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The *let-7* microRNA enhances heme oxygenase-1 by suppressing Bach1 and attenuates oxidant injury in human hepatocytes ☆

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ABSTRACT

The *let-7* microRNA (miRNA) plays important roles in human liver development and diseases such as hepatocellular carcinoma, liver fibrosis and hepatitis wherein oxidative stress accelerates the progression of these diseases. To date, the role of the *let-7* miRNA family in modulation of heme oxygenase 1 (HMOX1), a key cytoprotective enzyme, remains unknown. Our aims were to determine whether *let-7* miRNA directly regulates Bach1, a transcriptional repressor of the HMOX1 gene, and whether indirect up-regulation of HMOX1 by *let-7* miRNA attenuates oxidant injury in human hepatocytes. The effects of *let-7* miRNA on Bach1 and HMOX1 gene expression in Huh-7 and HepG2 cells were determined by real-time qRT-PCR, Western blot, and luciferase reporter assays. Dual luciferase reporter assays revealed that *let-7b*, *let-7c*, or miR-98 significantly decreased Bach1 3′-untranslated region (3′-UTR)-dependent luciferase activity but not mutant Bach1 3′-UTR-dependent luciferase activity, whereas mutant *let-7* miRNA containing base complementarity with mutant Bach1 3′-UTR restored its effect on mutant reporter activity. *let-7b*, *let-7c*, or miR-98 down-regulated Bach1 protein levels by 50–70%, and subsequently up-regulated HMOX1 gene expression by 3–4 fold, compared with non-specific controls. Furthermore, Huh-7 cells transfected with *let-7b*, *let-7c* or miR-98 mimic showed increased resistance against oxidant injury induced by tert-butyl-hydroperoxide (tBuOOH), whereas the protection was abrogated by over-expression of Bach1. In conclusion, *let-7* miRNA directly acts on the 3′-UTR of Bach1 and negatively regulates expression of this protein, and thereby up-regulates HMOX1 gene expression. Over-expression of the *let-7* miRNA family members may represent a novel approach to protecting human hepatocytes from oxidant injury.

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1. Introduction

Heme oxygenase 1 (HMOX1) is a key cytoprotective enzyme, catalyzing heme degradation to produce ferrous iron, carbon monoxide (CO) and biliverdin, which is further reduced to bilirubin in liver, with carbon monoxide, biliverdin and bilirubin acting as antioxidants [1–5]. The induction of HMOX1 has been observed as an important event in response to oxidative stress, and the molecular mechanisms underlying regulation of HMOX1 gene expression by oxidative stress, heme and other non-heme metalloporphyrins have been extensively investigated. Among key transcription factors associated with regulating HMOX1 gene expression, we and others have demonstrated that Bach1, a member of the cap n' collar family of zinc-containing basic leucine zipper (bZip) proteins, plays a key role in tonic repression of HMOX1 gene [6–12]. It does so by forming antagonizing heterodimers with the Maf-related oncogene family. These heterodimers bind to Maf recognition elements (MAREs) and suppress expression of genes [e.g., HMOX1 and NAD(P)H:quinone

Abbreviations: BCA, bicinchoninic acid; bZip, basic leucine zipper; CLIP, cross-linking immunoprecipitation; CO, carbon monoxide; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HITS, high-throughput sequencing; HMOX1, heme oxygenase1; MARE, Maf recognition element; miRNA, microRNA; MINC, microRNA inhibitor negative control; MMNC, microRNA mimic negative control; NQO1, NAD(P)H:quinone oxidoreductase; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; tBuOOH, tert-butyl hydroperoxide; UTR, untranslated region; WB, Western blot

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oxidoreductase (NQO1)] [6,8,9,13]. Down-regulation of Bach1 enhances HMOX1 gene expression, and thus has emerged as an effective approach to up-regulate HMOX1 and NQO1 gene expression in response to oxidative stress [12–14]. Our recent studies suggest that microRNAs (miRNAs), a new class of small non-coding regulatory RNAs (~22 nt), play a critical role in regulating Bach1 gene expression at a post-transcriptional level, indirectly enhancing HMOX1 gene expression in human hepatocytes [12,14]. Bach1 contains a 3′-untranslated region (UTR) 3313 nucleotides (nt) in length which provides a basis for candidate miRNA binding. However, to date, miRNAs that are involved in modulating Bach1 gene expression are largely unknown.

The *let-7* miRNA family is one of the first two miRNAs discovered in *Caenorhabdities elegans* (*C. elegans*), and the first known human miRNA, consisting of *let-7a*, *b*, *c*, *d*, *e*, *f*, *g*, *i* and miR-98 miRNAs [15,16]. Besides a pivotal role for the *let-7* miRNA family in embryonic development and cell maturation [17–20], the *let-7* miRNA family was found to play an important role in human liver development [21] and liver diseases such as hepatocellular carcinoma [22,23], hepatic fibrosis [24] and hepatitis [25], conditions known to be accelerated by oxidative stress and for which, in consequence, antioxidant therapy has been proposed as a treatment approach. Thus far, the role of *let-7* miRNA in regulating Bach1 and HMOX1, and the biological function of *let-7* miRNA in defending against oxidative stress in liver have not been investigated. We performed in silico analysis and discovered two highly conserved sites in the 3′-UTR of Bach1 mRNA that perfectly matched the seed region of *let-7* miRNA.

In the present work, we aimed to study whether the *let-7* miRNA family directly regulates Bach1, and whether indirect up-regulation of HMOX1 by *let-7* miRNA attenuates oxidant injury in human hepatocytes. Our focus was on the five representative *let-7* miRNA family members, *let-7a*, *let-7b*, *let-7c*, *let-7e* and miRNA-98. Our findings show that *let-7* miRNA directly acts on the 3′-UTR of Bach1, translationally represses expression of this protein, and thereby up-regulates HMOX1, suggesting that over-expression of *let-7* miRNA family members may represent a therapeutic approach for protection against oxidant injury induced by chronic liver diseases.

2. Materials and methods

2.1. miRNA target predications

Three algorithms, TargetScanHuman5.1 (http://www.targetscan.org), PicTar (http://pictar.mdcberlin.de) and miRanda (http://www.microrna.org) were used to predict putative binding sites in the Bach1 mRNA for members of the *let-7* miRNA family.

2.2. Cell culture

Human hepatoma cell lines, Huh-7 (Japan Health Research Resources Bank, Osaka, Japan) and HepG2 (American Type Culture Collection, Manassas, VA) were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA) [26]. All cells were maintained in a humidified atmosphere of 95% room air and 5% CO_2 at 37 °C.

2.3. Reporter constructs, co-transfection and luciferase reporter activity assays

pLSV40-Rluc and pLSV40-GL3/Bach1 reporter constructs were kindly provided by B. R. Cullen (Duke University, Durham, NC) [27]. pLSV40-Rluc contains a cDNA (R*luc*) encoding *Renilla* luciferase as an internal control reporter and pLSV40-GL3/Bach1 *firefly* luciferase reporter construct contains the full-length 3′-UTR of Bach1 mRNA [27]. Constructs were confirmed by restriction enzyme digestion and sequencing. Mutant pLSV40-GL3/Bach1-Mut was generated by GENEWIZ, Inc. (South Plainfield, NJ).

Co-transfection of miRNA mimics and reporter constructs were performed using Lipofectamine 2000 from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. Briefly, cells were co-transfected with 0.4 μg/mL pLSV40-GL3/Bach1 or mutant pLSV40-GL3/Bach1-Mut, 0.4 μg/mL of pLSV40-Rluc, and 10–50 nM miRNA mimics. 24–48 h after transfection, cells were harvested and lysed and luciferase reporter activities were measured using Dual-Glo® Luciferase Assay System. Firefly luciferase activity was normalized to *Renilla* luciferase activity and total protein, determined using the bicinchoninic acid (BCA) protein assay kit. Values for cells without miRNA mimic transfection were set equal to 1.

2.4. miRNA microarray analysis

The RNA samples were Poly (A) tailed and ligated to biotinylated signal molecules using the FlashTag™ Biotin RNA labeling Kit (Genisphere, LLC, Hatfield, PA). An enzyme linked oligosorbent assay (ELOSA) QC assay was performed to verify labeling prior to array hybridization. Hybridization, washing, staining and scanning were performed using Affymetrix GeneChip® system instruments (Affymetrix, Santa Clara, CA). Affymetrix GeneChip® Operating Software (GCOS) version 1.4 was used to analyze microarray image data and to compute intensity values. Affymetrix CEL files containing raw, probe-level signal intensities were analyzed using Partek Genomics Suite (Partek, St. Louis, MO). Robust multichip averaging (RMA) was used for background correction, quantile normalization and probeset summarization with median polish [28]. Statistical difference was calculated by two-way ANOVA analysis with false discovery rate (FDR). Cluster and Treeview software was used to perform miRNA hierarchical cluster analysis and visualization [29].

2.5. Transfections with miRNA mimics and inhibitors

The miRIDIAN miRNA mimics for *let-7b*, *let-7c*, miR-98, customized mutant *let-7b*, and miRNA mimic negative control (MMNC), and the miRIDIAN miRNA inhibitors for *let-7a*, *let-7c*, *let-7e* and miRNA inhibitor negative control (MINC) were obtained from Dharmacon (Lafayette, CO). Transfection of miRNA mimics and inhibitors were performed as described previously [12]. Briefly, Huh-7 or HepG2 cells were transfected with 10–50 nM of tested miRNA mimics or inhibitors using Lipofectamine 2000 from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. After 24–48 h transfection, cells were harvested for appropriate subsequent assays.

2.6. Human Bach1 expression vector and transfection

The human Bach1 expression vector [30], constructed by subcloning the human Bach1 full length cDNA into the mammalian expression vector pCMV6-XL4 (OriGene, Rockville, MD), was obtained from OriGene. For transfection of Bach1 expression vector pCMV-Bach1, Huh-7 cells were plated in 24-well plates one day prior to transfection and transfected at 50% confluence. Cells were transfected with 0.4 μg/well of pCMV-Bach1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.7. Real-time quantitative RT-PCR

Total RNA from treated cells was extracted and cDNA was synthesized. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using a CFX96™ six-channel Real-Time PCR Detection System from Bio-Rad (Hercules, CA) and iQ™ SYBR Green Supermix Real-Time PCR kit (Bio-Rad) as described previously [11,12]. Samples without template and without reverse transcriptase were included as negative controls, which, as expected,

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