MicroRNA-9 regulates fetal alcohol-induced changes in D2 receptor to promote prolactin production

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Abstract

Fetal alcohol exposure (FAE) is known to increase prolactin (PRL) secretion from the pituitary lactotrophs. In this study, we determined whether microRNAs (miRs) are involved in FAE-induced alteration in PRL release. We employed a rat animal model of FAE involving feeding pregnant Fisher 344 rats with a liquid diet containing 6.7% alcohol between gestational days 7–21 (AF). Both cyclic and estradiol-implanted FAE females showed increased levels of plasma PRL and pituitary *Prl* mRNA but reduced levels of pituitary dopamine D2 receptor (*D2r*) and its short spliced form (*D2s*). FAE increased the expression levels of miR-9 and miR-326 and did not produce any significant changes in miR-153 or miR-200a levels in the pituitary. Effects of FAE on miR-9 and miR-326 were associated with reduced levels of *D2r* and *D2s*, increased levels of *Prl* in the pituitary, and in plasma. These effects of FAE on *D2r*, *D2s* and *Prl* were enhanced following estradiol treatment. In PRL-producing MMQ cells, ethanol increased miR-9 but not miR-326, reduced levels of *D2r* and *D2s* and increased levels of *Prl*. Treatment of MMQ cells with an anti-miR-9 oligo reduced ethanol effects on miR-9, *D2r*, *D2s* and *Prl*. miR-9 mimic oligos reduced the luciferase activity of reporter vector containing *D2r* 3'UTR, but failed to reduce the mutant luciferase activity. These data suggest that FAE programs the pituitary to produce increased amounts of miR-9 expression that represses the *D2r* gene and its spliced variant *D2s* by targeting its 3'UTR leading to an increase in PRL production and secretion.

Key Words:

- ▶ fetal alcohol exposure
- ▶ prolactinomas
- ▶ estradiol
- ▶ dopamine D2 receptor
- ▶ D2R spliced variants
- ▶ microRNA-9

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Introduction

Dopaminergic neurons in the hypothalamus are important regulators of lactotropic cells in the pituitary gland. Dopamine released from these neurons travels to the pituitary gland and acts on D2 receptors located on the lactotrophs of the pituitary gland to reduce secretion of PRL hormone and inhibit control of the lactotropic cell growth (Ben-Jonathan & Hnasko 2001, Cristina *et al.* 2006, Sarkar 2006). Alcohol is known to alter alternative

splicing of the D2 receptor and by reducing the D2S variant, it is believed to suppress dopamine's ability to reduce PRL secretion and lactotropic cell proliferation (Mello *et al.* 1988, Seki *et al.* 1992, De *et al.* 2002). These effects of alcohol are enhanced by the hormone estradiol (Oomizu *et al.* 2003, Sengupta & Sarkar 2012), which is also known to reduce D2R activity in lactotrophs (Raymond *et al.* 1978, Sarkar 1982, 2006).

Recently, we found that the effects of alcohol on lactotropic cell functions are long lasting especially if the exposure period is during the fetal life (Gangisetty *et al.* 2015). Fetal alcohol induces epigenetic marks involving long-lasting changes in the expression of DNA methyl transferases (Dnmts) and MeCp2 in association with increased promoter methylation and reduced expression of the *D2r* gene that lasts through the adult period (Gangisetty *et al.* 2015). Thus, FAE induces hypermethylation of *D2r* gene that leads to reduction in *D2r* expression and its control of lactotrope's functions.

D2r splice variants *D2l* and *D2s* differ by inclusion or exclusion of exon 6, which encodes 29 amino acids in the third intracellular loop, which is essential for G-protein interactions (Guiramand *et al.* 1995). *D2L*-mediated signaling inhibits the AKT/protein kinase B activity, whereas *D2S*, in contrast, is required for the activation of the ERK 1/2 pathway (Iaccarino *et al.* 2002). Under normal conditions, presence of only one of the two *D2R* isoforms prevents hyperprolactinemia, formation of lactotrope's hyperplasia and tumorigenesis that is observed when these isoforms are deleted in *D2r*^{-/-} mice (Kelly *et al.* 1997). However, the protective function of the single *D2R* isoform is overridden when single isoform-knockout mice are challenged by chronic estrogen treatments as they show increased PRL production and lactotrope hyperplasia (Saiardi *et al.* 1997, Radl *et al.* 2013). The exact mechanism by which FAE alters *D2r* splicing is not clearly understood. One possibility is that miRs, small non-coding RNAs, which are about 20–22 nucleotides in length (Bartel 2004), may participate in ethanol-activated *D2r* splicing and expression changes. This is because an aberrant expression of miRs is associated with FAE (Wang *et al.* 2009) and is linked to the development of pituitary adenoma (Bottoni *et al.* 2007, Mao *et al.* 2010). We addressed whether FAE-induced changes in miRs expression alter the expression of *D2r* and its spliced isoform *D2s* and result in increased pituitary PRL production. We show here that miR-9 mediates FAE-induced changes in *D2r* and *D2s* expressions and PRL production and secretion from the pituitary.

Materials and methods

Animals

Fisher-344 strain rats were obtained from Harlan Laboratories (Indianapolis, IN, USA), housed and bred in a controlled condition of 12-h light/dark cycle at a

constant temperature of 22°C throughout the study. On gestational day 7–21, pregnant rats were fed rat chow *ad libitum* (AD), a liquid diet containing ethanol (AF) or pair-fed (PF) an isocaloric liquid diet. Both diets were purchased from Bioserve (Frenchtown NJ, USA). The concentration of ethanol varied in the diet for the first 4 days from 1.7 to 5.0% v/v to habituate the animals with the alcohol diet. After this habituation period, animals were fed the liquid diet containing ethanol at a concentration of 6.7% v/v (given fresh diet daily at 17:00 h). We have previously shown that in Fisher-344 rats alcohol feeding via the above-mentioned liquid diet paradigm in pregnant rats elevates the blood ethanol concentration in the range of 80–90 mg/dL at 2 h after the beginning of the dark period (De *et al.* 1995). At postnatal day 2 (PD2), AF and PF pups were cross-fostered to untreated lactating AD dams to prevent any compromised nurturing by the AF and PF moms. Litter size was reduced to 8 pups per dam. Pups were weaned on PD21 and housed by sex. Only one female offspring per litter was used in each treatment group to avoid any gene homogeneity. A group of AD, PF or AF animals at 30 days of age were ovariectomized and subcutaneously implanted with an estradiol-17β (Sigma-Aldrich) filled 1-cm silastic capsule (Dow Corning, Midland, MI, USA) under general anesthesia as described previously (Gangisetty *et al.* 2015). Ovariectomized rats with the estradiol implants were used at 60 days after the implant and about 90 days after birth (*n*=6/group). Estrogen levels in plasma of rats with estradiol implants was about 150 pg/mL (Supplementary Fig. 1, see section on supplementary data given at the end of this article). A group of cyclic AD, PF and AF female rats were used at diestrus stage of the estrous cycle around 90 days after birth (*n*=6/group). Estrogen levels in plasma of cyclic rats varied between 15 and 22 pg/mL (Supplementary Fig. 1). The stage of the estrous cycle was determined by microscopic examination of vaginal smears, and diestrus phase was characterized by the presence of many leukocytes. Animals were killed and trunk blood and pituitary samples were collected. Trunk blood samples were collected in tubes containing EDTA (0.1 mM), and plasma was isolated from the blood by centrifugation at 1500 *g* for 15 min. Pituitary and plasma samples were stored at –80°C until use. Trunk blood samples were used for estrogen and PRL assays and pituitary samples were used for gene expression measurements. Animal surgery and care were performed in accordance with institutional guidelines and compiled with NIH policy. Rutgers Institutional Animal Care and Use Committee (IACUC) approved this research protocol.

Cell culture and transfection

MMQ rat pituitary tumor cell line was purchased from American Type Culture Collection and was grown in DMEM/F12 medium (Sigma-Aldrich) with 12.5% horse serum, 2.5% fetal bovine serum and 5% Penn/strep antibiotics (Life Technologies) in 37°C incubator with 7.5% CO₂. The cells were cultured in DMEM/F12 medium containing serum supplements (20 nM apotransferrin, 0.6 μM sodium selenite, 2 μM putrescine and 5 ng/mL insulin) (Sigma-Aldrich) during experimentation. MMQ cells were used between passages 6–8. MMQ cells were seeded 200,000 cells per well in F12 medium with serum supplements in 6-well plates. Cells were treated with ethanol at a concentration of 50 mM or 100 mM for 24-h and 48-h time period by adding ethanol every 12 h ($n=6$ /group). Treated cells were centrifuged and extracted for miRs, RNA and protein.

MMQ cells were transfected with miR-9 mimic oligo or anti-miR-9 oligo or negative control oligo (Life Technologies) at final concentration of 50 nM using Lipofectamine LTX transfection reagent (Life Technologies) as per instructions from the manufacturer. After 12 h of transfection, cells were treated with 50 mM ethanol at 12-h intervals for a period of 24 h. Treated cells were extracted for miRs and total RNA using mirVana microRNA isolation kit (Life Technologies) and RNeasy kit (Qiagen) and determined the expression levels of miRs, *D2r*, *D2s* and *Prl*. Treated cells were also used for extraction of protein and measured the D2R expression by Western blot. The cell culture supernatant was used for PRL measurement by ELISA.

MicroRNA assay

MicroRNA was extracted from pituitary or MMQ cells using mirVana microRNA isolation kit (Life Technologies). The concentration of miR in each sample was measured using nanodrop and quality of miR was assessed by determining absorbance at 260/280 ratio, which was found to be in the range of 1.8–2.0. About 10 ng of miR was converted to cDNA using TaqMan miRNA reverse transcription kit using RT primer specific for each miR. RT reaction was performed in a thermocycler (Applied Biosystems) using the program (16°C for 30 min, 42°C for 30 min, 85°C for 5 min and finally at ~4°C). TaqMan assay for each miR was performed using a specific assay for miR-9 (Id 000583), miR-153 (Id 001191), miR-200a (Id 000502), miR-326 (Id 001061) and U6 snRNA (Id 001973). All miR kits were obtained from Life Technologies. TaqMan RT-PCR was

performed at 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in a 7500 Real-Time PCR machine (Applied Biosystem). The Ct values for all targeted miRs were observed in the range of 25–27 and U6 snRNA was observed at 22. None of the miRs or U6 snRNA required any pre-amplification step. Relative quantity of each miR was determined by standard curve method. Each miR/U6 snRNA RT sample was serially diluted and Ct value was determined. A standard curve was plotted using known concentrations of miR/U6 snRNAs versus Ct values. Results were presented as miR and U6 snRNA (internal control) ratios.

Real-time PCR for gene expression measurements

Gene expression levels of *Prl*, *D2r*, *D2s* in rat pituitaries and MMQ cells were measured by quantitative RT-PCR (SYBR green assay). Total RNA from pituitary or MMQ cells was extracted using RNeasy kit (Qiagen). The concentration of RNA in each sample was determined using nanodrop, and quality was assessed by measuring absorbance at 260/280 ratio, which was found to be in the range of 1.8–2.0. About 1 μg of RNA was converted to first-strand complementary DNA (cDNA) using high-capacity cDNA reverse transcription kit (Life Technologies) as per instructions from the manufacturer. All the primer sequences used for the study are given in Table 1. Real-time quantitative PCR was performed at 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 40 s in Applied Biosystems 7500 Real-Time PCR System. The quantity of target gene expression was measured using standard curve method. Control RT sample was serially diluted and Ct values of these diluted samples were measured. A standard curve was plotted using known concentrations of RNA versus Ct values. *Gapdh*, *18S rRNA* and *Rpl-19* were used as reference genes (internal controls) in the study. Values of these genes are shown in Supplementary Fig. 2. Gene expression levels in the text were presented as the ratios of target gene and *Gapdh*.

Western blot analysis for protein measurements

Protein levels of D2R were determined by Western blot analysis. About 30 μg of total protein from pituitary tissue or MMQ cells were run in 12% SDS PAGE and transferred to PVDF membrane (GE Health Care, Piscataway, NY, USA) at 30 V overnight at 4°C. The membranes were blocked in 5% non-fat dry milk-TBS-0.1% Tween 20

Table 1 Primer sequences used for the study.

Primer name	Sequence
Prl FP	5' CAGAAAGTCCCTCCGGAACCTT 3'
Prl RP	5' AGGAGCTTCATGGATTCCACC 3'
D2r FP	5' CCCAGAGAGGACCCGGTATAG 3'
D2r RP	5' CTGGTTTGGCAGGACTGTCA 3'
D2s FP	5' CCACTCAAGGATGCTGC 3'
Gapdh FP	5' AGACAGCCGCATCTTCTTGT 3'
Gapdh RP	5' CTTGCCGTGGGTAGAGTCAT 3'
D2r 3'UTR FP1	5' CCGCTCGAGGTCTGCCCTTGCC 3'
D2r 3'UTR RP	5' GACTCTTGCAAGGTTTTATT 3'
D2r 3'UTR miR-9 FP1	5' CCGCTCGAGCAAGCTGTGGGCAG 3'
D2r 3'UTR miR-9 RP1	5' TTGCGGCCGCTCCCTCCCACCACCT 3'
D2r 3'UTR mut1 FP	5' CCCTGTCTCCTTGGCAGGGAAGATGCAGCGGCCTT 3'
D2r 3'UTR mut1 RP	5' AAGGCCGTGCATCTTCCCTGCCAAGGAGACAGGG 3'
D2r 3'UTR mut2 FP	5' CCTGTCTCCTTGGCACCATTATGCAGCGGCCTTCTTG 3'
D2r 3'UTR mut2 RP	5' CAAGGAAGGCCGCTGCATAATGGTGCCAAGGAGACAGG 3'
D2r 3'UTR mut3 FP	5' ACCCTGTCTCCTTGGCAGGATTGATGCAGCGGCCTTCCT 3'
D2r 3'UTR mut3 RP	5' AGGAAGGCCGCTGCATCAATCCTGCCAAGGAGACAGGGT 3'
miR-9 inhibitor	5'-mU/ZEN/mCmA mUmAmC mAmGmC mUmAmG mAmUmA mAmCmC mAmAmA mG/3ZEN/-3'
NC5 Neg.con.inhibitor	5'-mG/ZEN/mCmG mAmCmU mAmUmA mCmGmC mGmCmA mAmUmA mUmGmG /3ZEN/-3'

Forward primer (FP), reverse primer (RP), 2'-O-methyl residue (m) with ZENTM modifications (ZEN) at or near the end of miRNA inhibitor oligos to confers resistance to nuclease degradation and increase the binding affinity.

(TBST) at room temp for 3 h and then incubated with primary antibody in the same buffer at 4°C for overnight. The primary antibodies used were rabbit polyclonal D2R (H-50) (1:200; cat# sc-9113; Santa Cruz Biotechnology) and mouse anti-β-actin monoclonal antibody (JLA20; cat# CP01; 1:5000, Calbiochem). The membranes were washed in TBST and then incubated with corresponding peroxidase conjugated secondary antibody (Vector Labs) at room temperature for 1 h. The membranes were washed in TBST and incubated with ECL reagent (Thermo Fisher Scientific) and were developed on the film by autoradiography. The protein band intensities were determined by Image Studio software (Image Studio, Lincoln, NE, USA) and normalized with corresponding β-actin band intensity.

PRL hormone enzyme immunoassay (EIA)

Plasma PRL levels were measured using rat PRL ELISA kit (Alpco Diagnostics, Salem, NH, USA). Each sample was assayed in duplicate. The lower detection limit for prolactin using this kit is about 0.6 ng/mL. The intra-assay coefficients of variation of the assay were found to be 3.85–5.32%.

Estrogen ELISA assay

Plasma estrogen levels were measured using rat estrogen ELISA kit (My Biosource, San Diego, CA, USA). Each sample

was assayed in duplicate. The detection range of the assay is 12.5 pg/mL–1000 pg/mL. The intra-assay coefficients of variation of the assay were found to be 4.8–5.3%.

In vivo knock down of miR-9

miR-9 and negative control inhibitor oligos were custom synthesized for *in vivo* experiments (IDT, Coralville, IA, USA) and sequences were listed in Table 1. miR-9 inhibitor and negative control inhibitor oligos were infused directly into the third ventricle as described previously (Gangisetty et al 2014, Jan et al. 2015). Rats were injected with 5 µL of oligos dissolved in artificial cerebrospinal fluid at a concentration of 1 nmol/µL directly into the third ventricle at the rate of 1 µL/min. After injection the syringe was kept in place for 5 min to avoid the oligo being sucked out during removal. The skin was closed with a wound clip. After 24 h, these rats were sacrificed and their blood and pituitary samples were collected. Plasma was extracted from the blood for PRL measurement. Pituitary samples were utilized for miRNA assay and gene expression measurement and protein expression.

Luciferase assay

The 3'UTR of rat *D2r* full length or short sequence containing miR-9 binding site were PCR amplified from pituitary genomic DNA using primers with restriction enzyme adopters as listed in Table 1. We initially cloned the PCR

product in to basic PCR2.1 vector (Invitrogen) and digested the plasmid with Xho1 and Not1 restriction enzymes and cloned in to psiCHECK 2.0 (Promega), a luciferase reporter vector downstream of renilla luciferase gene and the clones were validated by sequencing. In this vector, renilla luciferase was used as a target reporter and firefly luciferase was used as a normalization control. We constructed 3 different mutants with change in nucleotide sequences of miR-9-binding site using quick change site-directed mutagenesis kit (Agilent Technologies) according to manufacturer instructions. We used the psiCHECK 2.0 vector containing *D2r* 3'-UTR wild-type sequence as a template to make mutants, and primers used are given in the [Table 1](#).

MMQ cells (50,000 cells/well) were transfected with 100 ng of reporter vector or vector containing *D2R* 3'-UTR wild-type or mutant plasmid DNA along with either miR-9 mimic oligo or negative control oligo (50 nM at final concentration) using Lipofectamine LTX reagent. After 48 h post transfection we measured renilla and firefly luciferase activity using Dual Glo luciferase assay system (Promega) in BioTech model synergy HT plate reader (Winooski, VT, USA). Eight samples were used in each group in transfection studies. Luciferase activity was measured in duplicate.

Statistical analysis

The data shown in figures are mean $\pm$ S.E.M. The significant differences between two groups were analyzed using the unpaired *t* test, and two-tailed *P* value <0.05 was considered significant. The significant differences between different treatment groups with single factor (e.g. alcohol treatment) were analyzed by one-way analysis of variance (ANOVA) with Newman-Keuls *post hoc* test. The significant differences between different treatment groups with multiple factors (e.g., cyclic and estradiol treatment) were analyzed using two-way ANOVA with Bonferroni *post hoc* tests. Significance level was set at $P < 0.05$. F-statistics and *P* values of data shown in figures were presented in [Supplementary Table 1](#).

Results

Fetal alcohol exposure increases PRL levels in plasma and the pituitary but decreases *D2r* and *D2s* levels in the pituitary

To investigate whether rats that were exposed to alcohol in utero display any abnormalities in lactotropic hormone

secretion and dopamine receptor *D2R* regulation during adulthood, we analyzed PRL, *D2R* and *D2S* expression in ovariectomized and estradiol-treated rats. Data shown in this figure confirm our previous observations ([Gangisetty et al. 2015](#)) that estradiol administration increases plasma PRL ([Fig. 1A](#)) and pituitary *Prl* mRNA levels ([Fig. 1B](#)) while decreases pituitary *D2r* ([Fig. 1C](#)) and *D2s* mRNA levels ([Fig. 1D](#)). Furthermore, data shown in [Fig. 1A](#) and [B](#) indicate that both plasma levels of PRL and pituitary levels of *Prl* mRNA were elevated in cyclic and estrogen-implanted rats treated with alcohol in utero (AF) as compared to controls (AD and PF). In contrast, data shown in [Fig. 1C](#) and [D](#) indicate that *D2r* and *D2s* mRNA levels in the pituitary were reduced in both cyclic and estrogen-treated AF rats, as compared to PF and AD controls. Reference genes *Gapdh*, *18S rRNA* and *Rpl-19* expression levels were unaltered in any of the treatment conditions ([Supplementary Fig. 3A, C and E](#)). These data provide correlative evidence for a possible mediatory role of *D2R* and its spliced variant *D2S* in FAE-induced increases in PRL production and secretion from the pituitary gland.

MicroRNA target prediction in 3'UTR of dopamine receptor *D2* (*D2r*)

We used target scan web tool to predict the miRs targeting 3'UTR of *D2r*. The prediction analysis showed that 3'UTR of rat *D2r* has putative binding sites for miR-153, miR-200a, miR-9 and miR-326. The sequence alignment of *D2r* 3'UTR target and miR sequence showing 7–8-mer seed match ([Fig. 2A](#)). Of these four miRs, miR-9-, miR-200a- and miR-326-binding sites were conserved among different species such as mouse, human, chimpanzee and rhesus monkey ([Fig. 2B](#)).

Fetal alcohol exposure increases miR-9 and miR-326 expression in the pituitary gland

We measured the expression level of miRs targeting 3'UTR of *D2r* using TaqMan miRNA assay in the pituitary of both cyclic and estradiol-treated AF, AD and PF rats. We did not find any change in the expression of these miRs with estradiol treatment ([Fig. 3](#)). However, we found that in both cyclic and estradiol-treated rats, expression levels of miR-9 and miR-326 were significantly increased in the pituitary of AF rats compared to AD and PF controls ([Fig. 3A and D](#)). However, expression levels of miR-153 and 200a were unaltered in cyclic or estrogen-treated ovariectomized AF rats ([Fig. 3B and C](#)). The expression

Figure 1

Fetal alcohol exposure alters lactotropic cell function in the pituitary gland of cyclic and estradiol-treated ovariectomized rats. Alcohol-fed (AF), pair-fed (PF) or ad libitum-fed (AD) rats were used either on the day of diestrus (cyclic) or used 60 days after ovariectomy with an estradiol implant (E2). Levels of plasma PRL (A), pituitary *Prl* mRNA (B), *D2r* mRNA (C) and *D2s* mRNA (D) are shown in histograms. Data are mean $\pm$ S.E.M. of $n=6$ animals per groups and analyzed using two-way ANOVA with the Bonferroni *post hoc* test. $**P<0.01$ and $***P<0.001$ between AF and controls (AD or PF) in the similarly treated group (for F values please see [Supplementary Table 1](#)). Please note that AF-treated cyclic group did not achieve a significant level when compared to AD- and PF-treated cyclic group in two-way ANOVA test but did achieve a significant level when compared to AD and PF group in one-way ANOVA test (df-2, 15; F-32.22; $P<0.001$) and Newman-Keul *post hoc* test (AF vs AD and PF, $P<0.001$).

level of reference control U6 snRNA was unaltered in any of the treatment conditions ([Supplementary Fig. 2A](#)). These results indicate that FAE enhances production of some of the miRs that are known to target 3'UTR of *D2r*.

Alcohol increases miR-9 and reduces *D2r* and *D2s* expressions in pituitary tumor cell line MMQ

We determined the roles of miR-9 and miR-326 in ethanol-induced repression of *D2r* gene using a PRL-producing MMQ cell line with functional D2R. We treated the cells

with either 50mM or 100mM concentration of ethanol for 24h or 48h. We found that miR-9 expression was significantly increased in MMQ cells treated with both doses of ethanol at 24h and 48h compared to control ([Fig. 4A](#)). However, miR-326 expression was unchanged with ethanol treatment ([Fig. 4B](#)). These results suggest that in lactotropic cells, ethanol acts primarily on miR-9. We further evaluated ethanol-induced changes in D2R expression at both mRNA and protein levels in MMQ cells. We found that *D2r* mRNA level was significantly reduced in MMQ cells treated with ethanol compared to control ([Fig. 4C](#)). We also

Figure 2

MicroRNA target prediction in 3'UTR of dopamine receptor D2 (*D2r*). (A) *D2r* gene with 5'UTR and 3'UTR was represented. The putative miRNA-binding sites of *D2r* 3'UTR were also represented by thick vertical lines at 3'UTR region. The position of each miRNA-binding site of *D2r* 3'UTR, the sequence pairing of target region and miRNA seed match were described. (B) The conserved binding sequence of each miRNA in different species such as rat, human, mouse, chimpanzee and rhesus monkey were highlighted.

Figure 3

Fetal alcohol exposure increases miR-9 and miR-326 expressions in the pituitary gland. Alcohol-fed (AF), pair-fed (PF) or ad libitum-fed (AD) rat pituitaries were used either on the day of diestrus (cyclic) or used 60 days after ovariectomy and an estradiol implant (E2). MicroRNA levels were analyzed by TaqMan real-time PCR and were represented in the histograms; miR-9 (A), miR-153 (B), miR-200a (C), miR-326 (D). Each miR expression was normalized with U6 snRNA. Data are mean $\pm$ S.E.M. of $n=6$ animals per groups and were analyzed using two-way ANOVA with the Bonferroni *post hoc* test. ** $P < 0.01$ and *** $P < 0.001$ between AF and controls (AD or PF) in the similarly treated group.

found that D2R protein level was significantly reduced by ethanol treatment (Fig. 4D). Our results also indicate that *D2s* expression was reduced (Fig. 4E) while *Prl* expression was increased following both 50mM and 100mM doses of ethanol treatments (Fig. 4F). We did not observe any differences in *Prl* mRNA levels between 50mM and 100mM ethanol concentrations. These results demonstrate that, like in the pituitary tissue, ethanol exposure increases miR-9 and *Prl* levels and reduces *D2r* and *D2s* expressions in lactotropic cells in cultures.

miR-9 overexpression reduces *D2r*, *D2s* expression and increases PRL levels in MMQ cells

To test if miR-9 is involved in regulation of D2R, D2S and PRL, we first tested the effects of miR-9 mimic oligo on miR-9, *D2r*, *D2s* and *Prl* expression in MMQ cells. As expected, the miR-9 mimic oligo robustly increased miR-9 levels compared to the negative control oligo (Fig. 5A). Treatment with miR-9-specific oligo reduced the level of *D2r* (Fig. 5B) and *D2s* mRNA (Fig. 5C) but increased *Prl* mRNA (Fig. 5D). These data support a role of miR-9 in regulation of *D2r*, *D2s* and *Prl* expression in lactotropic cells in the pituitary.

miR-9 knockdown prevents ethanol actions on D2R, D2S and PRL expression in MMQ cells

Because alcohol exposure increases miR-9 and reduces *D2r* and *D2s* mRNA levels and miR-9 mimic suppresses

these receptor genes and increases *Prl* gene expression in the pituitary as well as MMQ cells, we hypothesized that miR-9 is involved in regulating *D2r*, *D2s* and *Prl* expression. To test this hypothesis, we knocked down miR-9 expression using anti-miR-9 oligo in MMQ cells treated with 50mM ethanol. The results show that anti-miR-9 oligo, but not control oligo, reduced miR-9 expression in control as well as ethanol-treated cells (Fig. 6A). We found that anti-miR-9 oligos, but not control oligo, increased *D2r* expression and prevented ethanol-suppressive action on *D2r* and *D2s* expression (Fig. 6B, C and D). Additionally, we found that *Prl* expression, which is increased with ethanol, was significantly reduced by anti-miR-9 oligos in cells treated with ethanol (Fig. 6E). However, we did not find any change in the expression of *Prl* in control cells transfected with anti-miR-9 oligo (Fig. 6E). We also found the anti-miR-9 oligo reduced PRL secretion in MMQ cell supernatants (Fig. 6F). These results suggest for a possible mediatory role of miR-9 in ethanol action on *D2r*, *D2s* and PRL expression and secretion.

In vivo knockdown of miR-9 increases D2R, D2S expression in FAE rat pituitary

We further confirmed the effect of miR-9 on *D2r* and *D2s* expression *in vivo* by knocking down miR-9 in FAE rat offspring. miR-9 inhibitor and negative control inhibitor oligos were injected into the third ventricles of AD, PF and AF rats and after 24-h pituitary tissues

Figure 4

Alcohol exposure increases miR-9, reduces *D2r* and *D2s* and increases *Prl* expression in MMQ cells in cultures. Lactotropes-derived MMQ cells were treated with 50 mM, 100 mM ethanol for 24 h and 48 h and analyzed for miRs, *D2r*, *D2s* and *Prl* expression and were represented in the histograms; miR-9 (A), miR-326 (B), *D2r* mRNA (C), *D2R* protein (D), *D2s* mRNA (E) and *Prl* mRNA (F). Data are mean $\pm$ s.e.m. of $n=6$ animals per groups and were analyzed using two-way ANOVA with Bonferroni *post hoc* test (Fig. 4A, B, C and E) and one-way ANOVA with the Newman–Keuls *post hoc* test (Fig. 4D and F). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ between ethanol treatment and controls at the same time period.

were collected and analyzed for the expression of miR-9, *D2r*, *D2s* and *Prl* expression and plasma samples were collected for measurement of PRL levels. We found that the miR-9 inhibitor oligo effectively reduced miR-9 expression in all three groups of rats compared to negative controls (Fig. 7A). We also observed that the miR-9 inhibitor prevented FAE-induced suppression of *D2r* mRNA and D2R protein levels (Fig. 7B and C). Also, FAE-induced suppression of *D2s* expression was normalized by the treatment of the anti-miR-9 oligo (Fig. 7D). Furthermore, miR-9 inhibitor suppressed FAE-induced increase in pituitary *Prl* mRNA levels (Fig. 7E). The plasma PRL levels after miR-9 knockdown in rats were significantly lower in all three groups of animals compared to negative control oligo (Fig. 7F). These results indicate that miR-9 may regulate FAE-induced

changes in *D2r* and *D2s* levels as well as *Prl* expression and secretion.

miR-9 directly targets *D2r* 3'UTR to repress its expression

In order to test the hypothesis that miR-9 may target 3'UTR of *D2r* to suppress its expression, we cloned the 3'UTR of *D2r* downstream of renilla luciferase in a reporter vector and measured luciferase activity. We made two different constructs one with the full-length 3'UTR and another with a short construct comprising of the miR-9-binding site to measure luciferase activity (Fig. 8A). We transfected these plasmids along with either the miR-9 mimic oligo or negative control oligo and determined the renilla luciferase activity as a measure of *D2r* 3'UTR function.

Figure 5

miR-9 overexpression by miR-9 mimic reduces *D2r*, *D2s* and increases *Prl* expression in MMQ cells in cultures. MMQ cells were transfected with miR-9 mimic oligo (miR-9 mimic) or negative control oligo (neg. contrl.) and expression levels of miR-9 (A), *D2r* (B), *D2s* (C) and *Prl* (D) were measured after 24 h. Data are presented as mean $\pm$ S.E.M. of $n=6$ per group and were analyzed using *t* test. * $P<0.05$ and *** $P<0.001$ between miR-9 mimic and neg. contrl.

We found that the miR-9 mimic oligo significantly reduced the luciferase activity compared to negative control oligo or mock transfection in both the plasmids transfected in MMQ cells (Fig. 8B). These results suggest that miR-9 represses *D2r* expression by binding to its 3'UTR and its binding site on *D2r* gene.

We further confirmed the specificity of miR-9 binding at 3'UTR by mutating the miR-9-binding site using site-directed mutagenesis. We made three different mutant constructs by altering the miR-9-binding site as it was shown in Fig. 8C. We found that miR-9 mimic oligo significantly reduced luciferase activity with wild-type plasmid compared to negative control oligo. However, the miR-9 mimic oligo did not alter luciferase activity of any of the three mutants transfected compared to the negative control oligo (Fig. 8D). Our results revealed that the miR-9-binding site is essential for miR-9 to reduce the *D2r* 3'UTR activity.

Discussion

We recently reported that FAE induces hyperprolactinemia by reducing D2R levels in the pituitary gland (Gangisetty *et al.* 2015). The data presented here confirm

the FAE effects on *D2r*, *D2s* and *Prl* and identify a role of miR9 in this process. This interpretation is based on TargetScan predictions of miRs targets on *D2r* 3'UTR, demonstrating two miRs (miR-9 and miR-326 with predicted binding sites on *D2r* 3'UTR) are altered in the pituitary following FAE treatment. Effects of FAE were stronger on *D2r*, *D2s* and *Prl* in estrogen-treated rats over cyclic animals, and also data showing miR-9 is altered in lactotrope-derived MMQ cells following ethanol treatment. We observed two miRs; miR-9 and miR-326 were increased in the pituitary following FAE treatment and only miR-9 expression was increased in MMQ cells following ethanol treatment. The cellular heterogeneity in the pituitary gland may have differential expression, which is not the same as MMQ cells although they were derived from estrogen-induced rat pituitary tumor and secrete prolactin and express functional D2R (Judd *et al.* 1988). Additional support for a mediatory role of miR-9 is the finding that overexpression of miR-9 using miR-9 mimic oligo suppresses *D2r* and *D2s* and increases *Prl* in MMQ cells, while reduction of miR-9 prevents ethanol/FAE's inhibitory actions on *D2r* and *D2s* and the stimulatory action on *Prl* in both MMQ cells *in vitro* and pituitary tissue *in vivo*. Also, we show that the site-directed mutagenesis of the miR-9-binding site of

Figure 6

miR-9 knockdown suppresses ethanol actions on *D2r*, *D2s* and *Prl* expressions in MMQ cells in cultures. MMQ cells were transfected with anti-miR-9 inhibitor oligo (anti-miR-9), negative control inhibitor oligo (Neg.control), and mock transfection (control) followed by treatment with ethanol (50 mM) for 24 h and measurement of expression levels of miR-9 (A), *D2r* mRNA (B), D2R protein (C), *D2s* mRNA (D), *Prl* mRNA (E) and PRL secretion (F). Data are mean $\pm$ S.E.M. of $n=6$ per group and were analyzed using one-way ANOVA with the Newman-Keuls *post hoc* test. *, **, *** significant at $P < 0.05$, 0.01, 0.001 vs control or neg. control. @, @@, @@@ significant at $P < 0.05$, 0.01, 0.001 vs Neg. control or Neg. control + ethanol.

D2r 3'UTR prevented miR-9 mimic's ability to alter *D2r* 3'UTR activity.

Lactotrope cell growth and PRL production are largely regulated by the tonic inhibitory control of the dopaminergic system. D2R is the predominant dopamine receptor subtype in the anterior pituitary and mediates dopamine's inhibitory action on lactotropes (Mansour *et al.* 1990), and a loss of D2R function is connected to the increased production of PRL, cell proliferation and tumorigenesis (Cristina *et al.* 2006, Sarkar 2006). Estrogen is known to stimulate the PRL synthesis and affect the growth of lactotropes by inhibiting the function of dopaminergic system. It downregulates dopamine production in the hypothalamus and suppresses the activity of D2R in lactotropes. It also alters the splicing of *D2r* to reduce its *D2s* isoform (Sarkar *et al.* 1982, Guivarc'h *et al.* 1998,

Oomizu *et al.* 2003). In addition, various growth factors released by lactotropes or other cells within the pituitary gland also influence PRL production and secretion. Accumulating evidences also suggest that PRL negatively regulates its own synthesis. It has been reported that PRL receptor-knockout mice display lactotrope hyperplasia and prolactinoma (Schuff *et al.* 2002). The data presented in this study indicate that *D2r* expression is negatively regulated by miR-9 in lactotropic cells of the pituitary. Previous studies have identified changes in various miRs during pituitary tumorigenesis. It has been reported that miR-15a, miR-16 and miR-132 are downregulated (Bottoni *et al.* 2007, Renjie & Haiqian 2015) while miR-200C is upregulated in pituitary adenoma (Mao *et al.* 2010), and miR-126 and miR-381 are downregulated in growth hormone-secreting pituitary adenomas (Mao *et al.* 2010) and miR-21, miR-141 and miR-150 are

Figure 7

In vivo knock down of miR-9 increases *D2r*, *D2s* mRNAs and reduces *Prl* mRNA levels in the pituitary and PRL levels in plasma of FAE rat offspring. AD, PF and AF rat offspring were treated with miR-9 inhibitor (anti-miR-9) or negative control oligo (neg.control) through intracerebroventricular (icv) injection. Pituitary miR-9 (A), *D2r* mRNA (B), D2R protein (C), *D2s* mRNA (D), *Prl* mRNA (E) and plasma PRL (F) levels were measured 24 h after the treatments. Data are presented as mean $\pm$ S.E.M. of $n=6$ animals per group and were analyzed using two-way ANOVA with the Bonferroni *post hoc* test. *, **, *** significant at $P<0.05$, 0.01, 0.001 vs AD or PF in neg. control group. @, @@, @@@ significant at $P<0.05$, 0.01, 0.001 vs corresponding AD or PF in neg. control group.

reduced in pituitary corticotropinoma (Amaral *et al.* 2009). Only a few studies illustrated the role of these miRs toward their target genes. miR-200C has known to regulate pituitary tumor formation through the PTEN/Akt signaling pathway (Liao *et al.* 2013). miR-132 and miR-15a/16 have shown to act as tumor suppressor genes by targeting Sox5, an oncogene, that is upregulated in pituitary tumor (Renjie & Haiqian 2015). We showed here that *D2r* is a direct target of miR-9, as evidenced by the reduction of the reporter luciferase activity of *D2r* 3'UTR following treatment with the miR-9 mimic oligo. Furthermore, we confirmed this by ameliorating the reporter luciferase activity of miR-9-binding site mutants using the miR-9 mimic oligo. These data suggest that *D2r* is a direct target of miR-9 in rat pituitary cells. In our study, FAE-induced miR-9 suppresses D2R and thereby increases PRL production in the pituitary gland. Alternative splicing of *D2r* results in two spliced

isoforms, *D2l* and *D2s*. These two isoforms differ by exon 6 inclusion or exclusion, which encodes 29 amino acids in the third intracellular loop, which is critical for G-protein coupling. Both the isoforms are expressed in pituitary lactotropes (Missale *et al.* 1998), and each may have its own specific physiological function. We report here that FAE reduces the *D2s* expression in the pituitary, which is in conformity with our previous observations (Oomizu *et al.* 2003). In our present study, we showed that miR-9 not only regulates ethanol-induced repression of D2R but also alters the level of spliced isoform *D2s*. However, the mechanism by which miR-9 regulates *D2r* splicing requires further investigation. It has been shown that several spliceosome factors such as polypyrimidine tract-binding protein PTBP1 (Sasabe *et al.* 2011), hnRNP M and Nova-1 (Park *et al.* 2011) play an important role in *D2r* splicing. The possible role of FAE-induced spliceosome factors in the regulation of

Figure 8

miR-9 directly targets *D2r* 3'UTR to repress its expression. (A) Schematic representation of *D2r* 3'UTR full length (*D2r* 3'UTR FL) and a short sequence containing miR-9 binding site (*D2r* 3'UTR miR-9 bs) were cloned down stream of renilla luciferase in a reporter vector. (B) *D2r* 3'UTR activity in MMQ cells transfected with a reporter plasmid harboring *D2r* 3'UTR FL or *D2r* 3'UTR miR-9 bs along with miR-9 mimic oligo (miR-9 mimic) or negative control oligo (NC mimic) or Mock transfection (Mock). (C) *D2r* 3'UTR containing wild-type miR-9-binding site and mutants with altered miR-9-binding site cloned in a reporter vector downstream of renilla luciferase was represented. (D) *D2r* 3'UTR activity of MMQ cells transfected with a reporter plasmid containing *D2r* 3'UTR with miR-9-binding site wild type (UTR WT) or mutant (UTR mut) along with miR-9 mimic oligo (miR-9 mimic) or negative control mimic oligo (NC mimic). *D2r* 3'UTR activity was measured as renilla luciferase activity and normalized with firefly luciferase. Data are presented as mean $\pm$ S.E.M. of $n=8$ per group and were analyzed using one-way ANOVA with the Newman-Keuls *post hoc* test. * $P<0.05$ and *** $P<0.001$ between miR-9 mimic and NC mimic or Mock. ** $P<0.01$ between UTR (WT) + miR-9 mimic and UTR (WT)+NC mimic.

D2r splicing needs further investigation. It should be mentioned that *D2r* expression in the pituitary is regulated by the endogenous ligand dopamine from the tuberoinfundibular dopaminergic (TIDA) neurons in the hypothalamus (Sarkar *et al.* 1982, Ben-Jonathan & Hnasko 2001). Since *D2R* controls the feedback loop of

TIDA neurons (Ben-Jonathan & Hnasko 2001), alcohol may similarly effect miR-9-regulated *D2R* expression and dopamine secretion from TIDA neurons and thereby dopamine action on the pituitary.

Dopamine agonist therapy has become the first-line treatment for prolactinomas. Accumulating evidence also suggests that less than 10% of prolactinomas do not respond to dopamine agonist therapy and are resistant to this drug (Oh & Aghi 2011). *D2* receptor inactivation and differential expression of its isoforms has been reported to be associated with dopamine agonist-resistant prolactinomas (Caccavelli *et al.* 1994, Gillam *et al.* 2006, Wu *et al.* 2010). Our present study showing a role of miR-9 in regulating *D2r* and its spliced isoform *D2s* expressions in FAE rat offspring identifies the novel possibility for the therapeutic use of miR-9 antagonists in dopamine-resistant prolactinomas.

In conclusion, the present study demonstrates that FAE programs the pituitary to differentially express miRNAs especially increasing the level of miR-9 causing the repression of *D2r* and its splice variant *D2s*, thereby regulating PRL production and secretion (Fig. 9).

Figure 9

Model diagram representing the role of miR-9 in regulation of FAE-induced changes in *D2R* expression and PRL production in the pituitary gland. In control rats, basal *D2R* expression maintains normal PRL production. FAE enhances miR-9 expression to suppress *D2R* production and its inhibitory control of PRL production.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-17-0135>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

O G helped in designing experiments and performed research, analyzed data, writing the paper; S J performed research and analyzed the data; O W performed research and analyzed data; D K S designed research, analyzed data and wrote the paper.

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