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Xenosensor CAR mediates down-regulation of miR-122 and up-regulation of miR-122 targets in the liver

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ABSTRACT

MiR-122 is a major hepatic microRNA, accounting for more than 70% of the total liver miRNA population. It has been shown that miR-122 is associated with liver diseases, including hepatocellular carcinoma. Mir-122 is an intergenic miRNA with its own promoter. *Pri-miR-122* expression is regulated by liver-enriched transcription factors, mainly by HNF4α, which mediates the expression via the interaction with a specific DR1 site. It has been shown that phenobarbital-mediated activation of constitutive androstane receptor (CAR), xenobiotic nuclear receptor, is associated with a decrease in miR-122 in the liver. In the present study, we investigated HNF4α–CAR cross-talk in the regulation of miR-122 levels and promitogenic signalling in mouse livers. The level of miR-122 was significantly repressed by treatment with 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), which is an agonist of mouse CAR. ChIP assays demonstrated that TCPOBOP-activated CAR inhibited HNF4α transactivation by competing with HNF4α for binding to the DR1 site in the *pri-miR-122* promoter. Such transcription factor replacement was strongly correlated with miR-122 down-regulation. Additionally, the decrease in miR-122 levels produced by CAR activation is accompanied by an increase in mRNA and cellular protein levels of E2f1 and its accumulation on the target *cMyc* gene promoter. The increase in accumulation of E2f1 on the target *cMyc* gene promoter is accompanied by an increase in cMyc levels and transcriptional activity. Thus, our results provide evidence to support the conclusion that CAR activation decreases miR-122 levels through suppression of HNF4α transcriptional activity and indirectly regulates the promitogenic protein cMyc. HNF4α–CAR cross-talk may provide new opportunities for understanding liver diseases and developing more effective therapeutic approaches to better drug treatments.

Introduction

MicroRNAs are small non-coding RNAs that provoke mRNA degradation or the blockade of mRNA translation by binding to the 3′-untranslated region (3′UTR) of target mRNAs (Bartel, 2004). MicroRNAs have been implicated in the regulation of a variety of complex biological functions. One single microRNA may have multiple gene targets, producing a physiological response via multiple perturbations (Lim et al., 2005). MiR-122 is a liver-specific microRNA representing 70% of liver microRNA population (Lagos-Quintana et al., 2002), and it has been characterised for its multiple roles in liver physiology and liver diseases. Functional studies showed that miR-122 was involved in lipid and cholesterol metabolism (Krützfeldt et al., 2005; Esau et al., 2006). MiR-122 levels are reduced in hepatocellular carcinoma (HCC) compared with normal liver cells, and low miR-122 expression correlates with poor prognoses (Kutay et al., 2006; Coulouarn et al., 2009). MiR-122 knockout mice spontaneously developed HCC (Tsai et al., 2012; Hsu et al., 2012). Further, overexpression of miR-122 reduces tumorigenic properties of HCC cells, indicating that it acts as tumour suppressor (Bai et al., 2009). Some of the miR-122 targets are involved in cell proliferation, differentiation, apoptosis, and angiogenesis (Xu et al., 2012; Nakao et al., 2014). Although miR-122 expression is reduced in HCC, the mechanism of this down-regulation is still unclear.

Transcription is one of the major regulatory steps involved in the biosynthesis of microRNAs. The features of microRNA gene promoters are similar to the promoters of protein-coding genes, and the transcription of miRs is regulated by similar transcription factors as those of protein-coding genes (Ul Hussain, 2012). MiR-122 is intergenic miR; its expression is regulated by its own promoter (Li et al., 2011). The core promoter is highly conserved across species and contains canonical elements of RNA polymerase II promoter. The promoter shows the highest liver-specific activity. It also contains conserved target sites for liver-enriched transcription factors, including members of the

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hepatocyte nuclear factor (HNF) family, HNF1α, HNF3β, HNF4α and HNF6α (Coulouarn et al., 2009; Xu et al., 2010; Laudadio et al., 2012). Among the HNF family, HNF4α seems to be the most important one in regulating *pri-miR-122* expression, which mediates the expression via the interaction with specific direct repeat (DR) 1 site (Xu et al., 2010; Li et al., 2011).

It was shown that xenochemicals can reduce miR-122 levels. For example, the sedative and antiepileptic drug phenobarbital (PB) induces a down-regulation of miR-122 in mouse liver, but regulators were not evaluated (Shizu et al., 2012). Gene regulation by PB is carried out by the nuclear constitutive androstane receptor (CAR, NR1I3). CAR, which is expressed primarily in the liver, was initially characterised as a xenosensor that regulates responses to xenochemicals. CAR mediates the up-regulation of xenobiotic/drug-metabolising enzymes, increasing the metabolic capability of the liver to protect cells from xenochemical toxicity (Kachaylo et al., 2011). Moreover, CAR regulates other physiologically important enzymes in the liver. For instance, CAR has been demonstrated not only to be a xenosensor but also to play a role in endogenous energy metabolism (Ueda et al., 2002; Kachaylo et al., 2012; Yarushkin et al., 2013). CAR activation by xenobiotics also causes liver hyperplasia in the short term (Huang et al., 2005; Blanco-Bose et al., 2008). Long-term treatments with these compounds cause liver tumours in rodents via a nongenotoxic mode of action, apparently through the induction of cell proliferation and suppression of apoptosis (Yamamoto et al., 2004; Huang et al., 2005; Dong et al., 2015). CAR activation is associated with the increased expression of a number of cell cycle regulators, including miR-122 targets. However, the entire mechanism of liver tumour formation promoted by CAR in rodents has not been fully elucidated.

In response to exposure to PB and other chemicals, CAR translocates from the cytoplasm into the nucleus, where it forms a heterodimer with the retinoid X receptor (RXR) and binds with responsive elements of gene promoters (Kachaylo et al., 2011). The first identified binding site for CAR was a DR5 in retinoic acid-sensitive gene promoters (Baes et al., 1994). Later studies indicated that most efficacious PB-responsive enhancers consisted of clusters of DR4 elements (Honkakoski et al., 1998). CAR is also able to bind to DR3 and everted repeat (ER) 6 elements in promoters (Goodwin et al., 2002). Moreover, activated CAR can repress gene expression by competing with HNF4α to bind to the DR1 motif (Miao et al., 2006; Kachaylo et al., 2012; Yarushkin et al., 2013). As HNF4α is the most important transcription regulator of miR-122 levels, in the present study we investigated HNF4α–CAR crosstalk in the regulation of miR-122 and its targets in mouse livers. Moreover, as cell proliferation is regulated by both CAR and miR-122, we examined if the liver hyperplasia promoted by CAR activation occurs through miR-122 repression. These targets were studied using a well-known strong primary chemical mitogen for the liver, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), which is an agonist of mouse CAR.

Materials and methods

Chemicals. TCPOBOP was obtained from Sigma-Aldrich (MO, USA). 3α-Hydroxy-5α-androstanol (Andr) was obtained from Steraloids (USA). All other analytical grade chemicals and solvents were obtained from commercial sources.

Experimental animals. Male C57BL mice (25–30 g) were supplied by the Institute of Clinical Immunology (Novosibirsk, Russia). Animals were acclimated for one week and allowed free access to food and water. All experimental procedures were approved by the Animal Care Committee for the Institute of Molecular Biology and Biophysics and were performed in strict accordance with the National Institutes of Health guidelines.

Experiment protocol 1. Animals were treated intraperitoneally (ip) with Andr (a single injection of 30 mg/kg body weight in corn oil) and/or TCPOBOP (a single injection of 3 mg/kg body weight in corn oil). Andr was injected ip 1 h before TCPOBOP administration. The control animals received an equal volume of corn oil. Animals were fasted and decapitated 24 h after treatment. For ChIP experiments animals were decapitated 6 h after treatment. Five mice were used for each treatment group.

Experiment protocol 2. Animals were treated ip with TCPOBOP (3 mg/kg body weight in corn oil as a single weekly dose) for 8 weeks. The control animals received an equal volume of corn oil. After 8 weeks, the animals were fasted and sacrificed 18 h after fasting began. Five mice were used for each treatment group.

RNA isolation, cDNA synthesis and real-time PCR. Total RNA was isolated from the livers frozen in liquid nitrogen using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm, with a correction for background at 320 nm, and RNA integrity was examined by visualising the 18S and 28S rRNA bands on a denaturating agarose (1%) gel. First-strand cDNA synthesis was carried out with a QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's protocol. For stem-loop RT-PCR, total RNA was reverse-transcribed using stem-loop primer (RT miR-122: 5′-GTCG TATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAAC-3′). Gene expression levels were measured by real-time PCR with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Lithuania). Specific oligonucleotide primers were used for miR-122, *Cyp2b10*, *E2f1*, *cMyc*, and *FoxM1* genes: miR-122 F: 5′-TGGAGTGTGACAATGGTGTT-3′ and R: 5′-CCAGTGCAGGGTCCGAGGT-3′; *Cyp2b10* F: 5′-CCCAGTGTTCCACGAGAC TT-3′ and R: 5′-GGTGCCGACAAAGAAGAGAG-3′; *E2f1* F: 5′-TCTGTACC ACACAGCTGCAA-3′ and R: 5′-GCACAGGAAAACATCAATGG-3′; *cMyc* F: 5′-ACGAGCACAAGCTCACCTCT-3′ and R: 5′-TCCAGCTCCTCCTCGAGT TA-3′; and *FoxM1* F: 5′-TCCAAGGCAAAGACAGGAGA-3′ and R: 5′-GCTCCTCAACCTTAACCCGA-3′. The expression levels of mRNA and miRNA were calculated on the basis of PCR efficiency (E) and C_t and were normalised with housekeeping gene *β-actin* for mRNA and with *U6* snRNA for miR-122.

Preparation of whole liver extracts, nuclear proteins and western blot analysis. Preparation of whole liver extracts and nuclear proteins from mouse livers and western blot analysis was performed as described previously (Kazantseva et al., 2013). Sixty micrograms of proteins was separated by SDS-PAGE, transferred onto nitrocellulose membranes, and exposed to the indicated antibodies before being visualised by Luminata Crescendo Western HRP Substrate (Millipore, MA, USA). Immunodetection was performed with anti-CAR (sc-13065, Santa Cruz Biotechnology, CA, USA), anti-HNF4α (sc-8987), anti-E2f1 (sc-22820), anti-cMyc (sc-788), anti-cyclin D1 (sc-718), anti-CDK4 (sc-260), anti-β-catenin (ab22656, Abcam, Cambridge, UK), anti-TBP (ab818), and anti-β-actin (Sigma-Aldrich, MO, USA) primary antibodies. The protein bands were analysed by a densitometric analysis programme. The intensities of the signals were determined from the area under the curve of each peak.

ChIP assay. ChIP assays were performed on mouse liver samples 6 h after TCPOBOP treatment in accordance with a previously described protocol (Pustylnyak et al., 2011). ChIP assays were performed using either the appropriate antibodies or normal rabbit IgG. The antibodies used for ChIP are the same as the aforementioned western blot analysis. Immunoprecipitated DNA was used as template for real-time PCR using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Lithuania). All PCR results were normalised to input controls. PCR amplification was performed with primers specific to the mouse *Cyp2b10* gene promoter (F: 5′-CGTGGACACAACCTTCAAG-3′ and R: 5′-GAGCAAGGTCCTGGTG