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Original article

Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response

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ABSTRACT

A nodal regulator of endoplasmic reticulum stress is the transcription factor, ATF6, which is activated by ischemia and protects the heart from ischemic damage, *in vivo*. To explore mechanisms of ATF6-mediated protection in the heart, a whole-genome microRNA (miRNA) array analysis of RNA from the hearts of ATF6 transgenic (TG) mice was performed. The array identified 13 ATF6-regulated miRNAs, eight of which were downregulated, suggesting that they could contribute to increasing levels of their mRNAs. The down-regulated miRNAs, including miR-455, were predicted to target 45 mRNAs that we had previously shown by microarray analysis to be up-regulated by ATF6 in the heart. One of the miR-455 targets was calreticulin (*Calr*), which is up-regulated in the pathologic heart, where it modulates hypertrophic growth, potentially reducing the impact of the pathology. To validate the effects of miR-455, we showed that Calr protein was increased by ATF6 in mouse hearts, *in vivo*. In cultured cardiac myocytes, treatment with the ER stressor, tunicamycin, or with adenovirus encoding activated ATF6 decreased miR-455 and increased Calr levels, consistent with the effects of ATF6 on miR-455 and Calr, *in vivo*. Moreover, transfection of cultured cardiac myocytes with a synthetic precursor, premiR-455, decreased Calr levels, while transfection with an antisense, antimiR-455, increased Calr levels. The results of this study suggest that ER stress can regulate gene expression *via* ATF6-mediated changes in micro-RNA levels. Moreover, these findings support the hypothesis that ATF6-mediated down-regulation of miR-455 augments *Calr* expression, which may contribute to the protective effects of ATF6 in the heart.

1. Introduction

For the efficient synthesis and folding of most secreted and membrane proteins, conditions in the endoplasmic reticulum (ER) must be optimal; suboptimal conditions lead to improper protein folding and eventual ER stress [1]. Initially, ER stress triggers protective aspects of the conserved signaling program known as the unfolded protein response (UPR), which are oriented toward restoring the ER environment [2–4]. However, if the stress continues, and ER protein folding is not restored, apoptotic aspects of the UPR ensue [5,6].

One of the proximal sensors of ER stress is activating transcription factor 6 (ATF6) [7], a transcription factor that binds to ER stress response elements (ERSEs) and transcriptionally induces numerous genes, a subset of which encodes proteins that restore ER protein folding, thus contributing to protection [8]. We previously showed that ischemia activates ER stress in the heart [9], and activates ATF6 in cultured cardiac myocytes [10]. To examine the function of the ATF6 branch of the ER stress response, *in vivo*, we generated a transgenic (TG) mouse line that expresses a conditionally activated form of ATF6 in the heart [11]. In this model, ATF6 decreased infarct size and apoptosis, and improved functional recovery upon reperfusion in an *ex vivo* mouse model of global myocardial ischemia. To examine how ATF6 might mediate this protection, a whole-genome microarray analysis revealed the identities of 607 ATF6-regulated genes, 381 of which were upregulated, mostly as a result of ATF6-mediated increases in transcription [12]. In the present study, we determined whether ATF6 regulates the expression of microRNAs (miRNAs).

MiRNAs are short, 20–23 nucleotide, non-coding RNAs that act as inhibitors of gene expression by forming partial duplexes with the 3′ UTR of mRNAs [13,14]. They act by either inhibiting mRNA translation, or by promoting the degradation of mRNAs [15]. It has been estimated that there are approximately 1000 microRNAs encoded by the human genome, and each can have numerous mRNA targets [16]. In addition, a single mRNA can be targeted by several different miRNAs, making gene regulation by miRNAs quite complex. Thus, it is possible that ATF6-mediated down-regulation of miRNAs that

Abbreviations: ATF6, activation of transcription factor 6; Calr, calreticulin; miRNA, microRNA; UPR, unfolded protein response; MER, mutant mouse estrogen receptor; MER-ATF6, fusion protein with the MER fused to the C-terminal of ATF6; TG, transgenic; NTG, non-transgenic; NRVMC, neonatal rat ventricular myocyte cultures; qRT-PCR, quantitative real time PCR; ERSE, ER stress response element.

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target ER stress response genes may be a mechanism by which the ATF6 could regulate gene expression, post-transcriptionally. This possibility was examined in the current study, where we determined the effects of ATF6 on miRNA expression in the hearts of ATF6 transgenic mice and in cultured cardiac myocytes.

2. Materials and methods

2.1. Animals

Approximately 12 adult male C57/BL6 mice (6 non-transgenic and 6 ATF6 transgenic mice), and 100 1–4 day-old Harlan Sprague–Dawley rats were used for this study. All procedures involving animals were carried out in accordance with the San Diego State University Institutional Animal Care and Use Committee.

2.2. ATF6-MER transgenic mice

The generation of ATF6-MER (*m*utant mouse *e*strogen *r*eceptor) transgenic mice (TG) featuring cardiomyocyte-specific transgene expression, has been described elsewhere [11]. Non-transgenic (NTG) and ATF6-MER transgenic (TG) mice were treated with vehicle or tamoxifen, n = 3 mice per treatment group, to activate Mer-ATF6 in the ATF6 transgenic mouse hearts. Tamoxifen was suspended at 10 mg/ml in 100 ml 95% ethanol and 900 ml sunflower oil and sonicated until clarified. Mice were injected intraperitoneally once/day with 20 mg/kg tamoxifen, or with vehicle only. After 5 d, RNA was extracted from mouse heart ventricles, as described previously [11].

2.3. ATF6 whole-genome miRNA array

Non-transgenic (NTG) and ATF6-MER transgenic (TG) mice [11] were treated ± tamoxifen for 5 days, n = 3 mice per group, and ventricular mRNA was subjected to miRNA array analysis. About 5 mg of total mouse ventricle RNA were submitted to LC Sciences (Houston, TX) for quality control, processing and miRNA expression analysis in accordance with their specifications. All samples met quality control standards. Samples were hybridized to individual mouse miRNA chips (LC Sciences, part #MRA-1002), which were current with Sanger miRBase version 14.0 and contained roughly 700 unique mature miRNA probes. The data reported in this publication have been deposited in NCBI's Gene Expression Omnibus [17] and are accessible through GEO Series accession number GSE33515 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33515).

2.4. miRNA array statistics and data analysis

Array statistics and data analysis for each of the 4 treatment groups were carried out essentially as described [12]. The miRNAs that exhibited significant changes in expression ($p<0.05$) and were differentially expressed in vehicle vs. tamoxifen-treated TG mouse hearts by 1.5-fold, or more, were included in this study. Since tamoxifen may affect miRNA levels independently of its ability to activate ATF6-MER, miRNAs that were differentially expressed in vehicle vs. tamoxifen-treated NTG mouse hearts were excluded from this study.

2.5. miRNA target prediction

Potential miRNA target sites were predicted by first searching for the miRNA of interest on Sanger miRBase (http://www.mirbase.org) [18], and then identifying putative targets using TargetScan Version 5.1 (http://www.targetscan.org) [19]. Targets for miR-455 were further confirmed using miRanda (http://www.microrna.org) [20].

2.6. Primary neonatal rat ventricular myocyte cultures

Neonatal rat ventricular myocyte cultures (NRVMCs) were prepared as previously described [12,21,22]. Following enzymatic dissociation with TrpE™ Express (Invitrogen Cat no. 12605), cells were plated onto plastic culture dishes and maintained in 10% FCS-containing DMEM/F-12 for ~2 h. During this time, cardiac fibroblasts adhere to the culture dishes, but cardiac myocytes remain in suspension. Following this pre-plating, cells that do not plate are mostly cardiac myocytes. After the pre-plating step, NRVMCs were plated onto fibronectin-coated 6-well plastic culture dishes at 1×10^6 cells/well in 10% FCS-containing DMEM/F-12. Twenty-four hours after plating, cells were washed with medium and used for experiments. Procedures for infection of NRVMCs with adenovirus to mediate efficient gene transfer have been described previously, as has the generation and characteristics of the recombinant adenovirus that encodes a constitutively active form of ATF6, ATF6(1–373) [12].

2.7. mRNA and miRNA quantification

For tissue samples, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA)[11]. For cultured cell samples, both total and small RNA-enriched RNA fractions were obtained using the miRNeasy and miRNA cleanup kits (Qiagen, Valencia, CA). mRNA and miRNA levels were analyzed using the TaqMan quantitative real-time PCR (qRT-PCR) method (10 ng/assay), and quantified with an ABI 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA). Taqman Primer assays for miRNAs and the reagents for reverse transcriptase and qRT-PCR reactions were obtained from Applied Biosystems. Relative expression was calculated using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$). mRNA levels were normalized to GAPDH, as previously described [11], while miRNA levels were normalized to U6 RNA. mRNA levels were measured as previously described [11] using custom primers and normalizing to GAPDH mRNA expression.

2.8. PCR primers

The following primer pairs were used for real time quantitative PCR (RT-qPCR):

Calreticulin (rat) NM_022399:

5′-AGCAGTTCTTGGACGGAGATG

3′-TGTTGGATTCGACCCAGC

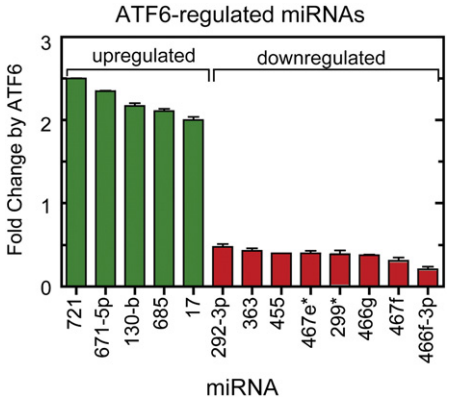

Fig. 1. ATF6 miRNA microarray. The 13 miRNAs found by miRNA microarray that were differentially expressed upon ATF6 activation in the heart are plotted as a function of the change in expression level. Those miRNAs that were upregulated are shown in green, and miRNAs that were downregulated are shown in red. The mean fold change in expression levels are shown (n = 3 mouse hearts per treatment group).