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Original Research

Expression profiles of miRNAs during ethanol-induced differentiation of neural stem cells

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Abstract MicroRNAs (miRNAs) are small non-coding RNAs that are gaining recognition as important regulators of gene expression with the capability to modulate cellular events. Although miRNAs are involved in a wide range of activities related to the development and differentiation of cells, evidence for miRNAs that control the ethanol-induced differentiation programs of specific neural cell types has been elusive. In this study, we isolated neural stem cells (NSC) from the forebrains of E14 embryos obtained from pregnant C57/BL6J mice. We used microarray technology to monitor the expression profiles of miRNAs during ethanol-induced differentiation of NSC. Comparison of the miRNA expression profiles of NSC and differentiated NSC showed that the expression levels of 58 miRNAs changed by twofold after differentiation. Among these differentially expressed miRNAs, we confirmed the downregulation of the two miRNAs miR-21 and miR-338-3p and the upregulation of miR-320 by real time RT-PCR. By computational analysis, we identified 48 target genes that are predicted to be regulated by miR-21, miR-338-3p and miR-320 during differentiation. We found that most of these predicted target genes are related to cellular processes, such as apoptosis, neural differentiation, cell cycle, metabolism, cell signaling and development. Finally, our findings may contribute to a better understanding of miRNA-regulated neural differentiation in response to ethanol.

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Introduction

Neural stem cells (NSC) are self-renewable cells that play a pivotal role in the development and maturation of the central nervous system (CNS). NSC and adult neurogenesis are thought to contribute to the structural integrity of the hippocampus, which is a limbic system region involved in learning, memory, behavioral control and mood. The differentiation of NSC into the various cell types of the adult CNS is regulated by a number of factors that are secreted by neighboring cells during CNS development[1]. The fate of NSC during differentiation is determined by various signal transduction components, including kinases, growth factors, transcription factors and certain genes that are specifically expressed in early NSC[2]. Moreover, these complex neurogenesis processes are affected by several neurotoxicants, such as ethanol, morphine, and cocaine. Ethanol is a well-known teratogen, and its deleterious effects are regulated by mechanisms, including the induction of apoptosis and the inhibition of proliferation, migration, differentiation, and other cellular functions during the developmental period[3-6]. Ethanol exposure also influences membrane-associated receptor signaling pathways, cell adhesion and the binding of transcription factors. Recent studies suggest that ethanol interferes with the migration and organization of brain cells, which may cause structural deformities or deficits in the brain. It has also been reported that developing brains are more susceptible to ethanol exposure and respond differently than adult brains[7,8]. Neu-

rons usually require tight control in several gene expression pathways. Dysregulation in any one of these pathways could have drastic effects on the expression of downstream genes and proteins that could eventually upset the balance and function of the neurons. A large number of genes and signaling mechanisms have been implicated in the deleterious effects of ethanol on neurons, leading to the suggestion that ethanol is an important teratogen. Recently, many scientists have focused their work on cellular transcriptomic and proteomic analyses to determine the best explanation for the pleiotropic effects of ethanol.

Small non-coding RNAs (ncRNAs) are recognized as key regulators of gene expression. MicroRNAs (miRNAs) are fast emerging as important regulators of gene expression because they control almost every activity of a cell from development to death[9,10]. MiRNAs are approximately 22 nucleotide-long, non-coding RNA molecules that are important regulators of mRNA transcript stability and translation[11]. Most miRNAs are associated with dozens of predicted protein targets and are believed to be major regulators of the protein machinery. Furthermore, many genes may have multiple miRNA binding sites that represent targets for one or more miRNAs. It has been predicted that the human genome contains 800 miRNAs[12]. Although relatively few in number, these miRNAs are predicted to control a large proportion of the tissue- and cell-specific transcriptome, thereby regulating important biological processes including mitosis, tissue-specific differentiation, and death[13]. MiRNAs also have a critical role in maintaining the pluripotent state of embryonic stem cells. It has been shown that certain miRNAs delay neuronal maturation, whereas others promote neuronal differentiation by suppressing the expression of non-neuronal mRNA transcripts[14,15]. MiRNAs that are specifically expressed and enriched in the brain are implicated in maintaining normal neuronal function and homeostasis, which is associated with memory, neuronal differentiation, synaptic plasticity, neurogenesis and neuronal degeneration[14,16,17]. The growing research on miRNA expression profiles for a variety of neurodegenerative diseases infers their importance in cellular responses to ethanol teratogenesis. In this study, we aimed to understand the function of miRNAs in ethanol-induced neural differentiation. We used microarray technology to monitor the expression profile of miRNAs during the differentiation of NSC. The results of this study indicated that there were significant differences between miRNA expression in NSC and differentiated NSC in response to ethanol.

Results

In this study, we isolated NSC from the fetal forebrains of E14 mice, and neurospheres were cultured in a defined medium up to passage 4. After the cells were harvested, they were allowed to differentiate either with or without ethanol for one day. First, we checked

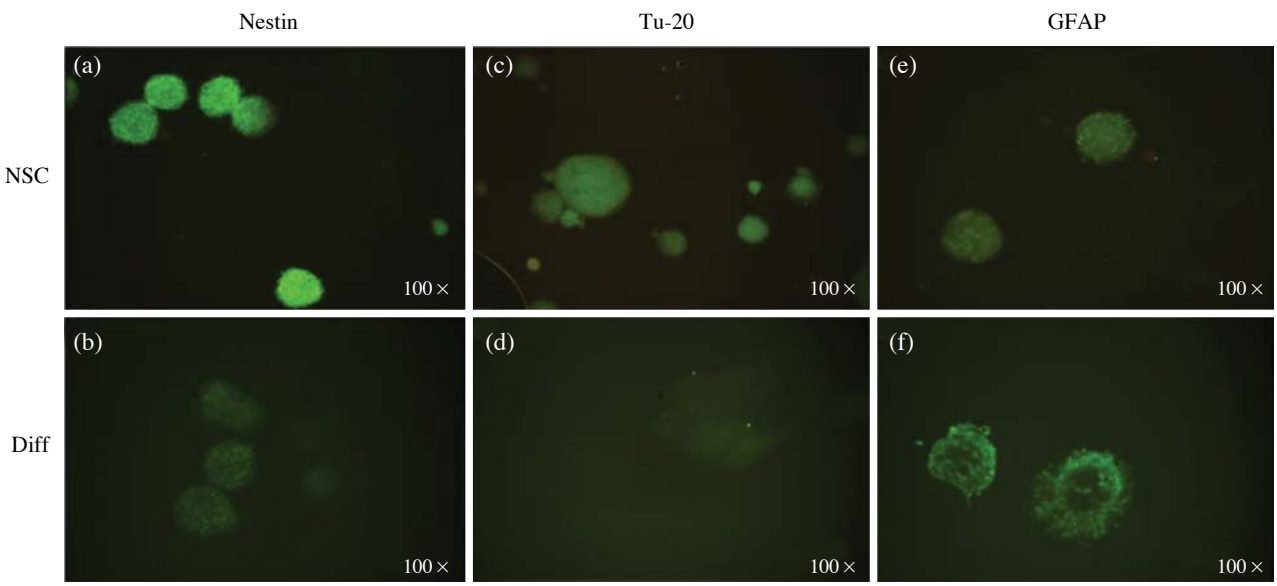

Figure 1. NSC were isolated from fetal E14 mice (see Materials and Methods) and grown as neurosphere cultures. Cells were allowed to differentiate for one day. In differentiated cells, nestin expression was decreased (a, b) compared with the controls. Neurosphere cultures were immunopositive for the astrocyte marker GFAP (e, f) compared with NSC. However, the neural marker Tu-20 was not expressed in differentiated cells (c, d).

NSC differentiation and found that nestin expression was decreased in differentiated NSC, whereas the expression of the astrocyte marker GFAP was increased in differentiated NSC compared with control NSC (Figure 1). We next used a microarray to profile the expression levels of miRNAs in control NSC and differentiated NSC in response to ethanol exposure.

Differentially expressed miRNAs in control NSC and differentiated NSC in response to ethanol

In order to identify miRNAs involved in neural differentiation, we assumed that these miRNAs would be differentially expressed between undifferentiated and differentiated cells in response to ethanol. We set the

Table 1. Comparative analysis of twofold upregulated miRNA expression in response to ethanol.

Name	NSC vs NSC+EtOH (log_2 ratio)	NSC vs NSC diff (log_2 ratio)	NSC vs NSC diff+EtOH (log_2 ratio)	NSC diff vs NSC diff+EtOH (log_2 ratio)
mmu-miR-466i	1.743	–	1.656	–
mmu-miR-467b	1.516	1.098	–	–
mmu-miR-669f	1.459	–	1.219	–
mmu-miR-467f	1.434	–	1.702	–
mmu-miR-467d	1.363	–	1.160	–
mmu-miR-467a-str	1.362	–	1.289	–
mmu-miR-466f-3p	1.360	–	1.727	–
mmu-miR-297a-star	1.349	1.065	–	–
mmu-miR-297b	1.328	–	–	–
mmu-miR-467a-1-star	1.307	–	1.651	1.142
mmu-miR-467g	1.201	–	–	–
mmu-miR-466g	1.177	–	1.020	–
mmu-miR-466d	1.160	–	–	–
mmu-miR-467e	1.009	–	–	–
mmu-miR-711	–	2.913	2.683	–
mmu-miR-1937a	–	1.860	1.489	–
mmu-miR-1937b	–	1.820	1.473	
mmu-miR-1224	–	1.612	1.933	–
mmu-miR-665	–	1.526	1.601	–
mmu-miR-3473	–	1.423	–	–
mmu-miR-1959	–	1.380	1.181	–
mmu-miR-206	–	1.254	1.381	–
mmu-miR-1937c	–	1.198	–	–
mmu-miR-1906	–	1.179	1.828	–
mmu-miR-467b	–	1.098	1.970	–
mmu-miR-2132	–	1.071	1.268	–
mmu-miR-2145	–	1.053	1.693	–
mmu-miR-181c	–	1.034	–	–
mmu-miR-150	–	1.014	–	–
mmu-miR-503	–	–	1.528	–
mmu-miR-714	–	–	1.42	–
mmu-miR-708	–	–	1.347	–
mmu-miR-675	–	–	1.303	–
mmu-miR-680	–	–	1.280	–
mmu-miR-1187	–	–	1.276	–
mmu-miR-1956	–	–	1.230	–
mmu-miR-290	–	–	1.168	–
mmu-miR-574	–	–	1.111	–
mmu-miR-690	–	–	1.108	–
mmu-miR-762	–	–	1.094	–
mmu-miR-138	–	–	1.067	–
mmu-miR-615	–	–	1.066	–
mmu-miR-466f-2	–	–	1.058	–
mmu-miR-2133	–	–	1.037	–
mmu-miR-466j	–	–	1.031	–
mmu-miR-466f	–	–	1.011	–
mmu-miR-466f-1	–	–	1.002	

NSC diff, differentiated NSC; EtOH, ethanol.

Table 2. Comparative analysis of 2-fold down-regulated miRNA expression in response to ethanol.

Name	NSC vs NSC+EtOH (log_2 ratio)	NSC vs NSC diff (log_2 ratio)	NSC vs NSC diff+EtOH (log_2 ratio)	NSC diff vs NSC diff+EtOH (log_2 ratio)
mmu-miR-703	1.664	–	1.937	–
mmu-miR-494	1.537	1.099	–	–
mmu-miR-31	1.036	–	–	–
mmu-miR-7a	–	1.863	2.423	–
mmu-miR-139	–	1.831	1.361	–
mmu-miR-132	–	1.777	1.006	–
mmu-miR-29b	–	1.695	1.992	–
mmu-miR-146a	–	1.402	1.594	–
mmu-miR-1195	–	1.312		–
mmu-miR-1946a	–	1.212		–
mmu-miR-1946b	–	1.131	1.134	–
mmu-miR-18a	–	1.088		–
mmu-miR-9-star	–	1.069		–
mmu-miR-425	–	1.060		–
mmu-miR-212	–	1.045		–
mmu-miR-34b	–	1.024	1.195	–
mmu-miR-146b	–	–	1.752	–
mmu-miR-335	–	–	1.411	–
mmu-miR-29a	–	–	1.355	
mmu-miR-450b	–	–	1.324	1.165
mmu-miR-501	–	–	1.298	
mmu-miR-199a-3p	–	–	1.212	–
mmu-miR-338	–	–	1.196	–
mmu-miR-98	–	–	1.157	–
mmu-miR-22	–	–	1.135	–
mmu-miR-30e	–	–	1.062	–
v11_mmu-miR-805	–	–	1.055	–
mmu-miR-1983	–	–	1.030	–
mmu-miR-155	–	–	1.013	–
mmu-miR-26b	–	–	–	1.443
mmu-miR-21	–	–	–	1.273
mmu-miR-340	–	–	–	1.230
mmu-miR-181c	–	–	–	1.092

NSC diff, differentiated NSC; EtOH, ethanol.

Table 3. Number of miRNA target genes that are differentially expressed between control NSC and differentiated NSC in response to ethanol.

Samples	Up-regulated target	Down-regulated target	Total target
NSC vs NSC+EtOH	27	13	40
NSC vs NSC diff	67	61	128
NSC vs NSC diff+EtOH	111	56	167
NSC diff vs NSC diff+EtOH	54	35	89

NSC diff, differentiated NSC; EtOH, ethanol.

criterion for differential expression as a twofold change in miRNA expression; miRNAs with expression that was changed by this amount were analyzed in the different experimental groups. Thus, we found that 14 miRNAs were upregulated and 3 miRNAs were downregulated when control NSC and ethanol-treated NSC were compared. Interestingly, among the upregulated miRNAs, the expression of 8 miRNAs also increased in differentiated NSC, even though they were treated with the same amount of ethanol. We found that the expression of only two miRNAs, miR-467b and miR-297a-star, was increased in differentiated NSC without ethanol (Table 1). In the case of downregulated miRNAs, only miR-703 showed a similar pattern of expression during differentiation in response to ethanol. Additionally, we counted 15 miRNAs that were upregulated between control NSC and differentiated NSC, and most of them also increased in differentiated NSC in response to ethanol. Only the expression of 4 miRNAs was downregulated due to the action of

Table 4. List of miRNAs validated by real-time RT-PCR.

Probe name	NSC diff signal	NSC diff +EtOH signal	Log_2 ratio	PCR log_2 ratio	Chip log_2/ PCR log_2
mmu-miR-21	11.448	10.174	−1.273	−1.575	0.8086
mmu-miR-338-3p	5.473	4.506	−0.967	−1.529	0.6325
mmu-miR-320	6.477	7.071	0.592	0.415	1.425

NSC diff, differentiated NSC; EtOH, ethanol; PCR, real-time RT-PCR

Table 5. List of miR-21 target genes; miR-21 is downregulated in NSC diffs in response to ethanol.

Gene symbol	Description	Functions
BOLL	Bol, boule-like (drosophila)	RNA-binding protein, which may be required during spermatogenesis. May act by binding to the 3′ UTR of mRNAs and regulating their translation.
CHD7	Chromodomain helicase DNA binding protein 7	This gene encodes a protein that contains several helicase family domains. Mutations in this gene have been found in some patients with the CHARGE syndrome.
CNTFR	Ciliary neurotrophic factor receptor	Plays a critical role in neuronal cell survival, differentiation and gene expression.
ELF2	E74-like factor 2 (ets domain transcription factor)	Isoform 1 transcriptionally activates the LYN and BLK promoters and acts synergistically with RUNX1 to transactivate the BLK promoter. Isoform 2 may function in repression of RUNX1-mediated transactivation.
NFIB	Nuclear factor i/b	Recognizes and binds the palindromic sequence 5′-TTGGCNNNNNGC CAA-3′ present in viral and cellular promoters and in the origin of replication of adenovirus type 2.
NTF3	Neurotrophin 3	Seems to promote the survival of visceral and proprioceptive sensory neurons, similarity: Belongs to the NGF-beta family.
PCBP1	Poly (rc) binding protein 1	Functions as translational coactivators of poliovirus RNA replication by binding to its 5′-terminal cloverleaf structure. It has also been implicated in translational control of the 15-lipoxygenase mRNA; human Papillomavirus type 16 L2 mRNA, and hepatitis A virus RNA.
RAB11A	Rab11a, member ras oncogene family	The protein encoded by this gene belongs to the Rab family of the small GTPase superfamily. It is associated with both constitutive and regulated secretory pathways, and may be involved in protein transport.
SFRS3	Splicing factor, arginine/serine-rich 3	This gene is a member of the serine/arginine (SR)-rich family of pre-mRNA splicing factors, which constitute part of the spliceosome. In addition to being critical for mRNA splicing, the SR proteins have also been shown to be involved in mRNA export from the nucleus and in translation.
SOX5	Sry (sex determining region y)-box 5	Binds specifically to the DNA sequence 5′-AACAAT-3′. Activates transcription of COL2A1 and AGC1 in vitro.
STAG2	Stromal antigen 2	Component of cohesin complex, a complex required for the cohesion of sister chromatids after DNA replication. At anaphase, the complex is cleaved and dissociates from chromatin, allowing sister chromatids to segregate. The cohesin complex may also play a role in spindle pole assembly during mitosis.
TGFBI	Transforming growth factor, beta-induced, 68 kda	Binds to type I, II, and IV collagens and play an important role in cell-collagen interactions. In cartilage, may be involved in endochondral bone formation.

ethanol. Surprisingly, among the different experimental groups, a large proportion of miRNAs exhibited increased expression between undifferentiated and differentiated NSC in response to ethanol. Moreover, this group showed a larger number of downregulated miRNAs than did the other combinations (Table 2). By analyzing differentially expressed miRNAs between distinct groups, we hypothesized that differentiated NSC are more prone to regulation by miRNAs due to the deleterious effects of ethanol.

Computational predictions of the putative targets of dysregulated miRNAs

Malfunction of the cellular machinery could lead to the alteration of miRNA expression, which would result in the aberrant expression of target mRNAs. This dysregulation could alter several downstream pathways and manifest effects like a deficiency in the clearance of cellular by products[9]. We used TargetScan (version 5.1) (http://www.targetscan.org/) algorithms to identify the targets of differentially expressed miRNAs during neural differentiation due to the effects of ethanol. We identified a number of target genes that could be regulated by a 2-fold change in miRNA expression. In the case of ethanol-treated NSC, 40 out of 1,203 differentially expressed genes were targeted by the dysregulated miRNAs. The numbers of up- and downregulated target genes are given in Table 3. Between control NSC and differentiated NSC, 128 out of 989 differentially expressed genes are targeted by the dysregu-

Table 6. List of miR-338-3p target genes; miR-338-3p is downregulated in NSC diffs in response to ethanol.

Gene	Description	Function
AGPAT5	1-acylglycerol-3-phosphate o-acyltransferase 5	Converts lysophosphatidic acid (LPA) into phosphatidic acid by incorporating acyl moiety at the 2 position.
CBFB	Core-binding factor, beta subunit	CBF binds to the core site, 5′-PYGPYGGT-3′, of a number of enhancers and promoters, T-cell receptor enhancers, LCK, IL-3 and GM-CSF promoter.
CELSR2	Cadherin, egf lag seven-pass g-type receptor 2	Important role in cell/cell signaling during nervous system formation.
CNOT6	Ccr4-not transcription complex, subunit 6	Poly (A) nuclease involved in mRNA decay mediated by the major-protein-coding determinant of instability (mCRD) of the FOS gene in the cytoplasm. Has 3′-5′ RNase activity.
DLG2	Discs, large homolog 2, chapsyn-110 (drosophila)	Regulates surface expression of NMDA receptors in dorsal horn neurons of the spinal cord. Involved in regulation of synaptic stability at cholinergic synapses.
EPB41L1	Erythrocyte membrane protein band 4.1-like 1	Confer stability and plasticity to neuronal membrane via multiple interactions, including the spectrin-actin-based cytoskeleton, integral membrane channels and membrane-associated guanylate kinases.
FGF7	Fibroblast growth factor-7	Growth factor active on keratinocytes. Possible major paracrine effector of normal epithelial cell proliferation.
GNG12	Guanine nucleotide binding protein (g protein), gamma 12	Involved as a modulator or transducer in various trans membrane signaling systems. The beta and gamma chains are required for the GTPase activity, for replacement of GDP by GTP, and for G protein-effector interaction.
NHS	Nance-horan syndrome (congenital cataracts and dental anomalies)	Function as a pan-neural transcription factor associated with neuronal differentiation. May play a role in the development of neurons and oligodendrogalia in the CNS.
NONO	Non-pou domain containing, octamer-binding	May have key functions in the regulation of eye, tooth, brain and craniofacial development.
NRP1	Neuropilin 1	Regulate RNA splicing or metabolism in a specific subset of developing neurons.
PAPPA	Pregnancy-associated plasma protein a	DNA polymerase, probably involved in DNA repair. May play a role in sister chromatid cohesion.
PPP4R1	Protein phosphatase 4 regulatory subunit 1	May be a downstream target for TGF-beta1 signaling cascade in endothelial cells.
SRGAP3	Slit-robo rho gtpase activating protein 3	Promotes cell proliferation.
SYN2	Synapsin ii	GTPase-activating protein for RAC1 and perhaps Cdc42, but not for RhoA small GTPase. May attenuate RAC1 signaling in neurons.
TANC1	Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1	Neuronal phosphoprotein that coats synaptic vesicles, binds to the cytoskeleton, and is believed to function in the regulation of neurotransmitter release.
TMEM20	Transmembrane protein 20	May be involved in apoptosis.
VAPB	Vamp (vesicle-associated membrane protein)	Involved in the targeting and/or fusion of transport vesicles to their target membrane.

Table 7. List of miR-320 target genes; miR-320 is upregulated in NSC diffs in response to ethanol.

Gene	Description	Function
PPP1R8	Protein phosphatase 1, regulatory (inhibitor) subunit 8	Involved in pre-mRNA splicing; binds DNA and might act as a transcriptional repressor; required for cell proliferation.
GPR177	Mesoderm induction early response 1 homolog (xenopus laevis)	Transcriptional repressor of a number of genes including Sp1 target genes.
ENAH	Enabled homolog (drosophila)	Involved in microfilament assembly and cell motility. Induces the formation of F-actin rich outgrowths in fibroblasts. Required for neurulation and commissure formation.
NR4A2	Nuclear receptor subfamily 4, group a, member 2	May function as a general co activator of gene transcription; associated with cell cycle progression.
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	May activate NF-kappa-B and JNK and promote apoptosis.
GLCCI1	Islet cell autoantigen 1, 69 kda	Play a role in neurotransmitter secretion; Also associated with synaptic vesicles and Golgi complex.
CDC2L5	Cell division cycle 2-like 5	Controller of the mitotic cell cycle; Involved in the blood cell development.
OLFM1	Olfactomedin 1	Play an important role in regulating the production of neural crest cells by the neural tube, miscellaneous.
ABCA2	Atp-binding cassette, sub-family a (abc1), member 2	May have a role in macrophage lipid metabolism and neural development.
SH3KBP1	Sh3-domain kinase binding protein 1	May enhance tumor necrosis factor mediated apoptosis; role in the regulation of receptor endocytosis and lysosomal degradation.
TSC22D3	Tsc22 domain family, member 3	Protects T-cells from IL2 deprivation-induced apoptosis; in macrophage, play role as anti-inflammatory.
RNF34	Ring finger protein 34 isoform 2	Regulates the levels of CASP8 and CASP10 by targeting them for proteasomal degradation. Protects cells against apoptosis induced by TNF.
CSNK1A1L	Casein kinase 1, alpha 1-like	It can phosphorylate a large number of proteins. Participates in Wnt signaling.
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3	Function as a regulator of vesicle transport, through interactions with the JNK-signaling components and motor proteins.
CDH2	Cadherin 2 [n-cadherin] [n-cadherin 1]	N-cadherin may be involved in neuronal recognition mechanism.
DAZAP1	Daz associated protein 1	RNA-binding protein, which may be required during spermatogenesis.
SON	Son dna binding protein	Might protect cells from apoptosis. Might be involved in pre-mRNA splicing, miscellaneous.
MAPK1	Mitogen-activated protein kinase 1	Phosphorylates microtubule-associated protein 2 (MAP2). Myelin basic protein (MBP), and Elk-1; may promote entry in the cell cycle.

lated miRNAs. Moreover, 167 out of 1968 differentially expressed genes are targeted by the dysregulated miRNA in both control NSC and differentiated NSC in response to ethanol. We also counted 89 target genes out of 387 differentially expressed genes in differentiated NSC compared with ethanol-treated NSC.

Validation of differentially expressed miRNAs by real-time RT-PCR

To validate the differentially expressed miRNAs obtained from the miRNA microarray analysis, we performed real-time reverse transcription (RT)-PCR. We selected the 10 top-scored miRNAs whose expression was changed due to ethanol exposure during differentiation of the NSC. We analyzed the expression of these miRNAs and found that only the expression of miR-21, miR-338-3p and miRNA-320 was consistent with their respective microarray intensity ratios. The expression of other miRNAs was not consistent with their microarray values that predicted their differential expression (Table 4). We recorded that miR-21 and miR-338-3p were downregulated by the effect of ethanol during differentiation, while miR-320 expression was increased during the differentiation of the NSC. We found a log intensity value of -1.575 for miR-21, which is notably similar to its microarray log ratio (Table 4). Similarly, the log intensity ratios of miR-338-3p and miRNA-320 are consistent with their microar-

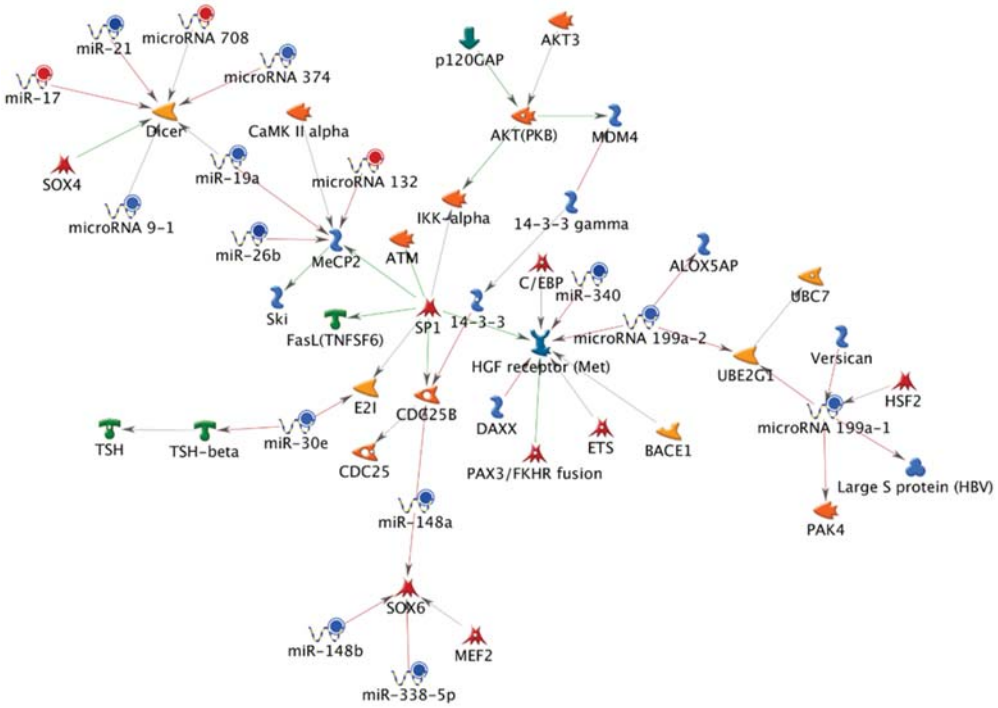

Figure 2. Network analysis for possible targets of miR-21 and miR-338, which were differentially expressed between control NSC and differentiated NSC in response to ethanol.

ray log ratios.

Predicted target genes of miR-21, miR-338-3p and miR-320 in response to ethanol

Because ethanol suppressed miR-21 and miR-338-3p expression, we initially set out to determine their predicted target genes. We also analyzed another upregulated miRNA (miR-320) to determine its predicted targets. Using miRNA target prediction programs (http://www.bioacademy.gr) in combination with previous mRNA profiling, we identified a number of potential target genes for miR-21, miR-338-3p and miR-320. We have listed 12 predicted target genes for miR-21 and analyzed their functions using bioinformatics tools. We determined that the majority of these genes are involved in cellular processes, such as apoptosis, cell cycle regulation, neuronal differentiation, transcriptional regulation, and development (Table 5). We also identified 18 predicted target genes for both miR-338-3p and miR-320 (Tables 6 and 7). The functions of these target genes are related to neural differentiation, apoptosis, metabolism, cell adhesion, cell cycle regulation, transcriptional regulation, and signal transduction pathways. Furthermore, we have proposed a hypothetical network for the predicted targets of miR-21 and miR-338-3p (Figure 2).

Discussion

Ethanol abuse could lead to ethanol-induced neurotoxicity, which changes the expression of genes implicated in myelination, ubiquitination, apoptosis, cell adhesion, neurogenesis and neurodegenerative diseases[18]. Some of the teratogenic and pleiotropic effects attributed to ethanol can be explained by the involvement of miRNAs as intermediaries because they regulate the stability and translation of large numbers of mRNAs involved in various biological processes. In this study, we reported the miRNA expression profile during neuronal differentiation in response to ethanol. We analyzed the differentially expressed miRNAs and observed that ethanol impairs large numbers of miRNAs during neural differentiation. We selected several top-scored miRNAs and confirmed their differential expression by real-time RT-PCR. We found that the expression of two miRNAs, namely, miR-21 and miR-338-3p, was decreased during ethanol-induced neural differentiation. We also validated that the expression of the one miRNA that was up-regulated in the microarray analysis, miR-320, was increased.

The upregulation of anti-apoptotic miR-21 has been shown to protect neurons from death in the cerebral ischemic model. MiR-21 was found to target FASLG, a member of the tumor necrosis factor-α family and a

cell death-inducing ligand[19]. In this study, we have demonstrated the ethanol-induced downregulation of miR-21. Previously, miR-21 was shown to be suppressed by ethanol, and this suppression of miR-21 accounted for the resistance of ethanol-exposed NSC/NPCs to apoptosis. The simultaneous triggering of proliferation and differentiation by ethanol would account for the promotion of NSC maturation and cell cycle induction without cell death via derepression of miRNA-inhibited neuronal identity factors[20]. In our study, ethanol changed the expression of miR-21 during neural differentiation. Many predicted targets of miR-21 have been identified by bioinformatics tools, and the majority of these genes are involved in various cellular functions, such as cell apoptosis, cell proliferation, neuronal differentiation and cell cycle regulation (Table 5). Our findings are similar to a previous study that showed that ethanol triggers cell proliferation and differentiation during neural differentiation. Moreover, we hypothesized that CNTFR is an important and critical predicted target gene of miR-21 (Table 5). CNTFR is an essential component of the receptor complex necessary for signaling by ciliary neurotrophic factor (CNTF), cardiotrophin-like cytokine and neuropoietin; this gene is expressed in neuronal precursors, neurons and astrocytes and is upregulated during in vitro neural differentiation[21]. CNTFR plays a vital role in modulating a cell's responsiveness to its environment via CNTF-mediated signaling and in affecting neural stem cell fate, survival, and the differentiation of neurons[22,23].

The miR-338 gene is located on chromosome 17 and produces two mature forms (miR-338-3p and miR-338 -5p). MiR-338-3p targets the apoptosis-associated tyrosine kinase (AATK) gene, which expresses a protein that plays an essential role in promoting neurite extension in developing neurons[24]. Our results showed that the expression of miR-338-3p was decreased during ethanol-induced neural differentiation. We next determined the potential targets of miR-338-3p by searching miRNA databases and found that miR-338-3p could regulate 18 genes. By a bioinformatics analysis, we found that these genes are mainly involved in neural differentiation, cell apoptosis, cell proliferation and development. Recently, one group showed that miR-338-3p regulates oligodendrocyte differentiation and that overexpression of this miRNA is sufficient to promote this process[25]. Previously, another study also showed that miR-338-3p is downregulated in prion-induced neurodegenerative diseases. Therefore, the dysregulation of miR-338-3p during ethanol-induced neural differentiation could explain the teratogenic effects of ethanol.

In addition, miR-320 is highly expressed in neurons and glial cells and found to be dysregulated in prion-induced neurodegenerative diseases[26]. In this study, we have demonstrated that miR-320 expression was upregulated during ethanol-induced neural differentiation. It has been shown that the knockdown of endogenous miR-320 expression reduced cell death and apoptosis, while the overexpression of miR-320 increased cell death in heart cells[27]. Next, we looked for the putative targets of miR-320 using bioinformatics tools and selected 18 genes that could be regulated by miR-320. We identified that these predicted targets are related to cellular functions, such as neural differentiation, apoptosis, cell cycle progression, cell death, and development. Among these target genes were OLFM1 and ABCA2, which are important neuronal genes that have been the focus of many studies. ABCA2 is a marker of neural progenitors because it is expressed in the subventricular zone of the lateral ventricle and the dentate gyrus of the hippocampal formation, which are sites of continual neurogenesis in the adult brain. ABCA2 shows a distinctive intracellular localization in lysosomal-related vesicles, which undergo morphological changes during neural differentiation. Dysregulation of this gene has serious consequences in neuropathological disorders[28]. Besides OLFM1 and ABCA2, there are many other predicted genes of miR-320 that could be analyzed as teratogenic targets of ethanol in future studies.

In conclusion, we have identified several miRNAs that appear to be dysregulated during ethanol-induced neural differentiation. A number of genes and signaling pathways that are important in neuronal differentiation are likely to be regulated, at least in part, by miRNAs. We predicted a total of 48 potential target genes for the three miRNAs that were dysregulated by ethanol during neural differentiation. There is a correlation between miRNA expression and the identified putative gene targets involved in signaling pathways related to cell cycle regulation, apoptosis, synapse function and neurogenesis. However, the mechanisms behind the regulation of miRNAs by these factors during neural development still need to be elucidated. Thus, these findings demonstrate the striking diversity of potential miRNA targets during ethanol-induced neural differentiation.

Materials and Methods

Isolation of cells from the forebrains of fetal mice

All experimental procedures were approved by the institutional animal care committee and were conducted in accordance with local ethics guidelines. The procedure for isolating cells from the forebrains of E14 embryos obtained from pregnant C57/BL6J mice for the

neurosphere cultures was performed as described in a recent publication from our group[29]. The isolated cells were seeded in 100-mm dishes containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, 1 : 1), 2% B-27 (Invitrogen), penicillin (100 U/mL)/streptomycin (100 μg/mL), 20 ng/mL bFGF (Invitrogen), 20 ng/mL EGF (Invitrogen) and 5 μg/mL heparin and maintained at 37˚C in a 5% CO_2 atmosphere during neurosphere formation. After the neurospheres had formed, they were subcultured once per week using 0.25% Trypsin-EDTA (1x). Neurospheres from the 4th passage were used for all of the experiments.

Neurosphere cultures and ethanol treatment

Neurospheres were harvested and dissociated into single cell suspensions. The resulting dissociated cells were subsequently plated on 100-mm dishes that were pre-coated with poly-$_L$-lysine (Sigma-Aldrich) containing DMEM, P/S, 2% B-27 and 1% FBS and differentiated in the absence or presence of 50 mM ethanol for 24 h. In order to prevent ethanol evaporation from the culture dishes, ethanol-treated cells were cultured in a separate CO_2 incubator that was saturated with 50 mM of ethanol.

RNA isolation and miRNA quality

Total RNA was isolated using TRIzol® according to the manufacturer's instructions (Invitrogen). RNA quality was assessed by using the Agilent 2100 Bioanalyzer with the RNA 6000 Nano Chip (Agilent Technologies), and the quantity was determined using the ND -1000 Spectrophotometer (NanoDrop Technologies). For the miRNA microarray analyses, freshly prepared RNA was used to ensure that the miRNA was of good quality.

Microarray hybridization

In the present study, we performed global miRNA gene expression analyses using the Affymetrix GeneChip® miRNA Array. The sample preparation was performed according to the manufacturer's instructions and recommendations. Approximately 1 μg of total RNA containing low molecular weight (LMW) RNA was polyadenylated and labeled with biotin using FlashTag Biotin for Affymetrix miRNA arrays. Eukaryotic hybridization controls (GeneChip® Eukaryotic Hybridization Control Kit, Affymetrix) were incubated at 65˚C for 5 min. Biotin-labeled RNA was suspended in a hybridization solution that had been incubated at 99˚C for 5 min and subsequently incubated at 45˚C for 5 min. The hybridization was performed at 48˚C for 16 h. The arrays were then washed and stained with the GeneChip® Hybridization, Wash, and Stain Kit (GeneChip Fluidics Station 450; Affymetrix, Inc.) and scanned with the GeneChip® Scanner 3000.

Microarray data analysis

The Affymetrix© miRNA QC Tool software (Affymetrix) was used for data summarization, normalization, and quality control. MiRNAs with $P<0.05$ ($q<0.001$) and fold changes >1.5 were defined as differentially expressed. Three biological replicates were performed for each chip hybridization experiment.

Real-time RT-PCR for validation of miRNA microarray data

We validated our miRNA microarray data by real-time RT-PCR using the ABI 7500 Real-Time PCR System (Applied Biosystems). Total RNA was isolated from the control NSC and the NSC that had differentiated in the presence of EtOH. To analyze gene expression, mRNA was first reverse-transcribed into cDNA using PrimeScript™ Reverse Transcriptase (Takara Bio). MiRNA-specific primers were purchased from Applied Biosystems, Inc., and miRNA real-time RT-PCR was performed according to the manufacturer's instructions.

MiRNA target prediction

To determine the gene targets of the differentially expressed miRNAs, we used three of the leading miRNA target prediction algorithms: miRanada (http://microrna.sanger.ac.uk/sequences/)[30], TargetScan (http://www.targetscan.org/) and GOmir (http://www.bioacademy.gr/bioinformatics/projects/GOmir)[31].

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