ARTICLE
Journal of Cellular Biochemistry 116:179–191 (2015)

Journal of Cellular Biochemistry

Inhibitory Effect of Hsa-miR-590-5p on Cardiosphere-derived Stem Cells Differentiation Through Downregulation of TGFB Signaling

Samaneh Ekhteraei-Tousi,[1] Bahram Mohammad-Soltani,[1] Majid Sadeghizadeh,[1] Seyed Javad Mowla,[1] Sepideh Parsi,[1] and Masoud Soleimani[2]

[1]*Molecular Genetics Department, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*

[2]*Hematology Department, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*

ABSTRACT

The cardiac cells generation via stem cells differentiation is a promising approach to restore the myocardial infarction. Promoted by our primary bioinformatics analysis as well as some previously published data on potential role of hsa-miR-590-3p in cardiogenesis, we have tried to decipher the role of miR-590-5p during the course of differentiation of cardiosphere-derived cells (CDCs). The differentiation induction of CDCs by TGFB1 was confirmed by real-time PCR, ICC, and flow cytometry. The expression pattern of hsa-miR-590-5p and some related genes were examined during the differentiation process. In order to study the role of miR-590-5p in cardiac differentiation, the effect of miR-590 overexpression in CDCs was studied. Evaluating the expression patterns of miR-590 and its potential targets (TGFBRs) during the course of differentiation, demonstrated a significant downregulation of miR-590 and an upregulation of TGFBR2, following the treatment of CDCs with TGFB1. Therefore, we proposed a model in which TGFB1 exerts its differentiation induction via downregulation of miR-590, and hence the higher transcriptional expression level of TGFBR2. In accordance with our proposed model, transfection of CDCs by a pLenti-III-hsa-mir-590-GFP expression vector before or after the first TGFB1 treatment caused a significant alteration in the expression levels of TGFBRs. Moreover, our data revealed that overexpression of miR-590 before TGFB1 induction was able to attenuate the CDCs differentiation probably via the reduction of TGFBR2 expression level. Altogether, our data suggest an inhibitory role of miR-590 during the cardiac differentiation of CDCs which its suppression might elevate the rate of differentiation. J. Cell. Biochem. 116: 179–191, 2015.

KEY WORDS: CARDIOSPHERE-DERIVED STEM CELLS; DIFFERENTIATION; hsa-miR-590-5p; TGFB1; TGFBR2

Several stem and progenitor cell types (c-kit$^+$, Sca-1$^+$, Isl-1$^+$, and MDR-1$^+$ progenitor cells, side population, and cardiosphere-derived stem cells) have been reported in the adult heart [Barile et al., 2013]. Cardiosphere-derived cells (CDCs) are a small population of multipotent primitive stem cells and committed progenitors toward the three major cardiac cell lineages; cardiomyocytes, endothelial cells (ECs), and smooth muscle cells (SMCs) [Barile et al., 2013]. The clonogenicity, self-renewal, and multipotentiality are the main properties of CDCs [Davis et al., 2009, 2010]. CDCs population is heterogeneous, comprising c-kit$^+$/CD105$^+$ cells, mesenchymal/stromal progenitor cells (CD90$^+$/CD105$^+$), and a small number of CD31$^+$ and CD34$^+$progenitor cells [Davis et al., 2009]. In animal studies, transplantation of CDCs to the infarcted hearts resulted in myocardial regeneration and functional improvement [Smith et al., 2007; Carr et al., 2011; Mishra et al., 2011; White et al., 2011]. Moreover, CDCs have been recently tested in a phase 1 clinical trial of patients with acute myocardial infarction (CADUCEUS trial) [Makkar et al., 2012].

Cardiac differentiation is highly conserved across the species, where its molecular regulation requires precise spatiotemporal controls [Cripps and Olson, 2002; Satou and Satoh, 2006]. MicroRNAs (miRNAs) are known to be important regulators of cardiogenesis process [Latronico et al., 2007; Zhang, 2008; Small and Olson, 2011], and hence are considered as promising candidates for therapeutic purposes [Pan et al., 2010]. The implications of miRNAs in some pathological process of the cardiovascular system, including cardiac

Conflict of Interest: The authors declare no competing financial interest.

*Correspondence to: Bahram Mohammad-Soltani, Molecular Genetics Department, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran. E-mail: soltanib@modares.ac.ir

Manuscript Received: 24 May 2014; Manuscript Accepted: 22 August 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 August 2014

DOI 10.1002/jcb.24957 • © 2014 Wiley Periodicals, Inc.

arrhythmia, cardiac hypertrophy, heart failure, cardiac fibrosis, cardiac ischemia, and vascular atherosclerosis, have been recently documented. For underscoring the therapeutic potential of miRNAs in cardiovascular diseases, the overexpression or downregulation of a single miRNA is enough to cause the pathological alteration [Pan et al., 2010]. Endogenously-made miRNAs (~22 nt) act by binding to the complementary sequences of their target transcripts, resulting in translational repression or mRNA degradation [Small and Olson, 2011]. As miRNAs could provide a new mean to manipulate stem and progenitor cells fate, understanding their pattern of expression and their roles during the cardiac differentiation is of crucial importance [Srivastava , 2006; Cordes and Srivastava, 2009]. Two miRNA profiling experiments have been recently reported for the cardiomyocytes derived from human embryonic stem cells [Wilson et al., 2010; Synnergren et al., 2011]. Moreover, the roles of some miRNAs such as miR-1, miR-499, and miR-133a in cardiac cell differentiation have been established [Liu et al., 2008; Sluijter et al., 2010]. Also, hsa-miR-590-3p has been reported to promote cardiomyocyte proliferation in animals [Eulalio et al., 2012].

In the present study, a combination of several bioinformatics approaches and literature studies has been employed to identify miRNAs with a potential regulatory role in cardiac differentiation. Initial analysis suggested a link between decreased expression level of hsa-miR-590-5p and TGFB1 induction, as an important cardiac related biological pathway [Behfar et al., 2002; Lim et al., 2007; Goumans et al., 2008]. Accordingly, a model was proposed and experimentally validated in which miR-590-5p exerts an inhibitory role in CDCs differentiation.

MATERIALS AND METHODS

PROPAGATION AND DIFFERENTIATION OF HUMAN CARDIOSPHERE-DERIVED STEM CELLS

Human cardiosphere-derived stem cell preparations (5th passage) from three different patients were obtained from Royan Stem Cell Bank (National code number: RSCB0180).

These human cell samples were approved by the ethical committees of the Royan Stem Cell Banks (RSCB). Individual permission using standard informed consent procedures from the patients was obtained. The preparation of the human samples in RSCB is based on the principles that are defined in the Declaration of Helsinki. The related flow cytometry data of some cell surface CD markers of the cells are summarized in Figure 1. Cardiac differentiation was performed according to a method described previously, in which co-culture of the CDCs with a feeder layers such as neonatal rat cardiomyocytes was excluded [Smits et al., 2009]. In each experiment, ~10^4 CDCs per cm^2 at passage 7 were seeded in duplicate onto gelatin-coated 6-well plate. To initiate the differentiation, cells were treated with 5 μM 5-azacytidine (5-aza, Sigma) for three consecutive days in differentiation medium (Iscove's Modified Dulbecco's Medium/Ham's F12 (1:1; GIBCO), supplemented with L-glutamine (GIBCO), 2% horse serum (GIBCO), and nonessential amino acids (Sigma).

Six days after the start of the differentiation process (two days after finishing the 5-aza induction), 1× ascorbic acid (10^{-4} M, Sigma), and 1 ng ml^{-1} TGFB1 (PeproTech) were added to the medium. Then, ascorbic acid (AA) and TGFB1 were added every other days and twice per week, respectively. Regular observation of cellular morphology was performed using an Olympus phase contrast microscope, during the course of differentiation.

IMMUNOCYTOCHEMISTRY (ICC) AND FLOW CYTOMETRY

ICC and flow cytometry techniques were carried out on CDCs, differentiating cells, and transfected cells after 28 days of the first seeding, using an antibody against cardiac TrnI (a sarcomer component [Smits et al., 2009]). For ICC, the cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), before being permeabilized (0.4% Triton X-100/PBS) and blocked with 2% bovine serum albumin (BSA), for 15–30 min. Subsequently, coverslips were incubated with the TrnI primary antibody (Hy Test Ltd, diluted 1/100) in PBS/10% normal goat serum (NGS), overnight at 4 °C. Next day, coverslips were incubated in blocking solution containing the secondary antibody (anti-mouse IgG, FITC conjugated goat antibody-RAY Biotech—for differentiated cells and anti-mouse IgG, PE conjugated goat antibody—Santa Cruz Biotechnology—for transfected cells, diluted 1/32) in PBS/10% NGS for two hours. DAPI dye was used to delineate the nuclei of the cells. In addition, mouse IgG1 isotype antibody (eBioscience) was used in ICC experiment. Incubation with the secondary antibody alone was also used as the negative control. ICC signals were visualized using a fluorescence microscope (Nikon Eclipse TE2000-U), and images acquired with a Nikon digital DXM 1200 camera.

For flow cytometry analysis, the cells were trypsinized, fixed in 4% PFA for 15 min, permeabilized, and washed with PBS, before being incubated with the TrnI antibody for 1 h at 4°C. The cells were then washed and incubated with the FITC conjugated or PE secondary antibody and intensity of the signals were measured using a flow cytometer (BD Biosciences). To quantify total cell numbers expressing TrnI, an isotype control (IgG1) was used to set the negative gate.

TOTAL RNA EXTRACTION AND REAL-TIME PCR

Total RNA was extracted from differentiating cells at 1, 2, 3, and 4 weeks post-plating, using Trizol reagent (Invitrogen), as instructed by the manufacturer. Undifferentiated cells (human CDCs, day 0) were used as the reference sample for real-time PCR, whereas the differentiated human cardiac myocytes (HCM, NCBI Code: C599, Pasteur) were used as a control for cardiac differentiation. In addition, total RNA from transfected cells with pLenti-III-hsa-miR-590-GFP and blank construct was extracted after 1, 2, and 3 weeks of transfection.

Briefly, one microgram of each DNase I (TAKARA) treated RNA samples was reverse transcribed using RevertAid^{TM} reverse transcriptase (Fermentas) and oligodT primer. Specific primers targeting the genes with a role in cardiomyocyte differentiation and TGFB signaling (TGFB1, TGFBR1, TGFBR2, MYCN, and EIF4H) were designed using the Primer 3.0 and were analyzed by OligoAnalyzer 3.1 in Integrated DNA Technologies (IDT) web server (Table I).

Real-time PCR reactions were performed using SYBR Green PCR master mix (TAKARA), on an Applied Biosystems 7500 real-time

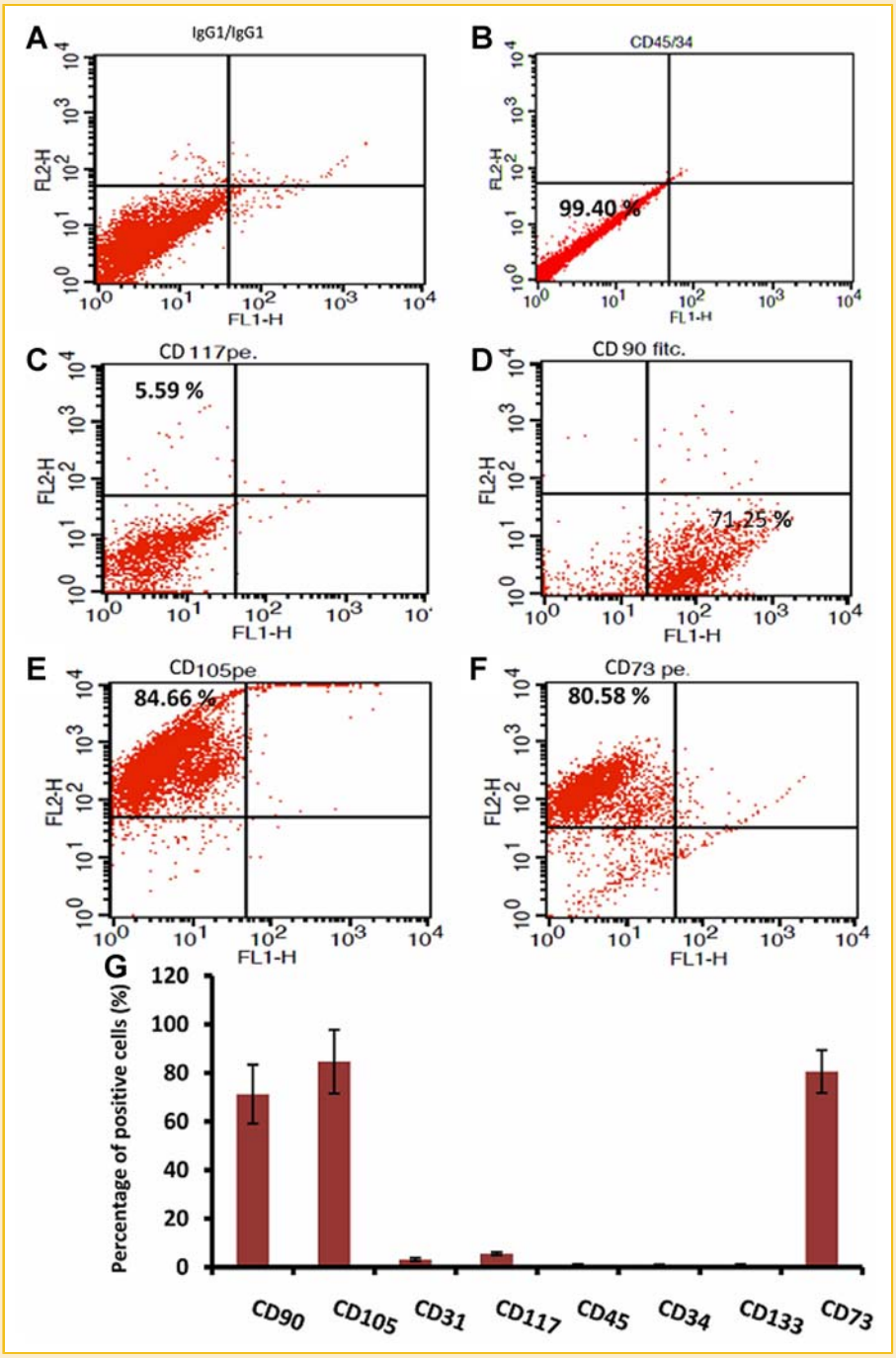

Fig. 1. Cell surface markers expression profile on undifferentiated hCDCs. High percentage of the cells which were positive for CD105, CD90, and CD73 (mesenchymal/stromal stem cell markers) and low percentage of $CD117^+$, $CD31^+$, $CD34^+$, and $CD133^+$ cells showed the cell preparations were heterogenous sources of cardiosphere-derived stem cells.

PCR machine. Cycling conditions were as follows: 95°C for 30 s followed by 40 cycles of amplification (denaturation at 95°C for 5 s, annealing at 51°C for 10 s and extension at 72°C for 40 s). All reverse transcriptase reactions, including no-template controls and RT minus controls, were run in duplicate. Quantified values were normalized against the HPRT1 housekeeping gene.

To analyze candidate miRNAs expression pattern, 20 ng of DNase-I treated total RNA was isolated from biological duplicates and used to synthesis cDNA using miRCURY LNA™ cDNA synthesis kit (Exiqon, Denmark). For amplification of each candidate miRNA, specific LNA™ PCR primer set (Exiqon) was used according to the manufacturer's instruction. The obtained CTs were normalized to that of endogenous control gene RNU48 [Wilson et al., 2010]. Cycling conditions of real-time PCR were as follows: 95°C for 10 min, followed by 40 cycles of amplification (denaturation at 95°C for 10 s, annealing and extension at 60°C for

TABLE I. Sequences of the Primers Used in Real-Time PCR

Primer	Forward sequences	Reverse sequences	Product size
Mef2c	5′-CTGGTGTAACACATCGACCTC -3′	5′-GATTGCCATACCCGTTCCCT-3′	105
ETS1	5′-TACACAGGCAGTGGACCAATC-3′	5′-CCCCGCTGTCTTGTGGATG-3′	237
GATA4	5′- CAGCAACTCCAGCAACG-3′	5′- ATCGCACTGACTGAGAACG-3′	126
GATA6	5′-CTGCGGGCTCTACAGCAAG-3′	5′-GTTGGCACAGGACAATCCAAG-3′	100
HPRT1	5′-CCTGGCGTCGTGATTAGTG-3′	5′-TCAGTCCTGTCCATAATTAGTCC-3′	125
Cnx43	5′-AGCTGCTGGACATGAATTAC-3′	5′-CTAGATCTCCAGGTCATCAG-3′	109
TrnT	5′-ACAGAGCGGAAAAGTGGGAAG-3′	5′-TCGTTGATCCTGTTTCGGAGA -3′	230
MYH7	5′-GGGGCAACAGGAAAGTTGG-3′	5′-CTCCTCAGCGTCATCAATGGA-3′	228
Actin	5′-TCACTGAGCGTGGCTACTCC-3′	5′-CTTGGTGATCCACATCTGCTG-3′	500
TGFB1	5′-CAATTCCTGGCGATACCTCAG-3′	5′-AACCACTGCCGCACAACT-3′	96
TGFBR1	5′-CATTTTTCCCAAGTGCCAGT-3′	5′-ACACCCCTAAGCATGTGGAG-3′	235
TGFBR2	5′-TTTGGATGGTGGAAGGTCTC-3′	5′-GCAACAGCTATTGGGATGGT-3′	193
MYCN	5′-TGATCCTCAAACGATGCCTTCCC-3′	5′-GGACGCCTCGCTCTTTATCTTCT-3′	113
EIF4H	5′-CACCTCCCCTTCAGTTCAGAGC-3′	5′-CAGTGTGTCCCCAAGATGTGG-3′	157

1 min). PCR efficiency was evaluated by LinRegPCR (12.x) software and raw data were introduced into DataAssist v3.0 software for further analysis.

OVEREXPRESSION OF MIR-590

As transduction of the CDCs might interfere with the timing of TGFB1 treatment and differentiation process, transient transfection strategy was performed instead of stable transduction of the cells. For that reason, 1 μg of pLenti-III-hsa-mir-590-GFP vector (ABM, Canada) was used for transfection of 5-aza treated CDCs with lipofectamin 2000 (Invitrogen) in 24-well plates, containing about 10^4 cells per well. Transfection was performed either at day 4 or 10 of post-plating, and a blank construct was used for transfection of negative control cells. The transfection efficiency was determined by flow cytometry which can accurately quantitate reporter gene expression (Green Fluorescent Protein, GFP) in each cell in a population being transfected. For selection of the transfected cells, the medium was replaced with fresh differentiation medium containing 4 μg/ml puromycin (Sigma), 24 h after transfection. The medium was refreshed with fresh puromycin-containing medium every other day. Ascorbic acid (AA) and TGFB1 were added to the medium according to the differentiation protocol [Smits et al., 2009].

DATA ANALYSIS AND STATISTICS

Data obtained from real-time PCR were analyzed with the one-way ANOVA (SPSS version 16.0). Post hoc multiple comparisons between samples for cardiac marker genes, candidate miRNAs and related target genes were made using Tukey's test. A P-value < 0.05 was considered as statistically significant, and error bars indicate standard deviation from the mean. All experiments were repeated at least twice with duplicate samples in each experiment.

RESULTS

DIFFERENTIATION OF CDCs TOWARD CARDIAC-LIKE CELLS

High percentage of the cells which were positive for CD105 (Endoglin, a part of the TGFB1 receptor complex), CD90 (Thy-1), and CD73 (lymphocyte-vascular adhesion-protein 2) as mesenchymal/stromal stem cell markers and low percentage of $CD117^+$ (c-kit), $CD31^+$, $CD34^+$, and $CD133^+$ cells showed that the cell preparations were heterogeneous sources of cardiosphere-derived stem cells (Fig. 1).

CDCs were first treated with 5-azacytidine (5-aza) demethylating agent and TGFB1, and then the differentiation process was weekly monitored up to four weeks. Our cellular and molecular data confirmed the process of cardiac differentiation. Firstly, as a result of differentiation induction, a fibroblast- like morphology of undifferentiated CDCs (Fig. 2A) changed into a branched and elongated morphology, mimicking the heart's mature cardiomyocyte morphology (Fig. 2B, C). Secondly, ICC was performed using a specific antibody against a sarcomeric component of the cardiomyocytes (TrnI), where accumulation of this protein was observed in the cytoplasm of differentiating cells, 28 days post plating (Fig. 2E). As expected, there was no considerable signal for the presence of TrnI protein in undifferentiated CDCs (Fig. 2D). Furthermore, flow cytometry results indicated that ~30% of the differentiated cells were $TrnI^+$ (Fig. 2F–I). Thirdly, the expression level of Mef2c, GATA4, GATA6, ETS1, Cnx43, TrnT, MYH7, and alpha-Sarcomeric actin were first decreased upon treatment of the cells with 5-aza, and increased following the induction of TGFB1 (Fig. 3, $P < 0.05$). The elevated expression of early cardiac transcription factors, as well as late cardiac differentiation markers (Cnx43, TrnT, MYH7, and alpha-Sarcomeric actin) mimicked the expression status of these markers in the human cardiomyocytes (Table II and Fig. 3). All together, our morphological and molecular data indicated a differentiation of CDCs toward cardiac-like cells.

BIOINFORMATICS ANALYSIS OF MIRNAS INVOLVED IN CARDIOGENESIS

Based on previously published miRNA expression profiles [Wilson et al., 2010; Synnergren et al., 2011], 272 miRNAs were found to be differentially expressed ($P < 0.05$) during the course of embryonic stem cells differentiation into cardiomyocytes. These miRNAs were then introduced to the Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com/products/ipa/try-ipa-for-free) and generated ~640 target genes (predicted or validated), with one or more assigned biological roles in the cardiovascular system. As the genes that have roles in the cardiovascular system are not necessarily involved in cardiogenesis, 76 of the aforementioned genes were further handpicked, as the candidate genes with known roles in cardiogenesis. These genes were targeted by 123 out of 272

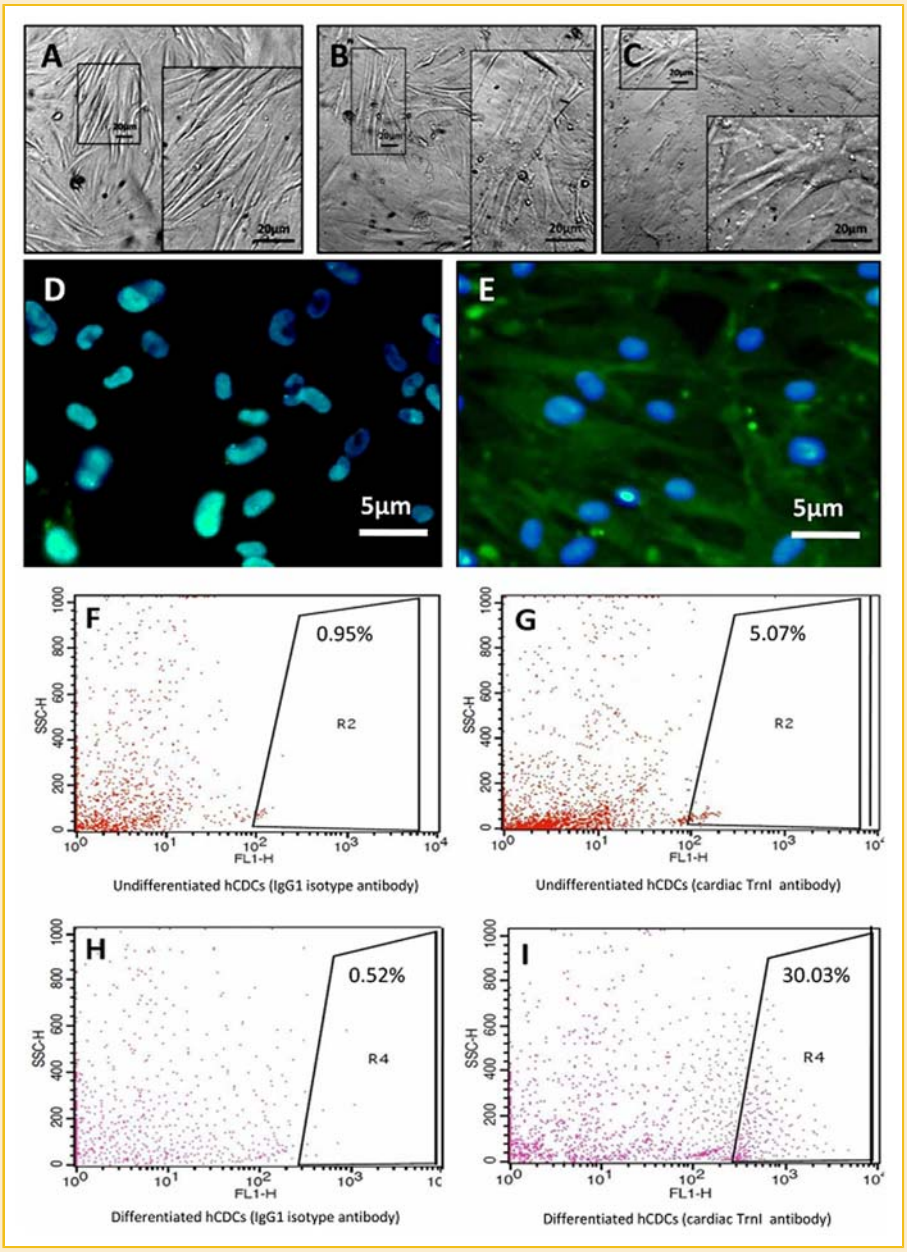

Fig. 2. Morphological and Immunocytochemical evidences of CDCs differentiation toward cardiac-like cells. A: Fibroblast-like undifferentiated CDCs. B and C: Differentiated cardiomyocyte-like cells with the unique branched and elongated morphology at 9th and 28th day post-plating, respectively. D: Shows undifferentiated CDCs without considerable ICC signal for TrnI protein, and E: Differentiated cells on 28 days after induction of differentiation. Also, (E) is only a small field of the chamber slide and is not representative of the whole culture. In the immunocytochemical analysis against TrnI, a specific first monoclonal antibody against TrnI and a (goat) FITC secondary antibody (green) were used. Blue fluorescence (DAPI) indicates nuclear staining. F and H: Flow cytometry analysis using IgG1 isotype antibody against undifferentiated and differentiated CDCs. G and I: Flow cytometry analysis against TrnI antibody in undifferentiated and differentiated CDCs, respectively. Results shows that ~30% of differentiating cells are positive for TrnI compared to undifferentiated CDCs.

aforementioned miRNAs, suggesting their potential biological roles in cardiogenesis (Table I). Two of these miRNAs (hsa-miR-590-5p and hsa-miR-125a-5p) were chosen for further studies, based on their transcriptional expression alteration during the course of cardiac differentiation, as well as the number of their targets with involvement in cardiogenesis. Furthermore, DIANA-mirPath (http://diana.cslab.ece.ntua.gr/pathways/) software suggested that these candidate miRNAs might be involved in some of the cardiac related biological pathways such as TGFB, MAPK, and WNT signaling pathways (Table III).

miR-590 AND ITS PUTATIVE TARGET GENES CO-EXPRESSION ANALYSIS

Following treatment of CDCs with exogenous TGFB1 protein at day 6th of differentiation process, the level of miR-590 was sharply declined (Fig. 5A, $P < 0.001$), whereas the level of miR-125a was

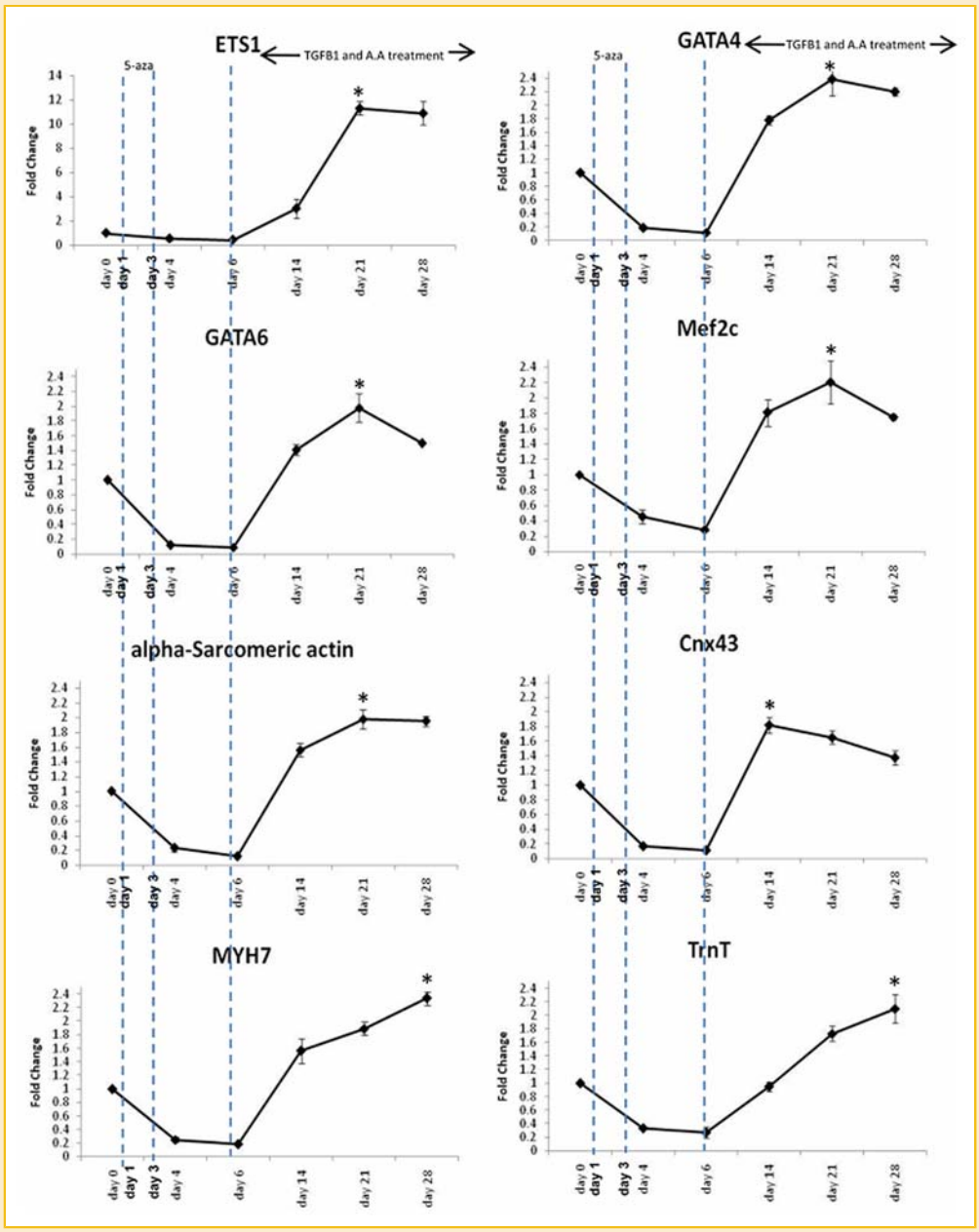

Fig. 3. Expression profiles of Mef2c, GATA4, GATA6, ETS1 transcription factors and Cnx43, TrnT, MYH7, and alpha-sarcomeric actin during the cardiac differentiation. Data shows significant up regulation of Mef2c, ETS1, GATA4, and GATA6 in the cells on day 21 compared to undifferentiated CDCs on day 4. Cnx43, TrnT, MYH7, and alpha-sarcomeric actin is also up regulated since day 6 after induction of differentiation. Error bars represent SD from the mean of four replicate experiments (*$P < 0.01$ compared with amounts on day 0).

slightly increased at the later stages (Fig. 5A, $P < 0.05$). The sharp decline of miR-590 expression level upon exogenous TGFB1 treatment suggests that it may be regulated by the TGFB signaling pathway. In addition to the DIANA microT3.0 (Fig. 4) bioinformatics algorithm's prediction, two recent reports by others [Shan et al., 2009; Jiang et al., 2012] indicated that TGFBR2 is targeted by miR-590. Based on the real-time PCR data, the expression of miR-590 was in its highest level before the 6th day of CDCs differentiation process (Fig. 5A, $P < 0.05$). Accordingly, the transcript levels of TGFB1 (endogenous), TGFBR1, and TGFBR2 were at their lowest levels (Fig. 5B, $P < 0.05$). Upon exogenous TGFB1 treatment of the CDCs, the transcript levels of miR-590 and TGFBR2 were significantly altered (Fig. 5B, $P < 0.05$). The later finding suggests that exogenous TGFB1 treatment inhibits miR-590 expression and induces TGFBR2 target gene stability. Transcription level of endogenous TGFB1 slightly increased since the 6th day of differentiation process (Fig. 5B, $P < 0.05$).

Real-time PCR results further demonstrated that EIF4H transcript (the gene in which miR-590 is located at 4th intron) was at the highest level at 4th day of differentiation process, while its level

TABLE II. The Fold Change of Studied Genes in Human Cardiac Myocyte (hcm, Ncbi Code: C599, Pasteur) Compared to CDCs (before Differentiation Induction) ± SD (standard deviation)

Genes	Fold change
Mef2c	2.56 ± 0.17
ETS1	3.50 ± 0.05
GATA4	0.98 ± 0.07
GATA6	1.54 ± 0.03
Cnx43	0.95 ± 0.05
TrnT	2.11 ± 0.24
MYH7	1.15 ± 0.19
alpha-Sarcomeric actin	2.67 ± 0.20
hsa-miR-590-5p	2.71 ± 0.10
hsa-miR-125a-5p	0.79 ± 0.03
TGFB1	1.07 ± 0.03
TGFBR1	0.59 ± 0.09
TGFBR2	7.57 ± 0.95

declined before TGFB1 treatment at day 6, independent of exogenous TGFB1 treatment (Fig. 5C, $P < 0.05$). Similar pattern of expression alteration was observed for MYCN, a positive regulator of both miR-590 and its host gene, which is also a target for TGFB1protein [Shohet et al., 2011].

THE INHIBITORY EFFECT OF miR-590 OVEREXPRESSION ON TGFB1-INDUCED CDCs DIFFERENTIATION

To induce CDCs differentiation towards the cardiac cell fate, these cells were first treated by 5-aza and then by TGFB1 at 6th day of plating. In order to examine the role of miR-590 in CDCs differentiation, pLenti-III-hsa-mir-590-GFP vector was used to overexpress miR-590 before or after TGFB1 treatment of the cells at 4th day or 10th day of the process, respectively. Then, the result was compared to the cells transfected with a CMV-blank vector. The transfection efficiency was about 65% (detected by flow cytometry) when the cells were transfected with pLenti-III-hsa-mir-590-GFP or CMV-blank vectors. The real-time PCR has been performed at all time points during the differentiation process. Real-time PCR data confirmed the exogenous expression of miR-590 at 14th day of differentiation, when the cells had been transfected either at 4th day (40 times, $P < 0.001$) or 10th day (10 times, $P < 0.001$) of induction (Fig. 6A). Coincident with miR-590 overexpression, a significant downregulation of TGFB1, TGFBR1, and TGFBR2 were detected (Table IV, Fig. 6B, C and D, $P < 0.05$). The effect of miR-590 overexpression before and after the first TGFB1 treatment on its target genes expression has been also compared. The results indicate that there was no statistically significant difference between them.

The effect of miR-590 overexpression on the differentiation statues of CDCs was also examined with ICC and flow cytometry against TrnI cardiac specific marker protein. These techniques demonstrated that in the CDCs overexpressing miR-590 since 4th day of differentiation, TrnI protein has been less abundant compared to the cells transfected at the 10th day of the process ($P < 0.05$, Fig. 7). Collectively, these results suggested that overexpression of miR-590 before TGFB1 induction of differentiation, is capable of attenuating CDCs differentiation towards a cardiac-like fate.

TABLE III. Predicted and Validated Target Genes of Two Candidate miRNAs That Are Involved in Cardiogenesis (Obtained from IPA and DIANA MicroT3.0)

Target gene symbol	hsa-miR-590-5p	hsa-miR-125a-5p
TGFBR3	+	-
TGFBR2	+	-
TGFBR1	+	+
ACVR1C	+	+
ACVR2B	+	+
ACVR1B	+	-
ACVR2A	+	-
APC	-	+
BMPR1B	+	+
BMPR2	+	+
BMP8A	-	-
BMPR1A	+	-
BMP3	+	-
BMP2	-	-
FZD1	-	+
SMAD1	+	-
SMAD2	+	+
SMAD4	+	+
SMAD5	+	+
SMAD9	+	-
CCNE1	-	-
GATA4	-	-
LRP6	+	-
MAP3K7	-	-
TCF3	-	-
E2F4	-	-
NODAL	-	-
DKK1	+	-
FZD6	+	-
LRP1	+	-
PRKCA	+	-
PRKCE	+	-
PRKCI	+	-
TCF4	+	-
TCF7L1	+	-

+ means the genes targeted by miRNAs.

DISCUSSION

Multiple lines of evidence have emphasized the essential roles of miRNAs in cardiac development and cardiac progenitor cells differentiation [Latronico et al., 2007; Zhang, 2008; Small and Olson, 2011]. The CDCs are a heterogeneous subtype of cardiac progenitor cells that are able to differentiate into three main cardiac cell lineages without requiring co-culture with neonatal cardiomyocytes [Barile et al., 2013]. CDCs have been reported to improve the function of infarcted rat myocardium [Carr et al., 2011]. The usage of CDCs in CADUCEUS trial has implicated the decreased scar formation by 30 to 70%, and increased left ventricular mass in ischemic adult patients [Makkar et al., 2012]. Also, the other clinical trial (SCIPIO trial) has reported the delivery of a pure population of c-kit$^+$ cardiac stem cells to adult patients with myocardial infarction which resulted in the reduction of infarct size (30%) over 12 months. The recent study didn't show any statistically significant change in ejection fraction with the treatment of adult-derived CDCs [Bolli et al., 2011]. Thus based on the mentioned study, c-kit$^+$ cells may be more effective than the heterogeneous CDCs population. In the other study, it has been shown that c-kit$^+$ subpopulation purified from CDCs produced lower levels of paracrine factors and mediated lower functional benefits compared with unsorted CDCs [Li et al., 2012]. Whether or not the purified cells populations are superior to CDCs for cell therapy approaches and cardiac regeneration remain to be more studied [Barile et al., 2013].

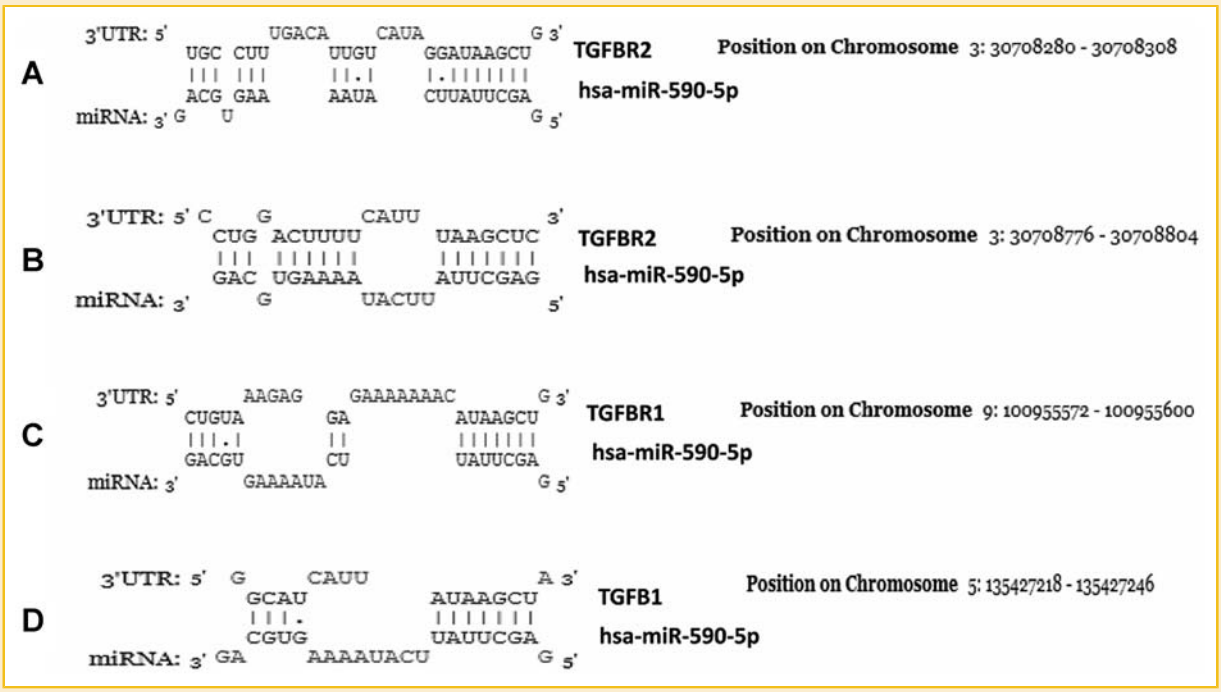

Fig. 4. Target prediction for miR-590-5p via DIANA-micro T version 3. Predicted data shows the interactions of miR-590-5p with TGFBR2 at two different positions on its 3′UTR. Also, miR-590-5p targets TGFBR1 and TGFB1.

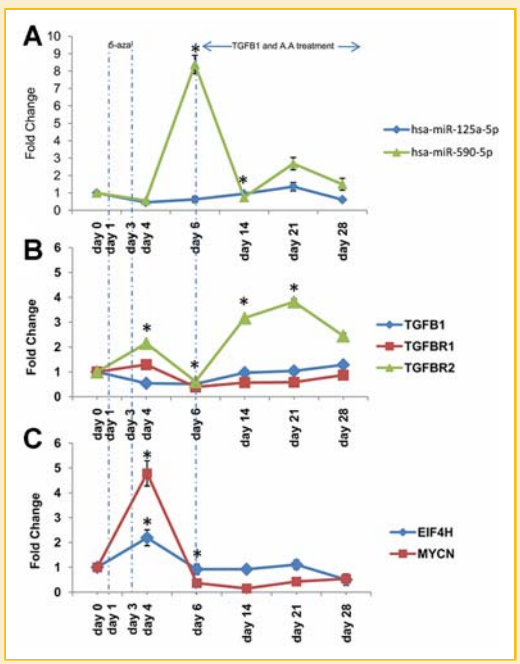

Fig. 5. Temporal alterations in the transcriptional expression of two candidate miRNAs and some of the miR-590 related genes. A: Unlike miR-125 and miR-590 expression pattern alteration during the CDCs differentiation upon TGFB1 treatment. B: Sharp TGFBR2 expression alteration upon TGFB1 treatment of the CDCs during the differentiation process, and C: miR- 590 host gene (EIF4H) and its positive regulator (MYCN) downregulation during the cardiac differentiation. Error bars represent SD from the mean of four replicate experiments (*$P < 0.01$ compared with amounts on day 0, Data obtained from miRNAs real-time PCR were normalized to the endogenous control gene RNU48).

Although some miRNAs such as miRNA-1, miRNA-499, and miRNA-133a are reported to be able to regulate cardiac differentiation and proliferation [Liu et al., 2008; Sluijter et al., 2010], the role of other individual miRNAs in controlling of proliferation and differentiation remains largely unknown. In the current study, our results demonstrated that overexpression of miR-590-5p attenuates the differentiation of CDCs.

DIFFERENTIATION OF CDCs

In the current research, several lines of evidence confirmed the differentiation of CDCs to the cardiomyocyte-like cells; 1) at the end of differentiation process, the shape of the fibroblast-like CDCs changed into a cylindrical and branched morphology, similar to the morphology of the cardiomyocyte cells isolated from the human fetal heart [Sawada and Kawamura, 1991; Okabe et al., 1999; Hosoda et al., 2011]. 2) The expression profile of cardiac cell markers: Mef2c, GATA4, GATA6, ETS1, TrnT, MYH7, Cnx43, and alpha-sarcomeric actin [Noseda et al., 2011], confirmed the differentiation process. GATA4 and GATA6 are regulators of gene expression in the cardiac muscle cells [Charron et al., 1999; Liang et al., 2001] and Mef2c regulates TrnI expression [Di Lisi et al., 1998]. Consistent with TrnI accumulation and acquisition of cardiac-like cell morphology, the transcription of these markers was increased from day 6th of differentiation process. ETS1 transcription factor is known to be expressed during the cardiac morphogenesis, a process accompanied by cell trans differentiation and vascular endothelial cell formation [Reisdorff et al., 2002; Anversa et al., 2007; Ye et al., 2010]. A sharp elevation in the expression level of ETS1 from day 6th of differentiation process might be attributed to the cardiac differentiation, probably differentiation to vascular endothelial cells. The

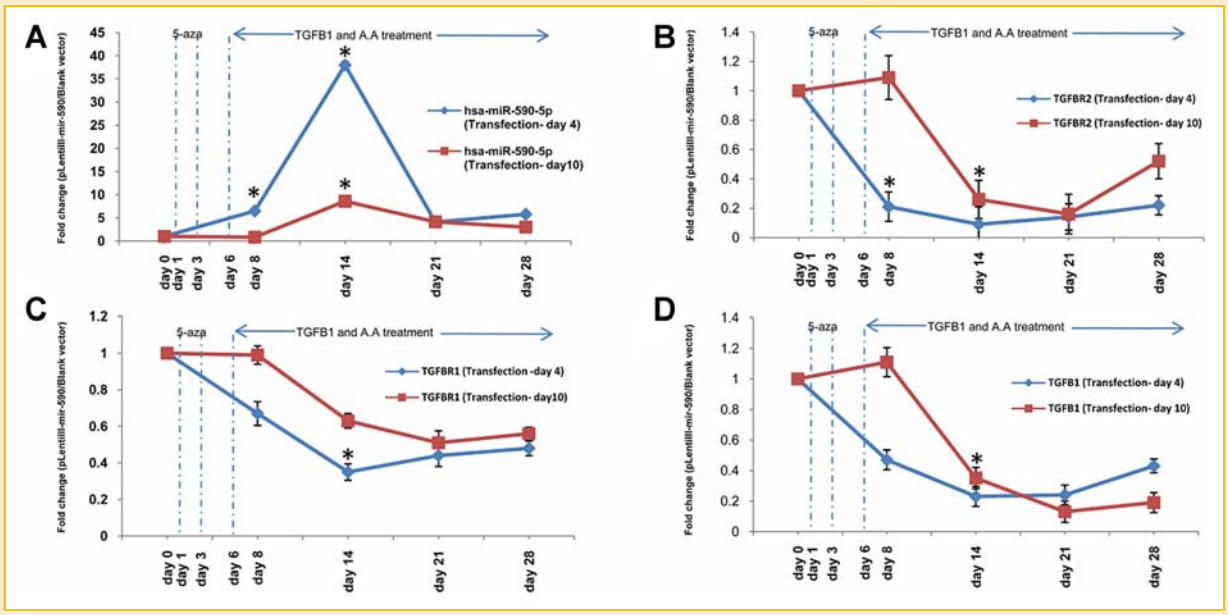

Fig. 6. The effect of miR-590 overexpression on its target genes and TGFB1 expression level. Each spot shows the fold change of intended gene transcription level, in the cells overexpressing miR-590 compared to the cells transfected by CMV- blank vector. The gene expression patterns of the cells transfected by overexpressing miR-590 vector at 4th and 10th day of differentiation process are compared in each graph. A: Overexpression of miR-590 after transfection of the CDCs. B, C, and D: Coordinated decreased transcription levels of TGFBR2, TGFBR1, and TGFB1 in the transfected CDCs following miR-590 overexpression (*$P < 0.01$ compared with amounts on day 0).

increased transcript level of ETS1 may be due to high percentage of $CD105^{+}$ cells in the used CDCs preparation with the fate of angiogenesis. Endoglin (CD105) is known to be involved in the heart angiogenesis [Duff et al., 2003; López-Novoa and Bernabeu, 2010]. The gap junctional connexin 43 (Cnx43) is involved in the cardiac syncytium formation [Brink et al., 2011] and its transcription alterations is potentially consistent with the appearance of branched shaped cells around the same time (Fig. 2B, C and Fig. 3). Comparing the flow cytometry results between two groups (differentiation-induced group and undifferentiated group without any treatment) indicated that ~30% of the differentiated cells were TrnI positive.

Altogether, the morphology changes of the differentiating cells, ICC plus flow cytometry results, as well as molecular marker expression data, confirmed the differentiation of CDCs toward a cardiac-like cell phenotype.

A POSITIVE FEED-BACK LOOP BETWEEN TGFB1 AND ITS RECEPTORS THROUGH miR-590

Two prominent miRNA profiling data have been reported during the differentiation process of human embryonic stem cells toward cardiomyocytes [Wilson et al., 2010; Synnergren et al., 2011]. Our bioinformatics analysis by using these miRNA profiling data indicated that 123 miRNAs target 76 genes with involvement in cardiogenesis. 25 of these genes are targeted by miR-590, from which some are involved in TGFB, MAPK, and other well-known signaling pathways (Table III). A significant alteration of miR-590 expression level has been already reported during the differentiation of embryonic stem cells to cardiomyocytes [Wilson et al., 2010]. Considering its targets category and expression profile during the cardiogenesis, miR-590 was further investigated during the course of CDCs differentiation.

TABLE IV. The Inhibitory Effect of miR-590 Overexpression on Its Target Genes Expression Level (*$P < 0.05$)

		Day 0	Day 8*	Day14	Day 21	Day 28
miR-590	Trans-Day 4[a]	1	6.48[c] ± 0.10	37.98 ± 0.12	4.09 ± 0.08	5.77 ± 0.09
	Trans-Day 10[b]	1	0.83 ± 0.09	8.59 ± 0.14	4.14 ± 0.16	3.01 ± 0.12
TGFBR2	Trans-Day 4	1	0.21 ± 0.08	0.12 ± 0.09	0.14 ± 0.06	0.22 ± 0.06
	Trans-Day 10	1	1.09 ± 0.15	0.26 ± 0.13	0.16 ± 0.13	0.52 ± 0.12
TGFBR1	Trans-Day 4	1	0.67 ± 0.05	0.35 ± 0.06	0.44 ± 0.07	0.48 ± 0.03
	Trans-Day 10	1	0.99 ± 0.06	0.63 ± 0.05	0.51 ± 0.06	0.56 ± 0.04
TGFB1	Trans-Day 4	1	0.47 ± 0.06	0.23 ± 0.06	0.24 ± 0.06	0.43 ± 0.04
	Trans-Day 10	1	1.11 ± 0.09	0.35 ± 0.07	0.13 ± 0.07	0.19 ± 0.06

[a]Transfected by overexpressing miR-590 vector at 4th day.
[b]Transfected by overexpressing miR-590 vector at 10th day.
[c]Each number shows the fold change of intended gene transcription level, in the cells overexpressing miR-590 compared to the cells transfected by CMV-blank vector.

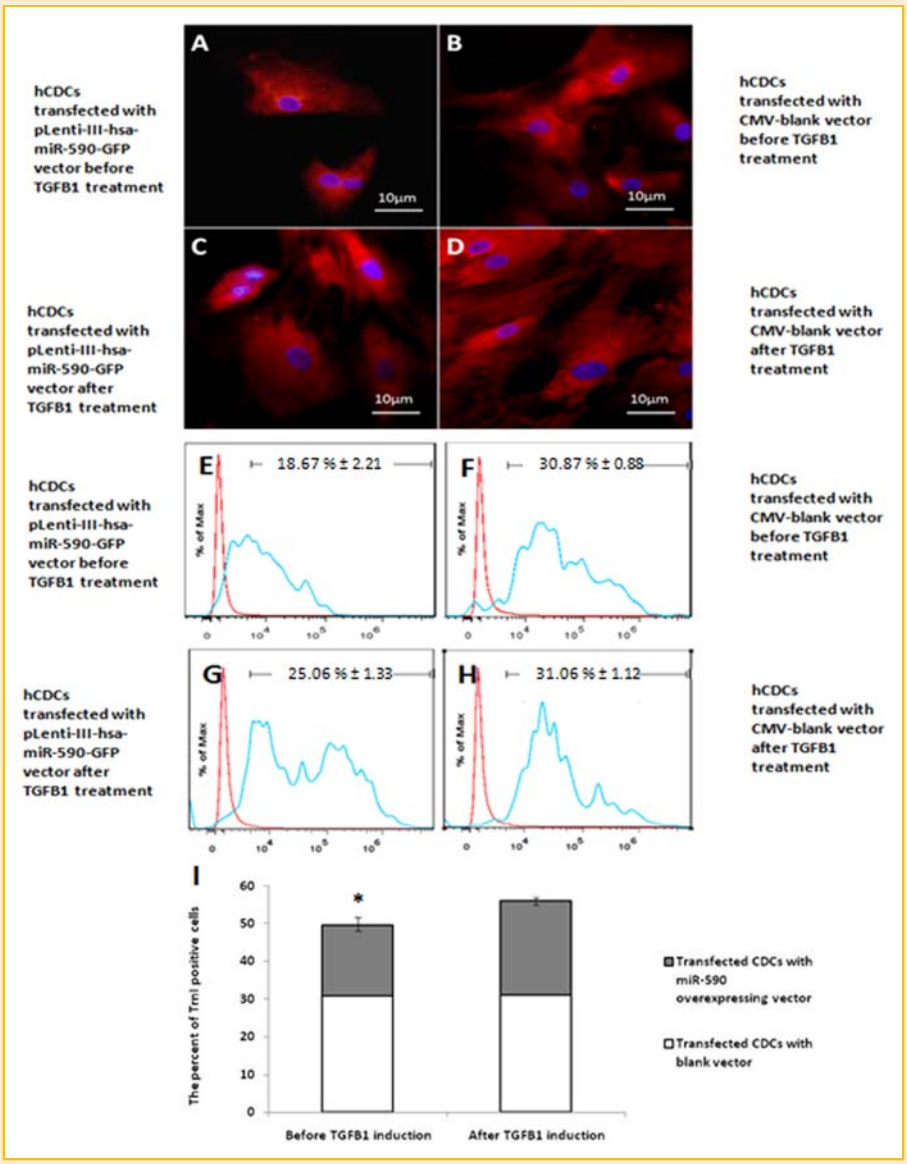

Fig. 7. TrnI protein evaluation in the cytoplasm of miR-590 transfected CDCs. Data obtained from ICC and flowcytometry illustrates the amounts of TrnI protein level in miR-590 transfected CDCs at 10th day is more compared to the miR-590 transfected CDCs at 4th day (A, C, E, and G). PE conjugated polyclonal goat anti-mouse IgG antibody (Santa Cruz Biotechnology) was used as secondary antibody. (I) A diagram to illustrate the statistical significant differences between two $TrnI^{+}$ cell groups that were transfected with miR-590 before or after TGFB1 induction (*$P < 0.05$).

Consistent with the results obtained in our study, TGFB1 has been reported to induce cardiac differentiation into cardiomyocytes [Goumans et al., 2008]. Here, we demonstrated that the expression level of miR-590 is sharply declined following TGFB1 treatment of CDCs. MiR-590 is located in the 4th intron of EIF4H host gene which is known to be upregulated by MYCN transcription factor [Shohet et al., 2011]. TGFB1 down-regulates MYC transcription factor [Chen et al., 2002]. Consistently, expression alteration of MYCN was related to the expression alterations of EIF4H at day 4. It seems the reduction of MYCN and EIF4H expression before TGFB1 treatment probably due to the activation of its upstream inhibitor genes via 5-aza treatment. Furthermore, decreased expression level of miR-590 following TGFB1 treatment may be related to an independent promoter of miR-590 from its host gene (EIF4H). The intronic promoter activity of miR-590 was experimentally validated by Monteys et al. [2010]. Recently, the other group demonstrated that miR-590 promoter is activated by phosphorylated STAT5 (Favreau and Sathyanarayana, 2012). Also, it is known that TGFB1 inhibits the expression of STAT5 at the protein level without any change in mRNA expression [Ryan et al., 2010]. TGFB1-induced activation of Smad2, -3, and -4 leads to a direct inhibition of STAT5 trans-activation and STAT5-mediated transcription of the downstream target genes such as miR-590 [Cocolakis et al., 2008]. Thus, decreased expression level of miR-590 upon TGFB1 treatment is expected due to inhibitory effect of TGFB1 on STAT5. Moreover, TGFB1 receptors regulate the specificity of TGFB signaling pathway

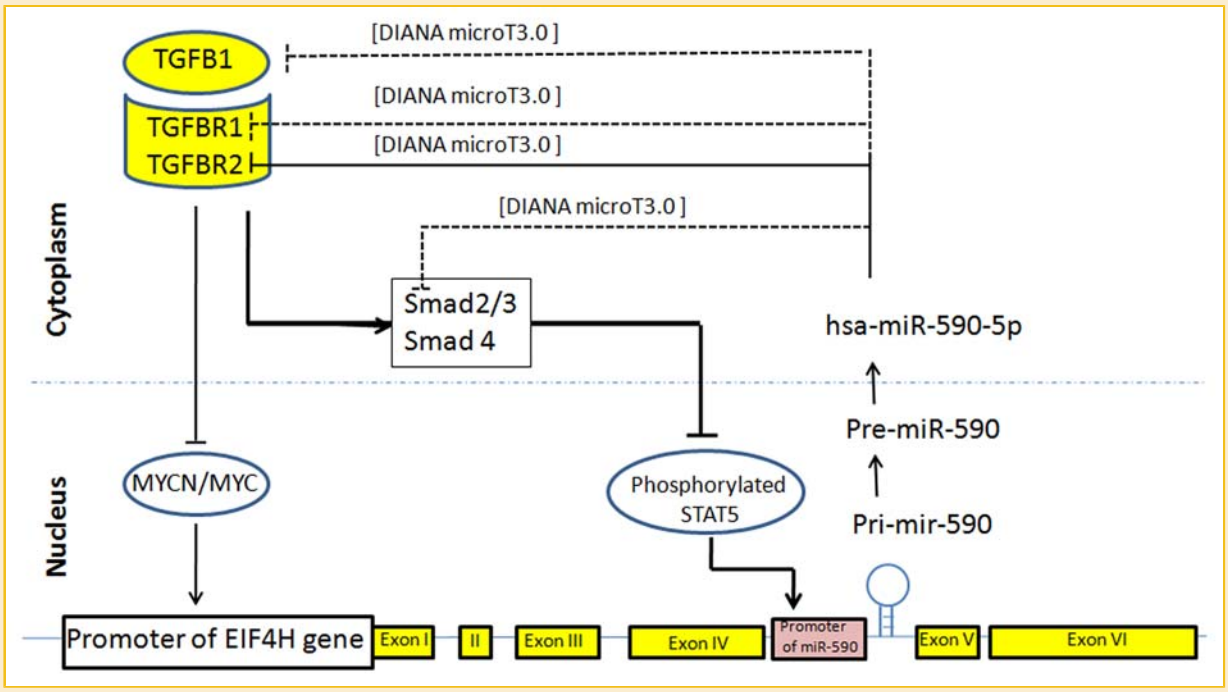

Fig. 8. Modeling of a positive feedback loop in TGFB signaling. Based on this model, TGFB1 elevates the expression level of its own receptors via down-regulation of hsa-miR-590-5p.

[Rojas et al., 2009]. Accordingly, TGFBR2 is one of the validated target genes of miR-590 [Shan et al., 2009; Jiang et al., 2012]. This receptor was shown to be upregulated following TGFB1 treatment and down-regulation of miR-590. Considering the expression pattern of TGFB signaling components, a positive feed-back loop between TGFB and its receptors with the mediation of miR-590 was hypothesized (Fig. 8). In this model, an exogenous addition of TGFB1 causes upregulation of TGFBRs via indirect suppression of miR-590, which in turn causes efficient TGFB signaling. To test this hypothesis, overexpression of mir-590 before or after TGFB1induction was carried out.

Overexpression of miR-590 in CDCs resulted in a significant downregulation of TGFBR2, TGFBR1, and TGFB1 target genes. This finding supported the bioinformatics prediction data suggesting that TGFBR1 and TGFB1 are also targeted by miR-590. Based on the proposed model, upon differentiation induction of CDCs by TGFB1 at 6th day, miR-590 level would be reduced, and hence TGFBR2 would be increased. However, overexpression of miR-590 at 4th day of the differentiation process caused a sharp reduction of TGFBRs expression levels and neutralized the induction effect of TGFB1 for CDCs differentiation.

The expanded cell morphology acquisition as well as TrnI protein synthesis is the fundamental character of differentiated cardiomyocytes. Overexpression of miR-590 before the first TGFB1 treatment probably resulted in the posttranscriptional repression of TGFBRs, reduction of cardiac TrnI protein accumulation in cytoplasm and lead to attenuation of cardiomyocytes maturation. Overexpression of miR-590 after the first TGFB1induction had similar but weaker effect on the cardiac TrnI protein expression. It seems the first treatment by TGFB1 would make CDCs more committed toward cardiogenesis fate.

CONCLUSIONS

Altogether, lowering the expression of miR-590 in the early stages of cardiac differentiation would have a potential role in determination of cardiac progenitor cell commitment. Finally, for determining the precise role of miR-590-5p during the cardiac differentiation, more analyses are needed.

ACKNOWLEDGMENTS

This work was supported by the Research Councils of Tarbiat Modares University (PhD Thesis) [1024862]. Also, the authors are grateful to Dr. Khosroshahi (Tarbiat Modares University), Dr. Attashi, Mr. Mosahebi, and Mrs. Gohari (Bon Yakhte Research Institute), Dr. Aghdami and Mr. Goudarzi (RSCB), PaniaTerraf (NIGEB) and Rezvan Mobashery (Tarbiat Modares University) for technical supports and advices.

REFERENCES

Anversa P, Leri A, Rota M, Hosoda T, Bearzi C, Urbanek K, et al. 2007. Stem cells, myocardial regeneration, and methodological. Stem Cells 25:589–601.

Barile L, Gherghiceanu M, Popescu LM, Moccetti T, Vassalli G. Human cardiospheres as a source of multipotent stem and progenitor cells. Stem Cells Int 1–10.

Behfar A, Zingman LV, Hodgson DM, Rauzier JM, Kane GC, Terzic A. 2002. Stem cell differentiation requires a paracrine pathway in the heart. FASEB J 16:1558–1566.

Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, et al. 2011. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): Initial results of a randomised phase 1 trial. Lancet 378:1847–1857.

Brink PR, Cohen IS, Mathias RT. 2011. Integration of stem cells into the cardiac syncytium: Formation of gap junctions. Stem Cell Biol Regen Med 2:301–320.

Carr CA, Stuckey DJ, Tan JJ, Tan SC, Gomes RSM, Camelliti P, et al. 2011. Cardiosphere-derived cells improve function in the infarcted rat heart for at least 16 weeks—An MRI study. PLoS ONE 6:e25669.

Charron F, Paradis P, Bronchain O, Nemer G, Nemer M. 1999. Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression. Mol Cell Biol 19:4355-4365.

Chen CR, Kang Y, Siegel PM, Massagué J. 2002. E2F4/5 and p107 as Smad cofactors linking the TGFb receptor to c-myc repression. Cell 110:19–32.

Cocolakis E, Dai M, Drevet L, Ho J, Haines E, Ali S, et al. 2008. Smad signaling antagonizes STAT5-mediated gene transcription and mammary epithelial cell differentiation. J Biol Chem 283:1293–1307.

Cordes KR, Srivastava D. 2009. MicroRNA regulation of cardiovascular development. Circ Res 104:724–732.

Cripps RM, Olson EN. 2002. Control of cardiac development by an evolutionarily conserved transcriptional network. Dev Biol 246:14–28.

Davis DR, Kizana E, Terrovitis J, Barth AS, Zhang Y, et al. 2010. Isolation and expansion of functionally-competent cardiac progenitor cells directly from heart biopsies. J Mol Cell Cardiol 49:312–321.

Davis DR, Zhang Y, Smith RR, Cheng K, Terrovitis J, et al. 2009. Validation of the cardiosphere method to culture cardiac progenitor cells from myocardial tissue. PLoS ONE 4:e7195.

Di Lisi R, Millino C, Calabria E, Altruda F, Schiaffino S, Ausoni S. 1998. Combinatorial cis-acting elements control tissue-specific activation of the cardiac troponin I gene in vitro and in vivo. J Biol Chem 273:25371–25380.

Duff S, Li C, Garland JM, Kumar S. 2003. CD105 is important for angiogenesis: evidence and potential applications. FASEB J 17:984–992.

Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M. 2012. Functional screening identifies miRNAs inducing cardiac regeneration. Nature 492:376–381.

Favreau A, Sathyanarayana P. 2012. MiR-590-5p, miR-219-5p, miR-15b and miR-628–5p are commonly regulated by IL-3, GM-CSF and G-CSF in acute myeloid leukemia. Leuk Res 36:334–341.

Goumans MJ, Boerb TP, Smits AM, Laake LW, Vlieta P, Metza CHG, et al. 2008. TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res 1:138–149.

Hosoda T, Zheng H, Cabral- da-Silva M, Sanada F, Ide-Iwata N, Ogórek B, et al. 2011. Human cardiac stem cell differentiation is regulated by a mircrine mechanism. Circulation 123:1287–1296.

Jiang X, Xiang G, Wang Y, Zhang L, Yang X, Cao L, et al. 2012. MicroRNA-590-5p regulates proliferation and invasion in human hepatocellular carcinoma cells by targeting TGF-β RII. Mol Cells 33:545–551.

Latronico MVG, Catalucci D, Condorelli G. 2007. Emerging role of micrornas in cardiovascular biology. Circ Res 101:1225–1236.

Li TS, Cheng K, Malliaras K, Smith RR, Zhang Y, Sun B, et al. 2012. Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere derived cells. J Am Coll Cardiol 59:942–953.

Liang Q, De Windt LJ, Witt SA, Kimball TR, Markham BE, Molkentin JD. 2001. The transcription factors GATA4 and GATA6 regulate cardiomyocyte hypertrophy in vitro and in vivo. J Biol Chem 276:30245–30253.

Lim JY, Kim WH, Kim J, Park SI. 2007. Involvement of TGF-beta1 signaling in cardiomyocyte differentiation from P19CL6 cells. Mol Cells 24:431–436.

Liu N, Bezprozvannaya S, Williams AH, Qi X, Richardson JA, Bassel-Duby R, et al. 2008. MicroRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes Dev 22:3242–3254.

López-Novoa JM, Bernabeu C. 2010. The physiological role of endoglin in the cardiovascular. Am J Physiol Heart Circ Physiol 299:959–974.

Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, et al. 2012. Intracoronary cardiosphere derived cells for heart regeneration after myocardial infarction (CADUCEUS): A prospective, randomised phase 1 trial. Lancet 10.1016/S0140–6736(12) 60195–0 .

Mishra R, Vijayan K, Colletti EJ, Harrington DA, Matthiesen TS, Simpson D, et al. 2011. Characterization and functionality of cardiac progenitor cells in congenital heart patients. Circulation 123:364–373.

Monteys AM, Spengler RM, Wan J, Tecedor L, Lennox AK, Xing Y, et al. 2010. Structure and activity of putative intronic miRNA promoters. RNA 16:495–505.

Noseda M, Peterkin T, Simões FC, Patient R, Schneider MD. 2011. Cardiopoietic factors: Extracellular signals for cardiac lineage commitment. Circ Res 108:129–152.

Okabe M, Kawamura K, Terasaki F, Hayashi T. 1999. Remodeling of cardiomyocytes and their branches in juvenile, adult, and senescent spontaneously hypertensive rats and Wistar Kyoto rats: Comparative morphometric analyses by scanning electron microscopy. Heart Vessels 14:15-28.

Pan Z, Lu Y, Yang B. 2010. MicroRNAs: A novel class of potential therapeutic targets for cardiovascular diseases. Acta Pharmacologica Sinica 31:1–9.

Reisdorff J, En- Nia A, Stefanidis I, Floege J, Lovett DH, Mertens PR. 2002. Transcription factor Ets-1 regulates gelatinase a gene expression in mesangial cells. J Am Soc Nephrol 13:1568–1578.

Rojas A, Padidam M, Cress D, Grady WM. 2009. TGF-β receptor levels regulate the specificity of signaling pathway activation and biological effects of TGF-β. Biochim Biophys Acta 7:1165–1173.

Ryan J, Fernando J, Barnstein B. 2010. TGFb1 suppresses the mast cell response in vitro and in vivo J Immunol 184:86.6.

Satou Y, Satoh N. 2006. Gene regulatory networks for the development and evolution of the chordate heart. Genes Dev 20:2634–2638.

Sawada K, Kawamura K. 1991. Architecture of myocardial cells in human cardiac ventricles with concentric and eccentric hypertrophy as demonstrated by quantitative scanning electron microscopy. Heart Vessels 6:129–142.

Shan H, Zhang Y, Lu Y, Zhang Y, Pan Z, Cai B, et al. 2009. Downregulation of miR-133 and miR-590 contributes to nicotine- induced atrial remodelling in canines. Cardiovasc Res 83:465–472.

Shohet JM, Ghosh R, Coarfa C, Ludwig A, Benham AL, Chen Z, et al. 2011. A genome-wide search for promoters that respond to increased MYCN reveals both new oncogenic and tumor suppressor microRNAs associated with aggressive neuroblastoma. Cancer Res 71:3841–3851.

Sluijter JPG, Mil A, Vliet P, Metz CHG, Liu J, Doevendans PA, et al. 2010. Microrna-1 and -499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. Arteriosclera Thromb Vasc Biol 30:859–868.

Small EM, Olson EN. 2011. Pervasive roles of microRNAs in cardiovascular biology. Nature 469:336–342.

Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, et al. 2007. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 115:896–908.

Smits AM, Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, et al. 2009. Human cardiomyocyte progenitor cells differentiate into functional

mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nat Protoc 4:232–243.

Srivastava D. 2006. Making or breaking the heart: From lineage determination to morphogenesis. Cell 126:1037–1048.

Synnergren J, Améen C, Lindahl A, Olsson B, Sartipy P. 2011. Expression of microRNAs and their target mRNAs in human stem cell-derived cardiomyocyte clusters and in heart tissue. Physiol Genomics 43:581–594.

White AJ, Smith RR, Matsushita S, Chakravarty T, Czer LSC, Burton K, et al. 2011. Intrinsic cardiac origin of human cardiosphere-derived cells. Eur Heart J 10.1093/eurheartj/ehr172.

Wilson KD, Hu S, Venkatasubrahmanyam S, Fu JD, Sun N, Abilez OJ, et al. 2010. Dynamic microRNA expression programs during cardiac differentiation of human embryonic stem cells: Role for miR-499. Circ Cardiovasc Genet 3:426–435.

Ye M, Coldren C, Liang X, Mattina T, Goldmuntz E, Benson DW, et al. 2010. Deletion of ETS-1, a gene in the Jacobsen syndrome critical region, causes ventricular septal defects and abnormal ventricular morphology in mice. Hum Mol Genet 19:648–656.

Zhang C. 2008. MicroRNAs: Role in cardiovascular biology and disease. Clin Sci 114:699–706.