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Alteration of the microRNA-122 regulatory network in rat models of hepatotoxicity

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ABSTRACT

MicroRNAs are small RNA molecules that post-transcriptionally regulate gene expression. MicroRNA-122 is the most abundant and specific liver microRNA. Hepatotoxicity involves a significant alteration of liver gene expression. The aim of this work was to evaluate the microRNA-122 regulatory network in models of hepatotoxicity induced by thioacetamide or carbon tetrachloride. We report that the toxins decreased the expression of microRNA-122, which corresponded with an increase in two target genes: Cyclin G1 and the cationic amino acid transporter CAT-1. We found a decreased expression of its precursor, pri-microRNA-122, and of the transcription factors that specifically bind its promoter: CCAAT/enhancer-binding protein alpha, and members of the hepatocyte nuclear factor family. Therefore, microRNA-122 expression levels are under transcriptional control during hepatotoxicity. We propose that the changes observed are associated with the liver response to cope with the injury caused by the hepatotoxins, likely through a cell proliferation process to repair the damaged tissue.

1. Introduction

MicroRNAs (miRNAs) are small (19–24 nucleotides) non-coding RNA molecules which act as post-transcriptional regulators of messenger RNAs (mRNA) translation and stability. Increasing evidence shows that miRNAs are involved in several biological processes, such as development, apoptosis, cell proliferation and differentiation (Huang et al., 2011). They are also implicated in numerous pathological situations (Huang et al., 2011) and abnormal patterns of miRNA expression have been observed in various hepatic diseases (Bala et al., 2009). In mammals, miRNAs interact with the 3′-untranslated region (3′-UTR) of their 'target' mRNAs (Bartel, 2009), and this interaction either blocks translation initiation and/or accelerates the decay of the target mRNA (Valencia-Sanchez et al., 2006).

Abbreviations: CT, carbon tetrachloride; TA, thioacetamide; LETF, liver enriched transcription factor; NRQ, normalized relative expression.

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Most mammalian miRNAs are initially transcribed by RNA polymerase II, generating a long primary miRNA (pri-miRNA). This pri-miRNA is further processed through a series of endonuclease cleavage reactions to generate the mature ~22 nt long miRNA, which is loaded onto the RNA Induced Silencing Complex (RISC), where it acts as a guide to repress target mRNAs. In mammals, the RNase III enzymes that catalyze the two sequential RNA processing reactions are DROSHA and DICER, which act in the nucleus and the cytoplasm, respectively (Siomi and Siomi, 2010). miRNAs, as mRNAs, can also be subjected to posttranscriptional modification, such as 3′adenylation, as a regulation to their turnover. The poly(A) polymerase germ-line development 2 (GLD-2) had initially been shown to act specifically on miRNA-122 (Katoh et al., 2009), but later it was demonstrated to have a more general function, acting promiscuously on other miRNAs (D'Ambrogio et al., 2012).

MiRNA-122 is a highly abundant liver-specific miRNA that accounts for 70% of the total liver miRNA population (Lagos-Quintana et al., 2002). It has been shown to be involved in lipid and cholesterol metabolism (Esau et al., 2006; Krutzfeldt et al., 2005), hepatocyte differentiation (Laudadio et al., 2012), and has been associated with liver diseases such as hepatitis C virus infection (Jopling et al., 2005) and hepatocellular carcinoma (Gramantieri et al., 2007; Jopling, 2012; Kutay et al., 2006). A number of studies postulate miRNA-122 as a circulating biomarker of liver injury (Wang et al., 2009; Yokoi and Nakajima, 2013). Several miRNA-122 target genes have been experimentally validated, including those that code for Cyclin G1 protein (Gramantieri et al., 2007) and the cationic amino acid transporter CAT-1 (Chang et al., 2004).

The levels of mature miRNAs are stringently controlled, and the miRNA maturation/stability can be globally or specifically regulated in different situations. In the first case, the expression and/or activity of proteins involved in the processing or stability of miRNAs can be altered. In the second case, individual miRNAs are independently regulated through factors that confer the specificity and may act at several points in the biogenesis pathway (Siomi and Siomi, 2010). MiRNA-122 expression is specifically regulated at the level of transcription initiation of pri-miRNA-122 through the specific binding of several liver-enriched transcription factors (LETFs) to its promoter region, such as: CCAAT/enhancer-binding protein alpha (C/EBPα; Zeng et al., 2010) and members of the hepatocyte nuclear factor (HNF) family, including HNF4α, HNF1α, HNF6α and HNF3β (Laudadio et al., 2012; Xu et al., 2010). Among the HNF family, HNF4α seems to be the most important one in regulating pri-miRNA-122 expression (Xu et al., 2010). The LETFs are evolutionary conserved transcriptional factors that are liver-enriched but not restricted to this tissue, and they stimulate cell-specific transcription to confine expression of liver genes in adult hepatocyte (Cereghini, 1996).

Hepatotoxicity is a pathology caused by the exposure of the liver to a drug or other noninfectious agent which produces damaged liver function or cell death. Experimental hepatotoxicity using whole animals is essential to demonstrate that a drug under study induces an adverse effect on the liver (Zimmerman, 1999). The outcome of toxicant-induced injury depends on the interplay among the process of liver injury and the compensatory cell proliferation and tissue regeneration, named liver repair (Mehendale, 2005). Carbon tetrachloride (CT) and thioacetamide (TA) are two known hepatotoxic compounds most commonly used in experimental models to study the liver repair after acute damage (Zimmerman, 1999). It has been found that several miRNAs change their expression in animal models of hepatotoxicity (Fukushima et al., 2007; Pogribny et al., 2007). The acute liver damage induced by acetaminophen or CT in rats was found to alter a number of miRNAs. Among those miRNAs, the miRNA-298 and miRNA-370 seem to be key molecules having a protective role during injury, since they are speculated to regulate an oxidative stress-related gene (thioredoxin reductase 3, Fukushima et al., 2007). Another miRNA presenting a protective role against CT toxicity is miRNA-29, whose induction has been found to inhibit CT-induced hepatic injury by significantly reducing hepatic fibrosis, in chronic mice models of CT hepatotoxicity (Kogure et al., 2012; Zhang et al., 2012).

Here, we report that miRNA-122 decreases during acute hepatotoxicity in rats induced by CT or TA, and that CAT-1 and Cyclin G1 are concomitantly increased. We further systematically analyzed the potential factors underlying the decrease of miRNA-122 during liver injury, and we observed that its transcription was affected. Furthermore, we found that the transcription factors C/EBPα and HNF4α were also decreased during acute hepatotoxicity. We propose that this miRNA-122 alteration in the hepatocyte is intended to activate cell proliferation to repair the injury produced.

2. Materials and methods

2.1. Animals and chemicals employed

Adult male Wistar rats (300–350 g; School of Biochemical and Pharmaceutical Sciences, National University of Rosario) were used in the study. The animals were provided a standard diet and water ad libitum and housed in a temperature (21–23 °C) and humidity (45–50%)-controlled room under a constant 12 h light, 12 h dark cycle. All animals received humane care, according to the Guide for the Care and Use of Laboratory Animals (School of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina) and the protocols were approved by the Animal Care and Use Committee of the School of Biochemical and Pharmaceutical Sciences. The hepatotoxins CT and TA were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Assessment of liver injury

Different animals received a unique intraperitoneal (i.p.) injection of vehicle and different doses of each hepatotoxin under study. The dosages of each chemical compound administered were as follows: CT (0.1, 0.4 and 1 ml/kg body weight; $n = 5$) and TA (10, 50 and 150 mg/kg body weight; $n = 5$). The vehicles used for each xenobiotic were: corn oil (4.4 ml/kg body weight, CT) and saline solution (6 ml/kg body weight, TA). 24 h later, the rats were anesthetized and sacrificed by pneumothorax and the liver and blood samples were collected. The correct establishment of the acute hepatotoxicity models by each toxin was confirmed by the determination of plasma

hepatotoxicity markers and a pathological examination. The plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were assessed using commercial kits (Roche Diagnostics, GmbH, D-68298, Mannheim, Germany) and a Roche-Hitachi Modular Autoanalyzer (Roche Diagnostics). Histologic sections of the liver were stained with hematoxylin–eosin (H&E).

2.3. Glutathione determination

The total glutathione (GSH) and oxidize glutathione (GSSG) assay was performed according to as described by Tietze (1969). Briefly, liver samples of treated and control rats were homogenized in 3% sulfosalycidic acid (SSA), and GSH and GSSG levels were determined in the supernatant through an enzymatic reaction using glutathion reductase. Quantification of GSSG was accomplished through derivatization of GSH using 2-vinylpyridine. A working solution of 0.3 mM β-NADPH and 6 mM DTNB was prepared in sodium dihydrogen phosphate assay buffer (125 mM sodium dihydrogen phosphate, pH 7.5 with 6.3 mM EDTA). 10 μl of SSA-homogenized samples was mixed with 400 μl β-NADPH/DTNB working solution, followed by incubation at 37 °C for 15 min. 0.5 U of glutathion reductase was added to the reaction mix, and Abs at 412 nm was measured. The total GSH content of samples was determined from standard curves generated with known amounts of GSH using the same procedure and expressed as a percentage of the control groups (which was set as 100%).

2.4. RNA purification and RT-qPCR

The compliance of the RT-qPCR experiments with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments, [http://www.rdml.org/miqe]) guidelines (Bustin et al., 2009) is shown in the MIQE checklist (Additional Table 1). In the following paragraphs we describe relevant information related to the RT-qPCR assays that were carried out.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.etap.2013.12.008.

Total RNA from 10 mg of liver samples of vehicle and hepatotoxin-treated rats was obtained through homogenization with a tissue-grinding tube and pestle in 1 ml of TRIzol reagent (Invitrogen, San Diego, CA, USA). The purification protocol was followed according to the instructions of the manufacturer. The quantification and purity valuation of the final RNA was assessed by measuring the absorbance at 260 and 280 nm using a NanoVue UV spectrophotometer (GE Healthcare, Piscataway, NJ, USA). The RNA integrity was assessed in 1.5% agarose gel electrophoresis stained with ethidium bromide. The RNA was stored at -70 °C for future use. Only those samples with a 260/280 ratio of approximately 2 (1.9–2.2) and a 28S/18S ratio >1.8 were used.

The expression levels of mature miRNA-122 and miRNA Let-7a were determined by Stem-Loop RT-qPCR (Chen et al., 2005), using miRNA-16 and the ribosomal RNA 5S (*5S*) as reference genes, as we have previously demonstrated that they are strongly and stably expressed in our experimental settings (Lardizábal et al., 2012). The expression levels of *CAT-1*, *CYCLIN G1*, pri-miRNA-122, *DICER*, *DROSHA*, *C/EBPα*, *HNF4α*, *HNF3β*, *HNF1α*, *GLD-2*, *ALB*, *CYP3A1*, *PCNA*, *CYCLIN D*, *CYP2E1*, *GS* and *β-ACTIN* were measured through standard RT-qPCR, using the stably expressed β-2 microglobulin (*B2M*) transcript and the ribosomal RNA 18S (*18S*) as reference genes (Lardizábal et al., 2012).

1 μg of the total RNA samples was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) conforming to the manufacturer protocol. The cDNA synthesis was performed using the Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase, according to the instructions of the manufacturer. For the cDNA synthesis of the miRNAs, the reaction mixture included Stem-Loop Oligos specific for the miRNAs and a specific primer for *5S*. The cDNA synthesis of the mRNAs was performed using both a poly-dT primer and a *18S* specific primer (see Tables 1 and 2 for the primer sequences). The reactions were incubated at 16 °C for 50 min, 37 °C for 50 min, 70 °C for 15 min and 25 °C for 1 min in the case of the miRNA and *5S* retrotranscription. The thermal protocol for mRNA retrotranscription was the same, except that the step at 16 °C was omitted. The cDNA samples were diluted 1/40 and stored at -20 °C for the subsequent qPCR reactions. The qPCR reactions were performed using an Mx3000P Real Time Thermocycler (Stratagene, La Jolla, CA, USA) using SYBR Green I (Invitrogen) as fluorescent dye, in a final volume of 20 μl. The reaction mixture consisted of 2 μl of 10× PCR buffer, 1.2 μl of 50 mM $MgCl_2$, 0.4 μl of 10 mM GeneAmp dNTP Mix (Applied Biosystems, Foster City, CA, USA), 0.8 μl of 10× SYBR Green I (Invitrogen), 0.1 μl of 5 U/ul *Platinum* Taq DNA Polymerase (Invitrogen), 4 μl of 2.5 μM primer mix (forward and reverse primers) and 5 μl of the diluted cDNA. The qPCR reactions were initiated with 1 min incubation at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 40 s. A melting curve was performed at the end of the qPCR run over the range of 55–95 °C, increasing the temperature stepwise by 0.5 °C every 2 s. The baseline and Cq were automatically determined using MxPro version 4.10 (Stratagene). No template controls were included for each primer pair reaction and each qPCR reaction was carried out in duplicate. Gene-specific amplification was confirmed by a single peak in the melting-curve analysis and a single band on a 2% agarose gel stained with ethidium bromide.

A dilution series was created with a pool of cDNA to construct standard curves for each primer pair. The mean Cq values for each serial dilution were plotted against the logarithm of the cDNA dilution factor. The amplification efficiency for each primer pair (Tables 1 and 2) was calculated from the expression $[10^{(1/-S)} - 1] \times 100\%$, where *S* represents the slope of the linear regression (Bustin et al., 2009).

The Normalized Relative Expression (NRQ) was calculated through the delta-delta Cq method (Livak and Schmittgen, 2001), incorporating the calculated amplification efficiency for each primer pair. The normalization factor was calculated as the geometric mean of the relative expression of 18S and B2M for mRNA quantification, and the geometric mean of 5S and miRNA-16 for miRNA quantification, as previously reported (Lardizábal et al., 2012). All the RT-qPCR experiments presented in this work meet the quality parameters M and CV,

Table 1 – Description of miRNA and small RNA-specific Real-Time PCR assays.

Gene symbol	Gene name	miRBase Acc. No.	Primer sequences	Tm (°C)	E (%)
5S	5S Ribosomal RNA	K01594.1	RT: AGCCTACAGCACCCGGTATT F: GCCCGATCTTGTCTGATCTC R: CCTGACCCTGCTTAGCTTCC	81.6	94
miRNA-16	MicroRNA 16	MI0000844	RT: GTCTCCTCTGGTGCAGGGTCCGAGGTATTCG-CACCAGAGGAGACCGCCAA F: CAGCCTAGCAGCACGTAAAT R: GAGGTATTCGCACCAGAGGA	83.5	91
miRNA-122	MicroRNA 122	MI000891	RT: GTCTCCTCTGGTGCAGGGTCCGAGGTATTCG-CACCAGAGGAGACCAAACA F: GGCTGTGGAGTGTGACAATG R: GAGGTATTCGCACCAGAGGA	83.4	97
miRNA-Let7a	MicroRNA Let 7a	MI0000827	RT: GTCTCCTCTGGTGCAGGGTCCGAGGTATTCG-CACCAGAGGAGACAACTAT F: CGCGCTGAGGTAGTAGGTTG R: GAGGTATTCGCACCAGAGGA	81.5	96

E: Assay efficiency; Tm: melting temperature; RT: retro-transcription primer; R: Reverse primer; F: Forward primer.

and the results in all cases were lower than the cut-off values (0.5 and 25%, respectively) established in a previous report (Hellemans et al., 2007).

2.5. *Western blot*

Liver total homogenates and liver nuclear fractions (20 μg of protein) were heated for 5 min at 90 °C in sample buffer (20 mM Tris, pH 6.8, 10% SDS, 400 μM DTT, 10% glycerol), subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. To ensure equal loading and transfer of proteins, membranes were reversely stained using Ponceau Red. After incubation in blocking buffer (10% fat-free milk in PBS buffer containing 3% Tween 20) for a minimum of 1 h and washing, blots were incubated overnight at 4 °C with rabbit antibodies against rat CAT-1, Cyclin G1, C/EBPα or HNF4α (0.4 μg/ml, Santa Cruz Biotechnology Inc.). The blots were then washed extensively and incubated with the horseradish peroxidase-conjugated corresponding secondary antibodies; bands were detected by the enhanced chemiluminescence detection system (ECL) and autoradiographs were obtained by exposing membranes to a radiographic film. Densitometric analysis of the developed bands of the proteins of interest and the Ponceau Red staining was performed using ImageJ Software Rasband, (1997–2011, http://imagej.nih.gov/ij/).

2.6. *Statistical analysis*

The results were expressed as mean ± SE. The one-way-ANOVA test was used when more than two groups were compared, followed by the Student–Newman–Keuls test for multiple comparisons. An unpaired two-tailed *t*-test was used to compare two separate sets of independent samples from the control and the treated rats. Normality was assessed using the Shapiro–Wilk test. In our previous work, we observed that under our experimental conditions, the expression data of all studied genes (total = 17) were distributed normally (Lardizábal et al., 2012). For all of the statistical analyses, the data were converted to logarithmic values to obtain symmetrical data. In all cases, p values of less than 0.05 were considered statistically significant. The statistical procedures were performed with the R program [http://www.R-project.org], v2.13.1.

3. Results

3.1. *Assessment of rat models of acute liver injury*

Two independent rat models of acute hepatotoxicity induced by CT and TA were established. The hepatotoxins were administered in a dose-response protocol in order to study the liver damage after 24-h exposure. The liver injury produced was characterized by evaluating plasma biochemical markers and through a histological examination of the liver sections stained with H&E. We found that a single i.p. injection of each drug induced a significant increase in the ALT activity levels at the highest doses administered (Fig. 1). The changes in the AST activities showed almost the same pattern as the ALT activities for all groups (data not shown). The liver of CT-exposed rats (1 ml/kg body weight) showed focuses of hepatic parenchymal necrosis and a marked cytoplasmic vacuolization, while the administration of TA (150 mg/kg body weight) induced a severe inflammatory infiltration of neutrophils, as well as liver cell necrosis and isolated apoptotic bodies (Fig. 1). The livers from rats treated with the vehicles of each hepatotoxin (Corn oil: 4.4 ml/kg body weight for CT and saline solution 6 ml/kg body weight for TA) did not show lesions. Based on the dose–response curves of the ALT activity levels, we decided to work with the administration of the doses of drugs that caused a significant damage.

Both CT and TA can cause oxidative stress as part of their mechanism of hepatotoxicity (Túnez et al., 2005; Weber et al., 2003), and glutathione (GSH) plays a central role in intracellular antioxidant defense against free radicals produced by xenobiotics. The extent of liver damage after 24 h-treatment with both hepatotoxins was evaluated through the determination of the GSSG and total GSH cellular content. No significant reduction in GSSG or GSH levels was observed (Fig. 2), indicating that the antioxidant reserve was not depleted because the liver injured induced was not massive.

Table 2 – Description of gene-specific Real-Time PCR assays.

Gene symbol	Gene name	GenBank Acc. No.	Primer sequences	Tm (°C)	E (%)
18S	18S Ribosomal RNA	V01270	RT: GAGCTGGAATTACCGCGGCT F: AAACGGCTACCACATCCAAG R: TTGCCCTCCAATGGATCCT	87.8	97
B2M	Beta 2 Microglobulin	NM_012512.1	F: ACATCCTGGCTCACACTGAA R: ATGTCTCGGTCCCAGGTG	87.1	94
PCNA	Proliferating Cell Nuclear Antigen	NM_022381.3	F: AGGACGGGGTGAAGTTTTCT R: CAGTGGAGTGGCTTTTGTGA	86.9	91
Cyclin D1	Cyclin D1	NM_171992.4	F: TGCAAATGGAACTGCTTCTG R: CTGGCATTTTGGAGAGGAAG	86.7	90
CYP2E1	Cytochrome P450 Fam.2, Subfam.E, polypeptide 1	NM_031543.1	F: AAAGCGTGTGTGTGTTGGAGAA R: AGAGACTTCAGGTTAAAATGCTGCA	86.9	90
GS	Glutamine synthetase	NM_017073.3	F: TTGGCTCTTAGGGGAACTGA R: ATGGAGGTGGGGTGTGTTT	79.3	90
CCNG1	Cyclin G1	NM_012923.2	F: AGTCTTAAGGGACGTCAGGAG R: GCTGAGGAGCTACCCACATT	86.1	92
CAT-1	Cationic aminoacid transporter	NM_013111.2	F: TTGTCCCCGTACTTCCTGTC R: CAGGGATGCTTCCTCACTGT	82.4	93
pri-miRNA-122	miRNA-122 precursor	NR_031864.1	RT: AGCGTCCAGAGCAAAATTGACCG F: GTTAGCACCCTTGTGCCTACA R: TAGACGGATAGCCTAGCAGTAGC	79	100
DICER	Dicer	XM_001068155.1	F: GTGGTCTGGCAGGTGTACTA R: ACTTCCACGGTGACTCTGAC	87.3	100
DROSHA	Drosha	NM_001107655.1	F: GGTGGCCGTTTACTTCAAAGG R: GTCCATTGCTGCTCCCATTT	85.8	95
GLD-2	Germ-line development 2	NM_001008372.1	F: GGCCAGATGATATGGAATGG R: TTCAGGGTAGCAGCTCTCAAA	78.6	98
C/EBPα	CCAAT/enhancer-binding protein alpha	NM_012524.2	F: GCCAAGAAGTCGGTGGATAA R: CGGTCATTGTCACTGGTCAA	90.4	95
ALB	Albumin	NM_134326.2	F: GATGCCGTGAAAGAGAAAGC R: CGTGACAGCACTCCTTGTTG	89.2	93
HNF4α	Hepatocyte Nuclear Factor 4 alpha	NM_022180.1	F: GCTGCTGCTCCTGCCCACTC R: GGCAGACCCTCCAAGCAGCA	88.9	100
HNF1α	Hepatocyte Nuclear Factor 1 alpha	NM_012669.1	F: ACAATGCCTGCCTGGTAAGT R: TTGCCTCCTGCGTAGTTACA	87.7	90
HNF3β	Hepatocyte Nuclear Factor 3 beta	NM_012743.1	F: CTTAAGGCCCCAAAGGAGCA R: CAGGTGCCTTGAGAAGCAGT	85.5	90
CYP3A1	Cytochrome P450 Fam.3, Subfam.a, polypeptide 1	NM_013105.2	F: GATGTTGAAATCAATGGTGTGT R: TAATTTCAGAGGTATCTGTGTTTCC	88.2	110
β-ACTIN	Beta-Actin	NM_013105.2	F: ATTGCTGACAGGATGCAGAA R: TAGAGCCACCAATCCACACAG	86.7	104

E: Assay efficiency; Tm: melting temperature; RT: retro-transcription primer; R: Reverse primer; F: Forward primer.

We determined the transcript levels of the proliferation markers *PCNA* and *Cyclin D1* through RT-qPCR in the liver of rats treated with CT and TA at the highest doses, in comparison with their respective control groups (Fig. 2). The increased levels of these transcripts show that liver proliferation increases after 24-h chemical exposure. We also measured the expression levels of cytochrome (*CYP*) *2E1* and glutamine synthetase (*GS*), two transcripts predominantly expressed in differentiated hepatocytes (Fig. 2). We found that acute liver damage induced by CT and TA decreases their expression, indicating that the number of differentiated hepatic cells diminishes after 24-h treatment.

3.2. *Mature miRNA-122 levels decrease during hepatotoxicity*

We measured the expression levels of mature miRNA-122 through Stem Loop RT-qPCR in the liver of rats after 24-h treatment with CT and TA at the highest doses, and rats treated with their corresponding vehicles. We found that miRNA-122 levels were significantly decreased in the treated groups under study, in comparison with their respective control groups (Fig. 3). We also determined the expression levels of two of miRNA-122 target genes, Cyclin G1 and CAT-1, through RT-qPCR and Western Blot. We observed that both expressions

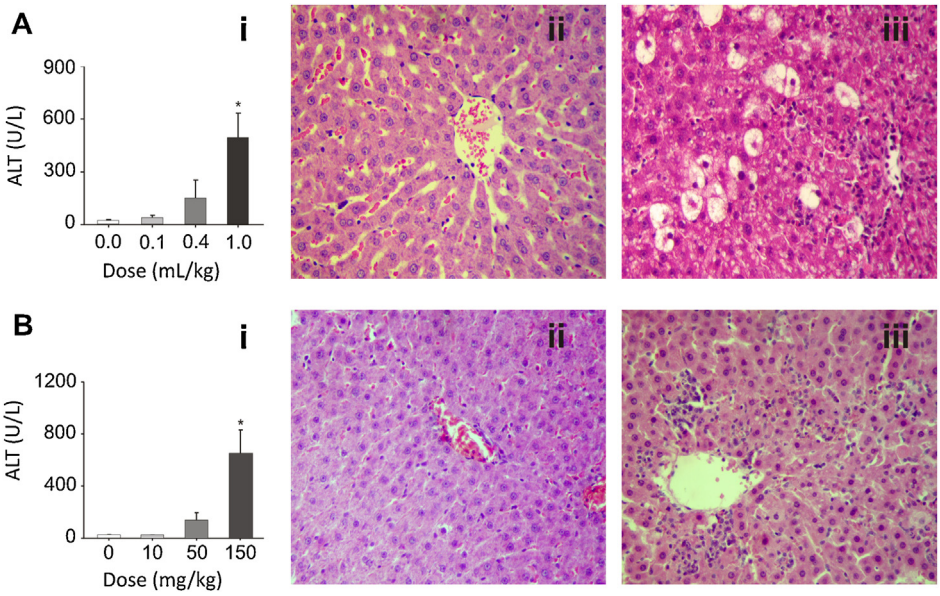

Fig. 1 – Validation of acute liver injury models by carbon tetrachloride and thioacetamide. Liver damage after 24-h treatment of rats with carbon tetrachloride (panel A) and thioacetamide (panel B). Plasma alanine aminotransferase (ALT) levels (i) are represented as the mean ± SD of 5 animals per group. The change in the plasma ALT levels in response to increasing doses of each hepatotoxin was tested by a one-way ANOVA followed by the Newman–Keuls test for multiple comparisons. An asterisk (*) indicates a significant difference ($p < 0.05$). Representative histological microphotographs of hematoxylin and eosin stained-liver sections after the administration of highest doses of the hepatotoxins (iii) and their respective vehicles (ii) are shown.

were significantly increased at the mRNA and protein levels, showing a coherent response of the miRNA-122 regulatory network during acute hepatotoxicity, with a decrease in the levels of the miRNA and an increase in its targets expression.

3.3. *The altered miRNA-122 levels are not associated with expression changes of processing enzymes*

We measured the expression levels of mRNA encoding *DICER*, *DROSHA* and *GLD-2* in the liver of rats treated with CT and TA

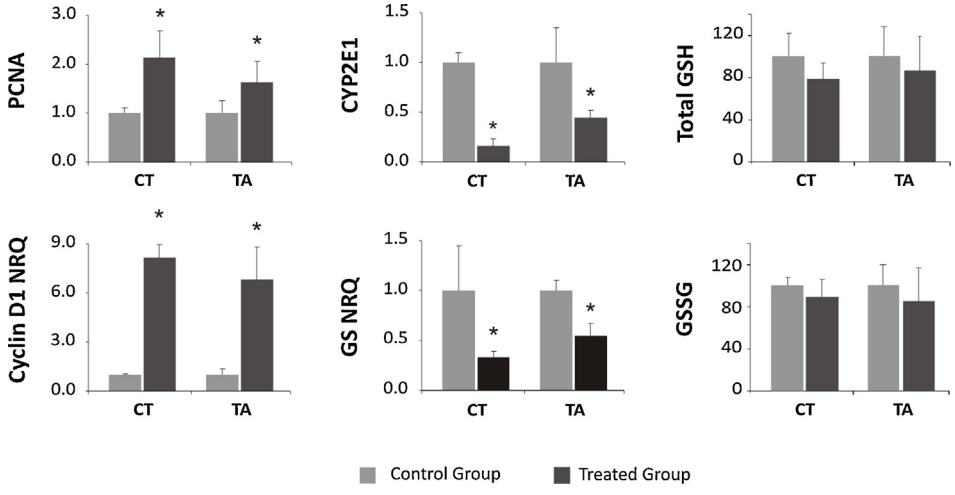

Fig. 2 – Increased proliferation of liver cells after moderate liver damage. The livers were evaluated 24 h after rats were intraperitoneally administered with: carbon tetrachloride (CT, 1 ml/kg body weight) or its vehicle (corn oil, 4.4 ml/kg body weight) and thioacetamide (TA, 150 mg/kg body weight) or its vehicle (saline solution, 6 ml/kg body weight). Steady-state levels of *Cyclin D*, *PCNA* (cell proliferation markers), *CYP2E1 and GS* (expressed in mature hepatocytes) were measured through RT-qPCR. The Normalized Relative Expression (NRQ) was obtained as described in Section 2. GSSG and total GSH content, expressed as a percentage of control groups, were determined through an enzymatic reaction using glutathion reductase and measuring absorbance at 412 nm. Results are represented as the mean ± SD of 5 animals per group. Student's t-test was used for statistical comparisons, and an asterisk (*) indicates a significant difference ($p < 0.05$).

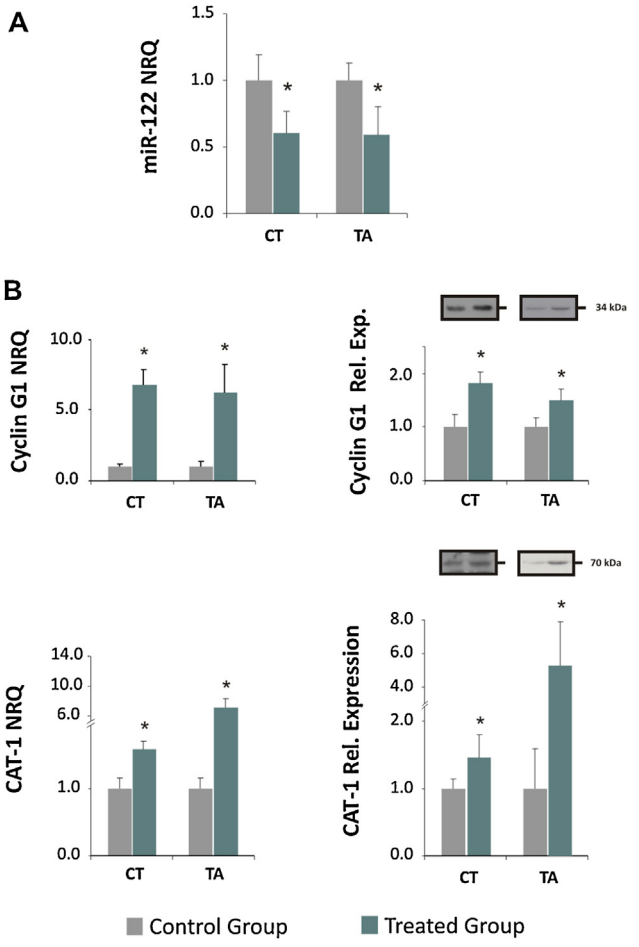

Fig. 3 – Alteration of the miRNA-122 regulatory network during liver injury. The livers were evaluated 24 h after rats were intraperitoneally administered with: carbon tetrachloride (CT, 1 ml/kg body weight) or its vehicle (corn oil, 4.4 ml/kg body weight) and thioacetamide (TA, 150 mg/kg body weight) or its vehicle (saline solution, 6 ml/kg body weight). (A) Steady-state levels of miRNA-122 were measured through Stem Loop RT-qPCR. (B) mRNA levels (left) and protein levels (right) of Cyclin G1 and CAT-1 were determined through RT-qPCR and Western blot, respectively. The Normalized Relative Expression (NRQ) was obtained as described in Section 2. Protein expression was normalized with Red Ponceau staining of membranes (not shown). In all cases, results are represented as the mean ± SD of 5 animals per group. Student's t-test was used for statistical comparisons, and an asterisk (*) indicates a significant difference ($p < 0.05$).

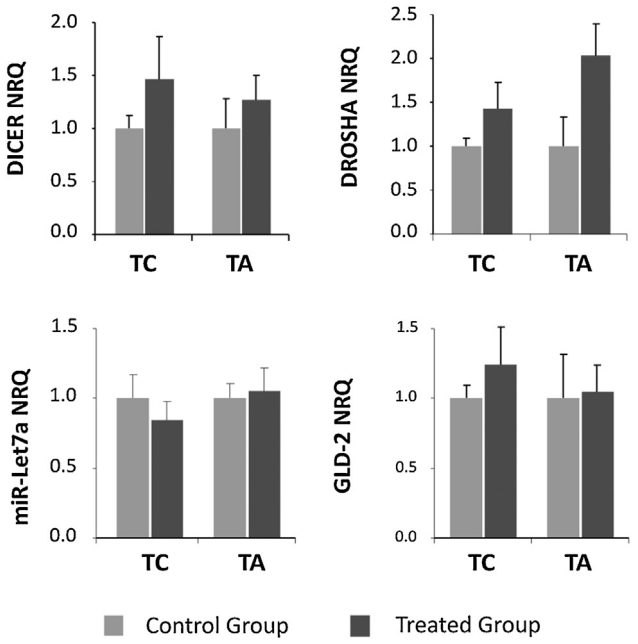

Fig. 4 – Down-regulation of miRNA-122 might not be the result of a global regulation. The livers were evaluated 24 h after rats were intraperitoneally administered with: carbon tetrachloride (CT, 1 ml/kg body weight) or its vehicle (corn oil, 4.4 ml/kg body weight) and thioacetamide (TA, 150 mg/kg body weight) or its vehicle (saline solution, 6 ml/kg body weight). Steady-state levels of *DICER*, *DROSHA* and *GLD-2* were measured through RT-qPCR, while levels of miRNA Let-7a were determined through Stem-Loop RT-qPCR. The Normalized Relative Expression (NRQ) was obtained as described in Section 2. Results are represented as the mean ± SD of 5 animals per group. Student's t-test was used for statistical comparisons.

in comparison with their respective control groups. We also determined the expression levels of the miRNA Let-7a, as it has been shown to target *DICER* (Forman et al., 2008). We did not find a decrease in the expression of these transcripts or miRNA in the liver of rats of our models of acute hepatotoxicity (Fig. 4). Considering these results, and also considering that several other miRNAs (miRNA-191; miRNA-103; miRNA Let-7a; miRNA-16) do not change its expression during hepatotoxicity (data not shown), we propose that miRNA-122 reduced expression levels are not a consequence of a general alteration in the processing or stability of miRNAs.

3.4. *Liver damage alters pri-miRNA-122 transcription*

We determined the expression levels of pri-miRNA-122 in the liver of rats treated with the two hepatotoxins in study and sacrificed 24 h later, compared with the corresponding vehicle-treated groups. We found that treatment of rats with CT and TA significantly reduced the pri-miRNA-122 levels (Fig. 5). These results suggest that the decreased expression of miRNA-122 induced by these drugs might be a consequence, at least in part, of a diminished transcription of its precursor. mRNA expression levels of *C/EBPα*, *HNF4α* were decreased in our acute hepatotoxicity models, as estimated by RT-qPCR (Fig. 5). Accordingly, protein levels of C/EBPα and HNF4α were diminished in these models, as well (Fig. 5), and the transcript levels of other members of the HNF family (Additional Figure 2). Finally, the transcript levels of two C/EBPα and HNF4α direct regulated genes, *ALB* and *CYP3A1*, respectively, were significantly reduced (Fig. 5), showing a consistent response of the transcription factor downregulation.

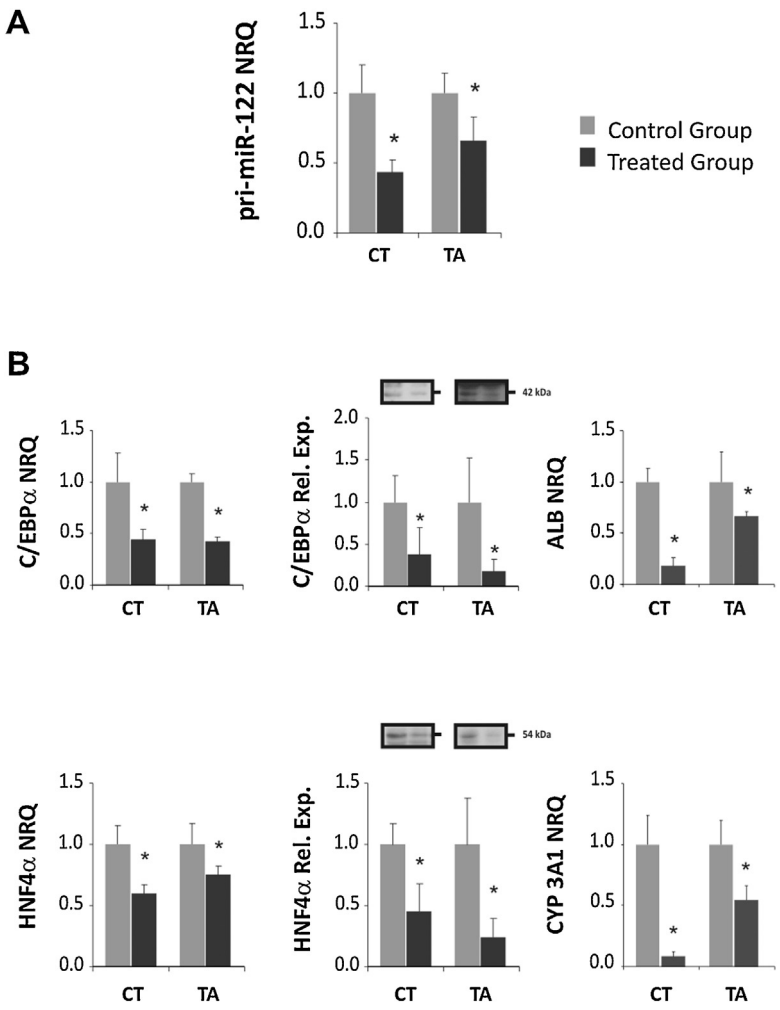

Fig. 5 – *MIR122* transcription is decreased in damaged liver. The livers were evaluated 24 h after rats were intraperitoneally administered with: carbon tetrachloride (CT, 1 ml/kg body weight) or its vehicle (corn oil, 4.4 ml/kg body weight) and thioacetamide (TA, 150 mg/kg body weight) or its vehicle (saline solution, 6 ml/kg body weight). (A) Steady-state levels of the precursor of miRNA-122 (pri-miRNA-122) were measured through RT-qPCR. (B) Expression levels (mRNA and protein) of the transcription factors C/EBPα and HNF4α, and their direct target genes (*ALB* and *CYP3A1* transcripts, respectively) were determined through RT-qPCR and Western blot. The Normalized Relative Expression (NRQ) was obtained as described in Section 2. Protein expression was normalized with Red Ponceau staining of membranes (not shown). In all cases, results are represented as the mean ± SD of 5 animals per group. Student's *t*-test was used for statistical comparisons, and an asterisk (*) indicates a significant difference ($p < 0.05$).

These results suggest that the resulting miRNA-122 levels in the liver of rats treated with CT and TA for 24 h are linked with a decrease in the expression of the liver transcription factors C/EBPα and HNF4α.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.etap.2013.12.008.

4. Discussion

Although in the past two decades the importance of the regulation by miRNAs has been demonstrated through an exponentially increasing number of reports, the factors that regulate them in specific situations, such as hepatotoxicity, has not been extensively characterized. CT and TA are classical hepatotoxins employed in toxicity studies of liver injury and compensatory tissue repair, to test hepatoprotective treatments and to identify potential biomarkers (Zimmerman, 1999). In murine models of acute or chronic liver injury, a number of miRNAs were associated with protective roles against different drug damage, suggesting that miRNAs may be key molecules in hepatotoxicity (Fukushima et al., 2007; Kogure et al., 2012; Zhang et al., 2012). In this work, we show that the levels of miRNA-122 are regulated at the level of its transcription in experimental models of acute liver injury induced by CT and TA, and we propose that this phenomenon is associated to a protective response of the liver to recover from the damage induced.

In order to precisely elucidate the liver response to xenobiotic agents, the development and validation of experimental models of liver injury is considered a main issue, since a

massive and non-specific damage (as secondary effects of the drugs) might obscure the expected results, and may impair the observation of specific pathway to be studied. We validated the correct establishment of our experimental models through: (i) the elevation of plasma transaminase levels; (ii) the description of the histopathological characteristics in the liver specimens, (iii) the conservation in the Coomassie pattern and the determination of expression levels of a group of transcripts whose expressions in hepatotoxicity models were previously reported (Lardizábal et al., 2012) and (iv) the non significant alteration in total GSH and GSSG cellular levels. We verified that xenobiotics, at the administrated doses, do not produce a massive and non-specific injury to the liver cells (Fig. 1), and that they are certainly responding to the exposure of the drug at the level of gene expression, through the finding that several transcripts are expressed very stably (and hence, were used as reference genes), while the expression of *β-ACTIN* was significantly increased (Additional Figure 1). The doses of drugs to administrate were chosen taking into account that the liver could conserve its ability to recover from the injury produced. Indeed, not sacrificing the animals 24 h post toxin dosage, allowed us to demonstrate that the animals recovered from the induced hepatotoxicity. Under our experimental conditions, the survival of rats was 100% in all vehicle and treated groups.

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It has been shown that liver exposure to certain hepatotoxic, such as tamoxifen, acetaminophen and CT lead to significant changes in the expression of a high number of miRNAs (Fukushima et al., 2007; Pogribny et al., 2007). We have found that miRNA-122 diminishes its expression levels in the liver of rats treated with the two hepatotoxicants under study. Consistently, the expression of its target genes Cyclin G1 and CAT-1 was increased (Fig. 3). The decreased miRNA-122 expression was observed using other hepatotoxins, such as dioxin, acetaminophen and phenobarbital, as it was previously reported (Shizu et al., 2012; Wang et al., 2009; Yoshioka et al., 2011), but its regulators were not evaluated. Since the increment of miRNA-122 in blood during hepatotoxicity is proposed as biomarker, its hepatocyte expression diminution and the comprehension of its regulatory network behavior in liver injury have important clinical applications.

It has been shown that the expression and activity of DICER and DROSHA might serve as important regulatory points in global miRNA biogenesis (Schmittgen, 2008). The general miRNA processing machinery (evaluated by the transcript expression of *DICER, DROSHA, GLD-2* and the miRNA Let7a levels) was unaffected by the treatment of rats with CT or TA (Fig. 4). This was consistent with the finding that other miRNAs do not change their expression during hepatotoxicity (Lardizábal et al., 2012).

Biogenesis of individual miRNAs can be independently regulated, and the transcription initiation by the RNA polymerase II is the first step that can be specifically regulated through the binding of transcription factors. We observed a transcriptional regulation of miRNA-122 expression, that is indicated by the diminution of its primary transcript, pri-miRNA-122. This miRNA-122 down-regulation is the result, at least in part, of a decreased transcript and protein expression of LETFs (i.e. C/EBPα and HNF4α; Fig. 5). MiRNA-122 is the most abundant miRNA in the liver, but it is almost non-detectable in other tissues. It has been shown that the amount of miRNA-122 increases over time with the embryonic development, reaching a plateau level just before birth (Chang et al., 2004), and that this increment is positively and strongly correlated with the expression of LETFs in livers of developing mice (Xu et al., 2010). Hepatic gene expression is controlled by a network of core LETFs (Cereghini, 1996), and it has recently been shown that miRNA-122 expression controls hepatocyte differentiation in an indirect way (Jopling, 2012; Laudadio et al., 2012). We propose that the decrease in LEFTs expression in our models of hepatotoxicity leads to a diminution of the miRNA-122, and both processes, in turn, induce a dedifferentiation of liver cells, with the concomitant loss of expression of liver specific genes (Fig. 5; Laudadio et al., 2012). Particularly, the downregulation of cytochrome CYP2E1 (Fig. 2) induced by the HNF family (Cheung et al., 2003), responsible for the liver biotransformation of CT and TA to their active metabolites (Wang et al., 2000; Wong et al., 1998), contributes to protecting the hepatocytes from the chemical exposure.

Down regulation of both C/EBPα and HNF4α is observed in human hepatocellular carcinoma (Xu et al., 2001; Ning et al., 2010; Zeng et al., 2010). Also, C/EBPα transcription is found to decrease in regenerating liver (Mischoulon et al., 1992), and HNF4α suppresses hepatocyte proliferation in mice and hepatoma cells (Bonzo et al., 2012; Yin et al., 2008). There are increasingly reports which show that reduced miRNA-122 levels are associated with liver tumorigenesis and cellular proliferation (Chen et al., 2011; Kutay et al., 2006; Tsai et al., 2009; Xu et al., 2010; Zeng et al., 2010). Besides, many validated target genes of miRNA-122 are involved in cell proliferation (Tsai et al., 2009). Particularly, CAT-1 is responsible for the transport of the cationic amino acids, and this intake stimulates DNA synthesis for cell replication (Byus and Wu, 1991). Cyclin G1, on the other hand, modulates the G2/M transition of the cell cycle, and experimental evidences obtained in cancer cell lines and tumor xenograft have shown that suppression of Cyclin G1 reduces cell proliferation and induces apoptosis, which leads to the inhibition of tumor growth (Chen et al., 1997; Gordon et al., 2000). Taking into consideration these antecedents, the increment of CAT-1 and Cyclin G1 observed (Fig. 3) and also considering the increased transcript levels of *PCNA* and *Cyclin D* in our models of hepatotoxicity (Fig. 2), we propose that the decrease in miRNA-122 expression after 24-h treatment with CT and TA is associated with a cellular proliferating status which is part of the regenerative response of the liver to repair the damage caused.

Liver tissue repair opposes progression of tissue injury, therefore playing a critical role in the ultimate outcome of acute hepatotoxicity. Tissue repair is a dynamic compensatory cell proliferation and regeneration response that recovers its structure and function (Mehendale, 2005). Hepatocytes are highly differentiated cells that are in quiescent phase (G0/G1 phase of the cell cycle), but they have the ability to divide to replace cells that have been damaged. Hepatocytes are able to clear the G1 checkpoint, thereby allowing S-phase DNA synthesis, a critical step in cell division. Cyclin D has been shown to be critical in preceding hepatocytes through the

G1 restriction point. The synthesis stimulation of this cyclin involves several intermediates, like ERK1/2 and p38 MAPK (Sawant et al., 2007). TA and CT are two of the most frequently used chemical models to study the interplay between repair and injury in hepatotoxicity. In this work, we propose that the diminished expression of the miRNA-122 and LETFs (i.e. C/EBPα and HNF4α) are part of the process that leads to liver recover. The interaction of this system with the Cyclin D regulation pathway remains to be elucidated, and it represents an interesting subject to fully understand the response of liver cells to hepatotoxic agents.

In summary, we have carried out an original integral study of the regulation of miRNA-122 expression in hepatotoxicity events, that involved the assessment of liver injury models, the evaluation of the expression of its primary transcript and validated targets, processing enzymes, stability enzyme, and transcription factors, that contribute to establish a general model to study similar situations. We conclude that an acute injury to the liver leads to a down-regulation of the miRNA-122, and that this phenomenon is the result of, at least in part, a diminished rate of transcription of the miRNA-122 precursor, due to a decrease in the expression of the transcription factors C/EBPα and HNF4α. We propose that the decrease in miRNA-122 expression, through the down-regulation of LETFs, is associated with the liver response of self protection against the chemical exposure and recovery from the damage caused, through the phenomenon of cell dedifferentiation and proliferation, respectively.

Conflict of interest

The authors have no potential conflicts of interest.

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Appendix A. Supplementary data

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