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Chronic ethanol feeding alters miRNA expression dynamics during liver regeneration

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Abstract

Background—Adaptation to chronic ethanol treatment of rats results in a changed functional state of the liver and greatly inhibits its regenerative ability, which may contribute to the progression of alcoholic liver disease.

Methods—In this study we investigated the effect of chronic ethanol intake on hepatic miRNA expression in male Sprague-Dawley rats during the initial 24 hrs of liver regeneration following 70% partial hepatectomy (PHx) using microRNA (miRNA) microarrays. miRNA expression during adaptation to ethanol was investigated using RT-qPCR. Nuclear Factor kappa B (NFκB) binding at target miRNA promoters was investigated with chromatin immunoprecipitation.

Results—Unsupervised clustering of miRNA expression profiles suggested that miRNA expression was more affected by chronic ethanol feeding than by the acute challenge of liver regeneration after PHx. Several miRNAs that were significantly altered by chronic ethanol feeding, including miRs-34a, -103, -107, and -122 have been reported to play a role in regulating hepatic metabolism and the onset of these miRNA changes occurred gradually during the time course of ethanol feeding. Chronic ethanol feeding also altered the dynamic miRNA profile during liver regeneration. Promoter analysis predicted a role for Nuclear Factor kappa B (NFκB) in the immediate early miRNA response to PHx. NFκB binding at target miRNA promoters in the chronic ethanol-fed group was significantly altered and these changes directly correlated with the observed expression dynamics of the target miRNA.

Conclusions—Chronic ethanol consumption alters the hepatic miRNA expression profile such that the response of the metabolism-associated miRNAs occurs during long-term adaptation to ethanol rather than as an acute transient response to ethanol metabolism. Additionally, the dynamic miRNA program during liver regeneration in response to PHx is altered in the chronically ethanol-fed liver and these differences reflect, in part, differences in miRNA expression between the ethanol-adapted and control livers at the baseline state prior to PHx.

Keywords

Alcohol; microRNA; Liver Regeneration; Nuclear Factor kappa B; Partial Hepatectomy

Introduction

Chronic ethanol consumption results in (mal)adaptive changes in multiple organs, including brain, liver, heart, skeletal muscle. Hepatic steatosis (fatty liver) occurs commonly and is thought to facilitate progression to more severe liver disease. Ethanol-induced steatosis may

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reflect a metabolic adaptation to the necessity for rapid ethanol disposal, resulting in marked alterations in lipid metabolism with accumulation of excess triglycerides (Crabb and Liangpunsakul, 2006). Adaptation to chronic ethanol use is reflected in relatively moderate changes to the hepatic transcriptome in rodents (Park *et al.*, 2008; Vadigepalli *et al.*, unpublished data), including genes involved in the metabolism of alcohol, cholesterol, lipid, carbohydrate, and protein.

MicroRNAs (miRNAs) are endogenous, ~22 nucleotide non-coding RNAs which post-transcriptionally regulate gene expression through semi-complementary association with mRNA targets (Bartel *et al.*, 2004). The potential for miRNA regulation of gene expression is extensive as most mRNAs are predicted to be regulated by miRNAs (Friedman *et al.*, 2010). miRNAs have been studied extensively for their roles in development and disease, and have been shown to regulate many cellular functions including metabolism and cell cycle progression.

Alterations in miRNA expression profiles occur in adaptation to stress and other external conditions. The adaptive functions of miRNAs have been studied extensively in plants, particularly in response to environmental conditions, including drought, salinity, and mechanical stress (e.g., Dalmay, 2006). Adaptation through altered miRNA expression has also been investigated in mammalian systems, such as skeletal muscle hypertrophy (McCarthy and Esser, 2007), hypoxia (Pocock, 2011), and chronic exposure to toxicants (Lema and Cunningham, 2010). Our data, presented here, and that of others (Dolganiuc *et al.*, 2009), indicate that chronic ethanol treatment also results in an altered hepatic miRNA expression profile that may contribute to maintaining an adapted state of the liver.

Hepatic miRNAs are also dynamically responsive to acute challenges, such as partial hepatectomy (Castro *et al.*, 2010; Marquez *et al.*, 2010; Song *et al.*, 2010). Liver regeneration is an important repair mechanism in response to toxic, viral or mechanical damage. In the partial hepatectomy (PHx) model of liver regeneration, the left lateral and medial lobes, together comprising ~70% of the rodent liver, are surgically removed, resulting in rapid and highly synchronized cell proliferation within the remnant lobes and restoration of liver mass within 1–2 weeks. Immediately following resection several transcription factors (TFs) are activated in the remnant, including NFκB, C/EBP-β, c-Fos, c-Jun, c-Myc, and Stat3 (Costa *et al.*, 2003), accompanied by an increased expression of immediate-early genes by one hour (Haber *et al.*, 1993; Taub, 1996). Six hours after PHx is marked by extensive changes in gene expression in the rat (Juskeviciute *et al.*, 2008; Vadigepalli *et al.*, unpublished data) and by 24 h after PHx the peak of hepatocyte S phase occurs for the first round of proliferation.

Chronic ethanol consumption impairs the hepatic regenerative response, which may contribute to the progression of alcoholic liver disease (ALD). In chronically ethanol-fed rats early activation of TFs is reduced (Zeldin *et al.*, 1996). DNA synthesis at 24 h is drastically inhibited in livers of ethanol-fed rats (Wands *et al.*, 1979) and delayed by 24 to 48 h compared to pair-fed rats (Duguay *et al.*, 1982; Diehl *et al.*, 1990). In a parallel study to be published separately, we found that this delay in proliferation in the ethanol-treated liver is associated with a lack of induction of cell cycle genes after PHx, though significant similarities were found in the gene expression program between the remnant livers of ethanol-fed and pair-fed rats. Therefore, this model allows for the examination of the miRNA expression response of liver to an acute challenge following adaptation to chronic ethanol feeding.

In the present study, we profiled miRNA expression in livers from chronically ethanol-fed rats compared to pair-fed controls and followed the dynamic changes during the first 24 h

following PHx. miRNA expression profiles were more significantly altered by adaptation to ethanol feeding than following PHx. Many of the miRNAs with altered expression upon chronic ethanol treatment have been implicated for their roles in liver metabolism. A time course analysis of the changes in miRNA expression during ethanol feeding suggests that miRNA expression changes are associated with adaptation to ethanol. Our data demonstrate that the ethanol-affected miRNA expression dynamics in response to PHx differs markedly from that of the pair-fed controls. We also provide evidence that the early miRNA expression changes in response to PHx are likely regulated by NFκB, an important early-response transcriptional regulator activated during liver regeneration.

Materials and Methods

Animal protocols

Male Sprague-Dawley rats (Charles River, Wilmington, MA) were maintained on a 12-h light and dark cycle. Animals received an ethanol-containing liquid diet (EtOH, 36% of calories from ethanol, 11% carbohydrate, 35% fat, 18% protein, Bio-Serv, Frenchtown, NJ) for 5 weeks, littermate control animals were pair-fed a carbohydrate control diet (CHO), or high fat control diet (HF) with maltose-dextran or corn oil isocalorically replacing ethanol (47% of calories as carbohydrate or 71% as fat, respectively) (DeCarli and Lieber, 1967; Lieber *et al.*, 1982) Animals on the ethanol diet were ramped up over 4 days before receiving the full strength ethanol diet. In shorter term ethanol feeding protocols (see Results), only CHO controls were used. All animal protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Two-thirds partial hepatectomy (PHx) was performed under isoflurane anesthesia by removing the left lateral and medial lobes (LLM) of the liver through surgical ligation (Higgins and Anderson, 1931). Surgeries were performed between 8:00 am and 11:00 am to avoid circadian rhythm effects. Following surgery, the rats were allowed to recover with access to their appropriate diets. LLM tissue was retained at time of surgery and used as t=0 biological controls and rats were sacrificed 1, 6, 12, and 24 h (n=4 pairs per time point) after PHx. Liver tissue was clamp frozen in liquid nitrogen (Palladino *et al.*, 1980).

RNA isolation

Frozen liver tissue (50–100 mg) was homogenized in TRIzol (Invitrogen, Carlsbad, CA) Total RNA was isolated following the manufacturer’s recommendations and concentration was determined by ND-1000 (NanoDrop, Wilmington, DE).

miRNA profiling

miRNA expression was profiled using Agilent rat miRNA microarrays v1 (Agilent, Santa Clara, CA) which contain probes for the 351 annotated rat miRNAs in miRBase release 10.1. Total RNA from four biological replicates was pooled in equal amounts to result in 16 pooled samples: 1, 6, 12, and 24 h, with LLM and PHx samples each for all four time points for both EtOH and CHO, respectively. 200 ng of pooled RNA was labeled using the Agilent miRNA Complete Label and Hyb Kit (Agilent) and hybridized per the manufacturer’s recommendations. All 16 pooled samples were processed concurrently, with paired samples (biological and diet pairing) hybridized to arrays on the same slide. Slides were scanned on an Agilent DNA Microarray Scanner (G2539A, Agilent) using recommended settings.

MIAME compliant microarray data were deposited in the Gene Expression Omnibus database: GEO# GSE34057.

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Microarray data analysis

miRNA microarray data were extracted with Agilent Feature Extraction Software 10.7.3.1 (Agilent) and quantile-normalized with GeneSpring GX version 11.0.2 (Agilent). An EtOH LLM sample found to be a technical outlier was not used in further analysis and was replaced in analysis with the mean of the three remaining EtOH LLM samples. miRNAs not considered "present" by the software were not used for analysis. A fold change cutoff of 1.5 was employed for miRNAs to be used in further analysis based on the following comparisons: EtOH LLM to CHO LLM; PHx to LLM within each diet group; EtOH PHx to CHO PHx. Unsupervised hierarchical clustering and principal component analysis were performed with MultiExperiment Viewer (MeV) software (version 4.3). Spearman rank correlation was used for clustering.

miRNA RT-qPCR

miRNA expression was measured by TaqMan RT-qPCR miRNA assays (Applied Biosystems, Foster City, CA) according to manufacturer's recommendations. Briefly, 10 ng total RNA was used for reverse transcription. For qPCR, 1 μl of the RT product was used for each reaction and TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) was used. All assays were performed in triplicate on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Four biological replicates were used per time point and miRNA expression was normalized to rat E2 small nucleolar RNA (rat snoRNA TaqMan assay, Applied Biosystems) expression. Relative expression was determined with the ΔΔCt method. Normalized expression in the LLM was subtracted from the normalized expression in the PHx from the same rat. Relative expression following ethanol treatment was determined by comparing EtOH LLM to CHO LLM or HF LLM, respectively.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) was performed with the Magna ChIP G kit (Millipore, Billerica, MA) following the manufacturer's recommendations, starting with ~50 mg frozen liver. Chromatin was sheared via sonication to 200–400 bp fragments, confirmed by Bioanalyzer (Agilent). Immunoprecipitation was performed using anti-NFκB (ab7970, Abcam, Cambridge, MA) or anti-rat normal IgG antibody (sc-2026, Santa Cruz Biotechnology, Santa Cruz, CA), followed by binding to Magna ChIP protein G beads. Purified ChIP DNA was amplified with GenomePlex Complete Whole Genome Amplification (Sigma), according to the manufacturer's directions.

Upstream sequences for rno-pre-miR-196a and -196c were retrieved from Ensembl and potential NF-kB binding sites were identified using TRANSFAC Pro version 2009.4 (Matys *et al.*, 2006). Primer sets were designed to investigate putative NFκB binding sites at 458 bp upstream of pre-miR-196a (5′-TTCCCAAGCTGTAGGGATTTGCCA-3′, 5′-ATGGGTGTCTAGACCACAGCGCCT-3′) and 668 bp upstream of pre-miR-196c (5′-TTGTAGAGGTGGAGGAGAGTGGGA-3′, 5′-GCTTGGACAGGCAGAGAACAAGTTT-3′). qPCR was performed with iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA). Abundance of a target DNA sequence was determined relative to its abundance in the input chromatin. Fold enrichment was determined with the ΔΔCt method as described above.

Statistical analysis

Statistical significance was determined with a two-tailed Student's *t*-test. Analysis of miRNA expression differences between PHx and LLM samples was paired by animal. All other observations were unpaired. A p-value of < 0.05 was considered significant.

Multiple testing correction based on estimated false discovery rate was performed using the q-value approach (Storey and Tibshirani, 2003) as implemented in the *qvalue* library for the R Project for Statistical Computing (http://www.r-project.org).

Spearman rank correlation coefficient was used to determine correlation between data obtained with microarray and RT-qPCR. R-squared values are reported for correlation between RT-qPCR results obtained for CHO and HF samples.

Results

Hepatic miRNA profile is affected more by chronic ethanol than by partial hepatectomy

We compared hepatic miRNA expression profiles in chronically ethanol-fed and pair-fed CHO control animals and assessed the effect of adaptation to ethanol on miRNA expression changes during the initial 24 H following PHx. Global miRNA expression profiling of liver regeneration in EtOH and CHO animals identified 52 miRNAs that showed a 1.5-fold or greater change in at least one of the following comparisons: chronic ethanol treatment versus control at time zero (LLM^{EtOH}/LLM^{CHO}); any time point after PHx in either diet (PHx/LLM); or ethanol treated liver compared to control liver at any of the time points following PHx (PHx^{EtOH}/PHx^{CHO}) (Figure 1). This set of 52 differentially expressed miRNAs was further analyzed.

Microarray results obtained with pooled samples were extensively validated by testing 194 points of comparison by RT-qPCR of individual samples with four biological replicates per condition (Figure 2). Results obtained with RT-qPCR were well correlated with those from the microarrays with a Spearman rank correlation coefficient ( ) of 0.70 ($p < 2.2\times10^{-16}$). Analysis of individual biological replicates by RT-qPCR also allowed for statistical testing of the differences in expressions ascertained by microarray profiling, many of which were determined to be significant (Figure 2, Supplemental Table S1). Using an established approach for estimating false discovery rate (FDR) (Storey and Tibshirani, 2003) that treats the miRNA expression values as independent uncorrelated measures we determined that this validation set may contain 22% of false positives. However, the individual measures tested in our validation were performed as confirmation of the changes determined by the microarray data, a selective approach that biases towards reducing false positives. Thus the FDR becomes unreliable and the alternative raw p-value is a valid alternative.

miRNA expression in high fat (HF) control liver was very similar to that in CHO liver in the chronic diet-adapted state as well as in response to PHx (Supplemental Figure S1, Supplemental Tables S2 and S3). Thus, global miRNA profiling was performed with the EtOH and CHO groups, latter serving as control. The correspondence in miRNA expression between HF and CHO livers supports the conclusion that differences observed in miRNA expression in the EtOH group reflect effects of adaptation to ethanol in the liver.

Unsupervised hierarchical clustering of samples based on their miRNA expression profiles demonstrates that samples cluster predominantly based on ethanol treatment and secondarily by the response to PHx (Figure 1). We employed Principal Component Analysis (PCA) to investigate the miRNA response across samples. PCA aligns the data such that the first axis, or principal component 1 (PC1), lies along the greatest variance in the data, the second component (PC2) lies along the second greatest variance, and so on. Samples separated along the first two principal components primarily based on ethanol treatment (Figure 3A, Supplemental Figures S2A and S2B). Loadings of miRNAs along the first two components demonstrate the contribution of individual miRNAs to this separation of samples (Figure 3B, Supplemental Figures S2C and S2D).

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This pattern contrasts with available data that indicate that chronic ethanol treatment results in relatively modest changes in hepatic gene expression (Park *et al.*, 2008) while liver regeneration induces a significantly greater extent of changes in the liver transcriptome (Fukuhara *et al.*, 2003; Lai *et al.*, 2005; Juskeviciute *et al.*, 2008). Indeed, gene expression profiles obtained from the same tissue extracts cluster predominantly based on response to PHx with ethanol having a lesser effect (Vadigepalli *et al.*, unpublished data). Thus, regulation by miRNAs may play a greater role in the adaptive condition of chronic alcoholic liver than in the acute dynamics of liver regeneration after PHx. Therefore, we analyzed the time course of the response to ethanol feeding in more detail over the course of the 5-week chronic adaptation time frame.

Alterations in hepatic miRNA expression are induced during ethanol adaptation

Nineteen miRNAs were identified as altered by chronic ethanol treatment in the liver by microarray analysis of the replicates of pooled LLM samples (n=4) and RT-qPCR of individual samples (Table 1). Interestingly, several of these miRNAs have been previously identified as playing a role in regulation of metabolism and homeostasis in the liver, processes which functional studies have demonstrated to be affected by chronic ethanol feeding. Our data demonstrate increased expression of the highly abundant, liver-specific miR-122, which plays a role in cholesterol metabolism (Krutzfeldt *et al.*, 2005; Esau *et al.*, 2006) and iron homeostasis (Castoldi *et al.*, 2011). The paralogous miRNAs-103 and -107 were also increased due to ethanol feeding, as was miR-34a. miRs-103 and -107 were recently found to regulate glucose homeostasis (Trajkovski *et al.*, 2011) while miR-34a has been shown to target genes involved in glucose and lipid metabolism (Kaller *et al.*, 2011; Lee *et al.*, 2010). These observations provide many possible roles for a miRNA contribution to the ethanol-induced dysregulation of hepatic metabolic processes.

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We determined the time course of select miRNA expression changes during adaptation to ethanol feeding. Four days of feeding on the full ethanol diet (36% of calories) marked the beginning of increased ethanol consumption per kg body weight, indicating an acclimation to ethanol feeding (Supplemental Figure S3). Short term ethanol consumption (4 and 7 days on full ethanol diet), did not significantly alter hepatic expression of any of the miRNAs examined compared to pair-fed control animals (Figure 4). However, after two weeks of consuming ethanol, expression of miRs-122, -103, and -107 were significantly increased, though significant changes in miRs-34a and -19b were not yet apparent at this time. These data suggest that the hepatic miRNA expression profile begins to change during a sustained exposure to ethanol, but not after short-term exposure of a week or less. Thus, the change in microRNA expression is not a response to the metabolic challenge of acute ethanol treatment but rather occurs over the course of an adaptive response to ethanol exposure.

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Adaptation to ethanol alters the dynamic miRNA profile during liver regeneration

In order to determine how adaptation to ethanol affects the miRNA response to PHx, we performed unsupervised hierarchical clustering of the differential expression of miRNAs due to ethanol treatment. We contrasted the baseline condition prior to PHx (average LLM^{EtOH}/LLM^{CHO}) with changes occurring through the first 24 h of liver regeneration in both EtOH and CHO livers (PHx/LLM). In addition, we directly compared the remnant EtOH and CHO livers during regeneration (PHx^{EtOH}/PHx^{CHO}) (Figure 5; Supplementary Table S4).

Common to EtOH and CHO groups was the lack of differential miRNA expression by one hour after PHx, with the exception of miRNAs -196a and -196c, both of which have increased expression at 1 h in the CHO liver (Figure 5). By 6 h after PHx, however, many miRNAs change their expression in the CHO liver. This time point corresponds with a surge

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of gene expression changes (Juskeviciute *et al*., 2008; Vadigepalli *et al*., unpublished data) that also occur in the ethanol-exposed liver (Vadigepalli *et al*., unpublished data). Interestingly, we detected very few changes in miRNA expression occurring by this time in the ethanol treated liver.

The miRNA response to PHx is no longer suppressed in the ethanol-treated liver by 12 h at which time many miRNAs exhibit differential expression, although these changes differ from the pattern observed in the CHO liver at that time. Changes in miRNA expression begin to decrease by 24 h in both CHO and EtOH livers. This is reflected in the differential expression between EtOH and CHO livers also being reduced at this time point. Notably, the majority of the miRNA dynamic expression profiles in the EtOH liver do not mimic those present in CHO liver regeneration, indicating that the miRNA response in ethanol is not merely delayed or inhibited, but is more fundamentally altered.

Six major clusters were distinguished which demonstrate broad trends in miRNA responses to PHx and how they are affected by ethanol. Multiple clusters portray a response to PHx in the EtOH liver that is opposite of the response in the CHO liver in miRNAs that have altered expression due to ethanol adaptation (Figure 5, clusters 1 and 4). The miRNAs in cluster 1 were at lower baseline expression levels in the EtOH liver prior to PHx and then increase in expression following PHx, opposite to what occurs in the CHO liver. The miRNAs in cluster 4 show a progressively increasing expression in the CHO group up to 12 h and were normalized by 24 h. In contrast, these miRNA were down regulated during PHx in the EtOH group, albeit starting from a higher baseline level. In cluster 2, the miRNAs are slightly decreased due to ethanol-treatment, then increase more robustly following PHx in the EtOH liver than in the CHO liver.

Only one cluster of miRNAs, cluster 5, demonstrates an inhibition of response to PHx in the EtOH liver compared to the CHO liver. The miRNAs in cluster 5 decrease in expression following PHx in both the CHO and EtOH livers, but decrease earlier and to a greater degree in the CHO group.

Of note, our microarray data (Figure 5, cluster 2) revealed an increase of miR-21 in both the CHO and the regeneration-inhibited EtOH liver following PHx. Increased expression of miR-21 during liver regeneration has been demonstrated previously and has been associated with proliferation during this process (Castro *et al*., 2010; Marquez *et al*., 2010; Song *et al*., 2010). The role of miR-21 in the regeneration of the EtOH liver will be further discussed in a separate publication.

Potential regulation of miRs-196a and -196c by NFκB during liver regeneration

Intriguingly, only two miRNAs, miRs-196a and -196c, were induced by 1 h following PHx in the CHO liver, but not in the ethanol-adapted liver. This finding prompted us to investigate the transcriptional regulation of these miRNAs, which show very similar expression profiles (Figure 6A). Both were induced by 1 h following PHx, and remained elevated at 6 h in the CHO liver. In addition, these miRNAs were not induced in the EtOH liver following PHx, but instead decrease at later time points, and were unchanged by chronic ethanol treatment alone. miRs-196a and -196c are paralogous miRNA family members which differ by a single base outside of their seed sequences, indicating that they may predominantly regulate the same targets.

To investigate the transcriptional regulation of these two miRNA, we located the corresponding precursors in the rat reference genome. The precursor for miR-196a (pre-miR-196a) is located on the positive strand of chromosome 7 (q36) and pre-miR-196c is on the positive strand of chromosome 10 (q31). Neither miRNA is reported by the Intragenic

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miRNA Browser (Astrid Research) to lie within another known gene. Thus, both are likely intergenic. Also, no annotated rat pre-miRNAs lie within 1 kb of either pre-miR-196a or -196c, indicating that these miRNAs are likely not clustered with other miRNAs and regulated as individual genes. Computational studies suggest that most intergenic miRNA promoters are within 500 bp of the pre-miRNA (Zhou *et al.*, 2007) and the majority of the transcription factor binding sites occur within 1 kb of the miRNA precursors (Saini *et al.*, 2007). Based on this, we used Transfac Pro (Matys et al., 2006) to identify putative TF binding sites within 1 kb upstream of pre-miR-196a and -196c, respectively.

Our analysis identified two putative NFκB sites within the 1 kb region upstream of pre-miR-196a and one putative site within 1 kb of pre-miR-196c on the opposite strand (Figure 6B). NFκB is latent in the quiescent liver but is activated immediately following PHx, with peak activation within 30 minutes (Cressman *et al.*, 1994; Zeldin *et al.*, 1996), and contributes to the induction of the immediate-early gene expression response in liver regeneration. Chronic ethanol feeding attenuates the increase of NFκB binding activity following PHx (Zeldin *et al.*, 1996). Thus, the profile of NFκB activation in the early phase of liver regeneration correlates with the expression changes of miRs-196a and -196c. We tested if this correlation extends to the promoter binding activity of NFκB at the computationally identified loci shown in Figure 5B.

Chromatin immunoprecipitation (ChIP) demonstrated an increase in NFκB association with the putative NFκB sites 485 bp upstream of miR-196a and the 668 bp upstream on the opposite strand of miR-196c at 1 h after PHx in the CHO liver (Figure 6C). NFκB association with either miRNA upstream sequence was not significantly altered by chronic ethanol treatment. Interestingly, NFκB association with miR-196c was significantly decreased in the EtOH liver at 1 h after PHx compared to LLM^{EtOH} tissue. These results correlate with the expression profiles of miRs-196a and -196c during the early phase of liver regeneration (Figure 5). We conclude from these results that NFκB binding to the promoter regions contributes to the early expression of these two miRNA family members during the onset of liver regeneration.

Discussion

In this study we profiled hepatic miRNA expression following adaptation to chronic ethanol feeding in rats and found altered expression in several miRNAs associated with regulation of metabolic processes. These changes are not an acute response to short-term ethanol treatment, but rather appear to occur over the course of an adaptive response to longer treatment periods. We also profiled the dynamic miRNA program during the regenerative response following PHx, a process that is known to be inhibited by ethanol treatment. The miRNA response profile in livers from chronically ethanol-fed rats differed markedly from that occuring in control livers.

Our data demonstrate a greater extent of chronic ethanol-induced alteration of hepatic miRNA expression in rat than previously reported in mice (Dolganiuc *et al.*, 2009). Of the miRNAs found to be dysregulated, there is little overlap between our data and those reported previously. The reasons for these discrepancies are not clear, but could be related to species differences or ethanol feeding protocols. There is also little consensus amongst prior studies that reported miRNA expression profiles during liver regeneration after PHx (Song *et al.*, 2010; Castro *et al.*, 2010; Raschzok *et al.*, 2011, Shu *et al.*, 2011). However, the extensive validation of our microarray results with RT-qPCR and the high correlation between data obtained with these two methods gives us confidence in the dataset presented here.

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Several miRNAs demonstrated here to be altered by ethanol adaptation have known functional roles in metabolism and hepatic homeostasis. Of note, expression of liver-specific miR-122 is significantly increased during ethanol adaptation. *In vivo* miR-122 inhibition studies demonstrated a role of miR-122 in regulating cholesterol metabolism (Krutzfeldt *et al.*, 2005; Esau *et al.*, 2006) and iron homeostasis (Castoldi *et al.*, 2011), both which are dysregulated due to chronic ethanol feeding (Wang *et al.*, 2010; Valerio *et al.*, 1996). miR-122 is also altered in non-alcoholic fatty liver disease (NAFLD), although the data is conflicting. Jin *et al.* (2009) report increased expression of miR-122 in a rat model of NAFLD and Cheung *et al.* (2008) report decreased levels of miR-122 in liver samples from humans with NAFLD.

The paralogous miRNAs-103 and -107 also showed increased expression upon ethanol feeding. These miRNAs regulate glucose homeostasis through negative regulation of insulin sensitivity (Trajkovski *et al.*, 2011) and are elevated in livers of both ob/ob and diet-induced obese mice and in humans with NAFLD and ALD (Trajkovski *et al.*, 2011). miR-34a is another miRNA elevated in the liver in our model of chronic ethanol feeding as well as in human NAFLD liver samples (Cheung *et al.*, 2008). A recent attempt at global identification of miR-34a targets provided evidence that miR-34a may regulate genes involved in glycolysis and lipid metabolism (Kaller *et al.*, 2011). Additionally, a hepatic increase of miR-34a in mice with diet-induced obesity has been correlated with a decrease in SIRT1, a key regulator of glucose and lipid metabolism in the liver and validated target of miR-34a (Lee *et al.*, 2010). A decrease in hepatic SIRT1 expression has also been reported in a chronic alcoholic rat model (Lieber *et al.*, 2008). Our data demonstrate that the response of these metabolism-associated miRNAs is due to adaptation to the ethanol-containing diet rather than to an acute response to metabolic effects of ethanol intake. These observations suggest multiple miRNA contributions to the dysregulation of hepatic metabolism occuring during adaptation to ethanol.

Ethanol adaptation greatly affects the miRNA response to the acute challenge of partial hepatectomy, as demonstrated by the very different expression profiles following PHx in ethanol-exposed and control livers. The ethanol-mediated change of the dynamic miRNA program during liver regeneration can be attributed, in part, to ethanol-altered miRNA expression prior to resection. This observation is somewhat unexpected given that the gene expression program during liver regeneration in the ethanol adapted liver is only moderately different from that of the control liver, whereas gene expression is much more dramatically affected by the acute challenge of PHx (Vadigepalli *et al.*, unpublished data). Nevertheless, the observations reported here suggest the conclusion that miRNAs play a greater role in regulating adaptative processes than in driving a response to an acute challenge.

Interestingly, the response of miRNAs to PHx is very dynamic, in both control and ethanol-exposed livers. These rapid changes in miRNA expression are intriguing in regard to the function of miRNAs in the liver regeneration process. The dynamics of miRNA expression parallel the massive gene expression changes during liver regeneration (Vadigepalli *et al.*, unpublished data), in agreement with a role of miRNAs in fine-tuning the transcriptional regulatory network. Thus, the difference in the miRNA program during regeneration in the ethanol-adapted liver may compensate for, or modulate the transcriptional regulatory program. Another possibility is that additional differences between the regenerative program in the ethanol adapted and control livers may be found at the protein level due to translational inhibition by miRNAs.

Determining transcriptional regulation of miRNAs themselves for a given process remains challenging and requires attention to the genetic contexts of individual miRNAs. We were, however, able to correlate expression of miRs-196a and -196c with transcriptional

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regulation by NFκB. miR-196a has been shown to be important in development through its regulation of HOX8 genes and sonic hedgehog (Hornstein *et al.*, 2005; Chen *et al.*, 2011), and by sequence similarity miR-196c likely has overlapping function. The HOX8 genes are not expressed in the adult liver (Vadigepalli *et al.*, unpublished data), but a role for hedgehog (Hh) signaling in liver regeneration has been demonstrated (Ochoa *et al.*, 2010). Hh signaling is also increased in ethanol exposed livers (Jung *et al.*, 2008), but the role of this pathway in the ethanol adapted liver following PHx has not been reported. Hh signaling in the liver in these contexts originates in several of the non-parenchymal cell types of the liver (Jung *et al.*, 2008; Ochoa *et al.*, 2010). This raises an important point that our study, like many others, examines changes that occur in liver tissue composed of multiple cell types, rather than identifying contributions from individual cell types.

Profiling miRNA expression in a model of an adapted state responding to an acute challenge provides numerous possibilities as well as many challenges in determining the functionality of miRNAs in these contexts. With each miRNA potentially regulating hundreds of mRNA targets, and many mRNAs potentially regulated by tens of miRNAs, networking the miRNA:mRNA interactions in a dynamic system becomes complex. Additionally, miRNA target prediction algorithms generate many false positives and false negatives, making system-wide network predictions less reliable. Newly developed biochemical assays for determining miRNA:mRNA interactions (reviewed in Thomson *et al.*, 2011) offer promising tools for mapping these connections. Deeper insight into miRNA regulation of gene expression, as well as into the transcriptional regulation of miRNA expression, will be required to obtain a better understanding of how ethanol adaptation affects liver regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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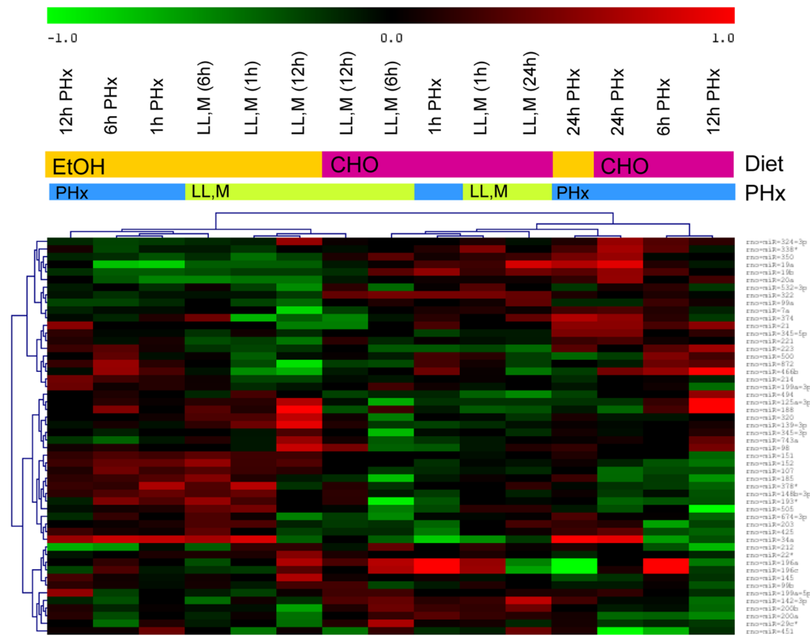

Figure 1. Clustering of miRNA expression profiles of pooled biological replicates

Unsupervised hierarchical clustering was used for both miRNAs and samples. Clustering was performed on miRNAs having a fold change of 1.5 due to ethanol treatment or PHx. Heat map coloring represents expression level of an individual miRNA compared to the mean expression across all samples for that miRNA.

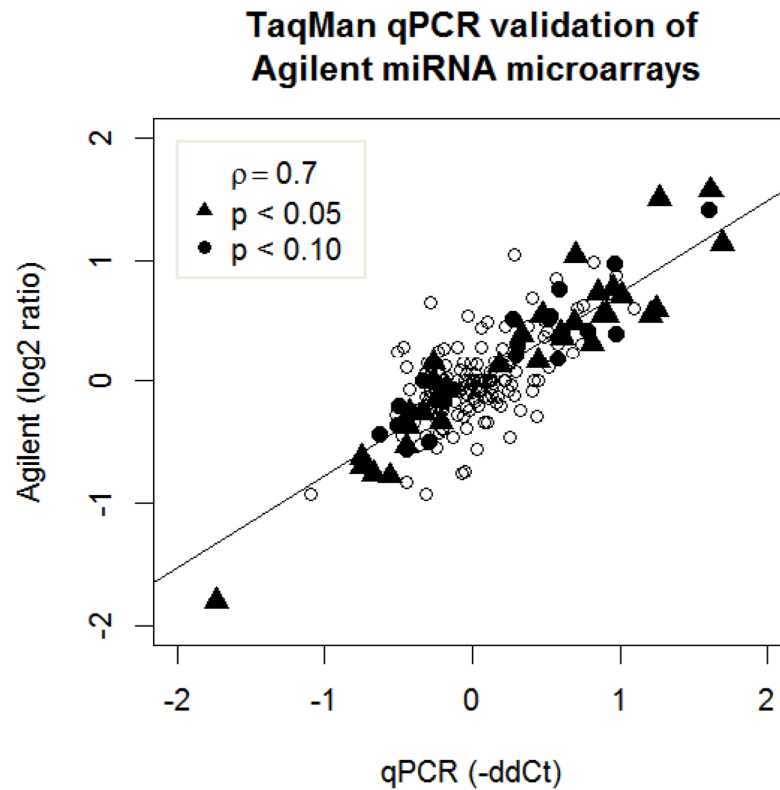

Figure 2. miRNA expression as measured with RT-qPCR highly correlate with microarray data
Over 200 points of comparison were tested with RT-qPCR with n=4 biological replicates. The correlation between the results obtained by the two methods was determined by Spearman rank correlation, with a correlation coefficient ( ) of 0.70 ($p < 2.2\times10^{-16}$). Statistical significance of expression changes determined by RT-qPCR are denoted by filled shapes: triangles, $p<0.05$; circles, $p<0.1$. Statistical significance was determined with Student's t-test.

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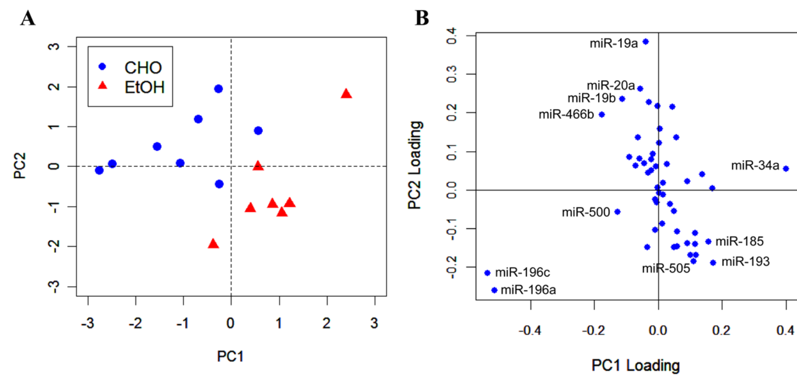

Figure 3. Principal component analysis (PCA) of samples based on miRNA expression profiles
A. Samples separate along the first two principal components (PC1 and PC2) based on ethanol treatment rather than response to partial hepatectomy. B. Loadings of miRNAs along the first two components reveal the contributions of individual miRNAs in the sample separation in A.

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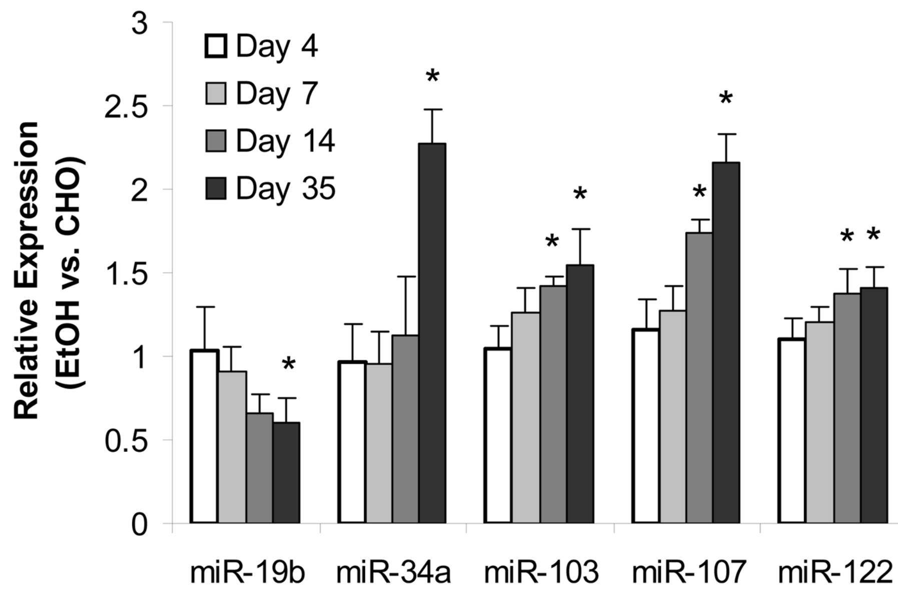

Figure 4. Alterations in hepatic miRNA expression during adaptation to ethanol

Several miRNAs demonstrated to be altered by chronic ethanol were investigated over the time course of ethanol adaptation. miRNA expression was determined by RT-qPCR. Expression in the EtOH liver was compared to expression in the CHO liver at each time point. Statistical significance was determined by Student's t-test. A p-value of <0.05 was considered significant (*).

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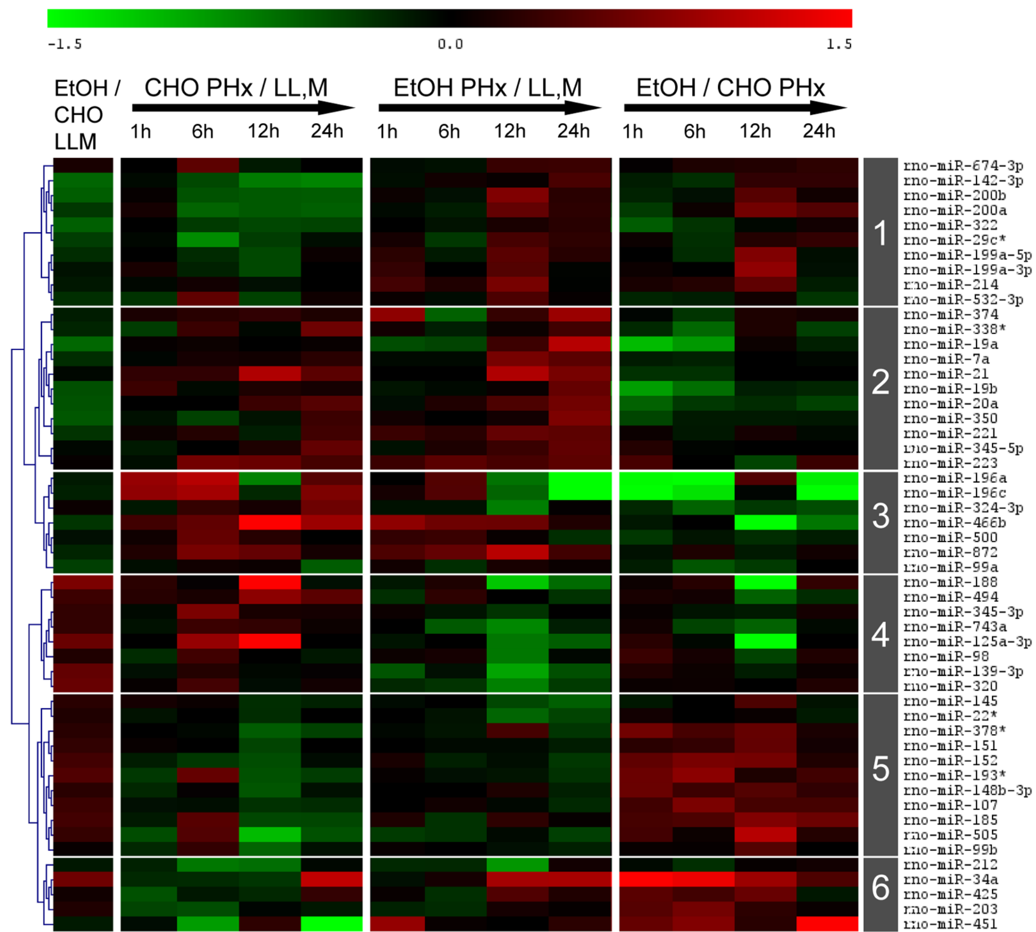

Figure 5. Dynamic miRNA expression profile during liver regeneration in CHO and EtOH livers

Differential expression (log2 ratio) of miRNAs due to ethanol (EtOH LLM / CHO LLM, average), following PHx in EtOH and CHO (PHx / LLM), and between EtOH and CHO after PHx (EtOH PHx / CHO PHx) is shown. Hierarchical clustering was performed resulting in 6 major profile clusters, as labeled and color-coded to the right of the heat map.

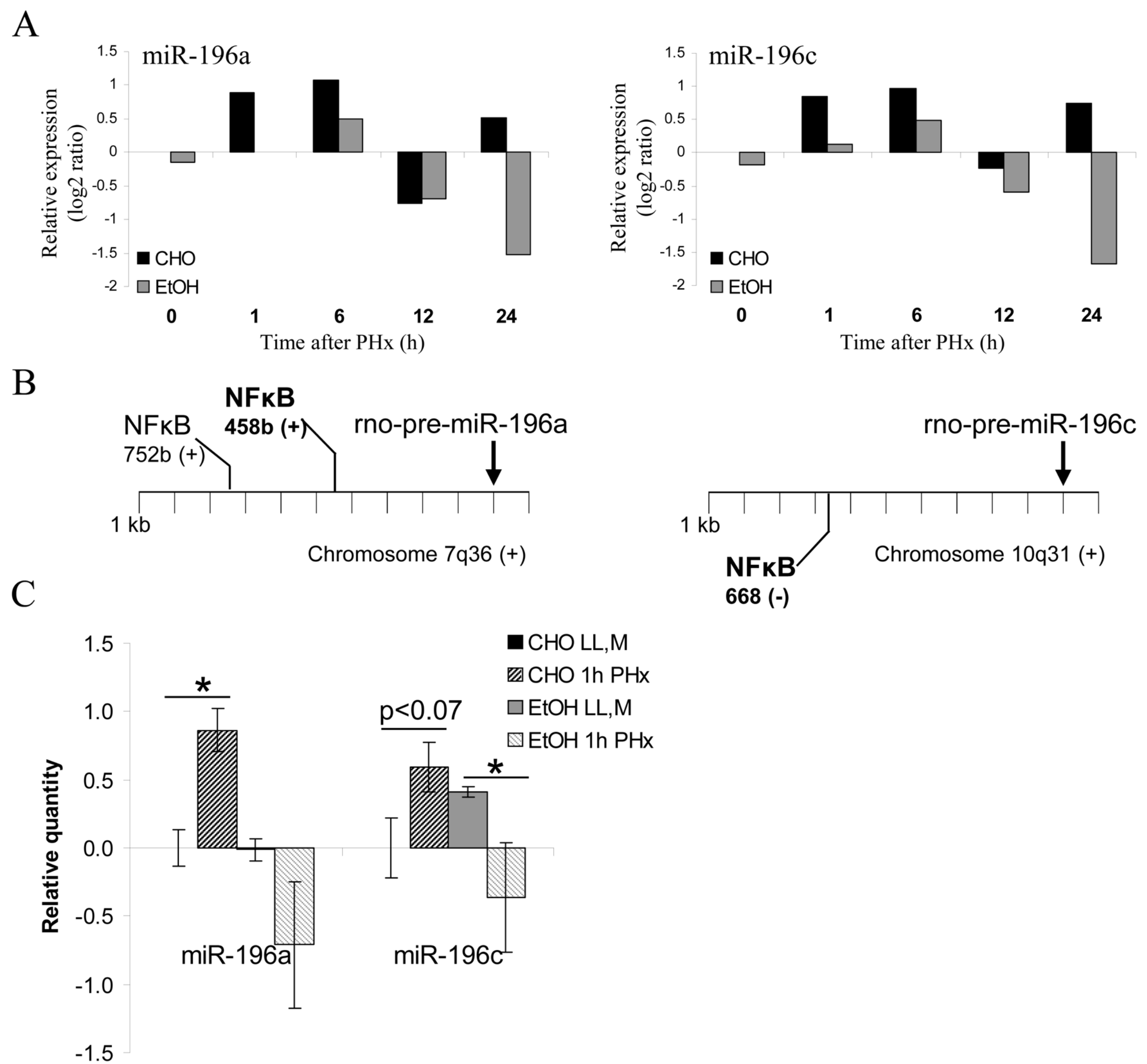

Figure 6. Regulation of early induction of miRs-196a and -196c during liver regeneration by NFκB

Differential expression profiles of miRs-196a and -196c at t=0 and following PHx in CHO and EtOH livers as measured by microarray. Both miRs-196a and -196c are induced by 1 h after PHx in CHO, but not EtOH livers. B. miRs-196a and -196c have upstream potential NFκB binding sites. Bold text denotes putative NFκB sites investigated with ChIP. C. NFκB ChIP followed by RT-qPCR demonstrates increased NFκB binding upstream of miRs-196a and -196c 1 h following PHx in the CHO liver, but not in the EtOH liver. Statistical significance determined with Student's t-test. (*) denotes $p < 0.05$.

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Table 1

Chronic ethanol alters hepatic miRNA expression. miRNAs were found to be altered by chronic ethanol treatment by analysis of microarray pooled sample replicates

RT-qPCR validated many of these expression changes and identified other miRNAs with significantly altered expression due to chronic ethanol using biological replicates. Expression in the EtOH LLM was compared to expression in the CHO LLM. Statistical significance was determined by Student's t-test. A p-value of < 0.05 was considered significant (bold text).

miRNA	qPCR	± SE	Array	± SE
rno-miR-34a	**2.27**	0.20	1.75	0.40
rno-miR-328	**1.83**	0.21		
rno-miR-103	**1.54**	0.20	**1.27**	0.05
rno-miR-365	**1.52**	0.17	**1.23**	0.13
rno-miR-107	**1.51**	0.20	**1.28**	0.13
rno-miR-122	**1.44**	0.15	1.00	0.00
rno-miR-125a-3p			**1.54**	0.19
rno-miR-320			**1.53**	0.16
rno-miR-139-3p			**1.51**	0.17
rno-miR-152			**1.32**	0.07
rno-miR-151			**1.23**	0.03
rno-miR-200a			**0.80**	0.11
rno-miR-125b-5p			**0.78**	0.06
rno-miR-10a-5p			**0.78**	0.07
rno-miR-99a			**0.77**	0.12
rno-miR-350			**0.70**	0.07
rno-miR-322			**0.68**	0.04
rno-miR-200b	**0.63**	0.32	**0.69**	0.16
rno-miR-19b	**0.60**	0.21	**0.72**	0.15