Gene 531 (2013) 235–242

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

mmu-miR-702 functions as an anti-apoptotic mirtron by mediating ATF6 inhibition in mice

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ARTICLE INFO

Article history:
Accepted 2 September 2013
Available online 10 September 2013

Keywords:
Apoptosis
MicroRNA
miR-702
Isoproterenol
Short hairpin introns

ABSTRACT

MicroRNAs (miRNAs) are a group of endogenous, small, noncoding RNAs that function as key post-transcriptional regulators. miRNAs are involved in many biological processes including apoptosis. In this study, mouse miR-702 (mmu-miR-702), a mirtron derived from the 13th intron of the Plod3 gene, was identified as a regulator of anti-apoptosis. mmu-miR-702 was down-regulated after treatment with the apoptosis-inducer isoproterenol both in vivo and in vitro. According to over-expression experiments, mmu-miR-702 inhibited apoptosis as well as the expression levels of a subset of apoptosis-related genes including activating transcription factor 6 (ATF6). An interaction between mmu-miR-702 and the ATF6 3′-UTR binding site was confirmed using luciferase reporter and western blot assays. This is the first report of ATF6 interaction with miRNA. Although the possible existence of miR-702 in the human genome is low, our results indicate that mirtrons also participate in the process of apoptosis and may provide a novel study strategy for apoptosis.

1. Introduction

Cell apoptosis, the main form of programmed cell death, plays an important role in the progression of various diseases, including cardiovascular disease, degenerative disease, and tumorigenesis. Although there are abundant studies about the molecular mechanisms of apoptosis, controversies and unknowns still exist, especially in the early period of apoptosis. For cardiac myocytes, apoptosis is triggered by a loss of homeostasis, such as changes in physiological stresses and increased sympathetic activity (Logue et al., 2013; Vaughan et al., 2002). Various pathways and hallmarks are involved in the complex molecular mechanisms of apoptosis and stress, including activating transcription factor 6 (ATF6) (Galindo et al., 2012; Nakanishi et al., 2005; Yao et al., 2013).

Activating transcription factor 6 (ATF6), a type II transmembrane protein embedded in the endoplasmic reticulum (ER), is a key stress sensor for initiation of the unfolded protein response (UPR) in ER stress (Haze et al., 1999; Lai et al., 2007). Under ER stress, ATF6 senses the accumulation of unfolded proteins in the ER through dissociation of the 78 kDa glucose-regulated protein (GRP78) from its lumenal domain. ATF6 then translocates from the ER to the Golgi where it is proteolytically processed from a 90 kDa protein to a 50 kDa protein to release its cytoplasmic domain. This truncated form enters the nucleus and binds the ER stress response element (ERSE) in target gene promoters and contributes to the reduction of misfolded protein levels (Adachi et al., 2008). The C/EBP homologous protein (CHOP; official symbol: DDIT3), which contains an ERSE in its promoter region, is an important target gene of ATF6. It also functions as a well-known transcription factor that mediates apoptosis (Tsukano et al., 2010; Yao et al., 2013). Although ATF6 is regarded as a protective protein in the ER stress response, its role in apoptosis has recently received increased attention (Galindo et al., 2012; Nakanishi et al., 2005; Yao et al., 2013).

MicroRNAs (miRNAs) are a group of endogenous, small, noncoding RNAs that are found in eukaryotes. They function as key post-transcriptional regulators through base pairing to partially complementary sites, mainly in the untranslated region of mRNA (Ambros, 2003; Lee and Ambros, 2001). Mirtrons, a subset of miRNAs derived from short hairpin introns, also work by targeting mRNAs (Okamura et al., 2008; Westholm and Lai, 2011). Mouse miR-702 (mmu-mir-702; miRBase accession: MI0004686) derives from the 13th intron of the Plod3 gene of mice and belongs to mirtrons. mmu-mir-702 is not expressed in mouse oocyte, but it appears in 8-cell stage embryos and has a significantly up-regulation during embryogenesis in mouse heart (Mineno et al., 2006). Although mmu-miR-702 was reported to promote proliferation in Dgcr8-deficient embryonic stem cells in recent research (Kim and Choi, 2012), the function of it remains a mystery. As a microRNA, a classical molecular mechanism involves targeting mRNAs to inhibit their expression and subsequent biological roles. However, to this day, mmu-miR-702 has not been investigated in this respect.

Stimulation of β-adrenergic receptors (β-AR) induces apoptosis in cardiac myocytes in vitro and in vivo (Iwai-Kanai et al., 1999; Singh

Abbreviations: mmu-miR-702, mouse microRNA-702; ATF6, activating transcription factor 6; ER, endoplasmic reticulum; UPR, unfolded protein response; GRP78, 78 kDa glucose-regulated protein; ERSE, endoplasmic reticulum stress response element; CHOP, C/EBP homologous protein; ISO, isoproterenol; XBP1, X-box binding protein 1; BCL2, B-cell CLL/lymphoma 2; BAX, BCL2-associated X protein; UTR, untranslated regions.

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http://dx.doi.org/10.1016/j.gene.2013.09.005

et al., 2000). Given that three distinct β-AR subtypes exist in mammals, isoproterenol (ISO), as a traditional nonselective agonist of the β-AR, is more frequently used as an apoptosis inducer in many researches (Hsu et al., 2013; Jin et al., 2007; Zhuo et al., 2013). Moreover, ISO has been indicated as an apoptosis inducer not only in cardiac myocytes, but also in other cells (Hudecova et al., 2013; Liao et al., 2010). In our study, an ISO-induced apoptosis model was established in vivo and in vitro with the aim of determining a possible molecular regulatory mechanism of mmu-miR-702 in this process.

2. Materials and methods

2.1. Establishment of animal model

Male C57Bl/6 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All procedures were approved by the regulations and protocols of the ethics committee of the Harbin Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

The animals were kept with water and fed a standard laboratory diet for 1 week. Thereafter, mice were randomly divided into four groups (n = 6/group), including three treatment groups and one control group. For the treatment groups, ISO (Sigma, St. Louis, MO, USA), dissolved in 0.2 ml 0.9% NaCl, was injected (10 mg/kg) into the subcutaneous tissue twice 24 h apart. For the control group, an equal volume of saline was injected. The animals in the three treatment groups were sacrificed at 6, 12, and 24 h after the last injection, while the animals in the control group were sacrificed at 24 h after the last injection. The left ventricles of every animal were excised for experimental samples.

2.2. Examination by electron microscopy

The left ventricles of mice were sliced into 1–3 mm cubes and fixed in 3% glutaraldehyde buffer overnight at 4 °C. Samples were processed by the Harbin Medical University Electron Microscopy Core facility. Sections were imaged on a Jeol 1200 EX TEM (Mitaka, Tokyo, Japan) at the indicated magnification.

Table 1
Amplification primers for PCR.

Gene	Amplification Primer (5′-3′)	Product size	Annealing temperature
mmu-miR-702	Forward: GAGTGCCCACCCTTTACC Reverse: CAGTGCGTGTCGTGGAGT	64 bp	62 °C
U6 small nuclear RNA	Forward: GCTTCGGCAGCACATATACTAAAAT Reverse: CGCTTCACGAATTTGCGTGTCAT	89 bp	60 °C
Bax	Forward: TTTGCTACAGGGTTTCATCCAG Reverse: TGTCCAGTTCATCTCCAATTCG	140 bp	61 °C
Bcl2	Forward: CCTACGGATTGACATTCTCAGT Reverse: ACATAAGGCAACCACACCATC	168 bp	61 °C
Casp3	Forward: GCTGACTTCCTGTATGCTTACT Reverse: CGTTGCCACCTTCCTGTTAA	165 bp	61 °C
CHOP	Forward: GCTGGAAGCCTGGTATG Reverse: CTTTGGGATGTGCGTGT	183 bp	56 °C
GRP78	Forward: ACGCACTTGGAATGACC Reverse: TTCTTTCCCAAATACGC	192 bp	48 °C
GAPDH	Forward: AGGGCATCTTGGGCTACAC Reverse: CATACCAGGAAATGAGCTTGA	132 bp	56 °C
XBP1s	Forward: GAACCAGGAGTTAAGAACACG Reverse: AGGCAACAGTGTCAGAGTCC	179 bp	63 °C
XBP1u	Forward: GAACCAGGAGTTAAGAACACG Reverse: AGGCAACAGTGTCAGAGTCC	205 bp	63 °C

Table 2
PCR amplification primers for obtaining the DNA insert fragment.

	Amplification primer (5′-3′)	Product size	Annealing temperature
ATF6 site 1	Forward: CGACGCGTGCAACTCCAGGGGAAGAGGAA Reverse: CCCAAGCTTCAGAACCAACACCAGGCGACA	155 bp	57 °C
ATF6 site 2	Forward: CGACGCGTGGTCCCATTCCAGTAAGCACAA Reverse: CCAAGCTTCCACAGCCACAGTCACATCCTC	423 bp	57 °C
ATF6 site 3	Forward: CGACGCGTCCAGCCAGTGTCACAAAGCAA Reverse: CCAAGCTTTTCGGGCACTCTAAGCAAGCA	504 bp	54 °C
ATF6 site 4	Forward: CGACGCGTATGGTGGATGGGGTATGTAG Reverse: CCCAAGCTTTATCCCTGGTTGACCCTTAA	180 bp	52 °C
DDIT3 site	Forward: CGACGCGTAGTGGGCATCACCTCCTGTC Reverse: CCCAAGCTTCAATGTACCGTCTATGTGCAAG	170 bp	55 °C

2.3. Cell culture

NIH 3T3 and HEK293T cell lines, obtained from the Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, China), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO_2. The NIH 3T3 cell line was transfected with mmu-miR-702 mimics or negative control RNA (miR-NC) with Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. ISO treatment was performed with medium containing 10 μM ISO at 24 h after transfection. RNA and protein were harvested 48 h after transfection or after ISO treatment for 0.5, 1, 2, and 24 h. The efficiency of mmu-miR-702 transfection was determined by quantitative real-time PCR. HEK293T cells were used for luciferase assays.

2.4. Caspase 3 activity detection

Caspase 3 activity in cardiac tissue extracts or NIH 3T3 cell proteins was detected by the Caspase 3 Activity Assay Kit (BestBio, Beijing, China), according to the manufacturer's protocol. The absorbance was measured at 405 nm, and the relative activity of Caspase 3 was determined.

2.5. RNA extraction and quantitative real-time PCR

Total RNA samples were isolated from tissues or from cell lines using TRIzol (Invitrogen Life Technologies) and reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The specific stem-loop primer designed for mmu-miR-702 reverse transcription was 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCG GCAATTGCACTGGATACGACGAGCGG-3′. The primer designed for U6 small nuclear RNA reverse transcription was 5′-CGCTTCACGAAT TTGCGTGTCAT-3′. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and the ABI 7500 Real-time PCR system (Applied Biosystems). Specific amplification primers were also designed for mmu-miR-702, U6 small nuclear RNA, BAX, BCL2, Caspase 3 (Casp3), CHOP, GRP78, and GAPDH (Table 1). U6 small nuclear RNA was used as a control for microRNA quantification, while GAPDH was employed as the endogenous control for other genes. PCR was performed according to the manufacturer's instructions. All assays, including those for the non-template control, were performed, at least, in triplicate. The relative expression levels were