Published in final edited form as:

Alcohol Clin Exp Res. 2012 June ; 36(6): 1058–1066. doi:10.1111/j.1530-0277.2011.01689.x.

Chronic Intermittent Ethanol Exposure and Its Removal Induce a Different miRNA Expression pattern in Primary Cortical Neuronal Cultures

Yingqiu Guo, M.D., Ph.D.¹, Yongxin Chen, M.D., Ph.D.², Stephanie Carreon, Bachelor¹, and Mei Qiang¹

¹Department of Pharmacology, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229

²Department of Pathology, Saint Louis University School of Medicine, Saint Louis, MO 63104, USA

Abstract

Background—Increasing evidence indicates that repeated exposure to and withdrawal from alcohol can result in persistent molecular and cellular adaptations. One molecular adaptation that occurs is the regulation of gene expression, which is thought to lead to the functional alterations that characterize addiction: tolerance, dependence, withdrawal, craving, and relapse. MicroRNAs (miRNAs) have been recently identified as master regulators of gene expression through post-transcriptional regulation. However, the role of miRNAs in the neuroadaptations after alcohol removal has not yet been directly addressed.

Methods—We employed a chronic intermittent ethanol (CIE) model in primary cortical neuronal cultures to examine the global extent of differential miRNA expression using a TaqMan real-time PCR miRNA array.

Results and Conclusions—Sixty-two miRNAs were differentially expressed after 10 days of CIE (CIE10) treatment ($n = 42$ with $FDR < 0.05$ and fold change > 2) and 5 days post-CIE (P5) treatment ($n = 26$) compared to untreated control values. Compared to CIE10, ethanol removal experience in P5 induced a distinct expression pattern, including 20 differentially expressed miRNAs, which did not exhibit a significant change at CIE10. The predicted target molecules of ethanol removal-induced miRNAs function mainly in the regulation of gene transcription, but also function in neuron differentiation, embryonic development, protein phosphorylation, and synaptic plasticity. Interestingly, some of the miRNAs differentially expressed 5 days after CIE treatment were found to cluster on chromosomes near CpG islands, suggesting that they share functional similarity by targeting alcohol-related genes. Taken together, these results suggest a potential role of differentially expressed miRNAs in mediating ethanol removal-related phenotypes.

Keywords

Chronic Intermittent Ethanol; Neuronal Culture; MicroRNAs; Alcohol; Neuroadaptation

Introduction

Alcoholism is a common disease resulting from the complex interaction of genetic and environmental factors. Prolonged alcohol consumption over a period of time results in alcohol dependence, and one essential criterion in the diagnosis of alcohol dependence is the appearance of an alcohol withdrawal syndrome when alcohol consumption ceases (Anton and Becker, 1995). The adaptation processes of the central nervous system in response to the effect of ethanol include an increase in the excitability of nerve cells, which remains in effect for a period of time after the alcohol has been eliminated from the body. Although the mechanisms for the development of alcohol withdrawal are not fully understood, increasing evidence indicates that repeated exposure to and withdrawal from alcohol has resulted in persistent molecular and cellular adaptations (Ron and Jurd, 2005). These molecular adaptations lead to the functional alterations that characterize addiction: tolerance, dependence, withdrawal, craving, and relapse (Volkow and Li, 2004). More recent advances in genetic and epigenetic studies have also provided new insights into ethanol-induced alterations in the regulation of gene expression, with important implications for alcoholism and alcohol-related problems (Hoffman and Tabakoff, 2005; Nestler, 2001; Pandey et al., 2008; Qiang et al., 2010; Ron and Jurd, 2005).

MicroRNAs (miRNAs) are recently discovered, small, noncoding RNA molecules that regulate gene expression through binding to complementary sequences in the 3' untranslated regions (3' UTRs) of target mRNA transcripts, which usually results in translation inhibition and/or mRNA cleavage (Ambros, 2001; Ambros, 2004). Previous studies have predicted that miRNAs may directly regulate the expression of more than half of all human protein-coding genes, and a single miRNA may interact with hundreds of mRNAs (Friedman et al., 2009). With such a regulation net, miRNAs may exert diverse roles in regulating a variety of cellular functions and signaling. In recent years, it has become apparent that numerous miRNAs are present in the developing and adult brain (Bak et al., 2008; Krichevsky et al., 2003; Mehler and Mattick, 2007; Miska et al., 2004), which suggests that miRNAs may have a regulatory role in the brain. As emerging regulatory molecules, miRNAs have attracted much attention as regulators of a variety of neurobiological processes, including brain developmental and neuronal function, such as synaptic plasticity and circadian rhythms (Klein et al., 2005; Schratt et al., 2006; Sempere et al., 2004). Moreover, links between miRNA dysfunction and neurological diseases have recently received more and more attention. For instance, miRNA has been shown to be involved in psychiatric disorders, such as schizophrenia and autism (Burmistrova et al., 2007; Hansen et al., 2007; Nomura et al., 2008; Perkins et al., 2007), and mood disorder (Zhou et al., 2009), as well as a variety of neurodegenerative disorders including Alzheimer's and Parkinson's disease (Barbato et al., 2009; Hebert and De Strooper, 2009; Hebert et al., 2009; Junn et al., 2009). More recently, the analysis has been extended to alterations in which miRNA levels are linked to mechanisms of drugs of abuse. For example, miR-9-dependent destabilization of the BK channel can mediate ethanol-induced tolerance (Pietrzykowski et al., 2008). Huang and Li (Huang and Li, 2009) demonstrated that miRNAs mediate the effects of nicotine on gene expression. In addition, prolonged exposure to morphine caused a dose-dependent increase in miR23b levels in neuronal cells (Wu et al., 2009), and striatal miR-212 signaling was shown to be involved in the regulation of vulnerability to cocaine addiction (Hollander et al., 2010). These findings suggest that alcohol and other drugs of abuse may regulate gene expression through a miRNA-based mechanism. However, the role of miRNAs in alcohol withdrawal-related adaptation has not yet been directly addressed.

Chronic intermittent ethanol (CIE) exposure, which is a model of long-term ethanol treatment, consists of repeated episodes of ethanol intoxication and withdrawal. Alcohol administration using a multiple intermittent paradigm in animals produces more persistent

signs of withdrawal, dependence, and changes in plasticity in the brain than one or a few periods of high alcohol administration (Becker and Hale, 1993; Cagetti et al., 2003; Kokka et al., 1993; McCown and Breese, 1993; Olsen et al., 2005). To explore the possible roles of miRNAs in the development of neuroadaptation induced by alcohol, we used a primary neuronal culture CIE model in this study in combination with a recently developed, highly sensitive quantitative qPCR array (Chen et al., 2009; Mestdagh et al., 2008) to profile the global expression of miRNAs. The results indicated a different miRNA expression pattern after CIE and its removal, suggesting that these miRNAs may be involved in the development of adaptation during ethanol removal. This study provides important insight for the further elucidation of miRNA function and related gene regulatory networks in alcohol consumption-related behavior.

METHODS

Cell Culture and CIE Model

Primary cortical neurons were prepared from C57BL/6 mouse fetuses as previously described (Follesa and Ticku, 1996; Qiang et al., 2005) with minor modifications. Briefly, cortices were isolated from E15 mice and cells were dissociated by trituration from the cortices. The cells were resuspended in minimal essential medium (MEM, Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, GA), 5% heat-inactivated horse serum (Gibco, Auckland, New Zealand), 100 μ M L-glutamine, 28 mM D-glucose, and 1X antibiotic-antimycotic solution (Sigma, St. Louis, MO). Cells were plated on poly-L-lysine coated tissue culture dishes and incubated under 5% CO₂ at 37°C. On the second day, a mixture of 5-fluoro-2'-deoxyuridine and uridine at a concentration of 20 μ g/ml and 40 μ g/ml, respectively, was added into the medium to inhibit non-neuronal cell proliferation. Starting from day 3 *in vitro* (DIV), the cells were switched to a serum-free medium system consisting of Neurobasal medium supplemented with B-27, 100 μ M Glutamine (Invitrogen), and 1 $\times$ antibiotic-antimycotic solution.

The cultured cells were assigned to one of three groups (Fig. 1): Control (Ctrl), where the neurons were kept in normal medium and subjected to a media change as with other groups; CIE10, where the neurons were exposed to 75 mM ethanol for 10 cycles, each cycle consisting of 14 h of ethanol exposure followed by a 10 h intermittent removal (neurons were harvested in the last cycle after 14 h ethanol treatment); and P5 (5 days post-CIE5), where the neurons received CIE for 5 days followed by 5 days of full removal of ethanol. The ethanol-treated neuronal cultures were maintained in an incubator saturated with ethanol, which maintained the ethanol concentration at the level added to the medium. The ethanol concentration was confirmed by constant monitoring using an Analox AM1 analyzer (Analox Instruments USA). During the removal cycles, the cultures were kept in a separate ethanol-free incubator. In view of the developmental changes of miRNA expression during culture, we started and harvested cells at the same time point (DIV3–13).

RNA Isolation

Total RNA was isolated from cultured primary neurons with or without CIE treatment using the mirVana™ miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA concentration and integrity were determined using the NanoDrop 2000c (Fisher Scientific, Hanover Park, IL).

TaqMan Real-time PCR microRNA Array

The stem-loop RT-PCR based TaqMan Rodent MicroRNA Arrays v2.0 (Applied Biosystems, Foster City, CA) were used and represent 518 mature miRNAs presenting in the Sanger miRBase v16. RT-PCR reactions were performed according to the manufacturer's

instructions as previously described (Chen et al., 2011). All reagents were obtained from Applied Biosystems. Briefly, 500 ng of total RNA was reverse-transcribed using Megaplex RT Primers and the TaqMan miRNA reverse transcription kit. The cDNA templates were amplified using Megaplex PreAmp Primers and TaqMan PreAmp Master Mix. Real-time PCR was performed using the ABI 7900HT system and a TaqMan Universal PCR Master Mix. Cycle threshold (Ct) values were calculated with SDS software v.2.3 using automatic baseline settings and a threshold of 0.2. The comparative Ct method was used to calculate the relative miRNA expression. The Ct value of an endogenous control gene (MammU6, snRNA) was subtracted from the corresponding Ct value for the target gene to calculate the Δ Ct value.

Real-time PCR

Real-time PCR was performed as previously described (Qiang et al., 2005). Using 1 μ g of total RNA as template, single-stranded cDNAs were synthesized using random hexamers and the TaqMan reverse transcription reagent kit (Applied Biosystems, Branchburg). Real-time PCR was performed with SyBr green using the ABI 7900 sequence detection system. The primers used to amplify MeCP2 were as follows: forward 5' ATA TTT GAT CAA TCC CCA GGG 3' and reverse 5' CTT AGG TGG TTT CTG CTC TC3' (Dura et al., 2008). Expression of the amplified products was calculated relative to the housekeeping gene GAPDH in each sample.

Western Blot

Equal amounts of protein (20 μ g) were separated by SDS-PAGE on 4–12% NuPAGE Bis-Tris gels and transferred to PVDF membranes. The expression levels of MeCP2 were probed using an anti-MeCP2 antibody (Active Motif). The chemiluminescence signal was developed using Western Lighting Plus reagent (Perkin Elmers, Boston, MA), and the band intensity was quantitated using Image J software. Data were normalized to the intensity of β -actin bands.

Data analysis

Since a Ct value of 35 represents single molecule template detection, Ct values >35 were considered to be below the detection level of the assay (Guthrie et al., 2008). miRNAs were included in analyses when at least 50% of the measurements were detectable (≥ 8 measurements with Ct ≤ 35). The JMP Genomics 4 package was used to estimate the log fold-change and False Discovery Rate (FDR) (Smyth, 2005). A miRNA was identified as differentially expressed when the FDR was < 0.05 and the fold change was > 2 , and subsequently selected for cluster analysis. Each time point was represented by four independent experimental samples. The clustering was performed using the hierarchical method with average linkage and Euclidean distance metric (de Hoon et al., 2004). The Log2-transformed intensity values were centered by subtracting the median Log2 values across all samples for individual miRNAs and used for cluster analysis to generate the heat map (Java Treeview) (Saldanha, 2004). The mRNA and protein levels of MeCP2 were analyzed by one-way ANOVA.

Identification of putative mRNA targets for differentially expressed miRNAs

The mRNA targets for differentially expressed miRNA were predicted using the computer program TargetScan, which is the database of conserved 3' UTR miRNA targets (Lewis et al., 2003). Gene ontology (GO) term annotation and functional enrichment of the predicted targets was performed using the DAVID gene annotation tool (<http://david.abcc.ncifcrf.gov/>) when $p < 0.01$.

RESULTS

The miRNA expression during a 10 day primary cortical neuronal culture

Although primary cortical neuronal cultures serve as a good model of neuronal population and have been used to study the effects of ethanol, these neurons are not fully matured when isolated from E15 fetuses. Several miRNAs have been reported to have distinct expression patterns within the developing neurons (Krichevsky et al., 2003; Miska et al., 2004; Sempere et al., 2003; Smirnova et al., 2005), suggesting a necessary to characterize the expression patterns in the cultured neurons. We therefore examined miRNA levels over time at DIV3, 6, 8, and 13 using a real-time PCR array. Among the 518 murine miRNAs present on the array, 385 were detectable in cultured cortical neurons across all groups. A total of 90 of the expressed miRNAs exhibited a significant change compared to DIV3 (Table 1), including 8 miRNAs (2.0%) at DIV6, 73 miRNAs (19.0%) at DIV8, and 84 miRNAs (21.8%) at DIV13 (Fig. 2). The majority of these changes showed up-regulation, but a small portion were down-regulated (Table 1). These results indicated that miRNAs are differentially expressed during neuronal cell maturation in culture. Thus, appropriate age-matched controls were used when evaluating the effects of CIE treatment (Fig. 1).

The profile of differentially expressed miRNAs by CIE treatment and removal

To determine the overall impact of ethanol treatment on miRNA expression patterns, we treated primary neuronal cultures with the CIE regimen (see Methods) and examined the miRNA expression profile with or without CIE treatment and removal (Fig.1). Figure 3 shows a graphical representation of the number of differentially expressed miRNAs in each of the treatment groups. Compared to the control value, the expression levels of 42 miRNAs after 10 days of CIE treatment were significantly affected ($FDR < 0.05$; fold change > 2), whereas 26 miRNAs were differentially expressed 5 days after CIE5.

Ethanol removal after 5 days of CIE treatment induced a differential expression of a group of miRNAs compared to 10 days of CIE treatment. Among 26 differentially expressed miRNAs in P5, only 23% (6/26) overlapped with those in CIE10 (Table 2), suggesting that these miRNAs may be involved in a general ethanol effect rather than being specific for ethanol removal. The other 20 miRNAs that were significantly altered in P5 but not in CIE10 may contribute to the mechanisms involved in alcohol removal-induced adaptation.

Characteristics of ethanol removal-related miRNAs

To gain insight into the types of miRNAs that had changes in expression at P5, the 26 differentially expressed miRNAs were selected for further analysis. Hierarchical analysis revealed two distinct patterns that were associated with ethanol removal: 5 miRNAs with decreasing expression and 21 miRNAs with increasing expression (Table 2). These distinct expression patterns of miRNA suggest complex regulatory roles during ethanol exposure and removal. Interestingly, the several differentially expressed miRNAs were located in close proximity to each other on chromosomes, such as miR-182 and -183/183* (within 1.5 kb) on chromosome 6, miR-27a/27a* and -24-2* (within 3 kb) on chromosome 8, and miR-322/322*, -450a-5p, 542-3p, and 503/503* (within 1.5 kb) on chromosome X (Fig. 4). These results suggest the possibility of polycistronic transcription.

Predicted potential targets of differentially expressed miRNAs in P5 and CIE10

To further understand the potential functional significance of the differentially expressed miRNAs, miRNA targets were investigated using *in silico* prediction tools. Since the number of experimentally validated miRNA targets is limited, we chose the widely used TargetScan algorithm to obtain a list of Entrez genes predicted to be targeted by the differentially expressed miRNAs during CIE exposure and removal. The expression of

miRNA in P5 and CIE10 exhibited a distinct pattern: 20 out of 26 differentially expressed miRNAs were specific in P5, 36 out of 42 differentially expressed miRNAs were specific in CIE10, and 6 of the miRNAs overlapped. We then used the NCBI DAVID server to identify the significantly enriched canonical pathways ($p < 0.01$) in these conserved targets, respectively. Due to the availability of the database, 14, 20, and 2 of the differentially expressed miRNAs from the P5, CIE10, and both groups were analyzed for target prediction, respectively. Interestingly, there was a great degree of convergence of the pathways regulated by the identified miRNAs. Transcription regulation was one of the most enriched pathways among the genes predicted to be regulated by both increasing and decreasing miRNAs in general, though they target different transcriptional regulators (Table S1, S2, and S3). Most of the miRNAs involved in P5 were up-regulated and involved with transcriptional regulation, whereas most miRNAs involved in CIE10 were down-regulated and enriched in more diverse aspects of transcriptional regulation and pathways, such as those involved in neuron differentiation, embryonic development, protein phosphorylation, and regulation of synaptic plasticity. Interestingly, two such miRNAs, miR-197 and -202b, were predicted to target the NR2B gene, which has been well documented as a target of ethanol action.

Taken together, these exploratory analyses suggest that variation in the expression level of key miRNAs may affect critical pathways involved in withdrawal, which is an important mechanism that warrants additional studies. Since the miRNA target prediction algorithm is known to contain both false positives and false negatives, and our *in silico* pathway enrichment analysis is based on mRNA genes predicted to be targeted by differentially expressed miRNAs, a full understanding of the potential functional role of these miRNAs can only be established using functional experiments.

Methyl CpG-binding protein-2 (MeCP2) could be one of the downstream targets of differential expressed miRNAs

Of the 26 miRNAs modulated by ethanol removal, miR-152, -199a-3p, and -685 appeared to be the most interesting and had the highest fold-change of the up-regulated miRNAs at P5 (Table 1). We found that multiple differentially expressed miRNAs; including miR-152, -199a-3p, and -685, were predicted to have a common target, MeCP2. Consistent with this regulatory relationship, we examined CIE-induced expression of MeCP2. As expected, both mRNA and protein levels for MeCP2 were significantly decreased in association with CIE exposure and removal (Fig. 5). This finding suggests that these three miRNAs may be involved in the regulation of a downstream gene involved in ethanol-induced epigenetic regulation.

DISCUSSION

In recent years, hundreds of miRNAs have been discovered. Although increasing evidence has revealed that miRNAs function in many cellular processes, the particular functions of individual miRNA with respect to their regulatory targets have yet to be uncovered (Liu et al., 2010). Recently, several studies have explored the impact of ethanol treatment on the expression of miRNAs using different models. For example, alcohol treatment was found to significantly alter the expression of four miRNAs, including miR-21, -335, -9, and -153, in neural progenitors using a neurosphere culture model. The expression of these ethanol-sensitive miRNAs differed in different ethanol concentrations and was dependent on the divergent regulatory mechanisms (Sathyan et al., 2007). Acute ethanol treatment was found to induce a rapid increase in miR-9 expression in cultured medium spiny neurons isolated from the striatum and dispersed supraoptic nucleus neurons (Pietrzykowski et al., 2008). A more recent analysis showed an up-regulation of 35 miRNAs in the prefrontal cortex of human alcoholics (Lewohl et al., 2011). These findings provide more direct evidence for the

role of miRNA in human alcoholism. In the present study, we profiled the global extent of differential miRNA expression associated with ethanol-induced neuroadaptation using a TaqMan Real-Time PCR miRNA array. In general, we found that the expression of 62 miRNAs was significantly altered after 10 days of CIE treatment as well as 5 days of CIE treatment followed by 5 days of ethanol removal. The findings in this study add new evidence to our understanding of the role of miRNA in mediating alcohol effects. Although the miRNAs described in these studies are different from each other, they nevertheless suggest that the effect of ethanol on miRNA expression is divergently regulated and dependent on the differences between development stages as well as the alcohol treatment regimens. Moreover, the methods used in different studies may also contribute to the differences observed with these results. In particular, we found that neurons responded to CIE treatment and its removal by differentially expressing miRNA. Only 6 out of the 26 differentially expressed miRNAs after CIE removal overlapped with those in the CIE10 group. In contrast, the remaining 20 miRNAs showed differential expression after CIE removal but not in the CIE10 treatment group, suggesting that ethanol removal is an independent factor that induces the expression of these miRNAs. Moreover, these specific miRNAs may be functionally important in the development of alcohol dependence.

The brain expresses more of the total genetic information encoded in DNA than any other part in the body. It is also a rich source of miRNAs. Increasing evidence indicates that the brain not only expresses numerous miRNAs that are common for all cell types, but also has been found to express a set of neuronal-specific miRNAs in mouse and human during neuronal differentiation (Fiore et al., 2008; Kim et al., 2007; Sempere et al., 2004). Therefore, it is not surprising that miRNA represents a powerful regulatory mechanism in CNS functions. In this study, we found that the majority of differentially expressed miRNAs after CIE and its removal were enriched in several functional categories and pathways, including the regulation of gene transcription, neuron differentiation, embryonic development, protein phosphorylation, and regulation of synaptic plasticity. Notably, these miRNAs were differentially expressed during CIE removal. This analysis showed that the highest ranked function was the regulation of gene transcription, especially for the miRNAs that were up-regulated. For example, among 20 up-regulated miRNAs, miR-152, -199a-3p, and -685 were found to have a common target, MeCP2, which is a methylated CpG binding protein that plays key roles in the formation of repressed chromatin (Jones et al., 1998; Nan et al., 1997; Nan et al., 1998). To examine a possible link between these three miRNAs and their potential target, we examined the concomitant expression level of MeCP2 after ethanol treatment and removal. The western blot results showed that CIE and its removal decreased MeCP2 expression at both the mRNA and protein levels, which is consistent with the predicted CIE removal-induced up-regulation of miR-152, -199a-3p, and -685. These results implicate MeCP2 as a possible target of these miRNAs. Recently, MeCP2 has been reported to be involved in regulating drug-taking behavior (Feng and Nestler, 2010; Im et al., 2010). Interestingly, a recent study by our group also indicated that CIE treatment induces a decrease in the occupancy of MeCP2 on DNA at the 5' regulatory region of the NR2B gene, which was especially apparent during ethanol removal (Qiang et al., 2010). Those findings suggest an epigenetic role of MeCP2 that underlies the CIE-induced long-lasting up-regulation of NR2B gene. Since the activity of NMDARs that contain the NR2B subunit mediate alcohol and other drug-withdrawal related behaviors (Bisaga et al., 2000; Nagy et al., 2004; Nagy and Laszlo, 2002; Narita et al., 2000; Wang et al., 2007), MeCP2 may contribute to the mechanism of withdrawal-induced CNS hyperexcitability. In addition, other recent studies have indicated that MeCP2-mediated epigenetic regulation is involved in cocaine-induced up-regulation of miRNA, including miR-212 (Feng and Nestler, 2010; Hollander et al., 2010; Im et al., 2010). Therefore, it is interesting to hypothesize that CIE treatment may induce increased expression of these miRNAs. Consequently, the affected miRNAs could knockdown MeCP2 expression resulting in a lower level of MeCP2. The

latter would in turn release MeCP2-mediated repression to the promoter of these miRNAs and thereby increase the expression of the involved miRNAs. This type of epigenetic-miRNA loop may explain the escalating compensatory (i.e., removal) responses by each consecutive episode of alcohol exposure, which may contribute to the long-lasting effect during withdrawal. In support of this hypothesis, a similar miRNA negative feedback circuit has been reported in midbrain dopamine neurons (Kim et al., 2007), and other feedback regulatory loops have been reported in diverse pathologies (Kaidi et al., 2007; Spizzo et al., 2010; Tessel et al., 2010). Further experimental validation is required to determine the role of the predicted targets.

A previously reported characteristic of miRNA is the organization of genes into clusters in genomes (Lagos-Quintana et al., 2001; Lee et al., 2002; Sempere et al., 2004; Sempere et al., 2003), which may allow for the co-expression or coordinated regulation of their target genes. For example, miR-140 and miR-140*, which have the same precursor, may be subjected to the same regulation (Huang and Li, 2009). Another study also identified a polycistronic, sensory organ-specific paralogous miRNA cluster that includes *miR-96*, *miR-182*, and *miR-183* on mouse chromosome 6qA3 (Xu et al., 2007). The co-expression of closely clustered miRNAs has also been used as evidence for the hypothesis that they are derived from a common primary transcript (Lau et al., 2001; Sempere et al., 2004). Similarly, in this study, we found several miRNAs out of the 26 differentially expressed miRNAs during CIE removal that were located in close proximity with each other on chromosomes, including miR-182 and -183/183* on chromosome 6, miR-27a/27a* and -24-2* on chromosome 8, and miR-322/322*, -450a-5p, 542-3p, and 503/503* on chromosome X. A search of the upstream sequences of the miRNA clusters found the existence of CpG islands within 2–3 kb of the genes, suggesting that ethanol removal may alter miRNA levels by the co-transcriptional regulation of primary transcripts. Taken together, these results suggest a potential role of differentially expressed miRNAs in mediating effects of ethanol, and underscore the importance of further investigations in alcohol addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors especially thank Dr. Alan Frazer for the helpful discussion of the results and critical reading of the manuscript. We thank Dr. Jonathan Gelfond for help with upload qPCR array data to NCBI/GEO. This research is supported by NIAAA AA017362 and NIAMS AR059096.

References

- Anton, RF.; Becker, HC. Pharmacotherapy and pathophysiology of alcohol withdrawal. In: Kranzler, HR., editor. *The Pharmacology of Alcohol Abuse*. Springer-Verlag; New York: 1995. p. 315-367.1995
- Ambros V. microRNAs: tiny regulators with great potential. *Cell*. 2001; 107(7):823–6. [PubMed: 11779458]
- Ambros V. The functions of animal microRNAs. *Nature*. 2004; 431(7006):350–5. [PubMed: 15372042]
- Bak M, Silahtaroglu A, Moller M, Christensen M, Rath MF, Skryabin B, Tommerup N, Kauppinen S. MicroRNA expression in the adult mouse central nervous system. *Rna*. 2008; 14(3):432–44. [PubMed: 18230762]
- Barbato C, Ruberti F, Cogoni C. Searching for MIND: microRNAs in neurodegenerative diseases. *J Biomed Biotechnol*. 2009; 2009:871313. [PubMed: 19707536]

- Becker HC, Hale RL. Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures: an animal model of alcohol withdrawal “kindling”. *Alcohol Clin Exp Res*. 1993; 17(1):94–8. [PubMed: 8452212]
- Bisaga A, Popik P, Beshpalov AY, Danysz W. Therapeutic potential of NMDA receptor antagonists in the treatment of alcohol and substance use disorders. *Expert Opin Investig Drugs*. 2000; 9(10): 2233–48.
- Burmistrova OA, Goltsov AY, Abramova LI, Kaleda VG, Orlova VA, Rogaev EI. MicroRNA in schizophrenia: genetic and expression analysis of miR-130b (22q11). *Biochemistry (Mosc)*. 2007; 72(5):578–82. [PubMed: 17573714]
- Cagetti E, Liang J, Spigelman I, Olsen RW. Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of GABAA receptors. *Mol Pharmacol*. 2003; 63(1):53–64. [PubMed: 12488536]
- Chen Y, Gelfond J, McManus LM, Shireman PK. Temporal MicroRNA Expression during in vitro Myogenic Progenitor Cell Proliferation and Differentiation: Regulation of Proliferation by miR-682. *Physiol Genomics*. 2011; 43:621–630. [PubMed: 20841498]
- Chen Y, Gelfond JA, McManus LM, Shireman PK. Reproducibility of quantitative RTPCR array in miRNA expression profiling and comparison with microarray analysis. *BMC Genomics*. 2009; 10:407. [PubMed: 19715577]
- de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. *Bioinformatics*. 2004; 20(9):1453–4. [PubMed: 14871861]
- Dura E, Villard L, Roux JC. Expression of methyl CpG binding protein 2 (MeCP2) during the postnatal development of the mouse brainstem. *Brain Res*. 2008; 1236:176–84. [PubMed: 18761004]
- Feng J, Nestler EJ. MeCP2 and drug addiction. *Nat Neurosci*. 2010; 13(9):1039–41. [PubMed: 20740030]
- Fiore R, Siegel G, Schratt G. MicroRNA function in neuronal development, plasticity and disease. *Biochim Biophys Acta*. 2008; 1779(8):471–8. [PubMed: 18194678]
- Follesa P, Ticku MK. Chronic ethanol-mediated up-regulation of the N-methyl-D-aspartate receptor polypeptide subunits in mouse cortical neurons in culture. *J Biol Chem*. 1996; 271(23):13297–9. [PubMed: 8663153]
- Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009; 19(1):92–105. [PubMed: 18955434]
- Guthrie JL, Seah C, Brown S, Tang P, Jamieson F, Drews SJ. Use of Bordetella pertussis BP3385 to establish a cutoff value for an IS481-targeted real-time PCR assay. *J Clin Microbiol*. 2008; 46(11): 3798–9. [PubMed: 18784312]
- Hansen T, Olsen L, Lindow M, Jakobsen KD, Ullum H, Jonsson E, Andreassen OA, Djurovic S, Melle I, Agartz I, Hall H, Timm S, Wang AG, Werge T. Brain expressed microRNAs implicated in schizophrenia etiology. *PLoS One*. 2007; 2(9):e873. [PubMed: 17849003]
- Hebert SS, De Strooper B. Alterations of the microRNA network cause neurodegenerative disease. *Trends Neurosci*. 2009; 32(4):199–206. [PubMed: 19268374]
- Hebert SS, Horre K, Nicolai L, Bergmans B, Papadopoulou AS, Delacourte A, De Strooper B. MicroRNA regulation of Alzheimer's Amyloid precursor protein expression. *Neurobiol Dis*. 2009; 33(3):422–8. [PubMed: 19110058]
- Hoffman P, Tabakoff B. Gene expression in animals with different acute responses to ethanol. *Addict Biol*. 2005; 10(1):63–9. [PubMed: 15849020]
- Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, Lu Q, Willoughby D, Wahlestedt C, Conkright MD, Kenny PJ. Striatal microRNA controls cocaine intake through CREB signalling. *Nature*. 2010; 466(7303):197–202. [PubMed: 20613834]
- Huang W, Li MD. Differential allelic expression of dopamine D1 receptor gene (DRD1) is modulated by microRNA miR-504. *Biol Psychiatry*. 2009; 65(8):702–5. [PubMed: 19135651]
- Im HI, Hollander JA, Bali P, Kenny PJ. MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. *Nat Neurosci*. 2010; 13(9):1120–7. [PubMed: 20711185]

- Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet.* 1998; 19(2):187–91. [PubMed: 9620779]
- Junn E, Lee KW, Jeong BS, Chan TW, Im JY, Mouradian MM. Repression of alpha-synuclein expression and toxicity by microRNA-7. *Proc Natl Acad Sci U S A.* 2009; 106(31):13052–7. [PubMed: 19628698]
- Kaidi A, Williams AC, Paraskeva C. Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nat Cell Biol.* 2007; 9(2):210–7. [PubMed: 17220880]
- Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, Hannon G, Abeliovich A. A MicroRNA feedback circuit in midbrain dopamine neurons. *Science.* 2007; 317(5842):1220–4. [PubMed: 17761882]
- Klein ME, Impey S, Goodman RH. Role reversal: the regulation of neuronal gene expression by microRNAs. *Curr Opin Neurobiol.* 2005; 15(5):507–13. [PubMed: 16150590]
- Kokka N, Sapp DW, Taylor AM, Olsen RW. The kindling model of alcohol dependence: similar persistent reduction in seizure threshold to pentylenetetrazol in animals receiving chronic ethanol or chronic pentylenetetrazol. *Alcohol Clin Exp Res.* 1993; 17(3):525–31. [PubMed: 8392817]
- Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. A microRNA array reveals extensive regulation of microRNAs during brain development. *Rna.* 2003; 9(10):1274–81. [PubMed: 13130141]
- Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science.* 2001; 294(5543):853–8. [PubMed: 11679670]
- Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science.* 2001; 294(5543):858–62. [PubMed: 11679671]
- Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J.* 2002; 21(17):4663–70. [PubMed: 12198168]
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell.* 2003; 115(7):787–98. [PubMed: 14697198]
- Lewohl JM, Nunez YO, Dodd PR, Tiwari GR, Harris RA, Mayfield RD. Up-Regulation of MicroRNAs in Brain of Human Alcoholics. *Alcohol Clin Exp Res.* 2011; 1928–1937. [PubMed: 21651580]
- Liu G, Ding M, Chen J, Huang J, Wang H, Jing Q, Shen B. Computational analysis of microRNA function in heart development. *Acta Biochim Biophys Sin (Shanghai).* 2010; 42(9):662–70. [PubMed: 20716610]
- McCown TJ, Breese GR. A potential contribution to ethanol withdrawal kindling: reduced GABA function in the inferior collicular cortex. *Alcohol Clin Exp Res.* 1993; 17(6):1290–4. [PubMed: 8116844]
- Mehler MF, Mattick JS. Noncoding RNAs and RNA editing in brain development, functional diversification, and neurological disease. *Physiol Rev.* 2007; 87(3):799–823. [PubMed: 17615389]
- Mestdagh P, Feys T, Bernard N, Guenther S, Chen C, Speleman F, Vandesompele J. High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. *Nucleic Acids Res.* 2008; 36(21):e143. [PubMed: 18940866]
- Miska EA, Alvarez-Saavedra E, Townsend M, Yoshii A, Sestan N, Rakic P, Constantine-Paton M, Horvitz HR. Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol.* 2004; 5(9):R68. [PubMed: 15345052]
- Nagy J, Horvath C, Farkas S, Kolok S, Szombathelyi Z. NR2B subunit selective NMDA antagonists inhibit neurotoxic effect of alcohol-withdrawal in primary cultures of rat cortical neurones. *Neurochem Int.* 2004; 44(1):17–23. [PubMed: 12963084]
- Nagy J, Laszlo L. Increased sensitivity to NMDA is involved in alcohol-withdrawal induced cytotoxicity observed in primary cultures of cortical neurones chronically pre-treated with ethanol. *Neurochem Int.* 2002; 40(7):585–91. [PubMed: 11900853]
- Nan X, Campoy FJ, Bird A. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell.* 1997; 88(4):471–81. [PubMed: 9038338]

- Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*. 1998; 393(6683):386–9. [PubMed: 9620804]
- Narita M, Soma M, Mizoguchi H, Tseng LF, Suzuki T. Implications of the NR2B subunit-containing NMDA receptor localized in mouse limbic forebrain in ethanol dependence. *Eur J Pharmacol*. 2000; 401(2):191–5. [PubMed: 10924926]
- Nestler EJ. Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci*. 2001; 2(2):119–28. [PubMed: 11252991]
- Nomura T, Kimura M, Horii T, Morita S, Soejima H, Kudo S, Hatada I. MeCP2-dependent repression of an imprinted miR-184 released by depolarization. *Hum Mol Genet*. 2008; 17(8):1192–9. [PubMed: 18203756]
- Olsen RW, Liang J, Cagetti E, Spigelman I. Plasticity of GABAA receptors in brains of rats treated with chronic intermittent ethanol. *Neurochem Res*. 2005; 30(12):1579–88. [PubMed: 16362777]
- Pandey SC, Ugale R, Zhang H, Tang L, Prakash A. Brain chromatin remodeling: a novel mechanism of alcoholism. *J Neurosci*. 2008; 28(14):3729–37. [PubMed: 18385331]
- Perkins DO, Jeffries CD, Jarskog LF, Thomson JM, Woods K, Newman MA, Parker JS, Jin J, Hammond SM. microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome Biol*. 2007; 8(2):R27. [PubMed: 17326821]
- Pietrzykowski AZ, Friesen RM, Martin GE, Puig SI, Nowak CL, Wynne PM, Siegelmann HT, Treistman SN. Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron*. 2008; 59(2):274–87. [PubMed: 18667155]
- Qiang M, Denny A, Chen J, Ticku MK, Yan B, Henderson G. The site specific demethylation in the 5'-regulatory area of NMDA receptor 2B subunit gene associated with CIE-induced up-regulation of transcription. *PLoS One*. 2010; 5(1):e8798. [PubMed: 20098704]
- Qiang M, Rani CS, Ticku MK. Neuron-restrictive silencer factor regulates the N-methyl-D-aspartate receptor 2B subunit gene in basal and ethanol-induced gene expression in fetal cortical neurons. *Mol Pharmacol*. 2005; 67(6):2115–25. [PubMed: 15755907]
- Ron D, Jurd R. The “ups and downs” of signaling cascades in addiction. *Sci STKE*. 2005; 2005(309):re14. [PubMed: 16278489]
- Saldanha AJ. Java Treeview--extensible visualization of microarray data. *Bioinformatics*. 2004; 20(17):3246–8. [PubMed: 15180930]
- Sathyan P, Golden HB, Miranda RC. Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. *J Neurosci*. 2007; 27(32):8546–57. [PubMed: 17687032]
- Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. A brain-specific microRNA regulates dendritic spine development. *Nature*. 2006; 439(7074):283–9. [PubMed: 16421561]
- Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol*. 2004; 5(3):R13. [PubMed: 15003116]
- Sempere LF, Sokol NS, Dubrovsky EB, Berger EM, Ambros V. Temporal regulation of microRNA expression in *Drosophila melanogaster* mediated by hormonal signals and broad-Complex gene activity. *Dev Biol*. 2003; 259(1):9–18. [PubMed: 12812784]
- Smirnova L, Grafe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG. Regulation of miRNA expression during neural cell specification. *Eur J Neurosci*. 2005; 21(6):1469–77. [PubMed: 15845075]
- Smyth GK. Limma: linear models for microarray data. In: Gentleman, VC.; Dudoit, S.; Irizarry, R.; Huber, W., editors. *Bioinformatics and Computational Biology Solutions using R and Bioconductor*, R. Springer; New York: 2005. p. 397–420.
- Spizzo R, Nicoloso MS, Lupini L, Lu Y, Fogarty J, Rossi S, Zagatti B, Fabbri M, Veronese A, Liu X, Davuluri R, Croce CM, Mills G, Negrini M, Calin GA. miR-145 participates with TP53 in a death-promoting regulatory loop and targets estrogen receptor- α in human breast cancer cells. *Cell Death Differ*. 2010; 17(2):246–54. [PubMed: 19730444]

- Tessel MA, Krett NL, Rosen ST. Steroid receptor and microRNA regulation in cancer. *Curr Opin Oncol.* 2010; 22(6):592–7. [PubMed: 20739888]
- Volkow ND, Li TK. Drug addiction: the neurobiology of behaviour gone awry. *Nat Rev Neurosci.* 2004; 5(12):963–70. [PubMed: 15550951]
- Wang J, Carnicella S, Phamluong K, Jeanblanc J, Ronesi JA, Chaudhri N, Janak PH, Lovinger DM, Ron D. Ethanol induces long-term facilitation of NR2B-NMDA receptor activity in the dorsal striatum: implications for alcohol drinking behavior. *J Neurosci.* 2007; 27(13):3593–602. [PubMed: 17392475]
- Wu Q, Zhang L, Law PY, Wei LN, Loh HH. Long-term morphine treatment decreases the association of mu-opioid receptor (MOR1) mRNA with polysomes through miRNA23b. *Mol Pharmacol.* 2009; 75(4):744–50. [PubMed: 19144786]
- Xu S, Witmer PD, Lumayag S, Kovacs B, Valle D. MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *J Biol Chem.* 2007; 282(34):25053–66. [PubMed: 17597072]
- Zhou R, Yuan P, Wang Y, Hunsberger JG, Elkahloun A, Wei Y, Damschroder-Williams P, Du J, Chen G, Manji HK. Evidence for selective microRNAs and their effectors as common long-term targets for the actions of mood stabilizers. *Neuropsychopharmacology.* 2009; 34(6):1395–405. [PubMed: 18704095]

Figure 1.
Experimental design.

Figure 2. Developmentally expressed miRNAs during a 10 day cell culture period

The results for a given miRNA were expressed in relation to the control. The control (Ctrl) consisted of neuronal cells at DIV3. Data were derived from 4 separate primary cultures at each time point.

Figure 3. Differentially expressed miRNAs during ethanol exposure and removal
Results for a given miRNA were expressed in relation to untreated neuronal cells as a Ctrl. Data were derived from 4 separate primary cultures in each treatment.

Figure 4. miRNA gene clusters

The precursor structure is indicated as a box. To the right, the chromosome location is indicated for mouse. The clusters are transcribed from either left to right (–) or right to left (+).

Figure 5. MeCP2 is down-regulated in association with CIE and removal

A, MeCP2 mRNA expression was determined by real-time PCR. Results are presented as the mean ratio to control $\pm$ SEM; B. Representative western blots for MeCP2 protein level (top) and quantitative analysis (bottom) are shown. β -actin was used as a loading control. * $p < 0.05$ vs. Ctrl. Data were derived from 6 separate experiments.

Table 1

Comparison of developmentally expressed miRNAs in DIV6, DIV8, and DIV13.

miRNA ID	DIV 6		DIV 8		DIV 13	
	Fold Change	FDR	Fold Change	FDR	Fold Change	FDR
mmu-miR-183	5.9	*	29.5	***	107.3	***
mmu-miR-339-3p	33.5	*	86.0	*	223.7	**
mmu-miR-380-3p	10.9	***	10.3	***	79.2	***
mmu-miR-383	2.9	*	5.2	***	11.7	***
mmu-miR-409-5p	7.4	***	26.2	***	113.1	***
mmu-miR-154	6.5	*	7.9	NS	15.0	**
mmu-let-7f	2.1	NS	3.1	*	4.4	**
mmu-miR-126-3p	1.3	NS	4.1	**	7.0	***
mmu-miR-126-5p	1.5	NS	4.8	*	11.3	**
mmu-miR-129-3p	3.5	NS	11.2	**	8.5	**
mmu-miR-129-5p	3.9	NS	7.6	*	12.7	**
mmu-miR-132	4.0	NS	13.7	***	30.6	***
mmu-miR-133b	-1.1	NS	4587.6	**	536.5	**
mmu-miR-134	1.4	NS	3.0	**	7.1	***
mmu-miR-136	2.6	NS	11.9	***	22.6	***
mmu-miR-138	1.7	NS	7.4	***	11.6	***
mmu-miR-139-5p	1.0	NS	3.8	*	9.5	***
mmu-miR-140	-1.1	NS	2.1	*	2.3	**
mmu-miR-182	3.2	NS	10.5	***	25.5	***
mmu-miR-187	1.4	NS	3.2	**	6.2	***
mmu-miR-188-5p	1.1	NS	3.8	**	4.0	**
mmu-miR-190	3.6	NS	7.2	**	22.5	***
mmu-miR-191	1.2	NS	2.2	***	2.1	**
mmu-miR-193b	1.3	NS	4.3	***	4.8	***
mmu-miR-215	2.1	NS	4.8	***	2.3	*
mmu-miR-222	-1.0	NS	5.9	**	11.3	***

miRNA ID	DIV 6		DIV 8		DIV 13	
	Fold Change	FDR	Fold Change	FDR	Fold Change	FDR
mmu-miR-29c	1.8	NS	7.2	*	28.0	***
mmu-miR-323-3p	1.7	NS	5.8	*	13.9	***
mmu-miR-331-5p	1.7	*	3.4	***	4.4	***
mmu-miR-337-3p	1.8	NS	5.8	*	11.4	**
mmu-miR-369-3p	5.2	NS	9.7	*	40.9	***
mmu-miR-370	1.6	NS	6.2	**	15.1	***
mmu-miR-376a	1.9	NS	5.7	**	18.2	***
mmu-miR-379	1.2	NS	5.1	*	16.6	***
mmu-miR-380-5p	1.7	NS	5.7	*	8.7	**
mmu-miR-382	2.2	NS	8.3	*	17.3	**
mmu-miR-384-5p	1.7	NS	5.0	**	5.7	**
mmu-miR-409-3p	1.4	NS	3.9	***	6.8	***
mmu-miR-410	2.3	NS	8.2	***	13.8	***
mmu-miR-411	1.6	NS	4.7	**	9.6	***
mmu-miR-429	-1.1	NS	51.6	***	1.4	NS
mmu-miR-431	1.9	NS	3.1	**	8.1	***
mmu-miR-433	1.9	*	4.7	***	12.8	***
mmu-miR-434-3p	1.9	NS	6.8	**	5.3	**
mmu-miR-434-5p	1.3	NS	4.0	*	9.5	***
mmu-miR-465b-5p	5.2	NS	10.2	***	10.6	***
mmu-miR-467b	1.1	NS	3.3	**	3.5	**
mmu-miR-467c	1.2	NS	3.2	*	10.1	***
mmu-miR-491	1.2	NS	3.8	*	8.9	***
mmu-miR-495	2.1	NS	5.3	*	9.7	**
mmu-miR-544	3.2	NS	10.0	**	27.1	***
mmu-miR-547	2.8	NS	10.7	***	19.1	***
mmu-miR-582-3p	2.7	NS	10.9	***	25.4	***
mmu-miR-598	3.7	NS	10.5	*	34.1	***

miRNA ID	DIV 6		DIV 8		DIV 13	
	Fold Change	FDR	Fold Change	FDR	Fold Change	FDR
mmu-miR-665	53.8	NS	126.8	*	395.9	**
mmu-miR-666-5p	2055.8	NS	1645.2	*	1077.3	***
mmu-miR-667	2.0	*	9.4	***	46.4	***
mmu-miR-668	3.6	NS	15.9	**	35.2	***
mmu-miR-770-3p	2.0	NS	3.2	***	11.3	***
mmu-miR-873	10.0	NS	30.1	***	166.3	***
mmu-miR-879	1.8	NS	7.2	***	26.1	***
mmu-miR-96	413.4	NS	393.1	*	1975.2	***
mmu-miR-98	-1.1	NS	3870.3	*	3986.3	***
mmu-miR-29b	2.1	NS	4.0	NS	19.2	***
mmu-miR-100	-1.1	NS	1.7	NS	8002.8	***
mmu-miR-99a	-1.1	NS	1.7	NS	7938.9	***
mmu-miR-218	1.6	NS	4.7	NS	10.6	**
mmu-miR-365	-1.1	NS	1.6	NS	5.1	**
mmu-miR-376b	2.0	NS	3.2	NS	12.1	***
mmu-miR-377	-1.1	NS	1.7	NS	521.2	***
mmu-miR-369-5p	2.6	NS	6.6	NS	37.7	***
mmu-miR-490	-1.1	NS	1.7	NS	2439.9	***
mmu-miR-92a	-2.4	*	-1.5	NS	-8.2	***
mmu-miR-145	-3.6	**	-2.3	*	-7.2	***
mmu-miR-216b	-1.6	NS	-2.8	*	-23.6	***
mmu-miR-217	1.7	NS	-8.9	***	-106.2	***
mmu-miR-223	-1.7	*	-30.4	***	-39.5	***
mmu-miR-199a-5p	2.8	NS	3.6	NS	-1942.7	***
mmu-miR-200b	2.1	NS	19.8	***	-8.8	***
mmu-miR-302d	-1.3	NS	-1.8	NS	-6.6	**
mmu-miR-486	1.8	NS	6.7	***	-1444.7	***
mmu-miR-487b	2.9	NS	10.7	***	-100.8	***

miRNA ID	DIV 6		DIV 8		DIV 13	
	Fold Change	FDR	Fold Change	FDR	Fold Change	FDR
mmu-miR-504	-1.4	NS	-1.2	NS	-19.6	***
mmu-miR-539	1.2	NS	3.3	*	-81.1	***
mmu-miR-540-3p	2.2	NS	10.3	**	-149.1	***

The significant difference of miRNA expression are shown as FDR < 0.05, Fold change > 2, up-regulation (red) or down-regulation (blue) in DIV6, DIV8, and DIV13 compared to DIV3. Data were derived from 4 separate primary cultures.

* FDR < 0.05

** FDR < 0.01

*** FDR < 0.001 compared with DIV3, respectively. NS, no significance.

Table 2

The change of miRNA expression in association with CIE and its removal.

miRNA ID	P5		CIE10	
	Fold Change	FDR	Fold Change	FDR
mmu-miR-668	-4.34	***	1.19	NS
mmu-miR-183 *	-2.7	*	1.1	NS
mmu-miR-694	-2.3	*	2.2	NS
mmu-miR-183	-2.82	*	-1.66	NS
mmu-miR-182	-2.07	*	-1.41	NS
mmu-miR-27a	2.43	*	-1.02	NS
mmu-miR-25	2.09	**	-1.12	NS
mmu-miR-125b-3p	2.21	*	-1.57	NS
mmu-miR-685	2.42	*	1.01	NS
mmu-miR-503 *	2.3	*	1.9	NS
mmu-miR-219	2.37	*	1.04	NS
mmu-miR-465b-5p	2.37	*	1.85	NS
mmu-miR-27a *	2.4	**	1.7	NS
mmu-miR-322	2.46	**	1.33	NS
mmu-miR-542-3p	2.54	*	1.21	NS
mmu-miR-497	2.72	**	1.06	NS
mmu-miR-152	2.74	*	1.17	NS
mmu-miR-322 *	3.2	**	2.0	NS
mmu-miR-199a-3p	3.96	*	1.31	NS
mmu-miR-450a-5p	8.70	*	3.10	NS
mmu-miR-28 *	2.1	**	4.1	***
mmu-miR-32	2.35	**	1.85	*
mmu-miR-24-2 *	2.6	*	2.4	*
mmu-miR-26b *	2.6	**	2.0	*
mmu-miR-15b *	3.1	***	2.3	**
mmu-miR-503	3.32	*	3.34	*

Comparison of differentially expressed miRNAs in CIE10 and P5.

The significance levels of 26 differentially expressed miRNAs in P5 were compared with those in CIE10. MiRNA expression is displayed as up-regulation (red) or down-regulation (blue), respectively.

Data were derived from 4 separate primary cultures.

*
FDR < 0.05

**
FDR < 0.01

FDR < 0.001 compared with their corresponding no-treatment controls, respectively. NS, no significance.