Materials and Methods

Materials

HepG2 cells, mouse hepatoma Hepa 1-6 cells, Hepa 1C-7, rat H9c2 cardiomyocytes and rat A7r5 vascular myocytes were obtained from ATCC. Mouse adrenal Y1 cells were provided by Dr. Salman Azhar (VA Palo Alto Health care System). Mouse primary hepatocytes were isolated from male C57BL/6J mouse at San Francisco General Hospital Liver Center. Flag-tagged mouse hnRNP D isoforms were generously provided by Dr. Robert J. Schneider (New York University School of Medicine). Berberine chloride was purchased from Sigma (Catalog number B325).

Generation of Alb-Luc-UTR mice

The plasmid pBlu-Alb was generously provided by Dr. Richard D. Palmiter (University of Washington, Seattle, WA) that contained a 2 kb fragment of the mouse albumin enhancer/promoter region (Ref. #1-3). This fragment was released from the vector by digesting with SacI and EcoRV, and was subsequently inserted into pGL4.23 vector at 5’ SacI and 3’ EcoRV sites to yield plasmid pAlb-Luc vector. A 2.4 kb DNA fragment containing human LDLR mRNA 3’UTR sequence from nucleotide (nt) 2677 to 5100 covering the entire 3’UTR without the 75 nt in the 3’ extreme end was released from pCMV-Luc-UTR vector (Ref. #4) by Xbal digestion and inserted at the Xbal site of pAlb-Luc to yield pAlb-Luc-UTR plasmid. After sequencing validation of the correct direction of UTR insert, the plasmid pAlb-Luc-UTR was digested with KpnI and SalI to release the 6.5 kb Alb-Luc-UTR fragment that was subsequently microinjected into fertilized oocytes of FVB mice at Transgenic Facility of Stanford University. Genomic DNA was isolated from the tail of founder and offspring and genotyping was performed by PCR to amplify a 360 bp fragment of Luc coding region with 5’ primer Luc2-F (5’-GTGGTGTGGTGTGTTGCTGGAC-3’) and the 3’ primer Luc2-R (5’-GTGGTGTGGTGTGTTGCTGGAC-3’). Initially five founders (three females and two males) were identified. Transgenic lines were generated by crossing the female and male founders, and their offspring were used in this study.

Bioluminescence imaging

Bioluminescence was detected with the In Vivo Imaging System (IVIS; Xenogen, Alameda, CA), consisting of a cooled charge-coupled device camera mounted on a light-tight specimen chamber, an anesthesia unit and a Windows computer system. D-luciferin potassium salt was purchased from Gold Biotechnology (St. Louis, MO) and prepared by dissolving the luciferin in phosphate buffered saline (PBS) to a final concentration of 10 mg/mL. The solution was sterilized by passing through a 0.2 μm filter. Mice received i.p. injection of 50 mg/kg D-luciferin 10 min before imaging and were anesthetized with isoflurane during imaging. Photons emitted from living mice were acquired as photons per second/cm² per steradian (sr) by using LIVINGIMAGE software (Xenogen) and were integrated over 60 sec. For photon quantification, a region of interest was manually selected and kept constant within all experiments. The signal intensity was converted into photons per s/cm² per sr.

BBR treatment of Alb-Luc-UTR mice

Animal use and experimental procedures including the generation of transgenic mouse lines and BBR treatment study were approved by the Institutional Animal Care and Use Committee of the VA Palo Alto Health Care System. All animals were fed a rodent normal chow diet ad
and housed in polycarbonate cages in a room with a 12-h light/12-h dark cycle, and maintained at a constant temperature of 22°C.

In the first in vivo experiment, nine to eleven month old Alb-Luc-UTR mice (male and female) were administered by oral gavage either BBR (200 mg/kg/day) or vehicle (0.2 ml of 0.5% carboxymethyl cellulose in PBS) for 14 days. Mice were maintained on a normal diet for the entire study. Before the treatment, the baseline bioluminescence imaging was obtained from all animals. After 14 days of treatment, bioluminescence imaging was recorded before sacrificing the animals. Bioluminescence was expressed as fold induction over baseline levels. At the end of treatment, animals were sacrificed and livers were immediately removed, cut into small pieces, and stored at -80°C for RNA isolation and protein isolation.

In the second in vivo experiment, Alb-Luc-UTR male mice of 8-12 week old were administered either vehicle (0.5% CMC) or BBR (200 mg/kg/day) orally for 3 days. Bioluminescence imaging was obtained from all animals before and after the treatment. Liver samples were collected and frozen at -80°C for further analysis.

Construction of plasmid and adeno-viral vectors

The U6 promoter based vector (pShuttle-shHNRNPD) that expresses a shRNA targeting mouse and human hNRNP D transcripts (5’-CGGAGAGTGTAGATAAGGTCA) was constructed followed by viral packaging, viral production and purification (Vector Biolab).

Construction of pLuc-ARE reporters and ARE mutated vectors

Utilizing pCMV-Luc-UTR plasmid as the template, a DNA fragment of 130 bp encompassing ARE1 site was amplified with primers entailed with XbaI site:

ARE1-F: 5’-CTAGTCTAGACGGCCTTGTGTTTTATTTCAAAG-3’
ARE1-R: 5’-CTAGTCTAGAATCCCAACCCCAAGCCATT-3’

A fragment of 95 bp surrounding ARE2 site was amplified with XbaI site entailed primers: ARE2-F: 5’-CTAGTCTAGACCCCAACTCCATTCAACAAATG-3’
ARE2-R: 5’-CTAGTCTAGAGTCTCCACGGGCAAGTAA-3’

A fragment of 135 bp surrounding ARE3 site was amplified with XbaI site entailed primers: ARE3-F: 5’-CTAGTCTAGACGGGCAACTTGGTTTCAAAATG-3’
ARE3-R: 5’-CTAGTCTAGAATTCATTGACACCGGCTTTT-3’

A fragment of 124 nt encompassing ARE4 site was amplified with XbaI-tailed primers:
ARE4-F: 5’-CTAGTCTAGACGGGCAACTTGGTTTCAAAATG-3’
ARE4-R: 5’-CTAGTCTAGAACCGGCCCAGAATAGT-3’.
The nucleotides of XbaI site are underlined. The amplified ARE fragments were individually inserted into pCMV-Luc vector at the 3’end of Luc coding sequence. After sequencing validation, plasmids with correct ARE orientations are used in transfection and luciferase assays.

pLuc-UTR-2 (Ref. 6) was used as the template to generate luciferase-UTR reporters with ARE sites mutations individually and combined.

**Transient transfection and luciferase activity assays**

Plasmids pLuc-LDLR3’UTR, pLuc-AREs and control vector pLuc were individually transfected into Hepa1-6 cells along with renilla luciferase (pRL-SV40) reporter as an internal transfection control as previously described (Ref.6). Two days after transfection, cells were lysed with 1 x passive lysis buffer, and firefly and renilla luciferase activities were measured by a 96-well plate reader (SpectraMax® L microplate luminometer, Molecular Devices). For BBR treatment, pLuc-UTR-2 wild-type or ARE mutated vectors were individually transfected into HepG2 cells along with pRL-SV40. One day after transfection, BBR or its diluents DMSO was added to cells for eight hours. Cells were lysed with 1 x passive lysis buffer, and firefly and renilla luciferase activities were measured.

**Tissue luciferase enzymatic assay**

Tissues from euthanized CMV-Luc-UTR mice were dissected, weighed, and homogenized in cell culture lysis buffer (Promega). Protein concentrations of tissue homogenates were determined using BCA™ protein assay reagent (PIERCE). Luciferase activity from tissue homogenate were measured and normalized to tissue protein.

**RNA isolation and real time quantitative RT-PCR (qRT-PCR)**

Total RNA was isolated from flash-frozen mouse liver tissue using an RNeasy kit (Qiagen, CA). RNA integrity was confirmed by agarose gel electrophoresis and ethidium bromide staining. Two µg of total RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) using random primers. Real-time qRT-PCR was performed on the ABI PRISM® 7900HT Sequence Detection System with SYBR PCR master mix (Applied Biosystems). Each cDNA sample was run in duplicate. The correct size of PCR product and the specificity of each primer pair were confirmed by examination of PCR product on agarose gel. Primer sequences of genes used in q-PCR are listed in Table 1 of supplemental materials.

**LDLR mRNA half-life determination**

To determine LDLR mRNA half-lives, HepG2, Hepa 1-6, Y1, A7r5 and H9C2 cells were seeded in 12-well plates at the density of 0.25x10⁶ cells/well in medium containing 10% FBS. Mouse primary hepatocytes were seeded in 24 well plates in Williams E Medium containing Cell Maintenance Cocktail (Cell Maintenance Supplement Pack, Invitrogen). The following day, actinomycin D (5 µg/ml) was added to cells for different intervals (0, 0.5, 1, 2, 4, and 6 h). At designated times, total RNA was extracted from cells.

**Western blot analyses of hnRNP D and LDLR in mouse liver tissues and cell lines**

Approximately 90-100 mg of frozen liver tissue from individual mice were homogenized in 1 ml RIPA buffer containing 1 mM PMSF and protease inhibitor cocktail (Roche). After protein quantification, aliquots of homogenates containing equal amounts of protein were used for SDS-
PAGE and Western blotting. Mouse hnRNP D was detected with rabbit anti-hnRNPD polyclonal antibody obtained from Novus Biological (NBP1-61684) and hnRNP D of HepG2 cells was detected by goat polyclonal antibody (sc-22368) from Santa Cruz Biotechnology. LDLR antibody was obtained from Biovision, Mountain View, CA. Membranes were reprobed with an anti-β-actin antibody (Sigma). Immunoreactive bands of predicted molecular mass were visualized using an ECL plus kit (GE Healthcare life Sciences, Piscataway, NJ) and quantified with the Alpha View Software with normalization by signals of β-actin.

miRNA gene arrays and miRNA functional evaluations

HepG2 cells were treated with BBR or DMSO at 4 h, 8 h, and 24 h. Total RNA was isolated using Norgen Total RNA Purification Kit (Norgen Biotek Corporation, Thorold, ON). Three sets of miRNA gene array were conducted at Protein and Nucleic Acid (PAN) Facility of Stanford University by using these RNA samples and Affymetrix miRNA 2.0 Array chips. For evaluation of potential miRNAs, specific anti-miRNA synthetic oligonucleotides were purchased from GeneCopoeia (Rockville, MD) and miRNA expression assay kits were purchased from Applied Biosystem (Foster City, CA).

Statistical analysis

All values are expressed as mean ± SEM. To determine statistical significance, one way ANOVA with Dunnett’s post hoc test was carried out using GraphPad Prism 5 software. For two group data analysis, unpaired two-tailed Student’s t test was applied. A p value of <0.05 was considered statistically significant.

Reference


