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RESEARCH ARTICLE

microRNA-1 Induces Transdifferentiation of Peripheral Blood CD34$^+$ Cells into Cardiomyocytes-like Cells

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Abstract

BACKGROUND: Transdifferentiation is a method to provide cells sources for cellular cardiomyoplasty. CD34$^+$ cells are potential cells sources because these cells can differentiate into cardiomyocytes through several mechanisms. MicroRNA (miR-1) is known to have the ability to inhibit the expression of histone deacetylase 4 (HDAC4). HDAC4 is a gene that essentially contributes in cardiomyocytes differentiation. However, the study reporting an evidence that miR-1 can induce transdifferentiation of CD34$^+$ peripheral blood cells into mature cardiomyocytes is limited.

METHODS: CD34$^+$ cells were taken from peripheral blood and isolated using a magnetic-activated cell sorting (MACS) method *in vitro*. Mature mimics of miR-1 were transfected into isolated CD34$^+$ cells and then incubated for 48 hours for quantification of HDAC4 mRNA using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). On the fifth day after miR-1 transfection, cardiomyocyte-like cells were identified based on their morphology and cardiac troponin expression using immunocytochemistry.

RESULTS: Transfection of miR-1 in CD34$^+$ isolated cells decreased HDAC4 gene expression by -0.54 fold at second day and caused a significant increase in percentage of cardiac troponin positive cells (median: 31.34; $p<0.05$) at fifth-day post-transfection. The efficiency of transdifferentiation was 32%. The miR-1 transfection had a significant negative relationship with HDAC4 gene expression (B=-1.000; $p=0.001$). HDAC4 gene expression had a negative and significant relationship with the percentage of cardiac troponin-positive cells (B=-0.701; $p=0.001$).

CONCLUSION: This study suggests that miR-1 can induce transdifferentiation of peripheral blood CD34$^+$ cells into cardiomyocytes-like cells by decreasing HDAC4 gene expression.

KEYWORDS: transdifferentiation, microRNA-1, CD34, cardiomyocyte, HDAC4

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Introduction

Coronary heart disease is the cause of myocardial infarction that leads to cardiomyocyte death and heart failure. To date, there is no cure for advanced heart failure except heart transplantation, which still has many limitations, such as the small number of donors and the possibility of immune rejection.(1,2) Development of alternative therapy is needed to overcome these problems, one of which is regenerative therapy.(3) Regeneration of dead myocardium can be done by replacing dead cardiomyocytes with new cells originating from stem cells or progenitor cells using a technique called cellular cardiomyoplasty.(4,5) Cellular cardiomyoplasty aims to replace damaged myocardial tissue and restore myocardial function.(6,7)

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Cellular reprogramming is one of the methods to provide cells sources for cellular cardiomyoplasty.(8) Transdifferentiation is a part of cellular reprogramming technique of converting certain differentiated cell types into other cells. Transdifferentiation has been proven safe and has a simple process that makes it more preferable to use than other techniques.(9) The success of the transdifferentiation is determined by the source cell type. Hematopoietic stem cells (HSCs) marked $CD34^+$ has the potential to become a cells sources because these cells can differentiate into cardiomyocytes. This differentiation process can be done because $CD34^+$ cells originate from the same embryonic layer as the heart, called the mesoderm layer.(10) $CD34^+$ cells can be easily extracted from peripheral blood through minimally invasive procedures.(11)

Cell transdifferentiation can be induced by microRNA (miRNA). miRNA is a non-coding RNA that works in the post-transcription stage by degrading or inhibiting the translation of messenger RNA (mRNA), resulting in the modification of gene expression.(12) Transdifferentiation using miRNA is easier to do in mature cells and has no risk of permanent genome change.(13) miRNA has a key role in the stage of differentiation of cardiomyocytes. The miRNA profile on cardiac tissue showed that microRNA-1 (miR-1) changes significantly during cardiomyocyte differentiation. miR-1 controls specific processes in cardiomyocyte differentiation through regulation of certain transcription factors and genes. This finding shows that miR-1 has an important role in cardiac differentiation.(9,14-17)

miR-1 is known to have the ability to inhibit the expression of the histone deacetylase 4 (HDAC4) gene. miR-1 has been proven to decrease HDAC4 gene expression by binding to the 3' end of the UTR.(18,19) miR-1 specifically targets 3' UTR in the CAUUCCA sequence at length 2334-2340 of HDAC4 gene.(20) HDAC4 is an enzyme that can trigger gene expression changes through histone protein modification. Modification of gene histone cause inhibition of the interaction between the gene and its transcription factor resulting in a decrease in gene expression. In myocyte differentiation activity, the reduction of HDAC4 will increase the expression of a muscle-specific gene called the myocyte enhancer factor 2 (MEF2). Increasing MEF2 is known to trigger cell differentiation into mature cardiomyocytes characterized by expression of cardiac troponin (c-troponin).(18,21,22) To the best of our knowledge, there is no study to investigate the induction of cardiomyocyte transdifferentiation from $CD34^+$ cells using miR-1. This study aims to prove the effect of miR-1 on HDAC4 expression in the process of inducing transdifferentiation of $CD34^+$ peripheral blood cells into mature cardiomyocytes.

Methods

Research Design and Samples

This research was an *in vitro* study with a true experimental post-test only design, which was conducted at the Research and Development Center for Stem Cell, Dr. Soetomo Hospital, Surabaya. The study was carried out from March to June 2020. Peripheral blood samples were taken from healthy male subjects aged 30 years old. The included subject had no human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus HCV infection; no history of acute myocardial infarction, heart failure, malignant arrhythmias, heart valve disease, stroke, transient ischemic attack, peripheral artery disease, hypertension, diabetes mellitus, and kidney failure; also had normal heart structure and function that was determined by echocardiography.

This study was approved by the Health Research Ethics Committee Universitas Airlangga School of Medicine, Surabaya, Indonesia (249/EC/KEPK/FKUA/2019). And all subjects involved in this study signed informed consent prior to the study.

Isolation and Expansion of $CD34^+$ Cells

$CD34^+$ isolation was carried out from peripheral blood used existing methods from STEM CELL Technologies (Vancouver, Canada) protocol.(23) Briefly, 40 mL venous blood samples were taken and then the preparations were made using Ficoll-Hypaque gradient centrifugation.(24) $CD34^+$ selective isolation was carried out using EasySepTM Human $CD34^+$ positive selection (STEM CELL Technologies), with cocktail with a volume of 50 µL per 1 mL sample. Cells were purified by magnetic-activated cell sorting (MACS) methods using the EasySepTM Magnet kit (STEM CELL Technologies). Immunocytochemistry and immunofluorescence were done to identify $CD34^+$ cells. The result of $CD34^+$ cell isolation was reproduced using an expansion medium and supplement. After expansion for 7 days, $CD34^+$ cells were randomly divided into two groups, namely control group and miR-1 treatment group.

miR-1 Transfection

This study used a mature mimic of miR-1, namely hsa-miR-1-1 MI0000651 (UGGGAAACAUACUUCUUUAU AUGCCCAUAUGGACCUGCUAAGCUAUGGAAUG

UAAAGAAGUAUGUAUCUCA). Transfection methods used the protocol of HiperFECT transfection reagent kit (Qiagen, Hilden, Germany). Before the transfection process, $1x10^5$ $CD34^+$ cells were seeded in a 24-well culture and incubated for 24 hours. As much as 37.5 ng of miR-1 and 3 μL HiperFECT transfection reagent was diluted with 100 μL culture medium. The resulting mixture was added to the $CD34^+$ cell culture plate. Then, the cells were incubated at 37°C in 5% CO_2 for 48 hours to analyze the mRNA.

Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) of HDAC4 Gene

The total mRNA of all samples was isolated according to the Total RNA purification kit protocol (Norgen Biotek Corp., Thorold, Canada). Total mRNA was counted using the NanoDrop 2000 spectrophotometer. The iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) was used for cDNA synthesis. The RT-qPCR using SsoFast™ EvaGreen® Supermix (Bio-Rad) was done to measure the mRNA of HDAC4. The total reaction mixture containing 50 ng cDNA sample, 0.25 μL HDAC4 primer (forward primer (5′–3′) CACACCTCTTGGAGGGTACAA and reverse primer (5′–3′) AGCCCATCAGCTGTTTTGTC), and 5 μL buffer PCR 10x, and nuclease-free water (NFW) were processed in a PCR machine with an annealing temperature of 64ºC for 45 cycles. β-actin was used for the control gene. Fold change was calculated using the ΔCt method, which compared Ct-value of the treated sample and control.(25)

Identification of Cardiac Troponin Expression

Identification of cardiomyocyte-like cells was based on the morphology and cardiac troponin expression by immunocytochemistry. Cardiac troponin was measured on the 5th day after miR-1 transfection. Confluent cells of each well plate were fixed with 4% paraformaldehyde and prepared with Triton X100/PBS, BSA, PBS, and Tween 20. Cells were exposed to a primary anti-cardiac troponin human monoclonal antibody (Termo Fisher, Waltham, MA, USA) and then incubated. After that, the secondary antibody was added and then incubated. Hematoxylin-eosin was used for counterstain. Result was observed using the light microscope with 400x magnification. We calculated the percentage of cells expressing cardiac troponin and divided it by the total cell count to determine the efficiency of transdifferentiation.(26)

Statistical Analysis

Data analysis was performed using SPSS 25.0 software (IBM Corporation, Armonk, NY, USA). One Way ANOVA analysis was used if the data were normally distributed and the Kruskal-Wallis analysis if the data were not normally distributed. The relationship between the independent and dependent variables was measured using regression analysis. Mean difference of $p<0.05$ was considered as significant.

Results

Isolation and Identification of $CD34^+$ Peripheral Blood

The isolation process resulted in a viable cell number of $8.72x10^6$ based on the cell counter count. The identification of isolated cells expressing CD34 using immunocytochemistry methods was shown by the presence of brown color expression in the cytoplasm of cells observed in a light microscope (Figure 1). CD34-expressing cells were also indicated by the presence of a green luminescence observed on a fluorescence microscope (Figure 1).

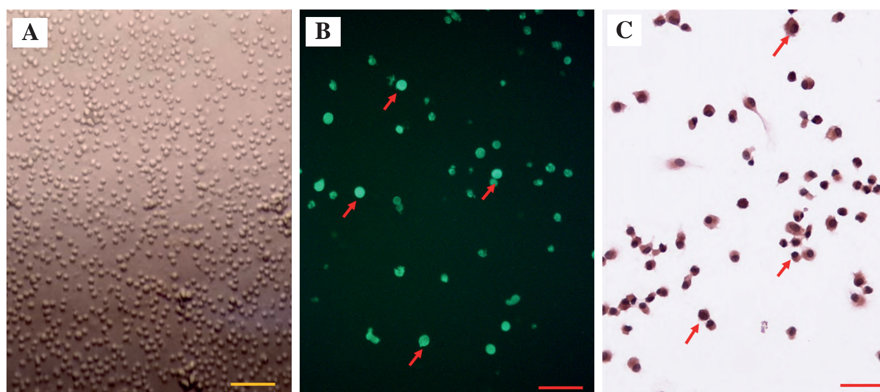


Figure 1. Observation of isolated $CD34^+$ cell from peripheral blood cells. A: Isolated $CD34^+$ cell observed with light microscope; B: Expression of CD34 in isolated cells (red arrow); C: Expression of CD34 in isolated cells (red arrow). Yellow bar: 10 μm; Red bar: 25 μm.

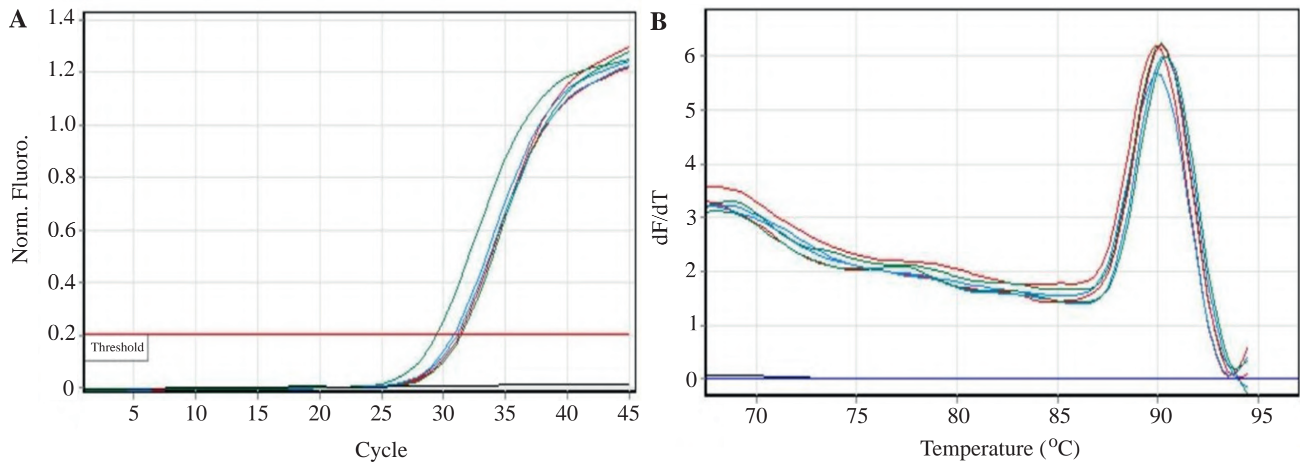


Figure 2. RT-qPCR amplification and melt curve of HDAC4. A: Curve of HDAC4 gene shows an optimum amplification condition characterized by exponential phase and a plateau phase at cycle ends; B: Melt curve of HDAC4 gene.

Expression of HDAC4

RT-qPCR of the HDAC4 gene was carried out with an annealing temperature of 64ºC for 45 cycles. The amplification curve of HDAC4 gene showed an exponential phase and a plateau phase at cycle ends (Figure 2A). Meanwhile, the melt curve of HDAC4 gene showed a single peak at temperature melting in the range of 85-90ºC (Figure 2B). Measurement of relative expression of HDCA4 based on the ΔCt method showed that transfection of miR-1 decreased HDAC4 gene expression by -0.54 fold at second day post-transfection. A representative graphic of HDAC4 relative expression was presented in Figure 3B.

Identification and Measurement of Cardiomyocyte-like Cells Percentage

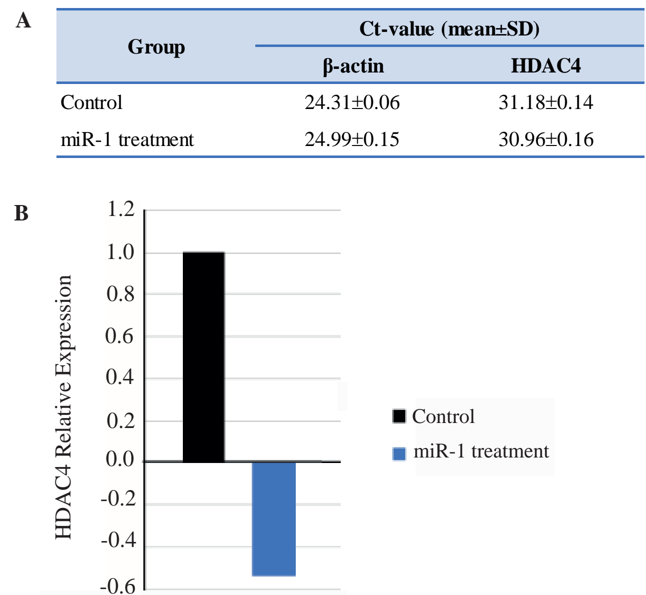
Cardiomyocyte-like cell percentage was calculated based on its expression of cardiac troponin. Transfection of miR-1 (median: 31.34) caused a significant increase in the percentage of cardiac troponin positive cells when compared to the control (median: 12.13) (p<0.05) at fifth day post-transfection. Immunocytochemistry of cardiac troponin and representative graphic of cardiac troponin positive cells percentages was presented in Figure 4. The efficiency of transdifferentiation of CD34^{+} peripheral blood cells into cardiomyocyte-like cells was 32%.

Relationships between Transfection of miR-1, HDAC4, and Cardiac Troponin Expression

The regression analysis showed that miR-1 transfection had a significant positive relationship with the percentage of cardiac troponin (B=0.701; p=0.001). The analysis also showed that miR-1 transfection had a significant negative relationship with HDAC4 gene expression (B=-1.000; p=0.001). HDAC4 gene expression has been shown to have a negative and significant relationship with the percentage of cardiac troponin (B=-0.701; p=0.001).

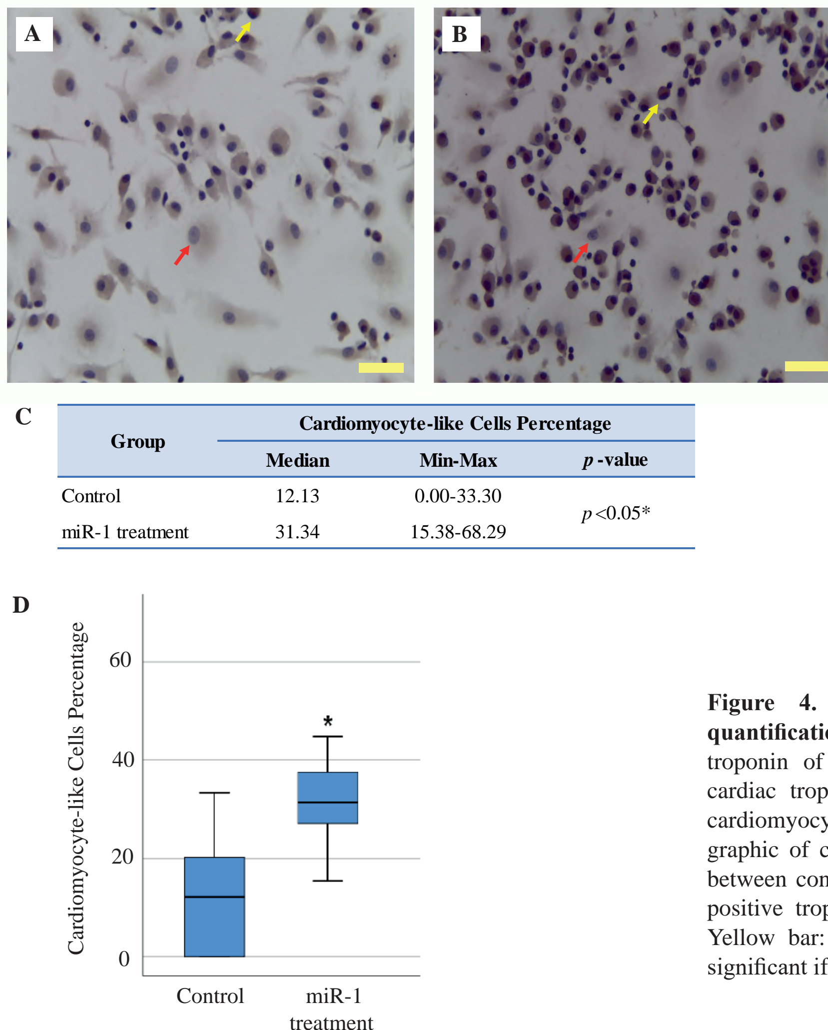
Discussion

In line with the development of cell regeneration therapy, there have been some studies conducted to produce cardiomyocytes through transdifferentiation. Production of


A

Group	Ct-value (mean±SD)	
	β-actin	HDAC4
Control	24.31±0.06	31.18±0.14
miR-1 treatment	24.99±0.15	30.96±0.16

Figure 3. RT-qPCR result and measurement of HDAC4 gene. A: Ct-value of HDAC4 and β-actin control gene; B: Representative graphic of fold change in HDAC4 relative expression. HDAC4 gene expression decreased by -0.54 fold at second day post-transfection.



Group	Cardiomyocyte-like Cells Percentage		
	Median	Min-Max	*p* -value
Control	12.13	0.00-33.30	$p<0.05$*
miR-1 treatment	31.34	15.38-68.29	

Figure 4. The result of cardiomyocyte-like cells quantification. A: Immuno-cytochemistry of cardiac troponin of control group; B: Immuno-cytochemistry of cardiac troponin of miR-1 treatment group; C: Table of cardiomyocyte-like cells percentage; D: Representative graphic of cardiomyocyte-like cells percentage comparison between control and miR-1 treatment group. Yellow arrow: positive troponin cell; Red arrow: negative troponin cell; Yellow bar: 25 μm. *Tested with Mann-Whitney U test, significant if p=0.05.

cardiomyocytes by transdifferentiation can be done using various agents such as transcription factors, small molecules, and miRNA.(27) Research about cardiomyocyte production by transdifferentiation has been carried out from various types of cells sources, including fibroblasts, adipocytes, and bone marrow hematopoietic cells.(28) However, studies on cardiomyocyte transdifferentiation from sources of peripheral blood CD34$^+$ cells using miRNA, especially miR-1, have never previously been done.

miR-1 is a specific miRNA that plays a dominant role in the process of cardiomyogenesis. The miR-1 has been found to propagate immature muscle cell genes expressions into mature cardiomyocytes, which is then followed by phenotypic and functional alterations.(15,16) This study showed that miR-1 transfection reduced HDAC4 gene expression. These results are in accordance with previous studies, which showed that there was a decrease in HDAC4 gene expression 24 hours after transfection of miR-1 in chondrocytes.(29) A decrease in HDAC4 gene expression in rat cardiomyocytes has also been found 24 hours after miR-1 transfection.(30) Contrary to those results, another study found that miR-1 transfection in mouse embryonic stem cells did not affect HDAC4 gene expression.(16) These different results indicate that the effect of miR-1 on HDAC4 gene expression depends on various factors such as the type of cells sources used in the treatment. Hence it is necessary to measure HDAC4 gene expression in CD34$^+$ peripheral blood cells after miR-1 transfection.

This study showed that miR-1 transfection could increase expression of cardiac troponin in cells, which indicates that CD34$^+$ cells were transdifferentiated into cardiomyocyte-like cells. Troponin expression of cardiomyocytes progenitor was proven to be increased in 3 days and 7 days after miR-1 transfection.(31) This is thought to be caused by a decrease in HDAC4 gene expression after miR-1 transfection which has been demonstrated in this study. This hypothesis is supported by a negative and significant relationship between HDAC4 gene expression and cardiac troponin expression based on regression analysis. It means that the reduction in HDAC4

gene expression causes an increase in cardiac troponin expression. Decreased expression of HDAC4 has been known to increase the expression of transcription factors that trigger muscle cell differentiation, MEF2. MEF2 plays a role in triggering the differentiation of muscle cells by increasing the expression of sarcomere genes and regulating the formation of muscle fibers.(32)

CD34+ cells' differentiation function is highly dependent on three factors; transcription factors, signaling pathways, and niche.(33) MEF2, regulated by a transcription repressor enzyme HDAC4, is one of the transcription factors having a role in cardiomyocytes differentiation. HDAC4 is a protein that regulates genes related to muscle cell differentiation, so this gene is found mostly in skeletal and cardiac muscle cells.(9,34,35) HDAC4 plays a role in the deacetylation process of histone proteins, causes gene structural modification, and affects its transcription process.(36) HDAC4 has been known to directly bind to and suppress MEF2 expression and function in embryonic stem cells (ESC). These findings were confirmed by evidence that MEF2 expression increased after HDAC4 inhibitor (sodium butyrate and trichostatin A) was given. HDAC4 inhibitor therapy also triggered production of other transcription factors such as Nkx2.5 and GATA4 then differentiated ESC into cardiomyoblast.(37) Fibroblast has also been shown to differentiate into cardiomyocytes which are indicated by an increase of MEF2, GATA4, HAND2, TBX5 and intracellular cardiac troponin.(38)

In this study, new mechanisms were found explaining the effect of miR-1 in the process of inducing transdifferentiation of peripheral blood CD34+ cells into cardiomyocytes-like cells. Regression analysis showed that miR-1 transfection increases the expression of cardiac troponin by decreasing the expression of the HDAC4 gene. There are limitations in this study that could be addressed in future research. First, this study did not measure levels of miR-1 mimic after transfection into CD34+ cells, so the levels of miR-1 mimic involved in the early stages of transdifferentiation were unknown. Second, it is known that histone deacetylation by HDAC4 in gene expression can be counteracted by histone acetyltransferases (HATs) and *vice versa*.(39) Measuring HAT acitivity may be useful to determine whether reduction of HDAC4 by miR-1 can trigger HAT reactivity, so that MEF2 gene expression may still occur. Last, determination of cardiomyocytes-like cells will have a higher validity if complemented with measurement of other mature cardiomyocyte markers such as α-actinin and α-MHC.

Conclusion

This study concludes that miR-1 transfection reduced HDAC4 gene expression and suggests that miR-1 can induce transdifferentiation of peripheral blood CD34+ cells into cardiomyocytes-like cells by decreasing HDAC4 gene expression.

Acknowledgments

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Authors Contribution

BSP and A were involved in the study conception and design; IGRS and HOH were involved in the data collection; A, HOH, and PMH analyzed and interpreted the study results; IGRS and PMH drafted manuscript preparation. All authors reviewed and approved the final version of the manuscript.

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