FEBS Letters xxx (2013) xxx–xxx

FEBS Letters

journal homepage: www.FEBSLetters.org

MicroRNA profiling during rat ventricular maturation: A role for miR-29a in regulating cardiomyocyte cell cycle re-entry

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ARTICLE INFO

Article history:
Received 10 November 2012
Revised 19 January 2013
Accepted 23 January 2013
Available online xxxx

Edited by Tamas Dalmay

Keywords:
MicroRNA
Cardiomyocyte
Proliferation
Microarray
MicroRNA-29a

ABSTRACT

Recent studies demonstrated that the mammalian heart possesses some capacity to proliferate. We observed cardiomyocyte proliferation within 4 weeks of age (P4W) in rats. We found 95 microRNAs that are differentially expressed in P4W cardiomyocytes. MicroRNA-29a was among the most highly up-regulated microRNAs in P4W cardiomyocytes. Overexpression of microRNA-29a suppressed the proliferation of H9c2 cell line. MicroRNA-29a inhibition induced cardiomyocytes to proliferate, accelerated the G1/S and G2/M transition, and up-regulated the cell cycle gene expression. Cyclin D2 (CCND2) was identified as a direct target of microRNA-29a. These findings indicate that microRNA-29a is involved in cardiomyocyte proliferation during postnatal development.

1. Introduction

It has long been thought that mammalian cardiomyocytes exit the cell cycle soon after birth and become terminally differentiated in the adult [1]. Recent studies indicated that postnatal mammalian cardiomyocytes are still capable of substantial plasticity [2]. The capability of the cardiomyocyte to proliferate is lost by 7 days of age in mouse [3] and a rapid switch from hyperplasia to hypertrophy in rat cardiomyocyte occurred within 3–5 days after birth [4]. In human heart, the capability to undergo proliferation is lost 3–6 months after birth [5]. Although many efforts have been made to understand the postnatal development and maturation in the heart [6], the potential mechanisms that regulate postnatal cardiomyocytes proliferation are still poorly understood.

MicroRNAs (miRNAs, miRs) are a class of endogenous evolutionarily conserved small (18–22 nucleotides) non-coding RNAs [6,7], which have emerged as pivotal regulators in cell proliferation, differentiation and apoptosis [7,8]. Some differentiation associated miRNAs have been reported to coordinately restrict oncogene-induced proliferation [9]. miR-29 has displayed the capacity for posttranscriptional regulation in myoblast [9] and rhabdomyosarcoma [10]. During cardiomyocyte postnatal development, miRNAs were reported to involve in cardiomyocyte proliferation [11,12].

In this study, we first characterized the proliferation pattern of cardiomyocytes during the postnatal development. Then we investigated the miRNA and mRNA expression profiles in isolated cardiomyocytes from postnatal day two (P2) and P4W groups. We identified miR-29a as one of the most robustly up-regulated miRNAs in P4W cardiomyocytes and found that miR-29a could suppress cardiomyocytes proliferation. CCND2 was identified as a miR-29a target which may be involved in the process of cardiomyocyte cell cycle and proliferation regulation.

Abbreviations: miRNA, microRNAs; CCND2, cyclin D2; CDK2, cyclin-dependent kinase 2; P2, postnatal day 2; P4W, 4 weeks of age; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; UTR, untranslated regions

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http://dx.doi.org/10.1016/j.febslet.2013.01.075

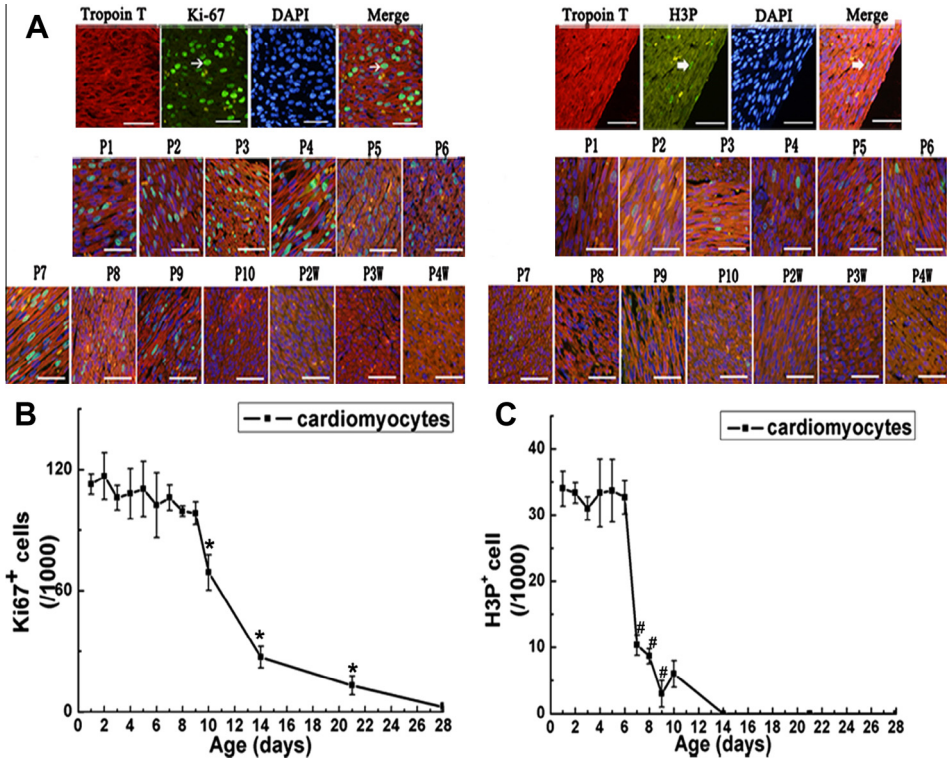

Fig. 1. Immunofluorescence staining of the expression of Ki-67 (green), H3P (green) and troponin T (red) in the heart of rats. (A) Sections from various postnatal stages were stained to label proliferating and mitotic cardiomyocytes (scale bars = 50 μm). Nuclei were stained by DAPI (blue). Quantification of the numbers of Ki-67 (B) and H3P (C) positive cardiomyocytes at sequential postnatal stages is shown. Values are presented as mean ± S.E.M., $^{*,\#}P$-value < 0.05 vs. P1, $n = 3$.

2. Materials and methods

2.1. Animals and cell lines

All animal procedures were conducted in accordance with humane animal care standards approved by the Institutional Animal Care Committee of Fuwai Hospital and Fuwai hospital Ethics Committee (Beijing, China). Ventricular cardiomyocytes were isolated from Sprague–Dawley rats (Animal Laboratory Center of Peking University Health Science Center, China) as described previously [13,14]. H9c2 cells (CRL-1446) were from ATCC (American Type Culture Collection, MD, USA).

2.2. Rat cardiomyocytes isolation and culture

Left ventricular cardiomyocytes were isolated with enzymatic dissociation of the whole heart from rats on a Langendorff apparatus as described before [15]. Briefly, cardiomyocytes were isolated by antegrade collagenase perfusion and purified by differential centrifugation as described [16,17]. After standard culture for 48 h, cardiomyocyte purity was analyzed on the BD FACSCalibur™ flow cytometer (Supplementary Fig. S1A) and immunofluorescence (Supplementary Fig. S1B) as previously described [18]. More than 95% of cardiomyocytes were positive for the cardiomyocyte specific marker.

2.3. miRNA microarray, mRNA microarray, and GO-network

Total RNA was extracted from cardiomyocytes using Trizol (Invitrogen, Carlsbad, CA, USA). miRNA and mRNA microarray hybridizations were performed in triplicate with total RNA using the Affymetrix miRNA microarray service (miRNA 2.0) and Rat Genome 230 2.0 Array (Affymetrix, CA, USA) in CapitalBio (Beijing, China) (Supplementary Materials and methods).

2.4. Cell culture and transfection

H9c2 and cardiomyocytes were cultured and a subcultivation ratio of 1:3 was used for further culture before cells researched the 70% confluent state [19]. Transfection was performed using the siPORT NeoFX transfection agent with miRNA mimics (a small, synthetic, double-stranded RNAs used to mimic endogenous mature miRNAs) or inhibitors (a small, synthetic, single-stranded RNAs used to inhibit mature miRNAs for functioning), according to the manufacturer's instructions (Applied Biosystems, CA, USA) (Supplementary Materials and methods).

2.5. Cell proliferation assay

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI, USA) was conducted to evaluate the effect of miRNAs on H9c2 proliferation, following the manufacturer's instructions. The absorption was determined at 490 nm on a microplate reader (Model 680; BioRad, Tokyo, Japan).

2.6. RNA extraction and quantitative reverse transcription-PCR

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). QRT-PCR was performed using an ABI 7300 cycle detection system. MicroRNA Reverse Transcription kit and TaqMan probes (Applied Biosystems) were used for miRNA RT-PCR. Data were analyzed using the $2^{-\Delta\Delta CT}$ method [20,21] and the fold changes of miRNA or mRNA expression were normalized to U6

Please cite this article in press as: Cao, X.-Q.P.c.t.g.n.a.s.h.b.i.c.>., et al. MicroRNA profiling during rat ventricular maturation: A role for miR-29a in regulating cardiomyocyte cell cycle re-entry. FEBS Lett. (2013), http://dx.doi.org/10.1016/j.febslet.2013.01.075