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MiR-125b protects against ethanol-induced apoptosis in neural crest cells and mouse embryos by targeting Bak 1 and PUMA

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ABSTRACT

MicroRNAs are a class of small noncoding RNAs that have been implicated in regulation of a broad range of cellular and physiologic processes, including apoptosis. The objective of this study is to elucidate the roles of miR-125b in modulating ethanol-induced apoptosis in neural crest cells (NCCs) and mouse embryos. We found that treatment with ethanol resulted in a significant decrease in miR-125b expression in NCCs and in mouse embryos. We also validated that Bcl-2 antagonist killer 1 (Bak1) and p53-upregulated modulator of apoptosis (PUMA) are the direct targets of miR-125b in NCCs. In addition, over-expression of miR-125b significantly reduced ethanol-induced increase in Bak1 and PUMA protein expression, caspase-3 activation, and apoptosis in NCCs, indicating that miR-125b can modulate ethanol-induced apoptosis by the regulation of Bcl-2 and p53 pathways. Furthermore, microinjection of miR-125b mimic resulted in a significant increase in miR-125b expression and a decrease in the protein expression of Bak1 and PUMA in ethanol-exposed mouse embryos. Up-regulation of miR-125b also significantly reduced ethanol-induced caspase-3 activation and diminished ethanol-induced growth retardation in mouse embryos. This is the first demonstration that miR-125b can prevent ethanol-induced apoptosis and that microinjection of miRNA mimic can prevent ethanol-induced embryotoxicity.

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Introduction

Fetal alcohol spectrum disorder (FASD) is one of the most common permanent birth defects caused by maternal consumption of alcohol during pregnancy. Prenatal alcohol exposure results in physical, behavioral, and cognitive defects, and it is considered to be the leading cause of mental retardation (Burd, 2004). While the pathogenesis of FASD is a complex, multifactorial process, apoptosis is considered to be one of the major mechanisms underlying the pathogenesis of FASD (Dunty et al., 2001; Ikonomidou et al., 2000; Kotch and Sulik, 1992). Among the vulnerable cell populations are neural crest cells (NCCs), a multipotent progenitor cell population that can give rise to a diversity of neural and non-neural cell types (Hall, 2008). Studies have shown that ethanol can diminish NCCs by inducing apoptosis and that apoptosis in NCCs contributes heavily to ethanol-induced abnormalities (Cartwright and Smith, 1995; Chen and Sulik, 1996; Kotch and Sulik, 1992).

MicroRNAs (miRNA) are small noncoding regulatory RNA molecules that modulate gene expression by binding to the 3′ untranslated region (3′-UTR) of mRNA, promoting RNA degradation and inhibiting mRNA translation (Flynt and Lai, 2008). miRNAs are expressed in a wide variety of tissues, and alterations in the abundance of these specific miRNAs can modulate certain miRNA-mediated transcriptional networks (Swarbrick et al., 2010), resulting in a profound impact on a wide array of biological processes, including apoptosis (Baek et al., 2008; Bartel, 2009). Studies have demonstrated that changes in miRNA expression are associated with ethanol-induced gut leakiness, tolerance, as well as neural stem cell proliferation and differentiation (Pietrzykowski et al., 2008; Sathyan et al., 2007; Tsang and Kwok, 2008). miRNAs are also involved in cell death induced by ethanol (Qi et al., 2014; Sathyan et al., 2007; Yadav et al., 2011).

miR-125b is a highly conserved miRNA that is expressed in many types of tissues, with the highest expression in the brain (Bak et al., 2008; Lagos-Quintana et al., 2002). It has been proposed that miR-125b regulates both apoptosis and proliferation (Le et al., 2011). Increase in the expression of miR-125b was observed in oligodendroglial tumors (Nelson et al., 2006). The down-regulation of miR-125b has been reported to suppress the proliferation of differentiated human neuroblastoma cells *in vitro* (Lee et al., 2005). Interestingly, decrease

Abbreviations: 3′-UTR, 3′-Untranslated region; Bak1, Bcl-2 antagonist killer 1; Bax, Bcl-2-associated X protein; CMV, Cytomegalovirus; FASD, Fetal alcohol spectrum disorders; GD, Gestation day; MTS, 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; NCCs, Neural crest cells; PUMA, p53-upregulated modulator of apoptosis.

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in miR-125b expression was associated with excessive apoptosis in rat embryos exposed to retinoic acid (Zhao et al., 2008). Down-regulation of miR-125b and an increased apoptosis were also observed in zebrafish embryos treated with gamma-irradiation or camptothecin (Le et al., 2009). However, the roles of miR-125b in ethanol-induced apoptosis and teratogenesis have never been investigated.

In the present study, we test the hypothesis that miR-125b modulates ethanol-induced apoptosis in NCCs and mouse embryos by the regulation of p53 and Bcl-2 signaling pathways and that overexpression of miR-125b can prevent ethanol-induced embryotoxicity. For the first time, our study demonstrates that miR-125b can modulate ethanol-induced apoptosis in NCCs by targeting its direct targets, Bcl-2 antagonist killer 1 (Bak1) and p53-upregulated modulator of apoptosis (PUMA). Furthermore, we demonstrate that microinjection of miRNA mimic into cultured mouse embryos can prevent ethanol-induced embryotoxicity.

Materials and methods

Cell culture and ethanol treatment

NCCs (JoMa1.3 cells) were cultured as previously described (Chen et al., 2013). Briefly, cells were grown on cell culture dishes coated with fibronectin and maintained in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1) at 37 °C in 5% CO2/95% air. For ethanol exposure, NCCs were cultured in medium containing 50 or 100 mM ethanol for 24 hours. Stable ethanol levels were maintained by placing the cell culture plates in a plastic desiccator containing corresponding ethanol concentration in distilled water as described previously (Chen et al., 2013).

Animal care and dosing

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were mated for 2 hours in the morning. The time of plug detection was considered 0 days, 0 hour of gestation (GD 0:0). Pregnant mice were given two intraperitoneal (i.p.) dose of ethanol, with 4 hours apart, in lactated Ringer's solution at a dosage of 1.9 g/kg or 2.9 g/kg maternal body weight (BW). The first injection was administered on GD 8:0. Control mice were administered lactated Ringer's solution alone. For miRNA analysis, pregnant mice were killed on GD 8: 6, GD 8:9 or GD 8:12 (6, 9, or 12 hours after the first ethanol treatment). Embryos were dissected free of their deciduas in lactated Ringer's solution and then staged by counting the number of somite pairs. Embryos with comparable developmental stage were pooled for mRNA preparation. All protocols used in this study were approved by the Institutional Animal Care and Use Committee.

Cell transfection

For transient transfection, miR-125b mimic, miRNA inhibitor, control mimic, or control inhibitor at a final concentration of 50 nM was transfected into NCCs according to the manufacturer's protocol (Ambion, Austin, TX). The cells were harvested 48 hours after transfection for additional treatments and analysis. The effects of miR-125b mimic or miR-125b inhibitor on the expression of miR-125b were verified using TaqMan® real-time PCR (Ambion, Austin, TX), as described in the next section.

Analysis of miRNA expression

To detect miRNA-125b expression, total RNA was isolated using the mirVana miRNA Isolation Kit (Ambion, Austin, TX), according to the manufacturer's instructions. Quantitative RT-PCR was performed using a stem-loop primer for reverse transcription followed by a sequence-specific real-time Taqman® probe. Briefly, total RNA (10 ng) was reversed transcribed with 100 mM dNTPs, 50 U of reverse transcriptase, 0.4 U of RNase inhibitor, and a specific stem-loop primer at a condition of 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. qRT-PCR reactions were performed using a standard protocol on a Rotor-Gene 6000 Real-Time PCR system (Corbett Life Science, Sydney, Australia). The reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 min and 60 °C for 1 min. All TaqMan microRNA assays were performed in triplicate. Data were normalized with snoRNA202 as endogenous controls. Relative expression was calculated with the comparative threshold cycle (Ct) method.

Construction of luciferase reporter plasmids and reporter assays

miR-125b target sites in the 3′-UTR regions of mRNAs were predicted by using the following online databases: Target Scan (http://www.targetscan.org/), microRNA (http://www.microrna.org/microrna/home/do), and PicTar (http://pictar.mde-berlin.de/). The 3′-UTR of Bak1 and PUMA containing putative miR-125b binding sites were amplified from mouse genomic and cloned into the pMIR-REPORT™ (Ambion, Austin, TX). Primers used to clone the DNA fragments containing the Bak1 3′-UTR and PUMA 3′-UTR were: 5′-actagtcgtggtacacagattcttcagatc-3′ and 5′-aagcttggagagcctgatggatgtgttcag-3′; 5′-actagttccgccttctgacaccctggcca-3′ and 5′-aagcttatctacagcagtgcatgtacagt-3′, respectively. The constructs (200 ng of plasmid/well of 24-well plates) were co-transfected with 20 ng Renilla luciferase pRL-TK control reporter vector (Promega, Madison, WI) and 50 nmol of miR-125b mimics or mimic control (Ambion, Austin, TX) into NCCs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase assay kit (Promega, Madison, WI) with a Lumat LB 9507 Ultra Sensitive Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany). The luciferase activity of each sample was normalized to the pRL/TK-driven Renilla luciferase activity.

Western blotting

Western blotting was performed as described previously (Dong et al., 2011). Briefly, NCCs were washed with phosphate-buffered saline (PBS) and then lysed in pre-cold RIPA lysis buffer (Cell Signaling, Beverly, MA) with 1 mM fresh-prepared PMSF (Sigma-Aldrich, St Louis, MO) and protease cocktail inhibitors (Roche Applied, Indianapolis, IN). Cell lysates were then centrifuged at 12,000 × *g* for 10 min at 4 °C and the supernatants were used for Western blot. The protein concentration in each sample was determined using BCA protein assay kit (Pierce, Rockford, IL) following the manufacturer's instructions. Western blots were performed by standard protocols. The levels of Bak1, PUMA, caspase-3, and β-actin were analyzed with the following antibodies, respectively: rabbit polyclonal anti-Bak antibody (Cell Signaling, Beverly, MA), rabbit polyclonal anti-PUMA antibody (Abcam, Cambridge, MA), rabbit monoclonal anti-cleaved caspase-3 antibody (Cell Signaling, Beverly, MA), and rabbit polyclonal anti-β-actin (Santa Cruz, Santa Cruz, CA). The membranes were developed on a Kodak X-OMAT 2000A imaging system (Kodak, Rochester, NY), and the intensity of the protein band was analyzed using the Adobe Photoshop CS software (Adobe Systems, San Jose, CA).

Determination of cell viability and apoptosis

Cell viability was measured using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) assay kit (Promega, Madison, WI) following the manufacturer's protocol. Apoptosis was determined by analysis of caspase-3 activation by Western blot as described previously (Dong et al., 2008).

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