Materials and methods

Isolation of human UCB-MSCs

MSCs were isolated and characterized according to a previously published protocol (Pham et al. 2014). UCB was collected from the umbilical cord vein with informed consent from the mother. The collection was performed in accordance with the ethical standards of the local ethics committee. Mononuclear cells (MNCs) and activated platelet-rich plasma (aPRP) were obtained from the same UCB sample. MNCs were then cultured in selective medium consisting of Iscove's modified Dulbecco medium (IMDM) containing 1 % antibiotic–antimycotic (Sigma-Aldrich, St. Louis, MO, USA) and 10 % aPRP. The medium was replaced every four days until the cells reached 70–80 % confluence. Then, the cells were cultured in IMDM proliferation medium containing 5 % aPRP and 1 % antibiotic–antimycotic. MSC candidates were confirmed based on the minimal criteria of MSCs suggested by Dominici et al. (2006).

For marker confirmation, MSC candidates were stained with the following antibodies: anti-CD13 conjugated with FITC, anti-CD14 conjugated with FITC, anti-CD34 conjugated with FITC, anti-CD44 conjugated with PE, anti-CD45 conjugated with FITC, anti-CD73 conjugated with FITC, anti-CD90 conjugated with PE, anti-CD105 conjugated with FITC, anti-CD106 conjugated with PE, anti-CD166 conjugated with PE, and anti-HLA-DR conjugated with

FITC (all purchased from BD Biosciences, San Jose, CA, USA). The stained cells were analyzed by a FACSCalibur flow cytometer. In order to establish differentiation potential, the cells were differentiated into adipocytes and osteoblasts according to previously published protocols (Pham et al. 2014).

Preparation of mouse fetal HE

Hearts were obtained from E.18.5 dpc mice, washed twice with phosphate-buffered saline (PBS), and cut into small pieces. These pieces were soaked in HEPES solution supplemented with proteinase inhibitor cocktail (HEI) (1:200 ratio, Sigma-Aldrich). Heart tissue was then finely minced. The mixture was then transferred to a cryotube and tissue completely crushed using liquid nitrogen. The solution was centrifuged at 13,000 rpm for 10 min. The supernatant was then collected in a 15 ml falcon tube, and the pellet was re-suspended in HEI solution. The pellet was homogenized using sonication and centrifuged at 13,000 rpm for 15 min. The HE supernatant was continuously collected in the same 15 ml falcon tube. The HE was stored at -80°C . Total protein of the extract was quantified using the Bradford method. All manipulations on mice were approved by the local ethical committee (Laboratory of Stem Cell Research and Application, University of Science, Vietnam National University, HCM, VN).

Differentiation of UCB-MSCs into cardiomyocytes

UCB-MSCs from passages 3–5 were used for the cardiomyocyte differentiation studies. Cells were seeded in 25-cm² Roux dishes with an average density of 1×10^5 cells/Roux. For in vitro differentiation by 5-azacytidine (Aza) treatment, UCB-MSCs were induced in DMEM supplemented with 10 % FBS, 1 % Penicillin/Streptomycin, 10 μM 5-Aza, 50 ng/ml activin A, and 0.1 mM ascorbic acid for 24 h. Then, the cells were washed in PBS twice and cultured in DMEM plus 15 % FBS, 1 % Penicillin/Streptomycin, 50 ng/ml activin A, and 0.1 mM ascorbic acid without Aza to avoid cell damage caused by long-term exposure to Aza. Fresh medium was replaced every 3 days for a total duration of 36 days. For in vitro differentiation by Aza and fetal heart extract (Aza + HE) treatment, the Aza amount was reduced

to 5 μM and 36 $\mu\text{g/ml}$ of mouse fetal HE was added to the medium.

The differentiation results were assessed based on four criteria: changes in cell morphology during differentiation, beating ability of induced cells, the expression of myocardial specific genes, and the expression of myocardial specific protein (sarcomeric α -actin protein).

Analysis of cell morphology changes and beating ability

Changes in cell phenotype and ability to beat without external stimulation were observed and recorded using an inverted microscope at the time points 0, 9, 18, 27, and 36 days after differentiation.

RT-PCR analysis

The expression of myocardial specific genes was analyzed at different time points: 0, 9, 18, 27 and 36 days during the differentiation process. Briefly, total RNA was extracted from cells of the control group, Aza group, or Aza + HE group at the different time points using an easy-BLUETM Total RNA Extraction Kit (iNtRON Biotechnology, Gyeonggi-do, Korea) according to the manufacturer's instructions. cDNA was synthesized from RNA using the Enhanced Avian First Strand Synthesis kit (Sigma-Aldrich) according to the manufacturer's protocol. PCR reactions were performed using iTaq DNA Polymerase (iNtRON Biotechnology) with primer sequences such as in Table 1. The following genes were examined: GATA binding protein 4 (GATA4), NK2 homeobox 5 (Nkx 2.5), myocyte enhancer factor 2C (Mef2c), potassium/sodium hyperpolarization-activated cyclic nucleotide-gated ion channel 2 (HCN2), brain natriuretic peptide coding gene (hBNP), α cardiac actin (α -Ca), cardiac troponin T (cTnT), Desmin (Des), and beta-myosin heavy chain (β -MHC). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Electrophoresis was performed on the RT-PCR products on a 2 % agarose gel at 100 V for 60 min. A 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used. The results were observed and recorded using the electrophoresis Gel Doc IT system (UVP, Upland, CA, USA). To quantify the expression of the gene of interest, RT-PCR products on a 2 % agarose gel after electrophoresis were analyzed for

Table 1 Primer sequences used in this study

Gene	Primer (5'–3')	Annealing temperature (Ta) (°C)	Cycles	Product size (bps)
GATA4	F: GACGGGTCACCTATCTGTGCAAC R: AGACATCGCACTGACTGAGAAC	55.5	30	475
Nkx2.5	F: CTTCAAGCCAGAGGCCTACG R: CCGCCTCTGTCTTCTCCAGC	54.5	30	233
Mef2c	F: CTGGGAAACCCCAACCTATT R: GCTGCCTGGTGGAATAAGAA	60	30	300
HCN2	F: CGCCTGATCCGCTACATCCAT R: AGTGCGAAGGAGTACAGTTCAC	61	30	230
hBNP	F: CATTTGCAGGGCAAACCTGTC R: CATCTTCCTCCCAAAGCAGC	61	30	206
α -Ca	F: TCTATGAGGGCTACGCTTTG R: GCCAATAGTGATGACTTGGC	54	30	260
cTnT	F: AGAGCGGAAAAGTGGGAAGA R: CTGGTTATCGTTGATCCTGT	55	30	235
Des	F: CCAACAAGAACAACGACG R: TGGTATGGACCTCAGAACC	54	30	408
β -MHC	F: GATCACCAACAACCCCTACG R: ATGCAGAGCTGCTCAAAGC	61	30	528
GAPDH	F: GTCAACGGATTTGGTCTGATTG R: CATGGGTGGAATCATATTGGAA	61	30	139

band density using the ImageJ software (NIH). The band density of the genes of interest between three groups (Control, AZA, and AZA + HE) was normalized to GAPDH.

Immunocytochemistry (ICC)

The cells were cultured on coverslips (Santa Cruz Biotechnology, Dallas, TX, USA) and prepared for ICC staining. Cells were fixed in 4 % paraformaldehyde solution (Santa Cruz Biotechnology) for 15 min and washed twice with PBS. Then, the samples were permeabilized for 10 min using PBS containing 0.25 % Triton X-100 (Sigma-Aldrich). They were then washed three times in PBS and blocked with 1 % BSA in PBS for 30 min. Next, the samples were incubated with the primary antibody, sarcomeric α -actin (1:400, Sigma-Aldrich), overnight at 4 °C. After 3 washes, the samples were incubated with the secondary antibody, rabbit anti-mouse IgG (1:500, Santa Cruz Biotechnology), and Hoechst 33342 (1:500, Sigma-Aldrich) for 45 min at room temperature. They

were rinsed three times with PBS, mounted, sealed with nail polish, and observed under a fluorescent microscope (Zeiss Axiovert, Oberkochen, Germany).

Results

Characterization of UCB-MSCs

UCB-MSCs were isolated from human UCB on the basis of cell phenotype selection, the expression of MSC-specific markers, and the ability to differentiate into adipocytes. After culturing in selection medium for 48 h, the UCB-MSC candidates attached and extended on the flask surface. From days 7 to 12, the cells proliferated and spread over the flask surface. In the secondary culture, the cells became fibroblast-like, spindle-shaped cells (Fig. 1a). UCB-MSC candidates were collected at passage 3 to detect the expression of MSC-specific markers by flow cytometry analysis. The candidate cells were negative for CD14, CD34, CD45, and HLA-DR; and positive for CD13, CD44,

Fig. 1 Mesenchymal stem cells isolated from umbilical cord blood. MSCs exhibited a fibroblast-like shape (a), successfully differentiated into osteoblasts (b), adipocytes (c), and expressed

the specific marker profiles of MSCs such as positive with CD13 (d), CD44 (g), CD73 (i), CD90 (j), and negative with CD14 (e), CD34 (f), CD 45 (h), HLA-DR (k)

CD73 and CD90 (Fig. 1). UCB-MSCs also showed the ability to differentiate into osteoblasts that were positive with alizarin red staining (Fig. 1b) and adipocytes that were positive with oil red (Fig. 1c). The analysis indicated that the candidate cells obtained from umbilical cord blood were MSCs.

The morphological changes of UCB-MSCs during differentiation

To assess the ability of UCB-MSCs to differentiate into cardiomyocytes using various induction media, the morphology of the cells was recorded at different

time points: 0, 9, 18, 27 and 36 days after differentiation. After treatment with inducers, a number of detached cells died whereas the adherent cells survived and continued to proliferate and differentiate. At day 0, cells of the control group, Aza group and Aza + HE group were spindle-shaped (Fig. 2a–c). At day 9, the cells of the Aza group maintained their MSC phenotype while the cells of the Aza + HE group showed little change in their shape. Specifically, several cells spread and seemed to begin to curl up (red arrow, Fig. 2f) while other cells elongated (blue arrow, Fig. 2f). At day 18, there were some cells that began rounding in the Aza group (red arrow, Fig. 2h).

Fig. 2 Phenotypic changes in induced cells. Induced cells changed their morphology during differentiation in both the Aza group (b, e, h, k, n) and the Aza + HE group (c, f, i, l, o).

This was different from the morphology of the cells in the control group. The changes in cell appearance were more noticeable in the Aza + HE group. Some

However, there was no change in cellular morphology observed in the control group (a, d, g, i, m). Scale bars 50 µm

cells of the Aza + HE group had a tube-like shape (blue arrow, Fig. 2i) and others were circular (red arrow, Fig. 2i). At day 27, in the Aza group some cells

Fig. 3 The expression of cardiomyocyte-specific genes in induced cells compared with UCB-MSCs. During differentiation from days 0 to 36, RT-PCR analysis showed the up-regulation of cardiac marker expression in induced cells. GAPDH was used as a housekeeping gene

formed a tube-like shape (blue arrow, Fig. 2k) and several cells had a round-like shape (red arrow, Fig. 2k) whereas in the Aza + HE group, the cells tended to connect with adjacent cells (Fig. 2l). At day 36, binuclear cells appeared in the Aza group (Fig. 2n) and the cells tended to gather together in the Aza + HE group (Fig. 2o). During the differentiation process, the UCB-MSCs of the control group did not change their morphology (Fig. 2a, d, g, j, m). This shows that there were differences in morphology between induced and control cells.

Cardiomyocyte-specific gene expression

Mef2c, *HCN2* and *hBNP* were expressed in both control and induced cells. However, they were more highly expressed in induced cells, and their expression increased from days 0 to 36 in the differentiated groups: Aza and Aza + HE (Figs. 3, 4 and Figure 1S). The transcription factor, *GATA4*, and structure gene, α -Ca, were not expressed in the control group but appeared in both the Aza and the Aza + HE groups on day 18 and increased their expression until day 36. Another transcription factor, *Nkx 2.5*, and structure gene, β -MHC, also were not expressed in the

control group. They were expressed in the Aza group on day 27 and were expressed earlier in the Aza + HE group on day 18. *cTnT* and *Des* were also not expressed in the control group. They were expressed in the Aza group on day 18 and day 9 in the Aza + HE group. *GAPDH*, an internal control gene, was expressed in all examined samples. In summary, the UCB-MSCs themselves expressed some cardiomyocyte genes such as *Mef2c*, *HCN2* and *hBNP*. The cells of the Aza group expressed all surveyed myocardial specific genes on day 27 of the differentiation process while the cells of the Aza + HE group expressed these genes earlier on day 18. The results suggest that mouse fetal HE accelerates the differentiation of UCB-MSCs into cardiomyocytes.

Expression of cardiomyocyte-specific proteins

Cells of the Aza and Aza + HE groups were stained with sarcomeric α -actin antibody at 0, 9, 18, 27 and 36 days after induction. The results indicated that the Aza group cells stained positive for sarcomeric α -actin on day 27 (Fig. 5k), some cells began to form multinuclear morphology (yellow arrow, Fig. 5k), and binuclear cells appeared on day 36 (yellow arrow,

Fig. 4 Relative expression of cardiomyocyte-specific genes in cells normalized to GAPDH. Cardiomyocyte-specific gene expression was evaluated on day 0 (a), day 9 (b), day 18 (c), day 27 (d) and day 36 (e). In combination with AZA, HE facilitated the cTNT and Des expression on day 9, and β -HMC on day 18. On day 27 and 36, these cardiomyocyte-specific

genes were expressed in both groups: AZA and AZA + HE. Control: cells untreated with differentiation factor (AZA) or HE; AZA: cells were differentiated by 5-azacytidine; AZA + HE: cells were differentiated by 5-azacytidine and murine fetal heart extract

Fig. 5n). When compared to the Aza group, the cells of the Aza + HE group expressed sarcomeric α -actin protein earlier, on day 18 (Fig. 5i). In addition, the Aza + HE cells associated with adjacent cells to form clusters from days 27 to 36 (Fig. 5l, o). Thus, at the protein level, the cells that were differentiated by Aza + HE expressed the cardiomyocyte-specific protein, α -actin, earlier than the cells differentiated using only Aza. The control cells, UCB-MSCs, did not show any evidence of sarcomeric α -actin expression (Fig. 5a, d, g, j, m) despite the expression of several genes specific to cardiomyocytes such as Mef2c, HCN2 and hBNP (Fig. 3, Fig. 4, Fig. 1S).

Contraction capacity of induced cells

Although differentiated cells expressed genes and protein specific for cardiomyocytes, they were not observed to beat spontaneously.

Discussion

The results show that UCB-MSCs can be differentiated into cardiomyocyte-like cells by medium supplemented with 5-azacytidine alone and 5-azacytidine plus fetal HE. However, cell differentiation occurs earlier in the Aza + HE group. Specifically, when induced by Aza alone, the cells began to express cardiomyocyte-specific genes and protein on day 27 of differentiation compared to day 18 in the Aza + HE group.

It is known that adult stem cells are maintained in an inactive state. They participate in the self-renewal process and differentiate only when induced by suitable stimuli. The reduction of histone acetylation leads to the phenomena of methylation and chromatin compaction that can silence gene expression in stem cells (Christman 2002; Yoshida et al. 1995). Aza is a synthetic pharmaceutical product

Fig. 5 The expression of the cardiomyocyte-specific protein in induced cells compared with UCB-MSCs. Immunofluorescence staining of induced cells revealed expression of cardiac marker, α -actin, on day 18 in the Aza + HE group and on day 27 in the

Aza group (red). Nuclei were stained with Hoechst 33342 (blue). There was no sarcomeric α -actin stain observed in the control group. Scale bars 50 μ m. (Color figure online)

capable of inhibiting DNA methylation (Palii et al. 2008). Therefore, Aza has been used to induce differentiation of stem cells into myocardial cells (Supokawej et al. 2013). Our study confirmed that UCB-MSCs can be induced to differentiate into myocardial cells using Aza.

During the differentiation, the Aza cells changed in morphology. The cells began rounding on day 18. By day 27, there were both round- and tube-shaped cells observed, and the appearance of binuclear cells was observed on day 36. This phenotypic change occurred earlier in the Aza + HE group. The Aza + HE cells began changing their shape on day nine instead of day 18. Similar to the Aza cells, some cells of the Aza + HE group also exhibited both round- and tube-like morphology. In contrast to the Aza group, on day 27 the Aza + HE cells tended to connect to form clusters. This cell clustering reflects the morphological changes that occur when cells differentiate into cardiomyocytes *in vivo*. During the development, early embryonic cells are circular (Baharvand et al. 2006). Incidentally, unorganized myofibrils of early myocardial cells are organized to form characteristic bands such as I, A and Z bands following the stages of embryonic development (Chacko 1976). From 3 to 4 weeks after birth, the cells elongate and form a tube-like shape (Angst et al. 1997). From 6 to 8 weeks after birth, the T-tube appears in adult myocardial cells (Baharvand et al. 2006; Ziman et al. 2010). Labovsky and colleagues differentiated human bone marrow-derived MSCs into cardiomyocytes by Aza or Strep-tolysin O with cardiac extract from neonatal rat cardiomyocytes (SLO + EX) (Labovsky et al. 2010). In their study, differentiated cells also changed their morphology but no differences between the two groups were observed. After 7 days of exposure, some round cells were found but no elongated cells were observed as in our study. At day 14, the induced cells elongated in one direction and formed a stick-like phenotype similar to the Aza + HE cells. From days 21 to 28, the induced cells connected with neighboring cells. Similar to the published results, the cells of the Aza + HE group also exhibited the cell–cell connection phenomena on day 27. In another study, Zhao et al. differentiated hMSCs into myocardial cells using protein phosphatase Slingshot-1 (SHH1L) gene transfection. The SHH1L-transfected cells increased in size and extended in the same direction at the beginning of differentiation. This was similar to the

behavior of the Aza + HE cells on day 9. Afterwards, SHH1L-transfected cells connected to adjacent cells, similar to what was observed in the cells of the Aza + HE group on day 27 (Zhao et al. 2012). The UCB-MSCs of the control group did not change their morphology during the experiment. Thus, we can provide an overview of the phenotypic changes of induced stem cells during the differentiation process. In the early period of the differentiation process, the cells shrink or stretch out. Then, in later periods, they form a circular or tubular phenotype and connect to adjacent cells.

Along with the phenotype changes, there was a change in gene expression in the induced cells. UCB-MSCs themselves have expressed transcription factor *Mef2c*, the *HCN2* gene coding for the potassium/sodium hyperpolarization-activated cyclic nucleotide-gated ion channel 2 protein related to sinoatrial node activities (Hofmann et al. 2005), and the hBNP-gene coding for Brain Natriuretic Peptide related to blood pressure reduction. Expression levels of these genes in differentiated cells increased chronologically in the Aza and Aza + HE groups. This result is consistent with the conclusions of Labovsky et al. (2010) and Mastitskaya and Denecke (2009). Mastitskaya and Denecke theorized that several cardiomyocyte genes are available in stem cells. Moreover, cells of the Aza + HE group expressed all surveyed genes on day 18. The Aza group cells did not express these genes until day 27. The transcription factor, *Nkx 2.5*, and structure gene, β -MHC, were expressed in the Aza + HE cells on day 18 and were not expressed in the Aza cells until day 27. Similarly, cTnT and Des were expressed in the Aza + HE cells on day 9 and not expressed in the Aza cells until day 18. The rest of the surveyed genes such as GATA4 and α -Ca were expressed on day 18 in both groups. *Nkx 2.5*, β -MHC, cTnT, Des, GATA4 and α -Ca were not expressed in the UCB-MSCs of the control group. These results are different from what has been reported in previous publications, especially in regards to the expression of GATA4. In our analysis, GATA4 was not expressed in the UCB-MSCs and was only expressed in induced cells at 18 days. In the Labovsky study, GATA4 was strongly expressed in both control MSCs and induced cells exposed to the neonatal rat HE. This expression was observed in MSCs themselves, so the use of the extract may not be a factor affecting GATA4 expression. In addition, Connell et al. reported that stem cells

derived from amniotic fluid did not express GATA4 even when exposed to rat HE (Connell et al. 2013). Thus, the combination of Aza and fetal mouse HE resulted in enhanced differentiation by allowing for GATA4 expression.

In addition, according to some recent publications, the combination of Aza with BMP2 and Wnt1, the combination of Aza and Angiotensin II, or co-culturing stem cells with myocardial cells in inducing medium with Wnt1 or BMP2 has resulted in enhanced differentiation of stem cells into myocardial cells when compared to using each ingredient separately (Hou et al. 2013; Xing et al. 2012; Zhang et al. 2012). Consistent with these reports, our study also showed that the addition of mouse fetal HE into Aza induction medium promoted the expression of cardiomyocyte-specific genes. Furthermore, the analysis of stem cell differentiation at the protein level also corresponded to the gene expression analysis. Myocardial specific protein, α -actin, was expressed in the induced cell groups. Particularly, the cells of the Aza + HE group expressed sarcomeric α -actin on day 18 after induction. This was earlier than in the Aza cells where sarcomeric α -actin expression was not observed until day 27.

These results may be attributed to the addition of mouse fetal HE. First of all, NO (nitric oxide) is in the fetal or neonatal myocardium (Massion et al. 2004). It plays an important role in the formation of heart muscle cells *in vivo* (Massion et al. 2004). NO promotes neonatal cardiomyocyte proliferation by inhibiting TIMP-3 expression through S-nitrosylation of AP-1 (Hammoud et al. 2007). cGMP-mediated NO signaling also plays an essential role in the differentiation of ES cells into myocardial cells (Mujoo et al. 2008). Second, rat fetal heart extract contains RA (retinoic acid) (DeJonge and Zachman 1995). RA is known to accelerate embryonic stem cell-derived cardiac differentiation and enhance development of Ventricular cardiomyocytes (Wobus et al. 1997). RA deficiency causes reductions in Fgf signaling reducing myocardial differentiation (Lin et al. 2010). In another signaling pathway, RA induces hepatic EPO resulting in IGF2 signaling activation leading to cardiomyocyte proliferation (Brade et al. 2011). Finally, some transcription factors were proven to enhance the cardio-inducing effect of GATA4, TBX5, MEF2C during the differentiation such as

MYOCD, SRF, Mesp1 and SMARCD3 (Christoforou et al. 2013).

Although induced cells expressed myocardial-characteristic genes and protein α -actin, they were not observed to beat spontaneously during differentiation. This result is similar to the observations made by Siegel et al. (2012) when differentiating BM-MSCs into cardiomyocytes and by Mastitskaya and Denecke (2009) when differentiating human spongiosa mesenchymal stem cells. However, some studies have reported that induced cells can beat after differentiation. Cao et al. (2011) induced rat embryonic stem cells into cardiomyocytes, and after differentiation the cells were capable of beating randomly. Similarly, Kuzmenkin et al. (2009) differentiated iPSCs into cardiomyocytes, and the induced cells demonstrated beating ability. Liao et al. (2012) offered the explanation that differentiated cells derived from multipotent stem cells have a poor sarcomere organization and therefore lack the ability to beat repeatedly. *In vivo*, myocardial cells undergo three developmental stages including a direction stage, precursor stage and mature stage. In the precursor stage, stem cells differentiate into myocardial progenitor cells. These cells express transcription factors such as Nkx 2.5, Mef2c, GATA 4 and a number of structure genes such as actin, troponin, and desmin. In the mature stage, the muscle pulsating structure, muscle fiber structure, tubular structure, and ion channels are formed to stimulate contractile activity (Chen et al. 2008). Hence, the differentiated cells in our study may be in the myocardial progenitor stage. The differentiated cells in this study had the same phenotypic changes observed in myocardial cell development and expressed the same gene and protein characteristics of cardiomyocytes. Specifically, the Aza + HE cells also tended to connect with adjacent cells to form cell clusters, but the induced cells did not beat randomly. To stimulate cells to beat like mature myocardial cells *in vivo* other factors may be needed. However, maintaining differentiated cells in a precursor stage prior to beating may be favorable for cell transplantation. It is known that the transplantation of cells with beating potential into the heart may cause arrhythmia (Gillum and Sarvazyan 2008; Menasché et al. 2008). We hypothesize that induced cells in the myocardial progenitor stage that do not beat can fuse with local cells, mature, and have the same

rhythm as the local beating cells when transplanted into the heart.

Conclusion

We have succeeded in differentiating UCB-MSCs into myocardial-like cells using two different induction media. Use of Aza plus mouse fetal HE inducing medium allowed for accelerated differentiation when compared to using Aza alone. After differentiation, the induced cells expressed genes and sarcomeric α -actin protein characteristic to cardiomyocytes but did not beat spontaneously, exhibiting the same properties as myocardial progenitor cells in vivo. These results contribute to research on the developmental biology of the heart by examining the differentiation potential of stem cells into cardiomyocytes. Understanding the development of the heart at the cellular level is critical for developing new methods for heart disease treatment.

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Conflict of interest The author's declare that they do not have any competing or financial interests.

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