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Research paper

MicroRNA-29c overexpression inhibits proliferation and promotes apoptosis and differentiation in P19 embryonal carcinoma cells

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ABSTRACT

Compared to healthy controls, microRNA-29c (miR-29c) is highly expressed in the heart during progression towards ventricular septal defect. However, studies on miR-29c function in heart development are scarce. We investigated the role of miR-29c in P19 cell proliferation, apoptosis, and differentiation and the underlying mechanisms. We evaluated proliferation and cell cycle progression, detected morphological changes; apoptosis rate; *BAX*, *BCL2*, GATA binding protein 4 (*GATA4*), cardiac troponin T (*cTnT*), and myocyte enhancer factor 2C (*MEF2C*) expression; and caspase-3, -8, and -9 activity in miR-29c-overexpressing P19 cells, and investigated whether *WNT4* was a miR-29c target. MiR-29c-overexpressing cells had decreased proliferation, increased G1 cells, and significantly higher apoptotic rate than the controls. Expression of the apoptosis-related *BAX* and *BCL2* genes and caspase-3, -8, and -9 activity were significantly increased in miR-29c-overexpressing cells. Expression of the cardiac-specific markers *GATA4*, *cTnT*, and *MEF2C* revealed promoted differentiation in miR-29c-overexpressing cells compared to the controls. Luciferase assay confirmed that *WNT4* is a miR-29c target. Wnt4 and β-catenin expression was decreased in miR-29c-overexpressing cells. MiR-29c inhibits P19 cell proliferation and promotes apoptosis and differentiation, possibly by suppressing Wnt4 signaling, whose deregulation contributes to congenital heart disease development.

1. Introduction

In vertebrate embryonic development, the heart is the first organ that becomes functional. Embryonic heart development involves cardiomyocyte specification, differentiation, or heart morphogenesis. The intricate process of embryonic development requires precise temporal and spatial regulation of gene expression and protein activity (Epstein, 2010; Liu and Olson, 2010). Errors in these processes can result in chamber hypoplasia and incorrect alignment of the atria and great arteries with the ventricles, which produces the worst forms of congenital heart disease (CHD). CHD is the most common congenital anomaly in newborns, affecting 0.8% of live births, making it the most frequent birth defect (Sissman, 2001; Hoffman and Kaplan, 2002). Although 40% of severe cases are diagnosed before birth, CHD remains a major cause of morbidity and mortality worldwide (Bruneau, 2008; Hill and Olson, 2008). Over the past decades, researchers have attempted to uncover the mechanism of CHD. The advent of fetal echocardiography as a screening tool for preventing CHD is a great achievement, but the genetic cause of the disease remains poorly understood.

MicroRNAs (miRNAs) have recently emerged as key players in various cardiovascular events through their regulation of heart gene expression (van Rooij and Olson, 2007; Cordes and Srivastava, 2009; Latronico and Condorelli, 2009; Liu and Olson, 2010; Small et al., 2010). MiRNAs regulate many aspects of cellular function, and their dysregulation is involved in heart disease (Mendell and Olson, 2012). MiRNAs are endogenous 18–22 nucleotide, stable noncoding RNAs that critically modulate post-transcriptional gene regulation by binding to the target mRNAs, resulting in repression of translation or degradation of the target mRNA, thus playing a pivotal role in many biological processes (Bartel, 2004; Bartel, 2009), including cell growth, proliferation, apoptosis, differentiation, and development (Shivdasani, 2006; Thum et al., 2008; Sluijter et al., 2010; Liu et al., 2011; Xiao et al.,

Abbreviations: α-MEM, alpha modification of Eagle's medium; CCK-8, Cell Counting Kit-8; CHD, congenital heart disease; cTnT, cardiac troponin T; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA4, GATA binding protein 4; GFP, green fluorescent protein; MEF2C, myocyte enhancer factor 2C; miRNAs, microRNAs; OD, optical density; PBS, phosphate buffered saline; qRT-PCR, quantitative real-time PCR; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween 20; UTR, untranslated region; V–APC, annexin V–allophycocyanin; VSD, ventricular septal defect; 7-AAD, 7-amino-actinomycin D.

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2011). MiRNAs are known to cause diseases; dysregulation of their expression has been implicated in CHD. There is a growing appreciation of the significance of miRNAs in developmental pathology, as reported by Zhao et al. (Zhao et al., 2005; Zhao et al., 2007), whose gain- and loss-of-function studies demonstrated that miRNA-1 (miR-1) targets the cardiac transcription factor Hand2, which is implicated in growth of the embryonic heart and regulation of cardiac morphogenesis, proliferation, and electrical conduction. Bittel et al. (2014) performed a miR-421 knockdown and overexpression study, reporting that miR-421 dysregulation was related to tetralogy of Fallot through regulation of the Notch pathway. Wang et al. (2010) provided evidence suggesting that the miR-17-92 cluster mediates myocardial differentiation of cardiac progenitors in the secondary heart field, but the role of miRNAs in modulating heart development has not been extensively established or investigated. We found that miR-29c was upregulated in ventricular septal defect (VSD), suggesting that increased miR-29c is related to VSD pathogenesis (Zhu et al., 2013). However, previous research of miR-29c so far has focused predominantly on cancer, such as hepatocellular carcinoma (Bae et al., 2014) and breast cancer (Nygren et al., 2014), where it regulates cancer cell growth and proliferation. In contrast, few studies have established that miR-29c is related to CHD pathogenesis.

Bioinformatics analysis determined that miR-29c is conserved evolutionarily in humans and rodents and predicted that Wnt4 is its direct target. The Wnt family plays a crucial role not only in cell proliferation and apoptosis, but also in regulating embryogenesis and development, in particular cell fate and patterning (Chien et al., 2009). Aberrant activation of Wnt signaling exhibits CHD (Zhou et al., 2007; Tian et al., 2010). P19 cells are pluripotent stem cells and are widely used as a model for molecular analysis of embryonic heart development. In this context, we aimed to investigate the effect of miR-29c overexpression in P19 cell proliferation, apoptosis, and differentiation. The effect of miR-29c overexpression on the target Wnt4 signaling-induced embryonic heart development malformations was also studied.

2. Materials and methods

2.1. P19 cells

Murine P19 embryonal carcinoma cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in alpha modification of Eagle's medium (α-MEM; Gibco-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. Dimethyl sulfoxide (DMSO) was diluted in culture medium (final concentration, 1.0%) and the cells allowed to aggregate for 4 days, during which the medium was replenished every 24 h. On day 4, the aggregates were transferred to 6-well plates without DMSO to allow subsequent cardiac differentiation. The medium was refreshed every 24 h for the duration of differentiation. Cell total RNAs and proteins were determined on day 0, 4, 6, and 10 throughout P19 cell differentiation.

2.2. Construction of miR-29c overexpression plasmids

To generate miR-29c overexpression plasmids, DNA fragments corresponding to precursor miR-29c were cloned into pGLV3 vectors conjugated with the green fluorescent protein (*GFP*) gene. The sequences of the amplification products were designed based on the cDNA sequence of the mouse miR-29c precursor and used the primers with indicated restriction sites, at the 5′ end of the sequence added the restriction enzyme sites of BamHI and at the 3′ end added the restriction sites of HindIII. Enzyme sites were given in the following. Forward: 5′-CATGGATCCTCGACACCATCAGTCTGCTC-3′, reverse: 5′-GCGAAGCTTCTGCTTTTCCCCCTACATCA-3′. The products were verified using fluorescence microscopy and quantitative real-time PCR (qRT-PCR).

2.3. Stable miR-29c overexpression cell line

The mmu-miR-29c overexpression and mmu-miR-negative control plasmids were purchased from GenePharma (Shanghai, China). P19 cells at 70–80% confluence were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells (2×10^6) were transfected with 4 μg plasmid in α-MEM according to the manufacturer's protocol, and the α-MEM was replaced after 8 h with complete medium. Gene transfection was characterized by GFP produced in the cells and imaged using fluorescence microscopy. The next day, stable cells were selected and screened using qRT-PCR in medium containing 2 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA) over 2 weeks.

2.4. Cell growth curve analysis

Cell Counting Kit-8 (CCK-8; Dojindo, Rockville, MD, USA) was used to evaluate the P19 cell proliferation rate. Briefly, miR-29c or vector control stable P19 cells were seeded in 96-well plates and incubated for 4 days according to the manufacturer's protocols. CCK-8 reagent (10 μl, 1 mg/ml) was added and incubated for 3 h at 37 °C in a humidified incubator containing 5% CO_2. The absorbance (optical density, OD) was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

2.5. Cell cycle analysis

Before analysis, the miR-29c stable or control cells were starved in serum-free α-MEM for 24 h to synchronize the cell cycle. Cells were incubated with complete medium for 0, 12, and 24 h after serum deprivation. Following incubation, floating cells were collected; adherent cells were harvested by trypsinization to detach the cells, and cells were centrifuged at 4 °C at 100 ×*g* for 5 min. Cells were washed twice with phosphate-buffered saline (PBS) at 4 °C and resuspended in 70% ethanol at −20 °C overnight. The cells were washed once with PBS, resuspended in 500 μl Propidium Iodide/RNase Staining Buffer (BD Bioscience, San Diego, CA, USA), and incubated for 15 min at room temperature. The DNA content of 10^4 cells was analyzed by a FACSCalibur flow cytometer (BD Bioscience); the population in each cell cycle phase was determined using FlowJo software (FlowJo, Ashland, OR, USA).

2.6. Hoechst staining

To detect apoptosis, miR-29c stable or control cells were grown and stained with Hoechst 33258 (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, P19 cells were fixed in 0.5 ml methanol for 15 min. After rinsing twice in PBS, the cells were stained with 1 g/ml Hoechst 33258 solution in the dark for 10 min at room temperature, and then rinsed with PBS. Analysis was conducted using a fluorescence microscope with ultraviolet excitation at 348 nm and emission at 480 nm. Apoptotic cells were characterized by pyknotic and fragmented nuclei emitting intense fluorescence.

2.7. Caspase activity assay

Caspase-3, -8, and -9 activity was determined using a Caspase Activity Kit (Beyotime Institute of Biotechnology, Shanghai, China). The miR-29c stable or control cell lysates were homogenized in 100 ml reaction buffer containing 10 ml caspase-3, -8, and -9 substrate (Ac-DEVD-pNA) (2 mM) and incubated at 37 °C for 2 h. Samples were measured with an enzyme-linked immunosorbent assay reader at 405 nm.