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WORD COUNT	4203	TIME SUBMITTED	01-AUG-2022 02:15PM
		PAPER ID	89041470

Overexpression of GATA binding protein 4 and myocyte enhancer factor 2C differentiate mesenchymal stem cells into cardiac-like cells

Razzaq SS *et al*. Differentiation of MSCs into the cardiomyocytes

Syeda Saima Razzaq, Irfan Khan, Nadia Naeem, Asmat Salim, Sumreen Begum, Kanwal Haneef

Abstract

BACKGROUND

Heart diseases are the primary cause of death all over the world. Following myocardial infarction, billions of cells die resulting in a huge loss of cardiac function. The discovery of stem cell-based therapies has appeared as a new area to support heart regeneration. The GATA binding protein 4 (GATA-4) and myocyte enhancer factor 2C (MEF2C) transcription factors are considered prominent factors in the development of the cardiovascular system.

AIM

To explore the potential of GATA-4 and MEF2C for the cardiac differentiation of human umbilical cord mesenchymal stem cells (hUC-MSCs).

METHODS

hUC-MSCs were characterized morphologically and immunologically by the presence of MSC's specific markers *via* immunocytochemistry, flow cytometry, and by their potential to differentiate into osteocytes and adipocytes. hUC-MSCs were transfected with GATA-4, MEF2C, and their combination to direct the differentiation. Cardiac differentiation was confirmed by semi quantitative real-time polymerase chain reaction and immunocytochemistry.

RESULTS

hUC-MSCs expressed specific cell surface markers CD105, CD90, CD44, and vimentin but lack the expression of CD45. Transcription factors GATA-4 or MEF2C, and their combination induced differentiation in hUC-MSCs with significant expression of cardiac genes *i.e.*, *GATA-4*, *MEF2C*, *NK2 homeobox 5* (*NKX2.5*), *MHC*, and *connexin-43*, and cardiac proteins GATA-4, NKX2.5, cardiac troponin T (cTnT), and connexin-43.

CONCLUSION

Transfection with GATA-4, MEF2C, and their combination, effectively induce cardiac differentiation in hUC-MSCs. These genetically modified MSCs could be a promising treatment option for heart diseases in the future.

Key Words: Heart disease; GATA binding protein 4; Myocyte enhancer factor 2C; Transcription factors; Differentiation; Human umbilical cord-mesenchymal stem cells

Razzaq SS, Khan I, Naeem N, Salim A, Begum S, Haneef K. Overexpression of GATA binding protein 4 and myocyte enhancer factor 2C differentiate mesenchymal stem cells into cardiac-like cells. *World J Stem Cells* 2022; In press

Core Tip: Transcription factors have great potential to direct cell fate decisions during embryonic development. In this study, we investigated the overexpression of cardiac transcription factors in human umbilical cord mesenchymal stem cells to enhance their differentiation into cardiac-like cells. The synergistic effect of GATA binding protein 4 and myocyte enhancer factor 2C transcription factors increased the expression of cardiac genes and proteins. The results of this study will aid in the development of new therapeutic strategies aimed at curing heart diseases.

INTRODUCTION

Heart failure is the most challenging issue after myocardial infarction[1,2]. The environmental and genetic risk factors cause the deregulation of cardiomyocytes, and

endothelial, smooth muscle, and inflammatory cells of heart tissue[3]. Cardiomyocytes largely fail in adult life to divide or enter the cell cycle[4,5]. Therefore, the adult heart has a limited endogenous repair and regeneration mechanism[6-8]. Current interventions rely on heart transplantation, mechanical assistance devices, and medicinal therapies for the management of damaged organ. However, these options cannot revert the normal functioning of the heart. The future therapeutic strategy for cardiac diseases is to regenerate damaged tissue for restoring complete heart function[9,10].

Cell based therapies are promising for damaged heart tissue. Stem cells possess the remarkable potential to stimulate endogenous myocardial repair and regeneration processes[11-15]. However, the low viability of transplanted stem cells due to inadequate supply of blood and inflamed myocardium has been a major challenge[12-14]. The adult mesenchymal stem cells (MSCs) have the potential to make bone, muscle, nerve, cardiac and fat cells[16,17]. Furthermore, MSCs help in the formation of new blood vessels, induce apoptotic resistance and provide anti-fibrotic effects[18,19]. One of the recently employed innovative approaches is the use of forward programming with tissue type-specific transcription factors for the differentiation of stem cells[20].

The successful cell fate reprogramming requires a temporospatial expression pattern of transcription factors[21]. Heart development is a complex process that requires the coordination of a series of events such as specification, proliferation, and differentiation[22]. The cardiac transcription factors, including GATA binding protein 4 (GATA-4), myocyte enhancer factor 2A (MEF2A), NK2 homeobox 5 (NKX2.5), and serum response factor (Srf) have a paradoxical role in the differentiation and homeostasis of myocardial cells[23]. It has been documented that three cardiac transcription factors, GATA-4, NKX2.5, and T-Box transcription factor 5 (TBX5) programmed extra-cardiac mesoderm of mouse embryo into cardiac tissue[24]. Also, a combination of GATA-4, NKX2.5, TBX5, and BAF60C can differentiate embryonic stem cells (ESCs) into cardiac lineage[25]. Altogether, these research studies display that transcription factor mediated stem cell reprogramming is a valuable strategy that directs cardiomyogenic differentiation of various stem cell types.

The current study aimed to examine the effects of overexpressing two cardiac transcription factors, GATA-4 and MEF2C in cardiac differentiation of human umbilical cord MSCs (hUC-MSCs). After introducing the transcription factors either individually or in combination, hUC-MSCs were analyzed for the expression of cardiac genes and proteins. These genetically modified MSCs could be a promising treatment option for cardiovascular diseases.

MATERIALS AND METHODS

Ethics committee approval

The current research project was approved by the institutional bioethical committee, University of Karachi (ICB, UoK) under protocol #: ICB KU-92/2020.

Human umbilical cord collection

Human umbilical cords (n = 12) were collected from healthy pregnant females at the Dow University of Health Science, OJHA campus, Karachi, Pakistan after taking the consent from the donors.

Isolation and propagation of hUC-MSCs

Human umbilical cord tissue was longitudinally cut and thoroughly washed with sterile phosphate-buffered saline (PBS). The human cord tissue was cut into 2-5 mm in size and placed in 1 × (0.25%) trypsin (GIBCO, United States) for 20 min at 37 °C. Partially digested cord tissues were kept in a T-25 tissue culture flask having 3-5 mL of DMEM (GIBCO, United States) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin, and 1 mmol sodium pyruvate. Explants were placed at 37 °C with 5% CO_2 (Heracell, United States). The medium was changed after every third day. MSCs attached to the tissue culture flask during 15-20 d of the first culture. After adhesion of the MSCs, tissues were discarded and fresh DMEM was added for the proliferation of cells. Once the MSCs reached 70% to 80% confluence, they were detached using 1 × (0.25%) trypsin. P1 to P2 hUC-MSCs were used for experiments.

Immunocytochemistry

Isolated hUC-MSCs were characterized by immunocytochemistry to detect the MSC specific markers. Briefly, 4% paraformaldehyde (PFA) was added to the cells and then incubated with 0.1% Triton X-100. The permeabilized cells were then kept in a blocking solution for 1 h. After incubation, the solution was discarded and cells were kept at 4 °C with anti-mouse primary antibodies CD90, CD105, vimentin, CD44, and CD45. After overnight incubation, cells were thoroughly washed 4-5 times with PBS. Alexa fluor 488 goat anti-mouse secondary antibody was added to each well. The negative control cells were incubated only with secondary antibody. DAPI (4′,6-diamidino-2-phenylindole) was used to stain the cell nuclei. Lastly, cells were mounted and observed under a fluorescence microscope (NIE, Nikon, Japan).

Flow cytometry

MSCs were washed 2-3 times with PBS and incubated with dissociation buffer at 37 °C for 40 min. The cells were pelleted down through centrifugation and then the cell pellet was mixed in FACS solution containing 1% BSA, 1 mmol EDTA, and 0.1% Na-azide. The tubes were centrifuged for 5 min and then the blocking solution was added to all the tubes. Primary antibodies including CD44, CD90, and CD73 were added and tubes were incubated at 4 °C. After washing with FACS solution, Alexa fluor 488 goat anti-mouse secondary antibody was added. Unlabeled and isotype labeled cells were used as controls. Data was analyzed using BD FACS Diva software.

Adipogenic and osteogenic differentiation

Approximately, 4 × 10^5 hUC-MSCs were seeded in a 6-well plate for 24 h. After confirming cell proliferation, cells were washed with sterile PBS. For osteogenesis, low glucose DMEM supplemented with 10 mmol glycerol-2-phosphate, 0.2 mmol ascorbic acid, 0.1 µmol dexamethasone, 10% FBS, 100 µg/mL streptomycin, 100 units/mL penicillin, and 2 mmol L-glutamine were added into the cell culture plate. The medium

was replaced every 4th day till 21 d. After the completion of 21 d incubation period, the ice cold 75% ethanol was used for cell fixation, and then the cells were stained with 2% Alizarin stain.

For adipogenesis, hUC-MSCs were cultured in adipogenic induction and maintenance medium for 21 d. Adipogenic induction medium contains 10 µg/mL insulin, 100 µmol indomethacin, 1 µmol dexamethasone, 10% FBS, 100 µg/mL streptomycin, and 100 units/mL penicillin in low glucose DMEM. After 21 d, 4% PFA was used for cell fixation and then cells were stained with 0.5% Oil Red O. Finally, images were taken under phase contrast microscope (CKX41, Olympus, Japan).

hUC-MSC transfection

GATA-4 and MEF2C plasmids were purchased from Addgene (plasmid No. 46030 and No. 46031, respectively). Plasmid DNA was isolated by using a maxiprep plasmid DNA isolation kit (Thermo Scientific, United States). Briefly, *Escherichia coli* were harvested by centrifugation at 5000 × g. The pellet was mixed in resuspension solution and then lysis solution was added. The suspension was incubated at room temperature for 3 min and a neutralization solution followed by endotoxin binding reagent was added to the tube. The tube was incubated at room temperature for a further 5 min and 96% ethanol was added. The supernatant was collected through centrifugation and mixed with 96% ethanol and then shifted to the purification column. The tube was centrifuged at 2000 × g for 3 min. Wash solution 1 was added to the column and centrifuged at 3000 × g. This step was repeated with wash solution 2. The plasmid DNA was eluted in elution buffer and quantified using a nano-drop spectrophotometer. hUC-MSCs were transfected separately with GATA-4 and MEF2C, and co-transfected with 1 µg each of GATA-4 and MEF2C plasmids using lipofectamine 3000 kit (Invitrogen, United States). Briefly, the plasmid vector (1 µg for GATA-4 or MEF2C) was diluted in serum free DMEM, and 2 µL of P3000 reagent was added per 1 µg of plasmid DNA. Lipofectamine TM 3000 reagent and DNA were mixed and kept at room temperature for 15 min. The 70%-80% confluent cells were incubated with DNA-lipid complex at 37 °C for 24 h. After 24 h,

lipofectamine was replaced with FBS containing DMEM. The cells were kept for two weeks at 37 °C using an air jacketed CO_2 incubator. The medium was changed after every 3 d to 4 d. The following experimental groups were used in this study; untreated control, GATA-4 transfected, MEF2C transfected, and combination group of GATA-4 + MEF2C transfected hUC-MSCs.

Gene expression analysis of transfected hUC-MSCs

The overexpression of the *GATA-4* and *MEF2C* genes in transfected hUC-MSCs were confirmed by quantitative real-time polymerase chain reaction (RT-PCR). The RNA from transfected and control hUC-MSCs was extracted using the TRIzol method. For RNA isolation, cells were harvested and the pellet was gently mixed with TRIzol reagent. In the next step, chloroform was added to the tube and incubated at room temperature for 15 min. The cell suspension was centrifuged at 12000 × g for 15 min. Isopropyl alcohol was added to the separated aqueous phase followed by centrifugation at 12000 × g. The RNA pellet was air dried and then resuspended in RNAase-free water. The RNA absorbance was calculated at 260 nm. The complementary DNA was synthesized using cDNA synthesis kit (Invitrogen, United States) and then amplified using primers corresponding to *GATA-4* and *MEF2C* genes. The human *beta-Actin* was used as a housekeeping gene. Reverse transcription reaction products were initially denatured for 30 s at 94 °C, followed by 40 cycles of amplification: denaturation at 94 °C for 3 s and annealing at 60°C for 3 s. Primer sequences and melting temperatures of each gene are enlisted in Table 1.

Analysis of cardiac genes and proteins

For gene expression, RT-PCR of untreated and transfected hUC-MSCs was performed at day 14 of transfection. For cardiac protein expression, immunocytochemistry of untreated and transfected hUC-MSCs was performed also on day 14 of transfection. Primary antibodies for cardiac specific proteins i.e. GATA-4, connexin-43, NKX2.5, and cTnT were used. The negative control cells were incubated only with the secondary

antibody. Finally, cells were mounted and images were taken under a fluorescence microscope (NIE, Nikon, Japan). The fluorescence intensities were calculated through Image J software (NIH, United States).

Statistical analysis

The data was analyzed by using IBM SPSS Statistics 20 software. One way ANOVA and Tukey's post hoc test were used for multiple groups comparisons. All data were collected from three independent experiments. *P*-value less than 0.05 ($^{a}P < 0.05$) was considered statistically significant.

RESULTS

Morphological features of MSCs derived from human umbilical cord tissue

Adherent cells started to grow during 15 d to 20 d of isolation and are termed passage zero cells (P0 cells), as shown in Figure 1. The P0 cells were sub-cultured once they reached 80% confluence and termed passage one cells (P1 cells). The hUC-MSCs appeared in colonies and showed fibroblast-like morphology (Figure 1). P1 to P2 passage cells were used in this study.

Characterization of hUC-MSCs

Immunocytochemistry analysis showed positive expression of MSCs markers CD105, CD90, CD44, and vimentin; while, a hematopoietic marker, CD45 did not express in these cells (Figure 2A). The immunophenotypic analysis showed that the expression was positive for CD90, CD73, and CD44 in hUC-MSCs (Figure 2B). The osteogenic and adipogenic differentiation was confirmed, respectively by Alizarin Red staining which revealed mineral deposits, and Oil Red O staining which revealed lipid droplets (Figure 2C).

Molecular analysis of transfected hUC-MSCs

hUC-MSCs were successfully transfected with *GATA-4* and *MEF2C* genes. RT-PCR analysis showed a significant increase in *GATA-4* and *MEF2C* expression after 24 h of transfection compared with control (Figure 3).

Morphological changes and gene expression analysis in transfected hUC-MSCs

After 14 d in culture, the transfected cells displayed extended cytoplasmic processes and myotube like structure which are the typical features of cardiomyocytes (Figure 4A). Gene expression pattern of hUC-MSCs transfected with GATA-4, MEF2C, and their combination showed significant expression of cardiac genes, *MEF2C*, *NKX2.5*, *GATA-4*, *connexin-43*, and *myosin heavy chain* (*MHC*) (Figure 4B). Moreover, gene expression of the combination group for the evaluation of the synergistic effect of both transcription factors showed significant expression of cardiac genes as compared to the individual groups (Figure 4B).

Expression of cardiac proteins in transfected hUC-MSCs

Cardiac differentiation of transfected hUC-MSCs was further confirmed using immunocytochemistry. hUC-MSCs treated with GATA-4, MEF2C or their combination exhibited positive expression of cardiac specific proteins, including GATA-4, connexin-43, and NKX2.5 as compared to the untreated control at day 14 (Figure 5A). Moreover, the fluorescence intensity of hUC-MSCs treated with GATA-4, MEF2C, and their combination was also calculated using Image J software. Statistical analysis showed significant up-regulation of GATA-4, connexin-43, and NKX2.5 in all three treatment groups as compared to the untreated control. However, late cardiac marker cTnT was not up-regulated at day 14 (Figure 5B).

DISCUSSION

This study determined the effects of two cardiac transcription factors, GATA-4 and MEF2C on the differentiation of hUC-MSCs towards cardiac lineage *in vitro*. GATA binding protein-4 (GATA-4) is an important transcription factor that regulates the cell

proliferation, survival, and fate commitment of many cell types[26]. Moreover, GATA-4 plays a vital role in the process of heart development[27]. The myocyte enhancer factor 2C (MEF2C) acts as a transcriptional regulator in cardiovascular growth[28]. It is demonstrated by various studies that MEF2C acts together with GATA factors to induce gene transcription in cardiomyocytes[27]. Based on their widely documented role in the structure and function of the heart, we hypothesized that GATA-4 and MEF2C overexpression may have the potential to differentiate hUC-MSCs into cardiac-like cells.

In this study, hUC-MSCs were isolated by the explant method[29]. The characterization studies of isolated cells were performed according to the standard criteria of the International Society for Stem Cell Research (ISSCR)[30]. The isolated cells showed fibroblast-like morphology and positive expression of CD105, CD90, CD44, and vimentin, whereas they lack the expression of hematopoietic marker CD45. MSCs specific markers CD73, CD90, and CD44 were also verified by flow cytometry analysis. Moreover, cord derived MSCs showed adipocytes and osteocytes differentiation potential. The results of our study confirmed that the cord derived cells possess the main characteristics of MSCs. Next, we analyzed the overexpression of *GATA-4* and *MEF2C* mRNA in control and transfected hUC-MSCs. The expression pattern of GATA-4, MEF2C, and their combination showed that the expression was maximum after 24 h of transfection. Based on this gene expression data, we selected 24 h transfected hUC-MSCs for further experiments. hUC-MSCs were transfected with GATA-4 and MEF2C separately and in combination for 24 h, and then analyzed their cardiac differentiation potential at day 14. We observed elongated cells with extended cytoplasmic processes in the transfected groups in comparison with the control group. The transfected cells have a morphology similar to the cardiomyocytes and these results are also in line with the earlier studies[31,32].

The cardiac differentiation of transfected cells at day 14 was analyzed *via* mRNA expression of early and late cardiac specific markers, such as *GATA-4*, *MEF2C*, *NKX2.5*, *connexin-43*, and *MHC*. Cardiac markers were initiated to express in the GATA-4 and MEF2C transfected cells, while their significant up-regulation was prominent in the

combination group. Cardiac transcription factor GATA-4 facilitates the binding of various transcriptional factors and co-activators including GATA-6, NKX2.5, Srf, MEF2, dHAND, YY1, and NFAT[33]. MEF2C participates in the growth and maturation of myocardial cells with GATA-4[34]. It has been found that the overexpression of transcription factors induces cardiomyocyte differentiation in stem cells[35,36]. The combination of precardiac mesodermal transcription factors (Csx/NKX2.5 and GATA-4) has been reported to induce cardiac differentiation of 9-15c stem cells[37]. It has been found that GATA-4, MEF2C, and TBX5 generated cardiomyocyte like cells from mouse heart fibroblast[38]. The gene expression data proposes that GATA-4, MEF2C, and their combination were capable to direct stem cell fate into cardiomyocytes *in vitro*. Additionally, in the combination group, significantly higher expression of cardiac specific genes indicates their synergistic effect on cardiac differentiation.

To complement gene expression data, we analyzed cardiac specific proteins in GATA-4, MEF2C, their combination, and control groups. GATA-4 and MEF2C combination group showed significant up-regulation of connexin-43, NKX2.5, and GATA-4 proteins at day 14 of transfection. Transcription factor NKX2.5 is expressed at the early and late stages of heart development[39]. The NKX2.5 transcription is regulated by binding with GATA-4 and MEF2C[40,41]. The late stage marker, troponin T regulates cardiac rhythm and maintains thin filaments in cardiac and skeletal muscles[42]. The heart rhythm regulation and coordinated contraction are controlled by a complex network of interconnected cardiomyocytes. Gap junction proteins help cardiomyocytes to communicate with their surrounding cells[43]. Connexin-43 is the major connexin protein involved in the propagation of electrical signals essential for the structural and functional maintenance of cardiac cells[44].

Collectively, the results of the current study demonstrate that hUC-MSCs overexpressing GATA-4 and MEF2C and their (GATA-4 + MEF2C) co-transfection have the potential to generate cardiac-like cells. These genetically modified MSCs may be used as a new therapeutic approach for the regeneration of heart tissue.

CONCLUSION

It is concluded from this study that overexpression of the cardiac transcription factors in hUC-MSCs enhanced their differentiation potential into cardiac-like cells. The expression of early and late cardiac genes was significantly higher in all treatment groups. However, the combination group showed enhanced synergistic effect on cardiac differentiation. GATA-4 and MEF2C delivery seem to have the potential for the development of a cell-based treatment approach for cardiovascular diseases. However, further research is needed to explore the therapeutic effects of transfected hUC-MSCs in the *in vivo* model.

ARTICLE HIGHLIGHTS

Research background

Myocardial infarction is the leading cause of death worldwide. Following myocardial infarction, billions of cardiomyocytes die resulting in a significant loss in cardiac function. The discovery of cell-based therapies has emerged as a new area to support heart regeneration. GATA binding protein 4 (GATA-4) and myocyte enhancer factor 2C (MEF2C) are considered important transcription factors in the formation of cardiac cells during the embryonic development.

Research motivation

Stem cell based therapies are considered a promising approach for repairing the damaged heart. However, underlying mechanisms that control stem cell mediated cardiac cell fate decisions are still in their infancy. Since GATA-4 and MEF2C are the critical regulators of cardiac differentiation, therefore, use of these factors for transfection of mesenchymal stem cells (MSCs) may enhance the potential of these stem cells for cardiac differentiation.

Research objectives

Considering the critical role of cardiac transcription factors in maintaining the structure and function of the heart during the development process, their role in cardiac differentiation is highly anticipated. These genetically modified MSCs could be a promising future therapeutic option for heart diseases.

Research methods

Human umbilical cord-MSCs (hUC-MSCs) were isolated and characterized morphologically and immunologically. The cord derived MSCs were identified by the presence of specific markers *via* immunocytochemistry, and flow cytometry, and by their potential for osteogenic and adipogenic differentiation. hUC-MSCs were transfected with GATA-4, MEF2C, and their combination to direct cardiac differentiation. Cardiac differentiation was confirmed by semi quantitative real-time polymerase chain reaction and immunocytochemistry.

Research results

Cardiac transcription factors GATA-4, MEF2C, and their combination induced differentiation in hUC-MSCs with significant expression of cardiac genes and proteins. Moreover, myotube like structure which is the main characteristic of cardiomyocytes was also observed in the transfected cells.

Research conclusions

The results of this study concluded that the overexpression of GATA-4 and MEF2C in hUC-MSCs differentiates stem cells into cardiac-like cells. This study is an attempt to provide deeper insights into the mechanism of transcription factors in the cardiac differentiation of stem cells.

Research perspectives

The knowledge of the current study offers a promising therapeutic approach to improve treatment strategies for heart diseases. The genetically modified MSCs may serve as an ideal source for cardiac tissue repair and regeneration.

Figure Legends

Figure 1 Isolation and morphology of human umbilical cord mesenchymal stem cells. A: B: C: D: A stepwise method of human umbilical cord mesenchymal stem cells (hUC-MSCs) isolation and proliferation, showing spindle-shaped fibroblast-like cell morphology under phase contrast microscope at passage P0 and P1. All images were captured under phase contrast microscope (scale bar: 100 µm).

Figure 2 Human umbilical cord mesenchymal stem cell characterization by immunocytochemical analysis, flow cytometry and lineage differentiation assays. A: Immunocytochemsitry of Human umbilical cord mesenchymal stem cell (hUC-MSC) showing positive expression of CD44, CD90, CD105, vimentin, and negative expression of CD45, a hematopoietic marker. Images were captured under flouresence microscope (scale bar: 100 µm); B: Flow cytometry of hUC-MSCs showing positive expression of CD44, CD73 and CD90. Data were analyzed using BD FACS Diva software; C: Adipogenic and osteogenic lineage differentiation of hUC-MSCs. Images were captured under phase contrast microscope (scale bar: 100 µm).

Figure 3 Gene expression analysis of GATA binding protein 4 and myocyte enhancer factor 2C transfected human umbilical cord mesenchymal stem cells. Semi quantitative real-time polymerase chain reaction (RT-PCR) analysis showing gene expression levels of GATA binding protein 4 and myocyte enhancer factor 2C transfected mesenchymal stem cells, separately and in combination, in comparison to

the control. Results are expressed as mean ± SE (n = 3). Differences between groups with $^{a}P < 0.05$ are considered statistically significant, where $^{b}P < 0.01$ and $^{c}P < 0.001$. GATA-4: GATA binding protein 4; MEF2C: Myocyte enhancer factor 2C.

Figure 4 Morphological changes and cardiac-specific gene expression in transfected human umbilical cord mesenchymal stem cells. A: Images showing human umbilical cord mesenchymal stem cells trasnfected with GATA binding protein 4 (GATA-4); B-D: Myocyte Enhancer Factor 2C (MEF2C)(C) GATA-4 +MEF2C and (D) the corresponding untreated control. All images were captured at day 14 under phase contrast microscope (scale bar: 100 μm); E: Bar diagrams showing fold change analysis of cardiac gene expression by semi quantitative real-time polymerase chain reaction (RT-PCR) in GATA-4 and MEF2C separately, and in combination in comparison to the control after 14 d of culture. Results are expressed as mean ± SE (n = 3). Differences between groups with $^{a}P < 0.05$ are considered statistically significant where $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. GATA-4: GATA binding protein 4; MEF2C: Myocyte enhancer factor 2C; MHC: myosin heavy chain; NKX2.5: NK2 homeobox 5.

Figure 5 Cardiac-specific protein expression in transfected human umbilical cord mesenchymal stem cells. A: Flouresence Images showing human umbilical cord mesenchymal stem cells (hUC-MSCs) transfected with GATA binding protein 4 (GATA-4), myocyte enhancer factor 2C (MEF2C) separately and in combination, in comparison to the control (scale bar: 50 μm); B: Bar diagrams showing quantification of positive cells using ImageJ software. Also shown is the comparison between the individual and combination groups. Results are expressed as mean ± SE (n = 5). Differences between groups with $^{a}P < 0.05$ are considered statistically significant where $^{a}P < 0.05$ and $^{c}P <$

0.001. GATA-4: GATA binding protein 4; MEF2C: Myocyte enhancer factor 2C; CTnT: Cardiac troponin T; NKX2.5: NK2 homeobox 5; NS: Not significant.

Table 1 Primer sequence and annealing temperature of cardiac genes

Genes	Primer sequence (5′-3′)	Annealing Temperature (°C)
Beta-Actin	Forward: 5′-TGGGCATGGGTCAGAAGGATTC-3′	60
	Reverse: 5′-AGGTGTGGTGCCAGATTTTCTC-3′	
Myocyte Enhancer Factor 2C	Forward: 5′-CGAGATGCCAGTCTCCATCC-3′	60
	Reverse: 5′-CAGAGAAGGGTGAGCCAGTG-3′	
NKX2.5	Forward: 5′-AGTGTGCGTCTGCCTTTCC-3′	60
	Reverse: 5′-CACAGCTCTTTCTTTTCGGCTC-3′	
MHC	Forward: 5′-GACAGGTGCAGCAAAA CAGG-3′	60
	Reverse: 5′-AAGGGTATCCTGCAACTGCC-3′	
Connexin-43	Forward: 5′-CTTCATGCTGGTGGTGTCC-3′	60
	Reverse: 5′-ACCACTGGTCGCATGGTAAG-3′	
GATA-4	Forward: 5′-CTGCCCTCCGTCTTCTGC-3′	60
	Reverse: 5′-CTCGCAGGTCAAGGAGCC-3′	

NKX2.5: NK2 homeobox 5; MEF2C: Myocyte enhancer factor 2C; MHC: myosin heavy chain; GATA-4: GATA binding protein 4.

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