MicroRNA 16 enhances differentiation of human bone marrow mesenchymal stem cells in a cardiac niche toward myogenic phenotypes in vitro

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Aim: Upregulation of microRNA 16 (miR-16) contributed to the differentiation of human bone marrow mesenchymal stem cells (hMSCs) toward myogenic phenotypes in a cardiac niche, the present study aimed to determine the role of miR-16 in this process.

Main methods: hMSCs and neonatal rat ventricular myocytes were co-cultured indirectly in two chambers to set up a cardiac microenvironment (niche). miRNA expression profile in cardiac-niche-induced hMSCs was detected by miRNA microarray. Cardiac marker expression and cell cycle analysis were determined in different treatment hMSCs. Quantitative real-time PCR and Western blot were used to identify the expression of miRNA, mature miRNA and protein of interest.

Key findings: miRNA dysregulation was shown in hMSCs after cardiac niche induction, miR-16 was upregulated in cardiac-niche-induced hMSCs. Overexpression of miR-16 significantly increased G1-phase arrest of the cell cycle in hMSCs and enhanced the expression of cardiac marker genes, including GATA4, NK2-5, MEF2C and TNNI3. Differentiation-inducing factor 3 (DIF-3), a G0/G1 cell cycle arrest compound, was used to induce G1 phase arrest in cardiac-niche-induced hMSCs, and the expression of cardiac marker genes was upregulated in DIF-3-treated hMSCs. The expression of CCND1, CCND2 and CDK6 was suppressed by miR-16 in hMSCs. CCND1, CCND2 or CCND2 knockdown resulted in G1 phase arrest in hMSCs and upregulation of cardiac marker gene expression in hMSCs in a cardiac niche.

Significance: miR-16 enhances G1 phase arrest in hMSCs, contributing to the differentiation of hMSCs toward myogenic phenotypes when in a cardiac niche. This mechanism provides a novel strategy for pre-modification of hMSCs before hMSC-based transplantation therapy for severe heart diseases.

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Introduction

MSCs or MSC-like cells have been recovered from a variety of tissues, including bone marrow, adipose tissue, umbilical-cord blood, amniotic fluid, synovium, dental pulp and skeletal muscle (Phinney and Prockop, 2007). MSCs have clinical potential as a natural system for tissue repair in humans and in experimental models, including bone and cartilage defects, chronic skin wounds, and ischemic heart injuries (Ohishi and Schipani, 2010).

Increasing evidence indicates that MSC transplantation shows promise for cardiac regeneration after myocardial infarction in both animal models and patients (Kawada et al., 2004; Nagaya et al., 2006; Katritsis et al., 2005). The therapeutic potential of MSCs in myocardial regeneration is based on the ability of MSCs to differentiate into cardiac tissue and the paracrine action of factors released from MSCs (Song et al., 2010).

MSCs have the potential to transdifferentiate into cardiomyocytes if an appropriate cardiac environment is provided (Xu et al., 2004; Li et al., 2007). Our previous study, in which hMSCs were cultured with neonatal rat ventricular cardiomyocytes (NRVCs) in two separate chambers of a trans-well culture plate (Li et al., 2007), showed that MSCs can be induced to differentiate toward a myogenic phenotype when in a cardiac microenvironment (niche) in vitro. The mechanism involved, however, remains to be elucidated.

MicroRNAs (miRNAs) are endogenous, non-coding, 20–23 nucleotide RNAs that negatively regulate gene expression in diverse biological and pathological processes including cell differentiation, proliferation, apoptosis, heart disease, neurological disorders and human cancers (Karp and Ambros, 2005; Kloosterman and Plasterk, 2006; van Rooij et al., 2006; Kim et al., 2007; Calin and Croce, 2006). A recent study has suggested that miRNAs play important and varied roles in the self-renewal and differentiation of MSCs; for example, miR-1, -206, -24, and -181 contribute to the differentiation...
of MSCs into myocardial cells, while miR-133 can block this process (Guo et al., 2011).

To our knowledge, there are no reports on the involvement of miRNAs in the induction of MSC differentiation toward a myogenic phenotype in a cardiac niche. In this study, we compared miRNA expression profiles of hMSCs with or without cardiac niche induction to determine whether miRNAs are mechanistically involved in MSC differentiation.

**Experimental procedures**

**Cell culture and treatment**

Bone marrow was obtained from 6 adult human donors (aged 20–35 years) following informed consent and in accordance with the terms of the ethics committee of Guangdong General Hospital. hMSCs were isolated according to our previous report (Li et al., 2007). In brief, bone marrow cells were flushed and centrifuged in a 1.073 g/ml percoll (Pharmacia, St. Louis, USA) density gradient. The enriched cells were collected from the interphase, resuspended, and transferred into culture flasks with MSC culture medium (OriCellTM; Cyagen, Guangzhou, China) at 37 °C in humid air with 5% CO2. After reaching confluence, cells were harvested and cryopreserved as primary hMSCs. Cells from the fourth to the fifth passages were used for experiments. hMSCs were transfected with 50 nM miR-16 mimics, CDK6 siRNA, CCND1-siRNA or CCND2-siRNA (Genharma, Shanghai, China) using Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA) at 24 h before co-culturing with rat cardiomyocytes. DIF-3 (30 μM) (Takahashi-Yanaga et al., 2003) (Sigma, St. Louis, MO, USA) was added in the culture medium for the co-culture of DIF-3-treated hMSCs with rat cardiomyocytes. Rat cardiomyocytes were isolated from the hearts of neonatal rats (1 to 3 d old) following previously reported methods (Reinecke et al., 1999). WST-1 assay for hMSC proliferation detection was performed according to procedures in the kit (Roche Molecular Biochemicals, Mannheim, Germany).

**Co-culture of bone marrow stem cells with cardiomyocytes**

hMSCs and neonatal rat ventricular myocytes were co-cultured indirectly in two chambers separated by Falcon Cell Culture Inserts (catalog: [35] 3493, BD, NJ, USA) to set up a cardiac niche, as detailed in our previous report (Li et al., 2007). The semi-permeable membrane of this insert (pore size 0.4 μm) allows the diffusion of secreted factors but prevents cell transport between the two cell populations. hMSCs were induced for 7 d by the cardiac niche in this study.

**miRNA microarray**

miRNA expression analysis was performed on total RNA extracted from a pool of 3 samples of untreated or treated hMSCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Microarray procedures and data analysis were performed at Shanghai Kangchen Corporation. Briefly, 5.0 μg of total RNA extracted from hMSCs was fluorescently labeled with Hy3 using the miRCURY Array Labeling kit (Exiqon, MA, USA). The labeled samples were then concentrated and hybridized with the microarray slide (Exiqon, USA). Arrays were scanned on a GenePix 4000B (Molecular Devices, Sunnyvale, CA), and image analysis was performed using GenePix Pro 6.0 software (Molecular Devices, CA).

**Real-time quantitative PCR**

Total cellular RNA was extracted using Trizol reagent (Gibco-BRL, Grand Island, NY, USA). Methods for coding gene expression detection were as follows: first-strand cDNAs were synthesized using a mixture of oligo(dT)15 and random primers with Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using the MJ Opticon II Quantitative PCR System (MJ Research, Waltham, MA, USA) with the following thermal cycling profile: 95 °C for 5 min, followed by 40 cycles of amplification (94 °C for 25 s, 59 °C for 25 s, and 72 °C for 25 s). The absorption values of the SYBR Green I fluorescence in each tube were detected at the end of each cycle. The housekeeping gene β-actin was used as an internal control. PCR primers for coding genes used in this study are shown in Supplementary Table 1. Methods for mature miRNA level detection were as follows: the total RNA was used to detect mature miRNA level using Hairin-it miRNAs real-time PCR kit (Shanghai GenePharma, China). In brief, the real-time miRNA assay has two steps: stem-loop RT reaction and real-time PCR detection. Stem-loop RT primers bind to the 3’ end of miRNA molecules and are transcribed with reverse transcriptase. RT product is quantified using real-time PCR that includes miRNA-specific forward primer, reverse primer, and a carboxyfluorescein (FAM) dye-labeled reporter probes. Real-time PCR was performed using the vii A7 Quantitative PCR System (Applied Biosystems, Carlsbad, CA, USA) with the following thermal cycling profile: 95 °C for 3 min, followed by 40 cycles of amplification (95 °C for 12 s, 62 °C for 40 s). To normalize RNA content, the U6 snRNA was the internal control. The relative expression values of coding genes and mature miRNAs of interest were calculated using the 2−ΔΔCt method (Pfaffl, 2001).

**Western blot analysis**

The protein extract (40 μg) prepared from hMSCs was separated using 12% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked for 1 h at room temperature with 5% milk in Tris-buffered saline solution (pH 7.6) containing 0.05% Tween-20 (TBS/T) and incubated with a high affinity anti-CDK6 antibody (1:1000), anti-CCND1 antibody (1:1000), anti-CCND2 antibody (1:1000), anti-TNNI3 antibody (1:1000), anti-GATA4 antibody (1:800), anti-NKX2-5 antibody (1:500), or anti-MEF2C antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Membranes were then washed extensively with TBS/T and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Proteins were visualized using the ECL Plus detection system (GE Healthcare, WI, USA). As an internal control, membranes were also immunoblotted with an anti-β-actin antibody (1: 2000) (Santa Cruz Biotechnology, Santa Cruz, USA).

**Fluorescence-activated cell sorting (FACS) and cell cycle analysis by flow cytometry**

For FACS analysis, antibodies against the following markers were either conjugated with fluorescein isothiocyanate (FITC) or PerCP (Pharmingen, USA): CD34, CD44, CD29, and CD45. hMSCs were trypsinized, washed twice with PBS and then incubated with antibody for 30 min on ice. Cell suspensions were fixed in ice-cold 4% paraformaldehyde and stored at −20 °C prior to analysis. Samples were analyzed with a Beckman Coulter Quanta SC MPL flow cytometer using Quanta SC MPL Analysis software (Beckman Coulter, Brea, CA, USA).

For cell cycle analysis, hMSCs were trypsinized and washed twice with PBS, followed by centrifugation at 500 × g for 5 min. hMSC pellets were resuspended in 1 ml nuclear isolation and staining solution (NPE systems, FL, USA) for 2 min in the dark. DNA content was determined with a Beckman Coulter Quanta SC MPL flow cytometer using the Multicycle software (Beckman, Brea, CA, USA). The results are presented as the percent of the cell population in each cell cycle phase.
**Statistical analysis**

The data represent the mean ± standard deviation (SD). In each experiment, all determinations were performed at least in triplicate. Differences between-group comparisons were performed by two-way ANOVA. A value of $p < 0.05$ indicated significance.

**Results**

**miRNA expression in cardiac-niche-induced hMSCs**

Human MSCs were successfully isolated and expanded for subsequent experiments. Of these cultured hMSCs, 92% expressed CD44, and 96% expressed CD29. The CD34-positive and CD45-positive cell populations were only 1.81% and 0.02%, respectively (Supplementary Fig. 1). These results indicated that most of the cells isolated from human bone marrow in this study were MSCs. The real-time PCR result showed that $DROSHA$, $DICER1$, cardiac marker genes $NKX2-5$, $GATA4$ and $MEF2C$, and the cardio-specific $SERCA2A$ gene were all significantly upregulated in hMSCs after a 7- or 10-day-induction by a cardiac niche (Fig. 1A).

A miRNA-profiling array revealed that miRNAs were dysregulated in hMSCs after a 7-day induction by the cardiac niche. The expression of let-7a, let-7e, and miR-16, -138, and -524 increased by over two-folds. In contrast, the expression of another 6 miRNAs, including miR-129, -500, -492, -422b, -208, and -378, decreased by more than two-folds in cardiac-niche-induced hMSCs.

The real-time PCR result revealed that $GATA4$ mRNA expression was significantly increased in let-7e-modified hMSCs and miR-16-modified hMSCs ($p < 0.05$, and $p < 0.01$, respectively) in a cardiac niche, and $NKX2-5$ mRNA expression was only significantly upregulated in miR-16-modified hMSCs ($p < 0.05$) (Fig. 1B). Human miR-16 has two hairpin precursor forms, miR-16-1 and miR-16-2, which are transcribed from two different genomic locations and can both generate the same mature sequence. Human miR-16-1 and miR-16-2 are also clustered with miR-15a and miR-15b, respectively (www.mirbase.org). Mature miR-16, let-7e and miR-524, but not miR-15a and -15b, were significantly upregulated in cardiac-niche-induced hMSCs as indicated by quantitative real-time PCR assays ($p < 0.05$, $p < 0.05$, and $p < 0.05$, respectively). The mature miR-526b, whose sequence expression was unchanged by cardiac niche induction, and the down-regulated miR-378 (as indicated by miRNA array) were used as controls in quantitative real-time PCR assays (Fig. 1C).

**miR-16 increased cardiac marker gene expression in cardiac-niche-induced hMSCs**

A confocal assay showed that small, double-stranded RNAs can be efficiently transfected into MSCs, and the quantitative real-time PCR results validated that miR-16 mimics were also efficiently transfected into MSCs using the Oligofectamine reagent (Supplementary Fig. 2).
Using this transfection reagent, MSCs were transfected with miR-16 mimics. Compared to the negative controls, Western-blot result showed that miR-16 dramatically enhanced the expression of cardiac markers, including GATA4, NKX2-5, MEF2C and TNNI3 at 7 d after co-culturing in a cardiac niche (Fig. 2A, Supplementary Fig. 3). The WST-1 assay showed that the proliferation activity of miR-16-modified hMSCs were not co-cultured with cardiac myocytes, for the co-NC and co-miR-16 groups, the modified hMSCs were co-cultured with cardiac myocytes.

![Fig. 2](image)

**Fig. 2.** Representative cardiac marker expression, proliferation, and cell population assay of hMSCs modified with miR-16. GATA4, NKX2-5, MEF2C, and TNNI3 expressions in miR-16 modified hMSCs were determined by Western-blot assay (A). hMSC proliferation was detected by WST-1 assay (B). Populations of miR-16-modified hMSCs were detected by flow cytometry (C). All data represent the mean ± standard deviation (SD) of three independent experiments with n = 3. *p < 0.05, #p < 0.01 compared to the negative control (NC). for NC and the miR-16 group, the modified hMSCs were not co-cultured with cardiac myocytes, for the co-NC and co-miR-16 groups, the modified hMSCs were co-cultured with cardiac myocytes.

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![Fig. 3](image)

**Fig. 3.** Cardiac marker expression and cell population assays of DIF-3-treated hMSCs. The expression of GATA4, NKX2-5, MEF2C, and TNNI3 in DIF-3-treated hMSCs after a 7-d cardiac niche induction was determined by Western-blot assay (A). hMSC proliferation was detected by WST-1 assay (B). Populations of DIF-3-treated hMSCs were detected by flow cytometry (B). All data represent the mean ± standard deviation (SD) of three independent experiments with n = 3. *p < 0.05, #p < 0.01 compared to the DMSO control.
hMSCs was significantly decreased independent of a cardiac niche induction \((p<0.01, \text{and } p<0.05, \text{respectively})\) (Fig. 2B). Flow cytometry assays revealed that in miR-16-modified hMSCs, the population of cells in the G1 phase was significantly greater than that in controls, while the population of cells in the S phase was significantly less than that in controls (Fig. 2C, Supplementary Fig. 4).

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**Fig. 4.** The modulative effect of miR-16 on the cell-cycle-related gene expression in hMSCs. CDK6, CCND1, and CCND2 expressions in miR-16-overexpressed hMSCs by real-time PCR (A) and by Western-blot assay (B). CDK6 expression was detected in hMSCs modified with CDK6-siRNA or miR-16 mimics by real-time PCR (C) and by Western-blot assay (D). Each value was expressed relative to the negative control duplex (NC), and the value of the NC control was set to 1. mRNA expression of cardiac marker genes in CDK6 siRNA-modified hMSCs by real-time PCR assay (E). \(\beta\)-actin served as an internal control in the real-time quantitative PCR and Western blot analyses. Cell population assay of CDK6 siRNA-modified hMSCs by flow cytometry (F). All data represent the mean ± standard deviation (SD) of three independent experiments with \(n=3\). *\(p<0.05\); #\(p<0.01\) compared to the NC or co-NC control.
DIF-3 promoted cardiac marker gene expression and induced G1 phase arrest in hMSCs

Western-blot assay showed that DIF-3, significantly enhanced the expression of the cardiac markers GATA4, MEF2C and TNNI3 protein over that of DMSO controls in cardiac-niche-induced hMSCs (Fig. 3A). The results of flow cytometry showed that in DIF-3-treated hMSCs, the population of cells in the G1 phase was significantly higher than that in controls, while populations of cells in the S and G2/M phases were significantly less than those in controls (Fig. 3B, Supplementary Fig. 5).

CCND1-, CCND2-, and Cdk6-mediated G1 phase arrest in miR-16-modified hMSCs

Real-time PCR result revealed that Cdk6, CCND1 and CCND2 mRNA expressions were not changed significantly in the mir-16-modified hMSCs (Fig. 4A). However, Western blot analysis showed that Cdk6, CCND1, and CCND2 expressions were significantly inhibited in miR-16-modified hMSCs (Fig. 4B). Quantitative real-time PCR and Western blot assays demonstrated that Cdk6 siRNA knocks down Cdk6 protein expression efficiently (p<0.01) and that miR-16 inhibits Cdk6 expression post-transcriptionally (p<0.05) (Fig. 4C, D). Real-time PCR result showed that GATA4, NKX2-5, and TNNI3 mRNA expressions were up-regulated in hMSCs modified by Cdk6-siRNA or miR-16 when in a cardiac niche. However, only the MEF2C gene was significantly upregulated in miR-16-modified hMSCs (Fig. 4E). The flow cytometry results revealed that the population of Cdk6-siRNA-modified hMSCs in the G1 phase was significantly increased; in contrast, the populations of cells in the S and G2/M phases were significantly reduced (Fig. 4F, Supplementary Fig. 6). The flow cytometry results reveal that the G1 phase arrest occurs in hMSCs modified with either CCND1-siRNA or CCND2-siRNA (Supplementary Fig. 7). In addition, cardiac marker gene mRNA expression increased significantly in hMSCs modified with either CCND1-siRNA or CCND2-siRNA when in a cardiac niche (Supplementary Fig. 7).

Discussion

The transcription factors GATA4, NKX2-5 and MEF2C are first expressed in cardiac precursor cells and have essential roles in cardiac muscle development and morphogenesis of the heart (Pu et al., 2004; Bruneau, 2002; Olson, 2006). SERC2A and TNNI3 are also two important myocardial markers (Li et al., 2007). In the present study, we found that GATA4, NKX2-5, MEF2C, and SERC2A mRNAs (Fig. 1A) as well as GATA4 and TNNI3 proteins (Fig. 2A, Supplementary Fig. 3) were significantly upregulated in hMSCs after cardiac niche induction; these results are consistent with our previous report (Li et al., 2007). DROSHA and DICER1 are two key enzyme genes involved in miRNA generation (Tsuchiya et al., 2006). To confirm the role of miRNAs in the differentiation of hMSCs toward myogenic phenotypes, we analyzed both the expression of DROSHA and DICER1 and the profiles of miRNAs in cardiac-niche-induced hMSCs. DROSHA and DICER1 mRNA levels were significantly elevated in cardiac-niche-induced hMSCs (Fig. 1A), with dysregulation of miRNA profiles based on the miRNA microarray. The cause of this up-regulation of DROSHA and DICER1 remains to be elucidated. The miRNA-profiling array revealed that miRNAs were dysregulated in hMSCs after a 7-day induction by the cardiac niche. In the present study, we focused on the potential role of the up-regulated miRNAs in the cardiac niche-induced hMSC differentiation toward myogenic phenotypes. And the real-time PCR result revealed that GATA4 and NKX2-5 mRNA expressions were significantly increased in miR-16-modified hMSCs, but not in other miRNA-modified hMSCs in a cardiac niche (Fig. 1B). Mature miR-16, but not miR-15a or miR-15b, was also shown to significantly increase in hMSCs at 7 d after culturing in a cardiac niche (Fig. 1C), so, miR-16 was chosen for further mechanism study.

A previous report showed that miR-322/424 and miR-503 promote cell cycle quiescence by inhibiting CDC25A and Cdk2, contributing to muscle differentiation. This result indicates that the differentiation of proliferating cells into specific tissue types is always accompanied by an arrest of the cell cycle in the G0/G1 stage (Sarkar et al., 2010). Based on our observation that the fraction of cells in the G1 phase was significantly increased in miR-16-modified hMSCs (Fig. 2C, Supplementary Fig. 4), we speculate that the G1 phase arrest contributes to the differentiation of hMSCs toward cardiac cells. DIF-3, which is a G0/G1 cell cycle arrest compound, was used as a positive control; it was confirmed to increase the fraction of the cell population in the G1 phase and enhanced the differentiation of hMSCs in a cardiac niche toward a myogenic phenotype (Fig. 3A, B, Supplementary Fig. 5). Consistent upregulation of miR-16 has also been reported in neural differentiation models (Aranha et al., 2010). Therefore, the role of miR-16 in the process of differentiation may be associated with cell cycle regulation by suppressing the proliferation of differentiating cells.

miR-16 has been implicated in cancer cell cycle regulation (Liu et al., 2008; Bandi et al., 2009; Wang et al., 2009); Cdk6, CCND1, and CCND2 were shown to be modulated by miR-16 (Liu et al., 2008; Bandi et al., 2009). In the present study, Cdk6, CCND1, and CCND2 expressions were also revealed to be suppressed by miR-16 in hMSCs post-transcriptionally (Fig. 4A, B). The consistent results reveal that the G1 phase arrest occurs in hMSCs modified with Cdk6-siRNA, CCND1-siRNA or CCND2-siRNA. In addition, cardiac marker gene expression increased significantly in hMSCs modified with Cdk6-siRNA, CCND1-siRNA or CCND2-siRNA when in a cardiac niche. Therefore, Cdk6, CCND1, and CCND2 mediate miR-16-modulated G1-phase arrest in hMSCs, contributing to the differentiation of hMSCs in a cardiac niche toward myogenic phenotypes (Fig. 5).

Conclusion

Evidence for the fact that miRNAs were involved in the differentiation of hMSCs in a cardiac niche toward myogenic phenotypes was revealed in the present study. miR-16 was significantly upregulated during the differentiation of hMSCs toward myocardial cells and enhanced the differentiation of cardiac niche-induced hMSCs toward myogenic phenotypes by enhancing the G1-phase arrest of the cell cycle in hMSCs. Overexpression of miR-16 in hMSCs may present a
promising approach for inducing the differentiation of hMSCs in a cardiac niche into myocardial cells. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2012.05.011.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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