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Discrimination of Stem Cell Fate via Targeting of *BAX, BAK1* and *BOK* Genes by *Hsa-Mir-765*

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Abstract

Apoptosis is a way for loss of unwanted cells during homeostasis, development, and integrity of tissues which is a process depending on caspases started through extrinsic and intrinsic or mitochondrial pathways. During a bioinformatics survey, important targeting miRs in both pathways have been identified. In the current study, due to common biochemical origin of apoptosis and differentiation, the role of *hsa-miR-765* during the course of apoptosis induction and cardiomyocytes differentiation of hESCs have been investigated. We found the expression of *BAX*, *BAK1*, and *BOK* as the key elements of mitochondrial membrane pores involved in apoptosis induction and targeted by this miRNA during apoptosis and differentiation. All data represented here may disclose the imperative function of *hsa-miR-765* in discrimination of biochemical aspect of differentiation and apoptosis of embryonic stem cells towards cardiomyocytes.

Keywords: Apoptosis • Differentiation • miR 765 • Cardiomyocytes

Introduction

Capability to differentiate and self-renewal of hESCs are critical characteristics of hESCs (Human embryonic stem cells) making these cells noteworthy for cell therapies and tissue regeneration [1-4].

Apoptosis as a type of programmed cell death for disposing of unsolicited cells in multicellular organisms ensuring development, integrity, homeostasis and tissues generation [5,6]. Apoptosis is largely depends on activation of caspases as a group of enzymes called cysteine proteases and activated through extrinsic (receptor mediated) and intrinsic (mitochondrial) pathways. In mitochondrial (intrinsic) pathway, activity of caspase 9 is dependent on cytochrome *c* releasing followed by formation of a large protein complex (apoptosome) brought about activation of caspase 3/7. Any other way, another alternative pathway mediated by T-cell cytotoxicity and perforin-granzyme-dependent cell killing [6-8].

Growing body of evidences has shown that the apoptotic mitochondrial pathway is activated in the earliest steps of cell differentiation in different cell types and ESC cardiac differentiation in mouse and human [9-17]. This pathway has critical function in cell differentiation process, such as cytochrome c releasing and caspase activity as well, as the main hallmarks for mitochondrial pathway of apoptosis involved in differentiation of lens fiber epithelial cells, sperm, myocytes, monocytes, keratinocytes, osteoclasts, erythrocytes, and embryo development starting at the blastocyst stage.

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Short time exposure and low expression level of factors involved in apoptotic has been reported as inducers of differentiation via intrinsic (mitochondrial) mediated apoptotic pathway [9,16]. Recently, Akbari *et al* have reported that cytochrome *c* releasing, followed by formation of apoptosome complex, is essential for mouse ESCs differentiation into cardiomyocytes. Moreover, delay in apoptosome formation brought about with protection of mouse embryonic stem cell from death during differentiation towards cardiomyocytes [6]. However, alteration of mitochondria and low level of Apaf-1 expression have been observed in differentiation of human ESCs during differentiation towards cardiomyocytes and neuron, respectively [17,18]. A common biochemical niche has been proposed for apoptosis and differentiation and even it has been revealed that differentiation progression is a altered form of cell death [19]. How a common pathway, during the activation of the apoptosis, can convey two different cellular fates and determining factors in cell death versus differentiation is a research question which has not been addressed yet clearly [6]. Since main molecular regulators of these two different cell fates are not reported to date, here we attempt to identify those. However, recently our team has reported hsa-miR-766 as a miRNA involved in differentiation and cell death regulation [20]. In order to achieve this goal for survey other implicated factors, we have focused on non-coding RNAs, especially miRNAs, as major controllers of apoptosis pathway.

MicroRNAs are a group of endogenous small non-coding RNA molecules regulating gene expression at both transcriptional and post transcriptional levels, playing a critical role in cellular proliferation, development, differentiation and apoptosis processes. The aberrant miRNA expression has been linked to numerous human cancers and progressively evidences points to their role as tumor suppressors and oncogenes [20-23].

In spite of several reports about miRNAs role in apoptosis through controlling the intrinsic and extrinsic pathway [21,22,24,25], no reported researches was found about miRNAs expressed by a postponed time or by an alteration in expression intensity for the duration of differentiation and cell death. However, we have recently reported

hsa-miR-766 as one the miRNAs controlling these two different cell fates [20]. Therefore, it was motivating for us to survey if there are any microRNAs regulating the delayed activation of mitochondrial apoptosis for the course of differentiation towards cardiomyocytes and programed cell death via their differential expression. Rising studies reveal that miRNAs regulate the expression of genes implicated in intrinsic pathway of apoptosis [25]. However, none of these evidences aim to target all *BAX*, *BAK1* and *BOK* concurrently. In the present study, we targeted to study the role of *hsa-miR-765* for the duration of apoptosis and differentiation of hESCs into cardiomyocytes. We found that *BAK1*, *BAX*, and *BOK* RNAs, as the key participants of mitochondrion membrane pores implicated in apoptosis induction, are targeted by hsa-miR-765. All the data that have been represented in this study may disclose the imperative role of *hsa-miR-765* in apoptosis and differentiation through mitochondrial apoptosis pathway.

Materials and Methods

Bioinformatics analysis

Retrieving of *BAX*, *BOK* and *BAK1* genes 3'-UTR sequences was performed by using Entrez (http://www.ncbi.nlm.nih.gov/Entrez/). miRNA targets prediction was achieved via RNAHybrid (https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid), TargetScan (http://www.targetscan.org/vert_72/), miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/), DIANA TOOLS (http://diana.imis.athena-innovation.gr/DianaTools/index.php), and miRmap (http://mirmap.ezlab.org/app/) online tools.

Cell lines

DMEM-HG and DMEM-F12 culture media which were complemented with 100 µg/ml streptomycin, 100U/ml penicillin, and 10% FBS were applied for SW480 and HEK293T cell lines culturing, respectively. Cells were incubated with 5% CO_2 at 37 °C. SW480 (ID# C506) and HEK293T (ID# IBRC C10683) cell lines were achieved from Pasteur Institute/Iran and National Center for Genetic and biological resources/Iran, respectively.

miRNA expression constructs

Overexpression of hsa-miR-765 was initiated by amplification of its sequence in the human genomic region by PCR, and then the amplified sequence was cloned into pEGFP-C1 (Clontech) expression vector. Two overlapping primers were applied for amplifying anti-hsa-miR-765 and then was cloned in pEGFP-C1 vector. 3'UTRs regions of *BAX*, *BAK1*, and *BOK* were cloned at downstream of luciferase gene in psiCHECK-2 vector. Scrambled control was constructed in pEGFP-C1 vector, as previously described [Dokanehiifard et al., 2015]. All prepared recombinant plasmids were sequenced for final confirmation of the precise insert.

Dual luciferase assay

Dual luciferase assay analysis was performed according to the Promega kit manufacture's protocol.

Caspase-9 activity assay

Activity of caspase-9 was measured by using luminescence assay kit according to the prorocol described by the manufacturer (Promega). Cell lysate was prepared 48h after transfection. Protein concentration was measured using bradford assay protocol, and then the lysates was subjected for caspase-9 activity detection.

Caspase-3/7 activity assay

Activity of caspase 3/7 was measured by the Caspase-Glo 3/7 luminescent assay reagent (Promega) according to the manufacturer protocol. Cell lysates were prepared 48h after transfection and then equal protein concentration was applied to determine the activity of caspase-3/7.

RNA extraction

Trizol (Invitrogen) was applied for isolation of total RNAaccording to the described manufacturer's protocol. Extracted RNA was treated with DNAaseI (Fermentas), and 1% agarose gel was then used for controlling RNA quality.

cDNA synthesis and RT-qPCR

In order to hsa-miR-765 expression detection, polyadenylated RNAs was prepared and then CDNAs were made using a reported protocol. Each cDNA sample was amplified using the following conditions for 45 cycles: 95°C for 5s; 60°C for 20s; followed by 72°C for 30s. RT-qPCR was performed using EvaGreen (Amplicon, Korea) according to MIQE guidelines in two repeats condition. GAPDH and U48 were used as endogenous reference genes for analysis of expression data. This data was normalized by using $2^{-\Delta\Delta Ct}$ method.

Transfection and hsa-miR-765 overexpression

1.0 µg of each pEGFP-C1 expression vector DNA having *hsa-miR-765* precursor and sh-RNA cossete against *hsa-miR-765* were immersed in TurboFect (Fermentas) and applied for transfection of interested cell lines. Fluorescence microscopy (by Nikon eclipse Te2000-s) were used to ensured successful transfection of GFP plasmids within 24 hours.

Cell cycle analysis

hsa-miR-765 **e**xpression constrauct was transfected into the cells and then 36 hours after that cells were harvested and used for propidium iodide (PI) Staining. Delivery of plasmids and sample analysis were done by means of a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences) and the final data was analysed by Flowing Software.

Differentiation of hESCs into cardiomyocytes

2×10^5 cells/ml by the static suspension culture was applied in non-attach petri dishes for starting the differentiation of the hESC line (Rh5). After five days, Spheroids with 175 µm ± 25 dimension were gathered and transported into the differentiation medium in the presence of 12 µM CHIR99021 for the additional 24h. CHIR99021 was then depleted from the media, and these cells were kept in this condition for one day as the differentiation cuture media. Subsequently, differentiation medium was then replaced with a media containing Purmorphamine, IWP2 and 5 µM of SB4321542, for more 48h. Afterwards, all these small molecules were depleted from the media. Cell media was renewed every other day and Spheroid cells started to beat at 5th days of differentiation and reached to their highest beating at day 12.

Induction of apoptosis in hESCs

In order to inducing apoptosis in hESC, doxorubicin (Ebendoxo, EBEWE Pharma Ges) was applied at a range of concentrations, however 1.0 µM of doxorubicin was finally used to be added to the undifferentiated hESCs, and then incubated for 24 h at 37°C.

MTT assay

The number of 8000 cells/well from interested cell lines were seeded in a 96-well plate and they were then transfected after 24h. In order to cell death induction, cells were treated with doxorubicin 32 h after transfection, and kept on this condition for 8h. Then 20 µL of 5 mg/mL MTT (Sigma) was added to each well (40h after transfection), followed by further incubation for 4 h at 37°C, after which the culture medium was discarded and 100 µL DMSO (Sigma) was used for each well in order to fromazan crystals dissolving, and then was followed by OD490 measurment by means of an ELISA Microplate Reader (Biotek).

Primer designing

All related oligo sequences and primers were designed by NCBI Primer-blast and IDT oligo analyzer online PCR primer design tools.

Statistical Analysis

All statistical analysis was done by GraphPad Prism 5.04 (San Diego, CA). For flow cytometry, data was analysed by Repeated Measures ANOVA test.

Results

Bioinformatics study for targeting of *BAX*, *BAK1*, and *BOK* genes by *hsa-miR-765*

For investigation of potential miRNAs which target *BAK1 BOK,* and *BAX,* several online prediction tools were applied including: miRwalk, Dianna lab, TargetScan, miRmap, and mirDB. These software predicted *hsa-miR-765* as a miRNA potentially targeting *BAX*, *BOK*, and *BAK1* (Figure 1). These predictions revealed 2, 4, and 6 moderately conserved *bona fide* MREs in the *BAX*, *BAK1*, and *BOK* 3'-UTRs, respectively (Figure 1).

hsa-miR-765 can target *BAX*, *BOK* and *BAK1*

Since HEK293T and SW480 cell lines express *BAX*, *BOK* and *BAK1* (detected by RT-PCR, data not shown), *hsa-miR-765* was overexpressed and then RT-qPCR was performed in these two cell lines. Result revealed that *hsa-miR-765* is successfully overexpressed (to 600 folds), and *BAX* (60%), and *BOK* (40%) are significantly downregulated in the HEK293T transfected cells, whereas the expression levels of *BAK1* and *BCL2* transcripts were not changed (Figures 2A and 2B). Consequently, a significant decrease was observed in *BAX/BCL2* ratio in HEK293T cells overexpressing *hsa-miR-765* compared to the cells containing the scrambled vector as a

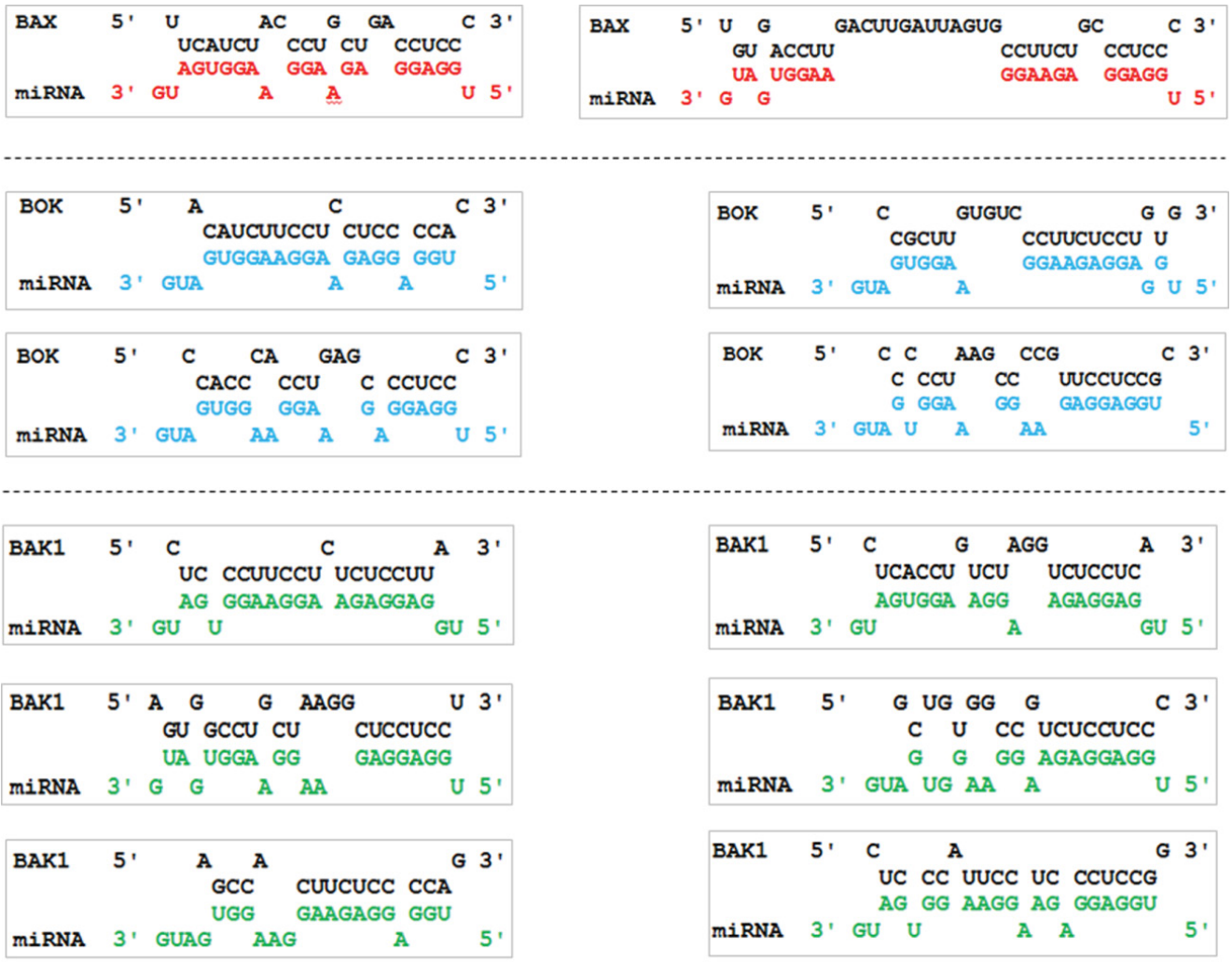

Illustration of predicted pairing status of MREs located in *BOK* (blue color), *BAX* (red color) and *BAK1* (green color) 3'UTRs with *hsa-miR-765*. *hsa-miR-765*sequence has been illustrated in color, and the predicted MREs sequences located in *BOK, BAX and BAK1* 3'-UTRs are displayed in black.

Figure 1. *In silico* prediction of hsa-miR-765 as a miRNA targeting the 3'-UTRs of BOK, BAK1 and BAX.

negative control (Figure 2C). Meanwhile, a significant reduction was observed in *BAK1* (80%) and *BOK* (60%) expression level detected through RT-qPCR in SW480 cells overexpressing *hsa-miR-765*, while no significant expression alteration was detected for *BAX* and *BCL2* (Figures 2D and 2E). As a result, no alteration was observed in *BAX*/*BCL2* ratio following overexpression of *hsa-miR-765* in SW480 cells compared to the cells having the scrambled cassette (Figure 2F).

hsa-miR-765 is directly interacting with BAX and BOK 3'UTRs

As *BAX*, *BOK* and *BAK1* were predicted to be targeted by *hsa-miR-765*, we examined to do dual luciferase assay. Therefore, 3' UTR of *BAX*, *BOK* and *BAK1* were individually cloned in psiCHECK vector downstream of luciferase ORF, and then co-expressed with *hsa-miR-765* in HEK293T cells. This data supported that *hsa-miR-765* interacts directly with *BAX and BOK* transcripts with 55% and 50% reduction in luciferase activity, respectively (Figures 3A and 3B). However, no significant result was obtained for *BAK1* 3'UTR sequence (Figure 3C).

Activity of caspase-3/7 and caspase-9 in SW480 cells overexpressing hsa-miR-765 and treated with an apoptotic inducer component

Mitochondrial apoptotic pathway is the pathway which is initiated by caspase-9 activation as the initiator caspase followed by activation of caspase 3/7. Doxorubicin is a cell death inducer component. For calculation of doxorubicin IC50 SW480 cells were treated with various concentrations of the doxorubicin, and 1.0 μM of doxorubicin was finally applied to be used as the half maximal inhibitory concentration

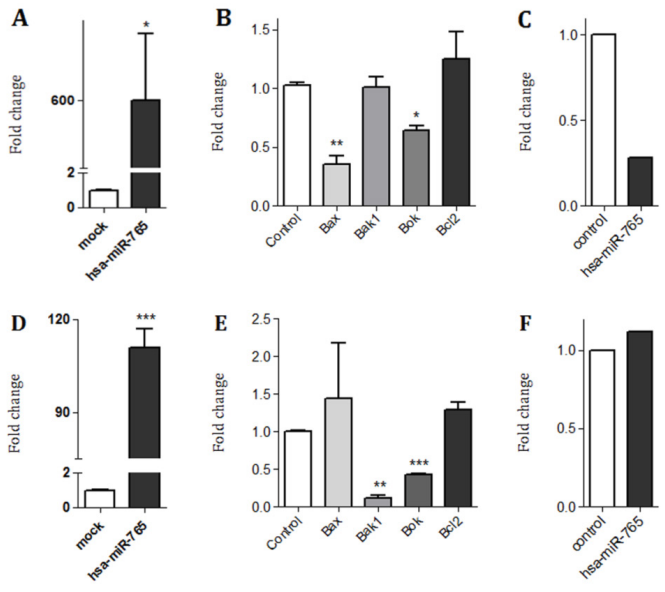

Figure 2. Analysis of potential ability of hsa-miR-765 for targeting BAK1, BOK and BAX 3'-UTRs through RT-qPCR.

A) *hsa-miR-765 was* successfully overexpressed in HEK293T cells.
B) Overexpression of *hsa-miR-765* resulted in significant downregulation of *BOK* and *BAX* detected through RT-qPCR in HEK293T cells; conversely *BCL2* and *BAK1* expressions were not significantly changed in these cells.
C) Effect of *hsa-miR-765* overexpression on *BAX*/*BCL2* ratio in HEK293T cells.
D) *hsa-miR-765* was successfully overexpressed in SW480 cells.
E) Effect of *hsa-miR-765* overexpression on downregulation of *BAK1* and *BOK* in SW480 cells detected by RT-qPCR; nevertheless the expression of *BAX* and *BCL2* were not significantly altered .
F) Analysis of *hsa-miR-765* overexpression effect on the ratio of *BAX*/*BCL2* in SW480 cells.

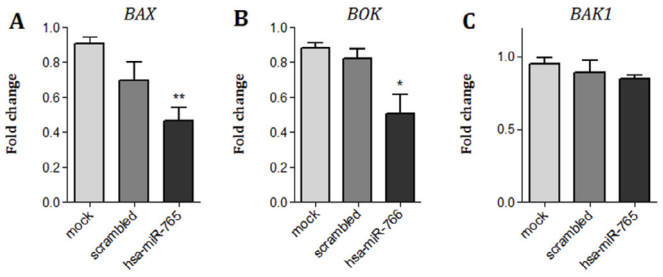

Figure 3

The cells which were transfected with the vector overexpressing *hsa-miR-765 revealed* lower luciferase activity in comparison with the cells overexpressing mock control vector, that supports *hsa-miR-765* direct interaction with *BAX* (A) *and BOK* (B) sequences. However, no significant result was obtained for *BAK1* (C) 3'-UTR sequence.

Figure 3. Analysis of direct interaction of hsa-miR-765 with BOK, BAK1 and BAX 3'-UTRs detected by dual luciferase assay.

(IC50). The activity of caspase-9 was considerably reduced after overexpression of *hsa-miR-765* in SW480 cell line in comparison with the SW480 cells containing the mock expression vector (Figure 4A). Nevertheless, no major reduction in caspase-3/7 activity was observed upon *hsa-miR-765* overexpression (Figure 4B).

hsa-miR-765 overexpression effect on the cell cycle in SW480 and HEK293T cell lines

For addressing this question if *hsa-miR-765* affects cell death, we transfected SW480 and HEK293T cells with the cassette overexpressing *hsa-miR-765*. S and sub-G1 cell cycle phases were significantly elevated following *hsa-miR-765* overexpression in HEK293T cells, while G0/G1 and G2/M cell populations were decreased (Figure 5A). On the other hands, sub-G1 and G2/M cell populations were significantly reduced in SW480 cells overexpressing *hsa-miR-765* in comparison with negative control; but, G0/G1 phase was significantly elevated (Figure 5B). MTT assay revealed that the viability of HEK293T cells is not changed after *hsa-miR-765* knocking down and overexpression at 48h and 72h time points compared to the cells transfected with mock and scrambled as negative controls (Figures 5C and 5D). Conversely, overexpression of this *miRNA* significantly elevated the rate of survival in SW480 cells compared to the ones transfected with the mock vector (Figure 5E).

Variation in the transcriptional expression of hsa-miR-765, BOK, BAX and BAK1for the duration of apoptosis and differentiation induction in hESCs

Since our in silico analysis predicted *BAK1, BOK* and *BAX* as *hsa-miR-765* target genes, we examined to analyze the expression alteration of these interested genes in several time points including 0, 2, 6, 12 and 24 hours after starting induction of apoptosis and differentiation. *BAX* expression was significantly reduced in 12h after induction of apoptosis and differentiation, and then started to be increased (Figure 6A). The expression alteration of *BAK* transcript and also *BOK* were comparable throughout induction of apoptosis and differentiation, as slightly elevated to 2h upon induction, and then started to decrease until 12h, and afterward somewhat increased to 24h post induction. RT-qPCR data revealed an oscillation pattern for *hsa-miR-765* expression throughout the differentiation and apoptosis induction course (Figures 6B, 6C and 6D). We also investigated *hsa-miR-765* expression alteration for the period of hESC differentiation into beating cardiomyocytes within 12 days. Results showed that the

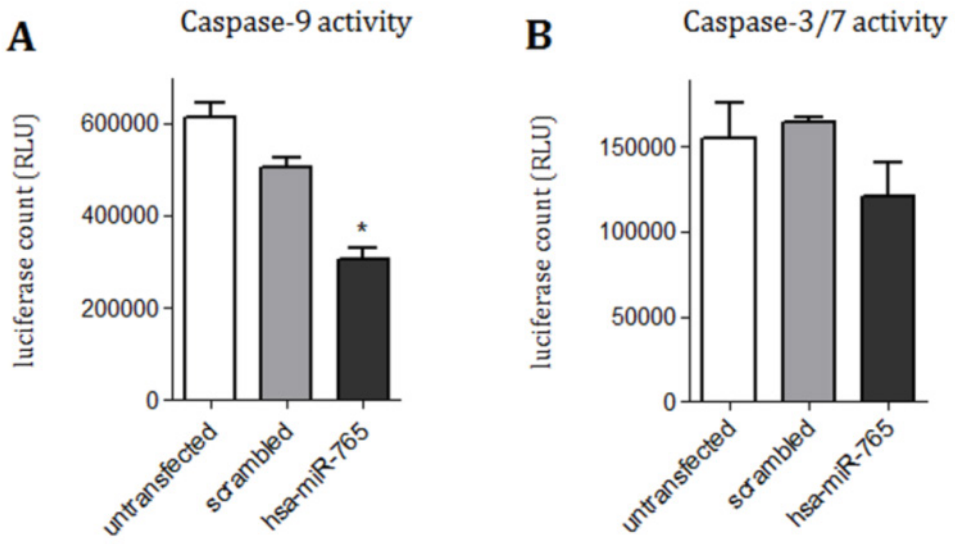

Figure 4

Figure 4. Analysis of caspase 9 and caspase 3/7 activity. Caspase 9 (A) and Capase 3/7 (B) activities following overexpression of hsa-miR-765 in SW480 cells treated with 1.0 μM doxorubicin compared to transfected cells with scrambled miR and untransfected cells.

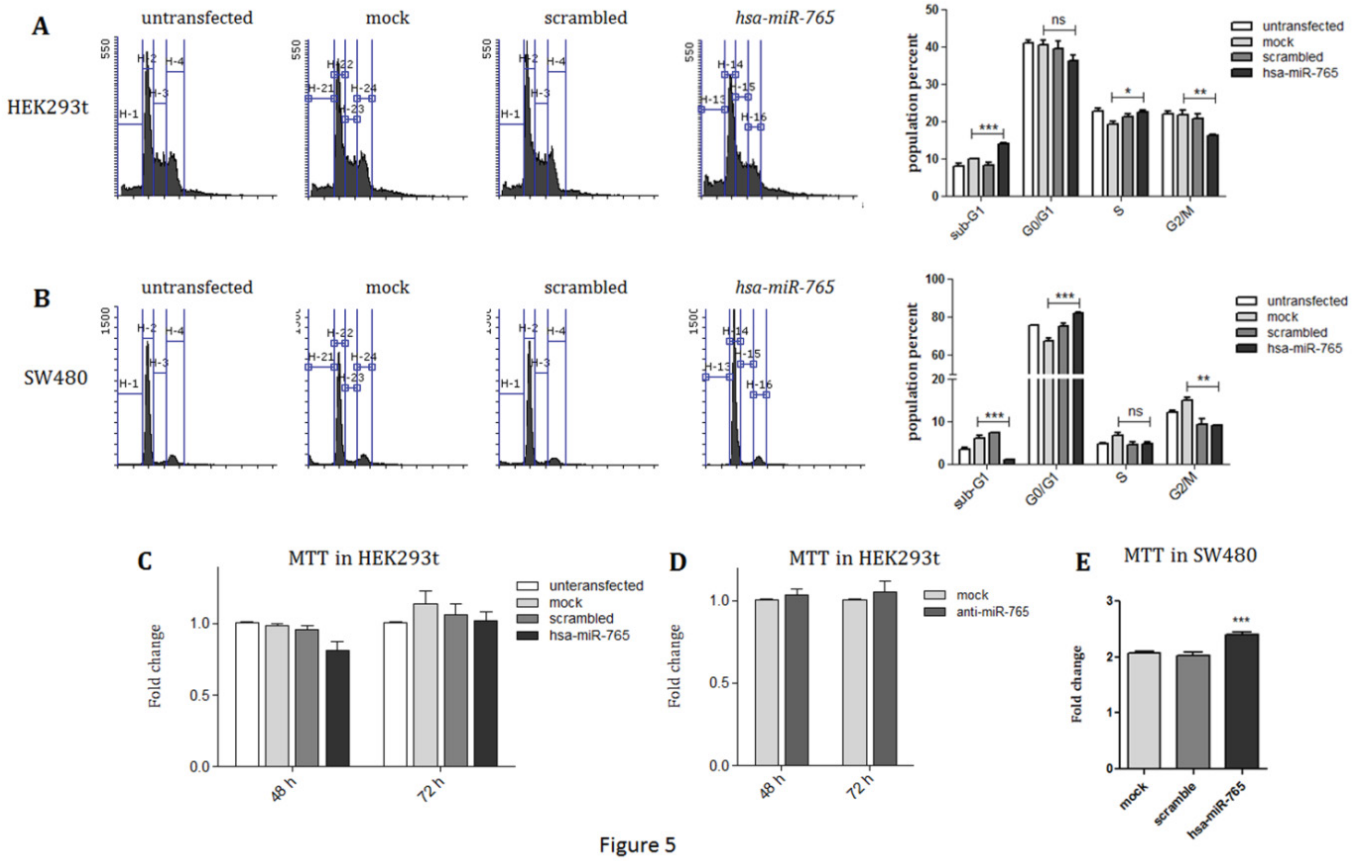

Figure 5

Figure 5. Cell cycle and cell viability analysis of HEK293T and SW480 cells overexpressing hsa-miR-765
Cell cycle analysis of HEK293T cells 40 hours after their transfection with the vector overexpressing *hsa-miR-765*. Result revealed a significant decrease in G2/M and G0/G1cell cycle distribution after *hsa-miR-765* overexpression compared to the control cells. However, sub-G1 and S phase populations were significantly increased.
Cell cycle analysis of SW480 cells 40 hours after their transfection with the vector overexpressing *hsa-miR-765*. In SW480 cells containing *hsa-miR-765* overexpression cassette, a significant decrease was detected in sub-G1 and G2/M phases, conversely G0/G1 cell cycle distribution was significantly elevated in these cells, compared to the control cells.
No significant cell viability alteration was observed in HEK293T cells 2 days after overexpression of *hsa-miR-765*, through MTT assay analysis.
No significant cell viability alteration was observed in HEK293T cells 2 days after down-regulation of *hsa-miR-765*, through MTT assay analysis.
E) A significant elevation in cell viability was observed in SW480 cells 2 days after overexpression of *hsa-miR-765*, detected through MTT assay analysis.

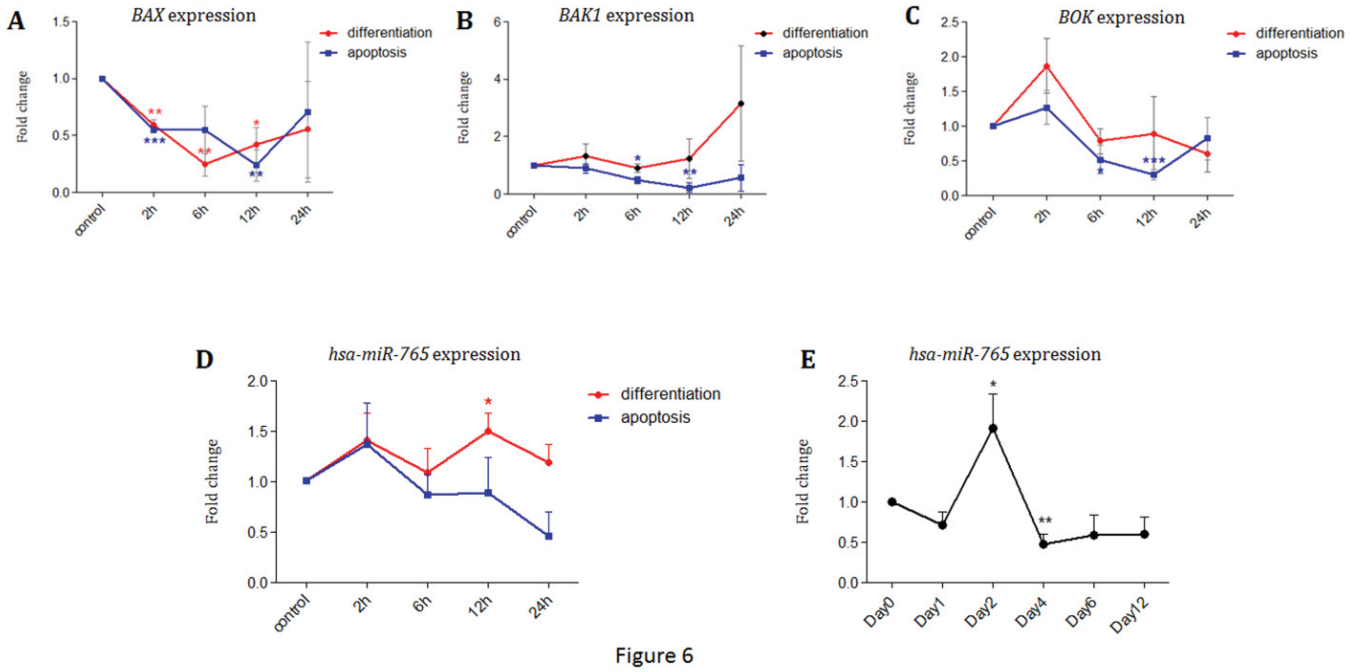

Figure 6

Analysis of the expression of *BAX (A)*, *BAK1 (B)*, *BOK (C)*, and *hsa-miR-765 (D)* for the period of the cardiac differentiation and apoptosis induction in a 24 hours' time point. (E) Expression analysis of *hsa-miR-765* all through the cardiomyocyte differentiation induction in 12 days.

Figure 6. Analysis of the expression of *BOK, BAK1, BAX* and *hsa-miR-765* throughout the course of differentiation and apoptosis induction of hESCs.

expression of *hsa-miR-765* was significantly increased on day 2, and then significantly reduced to day 4, afterward no significant alteration was detected (Figure 5E).

Discussion

Mitochondrial pathway of apoptosis has critical implication in both stem cell differentiation and apoptosis [4,6,9,10,12-17,19]. Activation of mitochondrial apoptosis pathway with delay, not only is involved in cell differentiation towards neuron and cardiomyocytes, but also has a function in cell death [6]. However, it seems that time point and intensity of caspases activation presumably through mitochondrial membrane alterations may determine whether a cell dies or differentiates Ghiasi, et al. [17]. Although a growing body of investigations has been published about apoptosis and differentiation, the strong molecular controllers of mitochondrial pathway of apoptosis are not identified yet. However, we have recently published one of this regulator, hsa-miR-766, via targeting of three critical gene transcripts involved in mitochondrial apoptosis. Although Akbari-Birgani et al. and Ghiasi et al. performed efforts in order to describe the cellular features which are key in human and mouse embryonic stem cell differentiation the genes which their expression are affected at the first steps of differentiation were not identified [6,17]. In this present study, we again applied a computational study for predicting new miRNAs and showed that still there are another miRNAs which can target many of the genes involved in mitochondrial apoptosis and subsequently can differentially control two different fates in embryonic stem cells, apoptosis and differentiation.

In the present study, bioinformatics analysis predicted hsa-miR-765 as a miRNA that simultaneously targets 3'UTRs of *BOK, BAK1* and *BAX* as the pro-apoptotic member's genes by having roles in outer membrane permeabilization of mitochondria (Figure 1) [26-28]. Therefore, this miRNA was chosen for further experimental validation.

Consequently, we selected HEK293T and SW480 cells to investigate the functional studies related to *hsa-miR-765* overexpression and knocking down role on the expression of *BAK1, BOK* and *BAX* (Figure 2). HEK293T and SW480 cells both express *BAK1, BOK* and *BAX*, was detectable through RT-PCR (data not shown), and also high rate of transfection has been reported in these two cell lines [20,22].

Although diverse behaviors of targeting *BAK1, BOK* and *BAX* by *hsa-miR-765* were detected in HEK293T and SW480 cells, which probably is related to diverse cell contents and diverse physiological conditions, RT-qPCR results showed a dramatically *BAK1, BOK* and *BAX* downregulation after overexpression of this miRNA meaning a nice targeting of these interested genes with this miRNA (Figure 2) [29-31].

As presenting another evidence for showing the interaction of *hsa-miR-765* with interested candidate genes, we performed dual luciferase assay in HEK293T cells. Results showed a significant reduction of luciferase activity in the cells containing *BAX* and *BOK* 3'UTRs which support *hsa-miR-765* direct interaction with *BOK* and *BAX* (Figure 3). However, no significant result was obtained for *BAK1* which is in accordance with non-alteration of BAK1expression in HEK293T cell line after overexpression of *hsa-miR-765* (Figure 2B). In the other side, the result might be addressed to the targeting of *BAK1* by *hsa-miR-765* at its protein level. Therefore it is continued to be verified if *BAK1* protein level is affected by *hsa-miR-765* in the cells.

In this present study for activation of caspases and apoptosis, we treated SW480 cells with a range of doxorubicin concentrations for 24h time line, and finally 1 μM of doxorubicin concentration was selected to induce apoptosis and activate caspase in these cells (data not shown) before performing caspase-3/7 and caspase-9 assays [6].

Caspase-9 and caspase-3/7 as the major initiator and executioner

apoptosis pathway caspases, respectively are the enzymes activated through differentiation of mouse and human osteoclasts, myocytes, keratinocytes, cardiomyocytes, erythrocytes and in other cells [6,9,10,12,15,17,32]. Therefore in our research, we examined to measure the activity of caspase-9 and caspase-3/7 in SW480 cell line after *hsa-miR-765* overexpression (Figure 4). As illustrated in Figure 4, the activity of caspases-3/7 and caspase-9 are reduced after *hsa-miR-765* overexpression suggesting the clear involvement of *hsa-miR-765* in the controlling of mitochondrial apoptotic pathway (Figure 4).

In a previous report, the function of miRNA-765 in the arterial stiffness by modulating the expression of apelin have been shown which works as an important element for preventing growth, migration and invasion in fulvestrant-treated prostate cancer and promotes proliferation of human hepatocellular carcinoma cell by down-regulation of INPP4B expression [33,34]. Moreover, it has been shown that microRNA-765 controls the proliferation of neural stem cells and their differentiation via Hes1 expression modification and increases CDDP anti-angiogenic outcome through APE1 in osteosarcoma [35,36]. This miRNA mediates in invasion of primary breast carcinoma via effecting on oncogenic protein EMP3 (Epithelial membrane protein 3) [37]. In this study, an increase rate of proliferation and also a significant sub-G1 population reduction was observed upon *hsa-miR-765* overexpression in SW480 cells which is in accordance with the recently reported oncogenic role of *hsa-miR-765* (Figures 5B and 5E) [34,37,38]. Alternatively, mentioned cell cycle data are correlated to the hsa-miR-765 function via targeting of BOK, BAK1 and BAX estimated via computational analysis, dual luciferase assay, caspase assay, and RT-qPCR technologies (Figures 1-4). However the data achieved in HEK293T cells was in opposite (Figures 5A, 5C and 5D). Elevation of sub-G1 in parallel with G0/G1 and G2/M populations' reduction in HEK293T cell line after *hsa-miR-765* overexpression is in accordance with some apoptogenic role of hsa-miR-765 in some cells [39]. Meanwhile, the data we have got for this miRNA function is comparable with our previous publication focused on the role of *hsa-miR-766* in mitochondrial apoptosis regulation [20].

For investigation of the function of *hsa-miR-765* in discriminating of the early steps of apoptosis and differentiation, we used hESCs and induced apoptosis and cardiomyocytes differentiation in these cells, and subsequently analyzed the alteration in the expression profile of this miRNA, by using the protocol previously described (Figure 6) [6,14,17].

Expression analysis of *BAX*, *BAK1* and *BOK* was investigated which showed *BAK1* and *Bok* a similar alteration in the pattern of expression with *hsa-miR-765* in the course of both apoptosis and differentiation of cardiomyocytes (Figures 6A-6D). Since expression of *hsa-miR-765* and *BAX* are in reverse at some time points, it seems that *hsa-miR-765* functions via *BAX* targeting during these processes. A zigzag or oscillation pattern of *hsa-miR-765* expression observed during apoptosis and differentiation induction which demonstrated its role during the cardiac development which is a common feature of the genes involved in cardiac function (Figure 6D) [40,41]. Expression pattern of *hsa-miR-765* is very similar throughout the course of the induction of differentiation and apoptosis however its intensity is different which may be in accord with the information described by Ghiasi et al. [17]. Then again, a significant increase in *hsa-miR-765* expression was demonstrated at day 4 and followed by its sharp significant decrease while we was tracking the expression of this miRNA for the duration of hESCs differentiation into cardiomyocytes (Figure 6E). The amazing data that we have got on increasing of *hsa-miR-765* expression at fourth day after starting cardiomyocyte differentiation was comparable with the data we published on *hsa-miR-766*, which might be attributed to the other key signaling pathways; TGFβ and Wnt signaling with important function in controlling the cardiomyocytes differentiation regulated on this time point [17,20,42,43]. It is still under consideration to be answered if TGFβ and Wnt signaling pathways are under control of *hsa-miR-765*, like the question that we are still figuring out about our previous study on *hsa-miR-766* as well [20].

Conclusion

In conclusion, according to the results presented in this communication, it may be concluded that miR765 is one of the key players in the upstream of mitochondrial pathway of apoptosis. In fact, this regulator can affect the fate of stem cells through regulating of main involving protein in permeabilization of outer membrane of mitochondria like *Bak1, Bok* and *Bax* thereby rendering cell towards cardiomyocytes or apoptosis.

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Conflict of Interest

The authors declare that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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