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MicroRNA-9 and MicroRNA-326 Regulate Human Dopamine D2 Receptor Expression, and the MicroRNA-mediated Expression Regulation Is Altered by a Genetic Variant*[S]

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Background: Regulation of dopamine D2 receptor (*DRD2*) is pathophysiologically and pharmacologically important.
Results: miR-9 and miR-326 target to the 3′-UTR of *DRD2*, and endogenously inhibit *DRD2* expression. A functional single nucleotide polymorphism alters such regulation.
Conclusion: *DRD2* is post-transcriptionally regulated by miR-326 and miR-9.
Significance: The study suggests a pathophysiological and pharmacological role of miR-9 and miR-326 in neuropsychiatric disorders.

The human *dopamine receptor D2* (*DRD2*) has been implicated in the pathophysiology of schizophrenia and other neuropsychiatric disorders. Most antipsychotic drugs influence dopaminergic transmission through blocking dopamine receptors, primarily DRD2. We report here the post-transcriptional regulation of DRD2 expression by two brain-expressed microRNAs (miRs), miR-326 and miR-9, in an *ex vivo* mode, and show the relevance of miR-mediated DRD2 expression regulation in human dopaminergic neurons and in developing human brains. Both miRs targeted the 3′-UTR (untranslated region) of *DRD2* in NT2 (neuron-committed teratocarcinoma, which endogenously expresses *DRD2*) and CHO (Chinese hamster ovary) cell lines, decreasing luciferase activity measured by a luciferase reporter gene assay. miR-326 overexpression reduced *DRD2* mRNA and DRD2 receptor synthesis. Both antisense miR-326 and antisense miR-9 increased DRD2 protein abundance, suggesting an endogenous repression of *DRD2* expression by both miRs. Furthermore, a genetic variant (rs1130354) within the *DRD2* 3′-UTR miR-targeting site interferes with miR-326-mediated repression of *DRD2* expression. Finally, co-expression analysis identified an inverse correlation of *DRD2* expression with both miR-326 and miR-9 in differentiating dopaminergic neurons derived from human induced pluripotent stem cells (iPSCs) and in developing human brain regions implicated in schizophrenia. Our study provides empirical evidence suggesting that miR-326 and miR-9 may regulate dopaminergic signaling, and miR-326 and miR-9 may be considered as potential drug targets for the treatment of disorders involving abnormal DRD2 function, such as schizophrenia.

The dopaminergic hypothesis of SZ postulates that dopaminergic overactivity causes psychosis. Underlying this hypothesis are the observations that psychotogenic stimulants (*e.g.* amphetamines) activate dopamine receptors (1) and that most antipsychotic drugs influence dopaminergic transmission through blocking dopamine (primarily D2) receptors (2–9). Besides the essential role of DRD2 affinity for antipsychotic effects (10), further support derives from the study of postmortem brains, live brain imaging, and animal models. Postmortem studies of SZ cases consistently found increased density of striatal D2 receptors (8, 11–15). Brain imaging studies have shown that amphetamine-induced increase of dopamine response is correlated with positive symptoms of SZ (16–18) and elevation of striatal D2 receptors (8, 19). Mice overexpressing striatal DRD2 exhibit selective cognitive impairments in working memory tasks (20, 21). Three *DRD2* missense variants ($Val^{96} \rightarrow$ Ala, $Pro^{310} \rightarrow$ Ser, and $Ser^{311} \rightarrow$ Cys) impair receptor function (22, 23), and the minor allele of rs1801028 ($Ser^{311} \rightarrow$ Cys) is associated with SZ[2] in a meta-analysis (24). However, these missense changes are uncommon, and their function does not seem congruent with a hyperdopaminergic hypothesis of SZ. Therefore, it is conceivable that for *DRD2*, increased expression (rather than structural changes) may play an important role in SZ pathogenesis. *DRD2* is also hypothesized to be involved in multiple other neuropsychiatric disorders, *e.g.* drug addiction (25), neuroticism, and anxiety disorders (26, 27), which highlights additional potential importance of studying its regulation.

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[S] This article contains supplemental Table 1.

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[2] The abbreviations used are: SZ, schizophrenia; SNP, single nucleotide polymorphism; TH, tyrosine hydroxylase; LD, linkage disequilibrium; miR, microRNA; MGS, Molecular Genetics of Schizophrenia; DA, dopaminergic; iPSC, human induced pluripotent stem cell; ANOVA, analysis of variance.

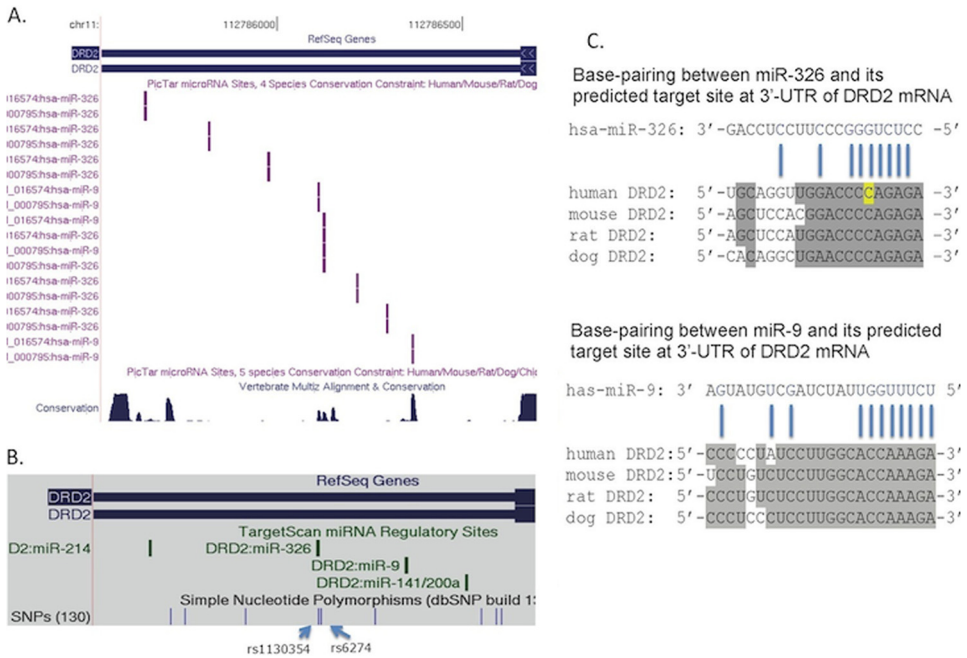

FIGURE 1. **DRD2 3′-UTR (chr11:113,281,399–113,280,217; hg19) has multiple miR target sites for miR-9 and miR-326 as predicted by PicTar and TargetScan.** *A*, PicTar prediction and DNA sequence conservation. *B*, TargetScan prediction of miR target sites. *C*, base pairing between miR and target sequences of *DRD2*. Conserved sequences (*shaded*) among human, mouse, rat and dog for the targeting sequences are also shown. The allele highlighted in *yellow* indicates the SNP site (rs1130354) within the 7-nucleotide seed sequence.

Multiple *DRD2* genetic variants influence *DRD2* expression at both the transcriptional and the post-transcriptional levels. At the transcriptional level, the insertion allele of the promoter variant rs1799732 (−141C Ins/Del) increases *in vitro DRD2* transcription and is reported to be nominally associated with SZ in a small Japanese sample (260 cases *versus* 312 controls) (28). Post-transcriptionally, two intronic SNPs, rs2283265 (intron 5) and rs1076560 (intron 6), in high linkage disequilibrium (LD) with each other, alter *DRD2* splicing (29) and are associated with reduced performance in working memory in controls (29) and with cocaine abuse (30, 31). We and others have also previously shown that allele C of a functional synonymous variant rs6277 (C957T) increases *DRD2* mRNA stability and receptor synthesis *in vitro* (32) and *in vivo* (33). Furthermore, rs6277 has been reported to be associated with cognitive performance (34–36), response to antipsychotic medication (37), and depressive rumination (38). Identification of these functional *DRD2* variants highlights the potential importance of noncoding regulatory sequences.

miRs have emerged as important post-transcriptional regulators of gene expression. miRs are ~22-nucleotide small noncoding RNAs processed from pre-miRs (~100 bp) with a typical folding structure containing a mature miR region and a stem-loop region (39, 40). miRs mediate translational repression and/or mRNA decay of a target gene through sequence complementation with the 3′-UTRs of their target genes (41, 42). There is evidence suggesting that the number of miR target sites at the 3′-UTRs of a gene is negatively correlated with RNA stability (43). The 3′-UTR of human *DRD2* mRNA is predicted (PicTar, TargetScan) to have multiple miR binding sites (Fig. 1, *A* and *B*), including those for miR-326 and miR-9, which are brain-expressed and involved in neurogenesis and neurodifferentiation (44–46). There are two known SNPs, rs1130354 and rs6274, within a predicted (PicTar) targeting region shared by miR-326 and miR-9 (Fig. 1, *B* and *C*). Polymorphisms within the target region may impair the base-pairing between an miR and its target, hence affecting target gene expression. In this study we used a cellular model to examine the effects of miR-326 and of miR-9 on *DRD2* receptor expression and whether genetic variation within the 3′-UTR of *DRD2* mRNA alters the targeting of miRs.

EXPERIMENTAL PROCEDURES

Cell Culture—We purchased CHO-K1 (hereafter referred to as CHO) and human NT2 cell lines from ATCC. We cultured CHO in F-12K and NT2 in Dulbecco's modified Eagle's medium (DMEM; from ATCC) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin 100 units/ml (from ATCC). We maintained cells in 15 ml of growth medium in a 75T flask with a cell density of $<1 \times 10^6$/ml in a 37 °C incubator with 5% CO_2, changing cell culture media every other day until the experiment day. We used both NT2 and CHO cell lines for reporter gene assay but used only the NT2 cell line for studying miR-mediated inhibition on endogenous *DRD2* expression, because CHO cells do not endogenously express DRD2 (23, 32, 47).

Samples and Genotyping—We studied 1870 SZ cases and 2002 controls from the Molecular Genetics of Schizophrenia (MGS) sample of European ancestry (48, 49) to evaluate the LD between functional SNPs and for estimating allele frequency of rs1130354. We previously described in detail the recruitment procedures and clinical phenotypes (48, 49). We used the TaqMan Pre-Designed SNP Genotyping Assay (Invitrogen) to genotype rs1130354 in the MGS European ancestry sample. Briefly, we used 4 ng of genomic DNAs in a total of 5 μl of a polymerase chain reaction (PCR) reaction in each well on a 384-well plate.

We carried out PCR on a 7900HT instrument (Invitrogen) with standard real-time PCR cycling conditions. We made automatic genotype calls with SDS 2.2 software (Invitrogen). The genotyping completion rate was 98 and 96% for cases and controls, respectively. There was no significant deviation from Hardy-Weinberg disequilibrium. The NorthShore University HealthSystem Institutional Review Board approved the study.

DNA Cloning and Sequence Mutagenesis—We used a PCR to amplify from MGS DNAs the *DRD2* 3′-UTR sequences (830 bp; chr11:113,280,381–113,281,210; hg19) spanning the predicted target sites of miR-9 and miR-326. The DNA sequences of the amplified fragments were confirmed by Sanger sequencing. We selected the amplified wild-type (WT) sequence as those with the same sequence as the hg19 reference sequence. We then cloned the wt sequence fragment into Xho1 and Pme1 sites downstream of the firefly luciferase coding region in the psiCHECK™-2 Vector (Promega). psiCHECK™-2 utilizes firefly luciferase as a reporter gene and *Renilla* luciferase in the same vector as a control to normalize transfection efficiency and cell number in the reporter gene assay. With the recombined reporter gene vector containing *DRD2* 3′-UTR wt sequence as a backbone vector, we used the QuikChange II Site-Directed Mutagenesis kit (Agilent) to change the nucleotide sequence to construct the mutant alleles of the two known SNPs (rs1130354 and rs6274) within the targeting sites of miR-9 and miR-326. We designed PCR primers (sequences available upon request) for cloning *DRD2* 3′-UTR and for mutagenesis with Primer3 and purchased primer oligos from IDT (Integrated DNA Technologies).

Reporter Gene Assay—We transformed reporter gene DNA constructs containing the *DRD2* 3′-UTR wt sequence (pD2UTR-wt) with rs6274 mutant allele G (pD2UTR-rs6274-G) and with rs1130354 mutant allele G (pD2UTR-rs1130354-G) (Fig. 2) into *Escherichia coli* DH5α and prepared endotoxin-free plasmid DNAs with the StrataPrep® EF Plasmid Midiprep kit (Agilent). We next transfected reporter gene plasmid DNAs into CHO or NT2 cells. Briefly, we plated 3.5×10^4 cells per well into 24-well plates 24 h before transfection. The next day we transfected ~100 ng of reporter gene vector and varying amounts of miR precursors into CHO and NT2 cells at 90% confluence, respectively. We used Lipofectamine 2000 (Invitrogen) for transfection. At 48 h post-transfection, we measured *Renilla* and firefly luciferase activity in transfected cells using a Dual-Luciferase Reporter Assay system (Promega) in a 96-well plate.

Overexpression of miRs and Anti-miRs in NT2 Cells—We plated NT2 cells at 2.5×10^5 cells per well on a 6-well plate 24 h before transfection. At 90% of cell confluency we transfected NT2 cells with Ambion® Pre-miR™ miR precursors miR-9 and miR-326 (Invitrogen) at final concentrations of 20, 50, and 100 nM. We also transfected Ambion® Anti-miR™ miRNA Inhibitors (Invitrogen) for miR-9 and miR-326. We used DMEM supplemented with 10% FBS without antibiotics to culture the transfected cells for 48 h. We then collected cells for RNA and protein extraction using the mirVana PARIS kit (Invitrogen).

Human iPSCs and Dopaminergic (DA) Neuron Differentiation—We generated three iPSC lines from fibroblasts cells of two individuals (GM01835 and GM03652) (from Coriell) and from skin biopsy of a healthy adult volunteer. The NorthShore University HealthSystem Institutional Review Board approved the study. We used a polycistron lentivirus vector (STEMCCA) containing four standard reprogramming factors (*OCT4*, *SOX2*, *c-MYC*, and *KLF4*) to infect the fibroblasts for generating iPSCs as previously described (50, 51). The iPSC clones were identified by stem cell-like morphology and positive immune fluorescence staining for pluripotent stem cell markers (TRA-1–60, OCT4, NANOG, and SSEA4). The iPSC clones were further evaluated for pluripotency by testing the formation of embryoid bodies that spontaneously differentiate into three germ layers (52). The three germ layers were characterized by immune fluorescence staining of α-fetoprotein (endoderm), tubulin β-III (ectoderm), and α-smooth muscle actin

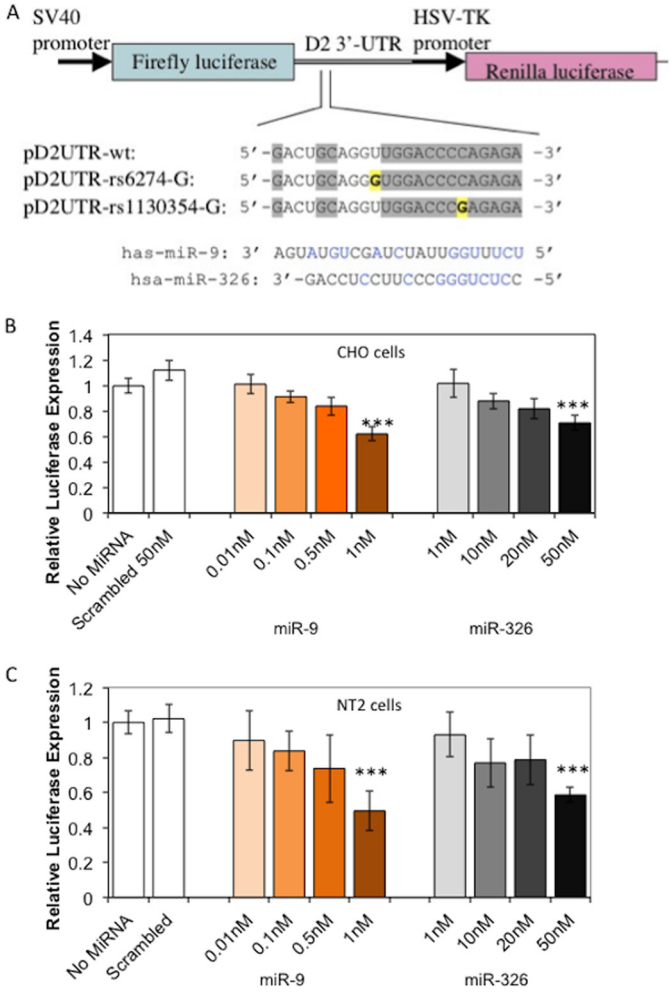

FIGURE 2. **Luciferase reporter constructs and reporter gene assay.** *A*, the reporter gene vector (psiCHECK) containing the 3′-UTR of *DRD2*, showing one putative target site shared by miR-9/miR-326. *Gray-shaded letters* in each construct represent conserved sequences, and *yellow letters* indicate the minor alleles of rs6274 and rs1130354. *Letters in blue* represent the miR nucleotides complementary to the target sequence. miR-9 and miR-326 inhibited the reporter gene (luciferase) expression through targeting the downstream *DRD2* 3′-UTR in transfected CHO cells (*B*) and NT2 cells (*C*). Concentration of both miRs had significant effect on reporter gene expression in both CHO cells (ANOVA $p < 0.0001$) and in NT2 cells (ANOVA $p < 0.0001$ for miR-9 and <0.0002 for miR-326). We co-transfected cells with pD2UTR-wt and synthetic pre-miRs. We measured luciferase activity at 48 h post-transfection and present data as the average ± S.D. from at least 3 independent experiments, each with 3 measurements for each condition. *** indicates $p < 0.0001$ from Student's *t* test.

(Mesoderm). Human iPSCs were cultured on mouse embryonic fibroblasts (Global Stem) in standard human embryo stem cell media (53). For DA neuron differentiation, we adopted a floor-plate-based midbrain DA differentiation method as previously described (53). In brief, we grew ~2.2 million iPSCs on a 10-cm dish pre-coated with Matrigel (BD Biosciences) for 11 days for floor-plate induction by exposing cells to a combination of LDN193189, SB431542, SHH C25II, Purmorphamine, FGF8 and CHIR99021. On day 11 we changed the media to Neurobasal/B27/L-Glut containing medium (NB/B27) (Invitrogen). On day 13 we changed the media to differentiation media (NB/B271, BDNF, ascorbic acid, glial cell-derived neurotrophic factor, dibutyryl-cAMP, TGFb3 and DAPT). On day 20, we dissociated and replated the cells at high density ($300-400 \times 10^3$ cells/cm^2) on 6-well plates and coverslips precoated with poly-D-lysine/laminin in differentiation medium. We kept the neurons in differentiation until the day for a certain experiment. For single neuron gene expression analysis to determine the percentage of DA neurons (TH+ neurons), we manually picked single neurons by serial dilution into wells on 96-well plate and directly lysed cells and carried out reverse transcription using CellsDirect™ One-Step qRT-PCR kit (Invitrogen). Quantitative PCR was performed on Fluidigm BioMark system as described below. We assayed expression of TH, MAP2, and GAPDH, and only samples with all three replicates of a gene assay passing the Ct threshold were considered "expressed."

Real-time Quantitative PCR—We reverse-transcribed total RNAs extracted from transfected cells or iPSC/neurons using TaqMan® Reverse Transcription Reagents (Invitrogen). We used 350 ng of RNA per 20-μl reverse transcription reaction with both random hexamers and oligo(dT)$_{16}$ to prime the reaction. To quantify gene expression, we used 2 μl of the reverse-transcribed cDNA in the subsequent real-time PCR using TaqMan gene expression assays (Invitrogen) in combination of TaqMan universal PCR Mastermix (Invitrogen). For miR quantification, we used the TaqMan® MicroRNA Reverse Transcription kit in the reverse transcription reaction and the TaqMan® MicroRNA Assay (for miR-9, miR-326 and RNU48) in real-time PCR. For expression quantification in NT2 cells, we carried out PCR in a 20-μl reaction with 4 replicates on a 384-well plate on a 7900HT (Invitrogen) with standard real-time PCR cycling conditions. For expression quantification in iPSCs and DA neurons, we carried out multiplex quantitative PCR on BioMark microfluid Dynamic Gene Expression Array (Fluidigm). We performed pre-amplifications of the reverse-transcribed cDNA products (also known as Specific Target Amplification) separately for miRNA and mRNAs (DRD2 and other genes) following the protocol recommended by Fluidigm. The preamplified products were then diluted (1:4 for miRNA and 1:2.5 for mRNA) and pooled together with equal amounts followed by multiplex PCR on a 48.48 Dynamic Gene Expression Array (Fluidigm) with three technical replicates. We used the standard Δ-Ct method (Invitrogen) for relative expression quantification. Expression of *GAPDH* was used as an endogenous control to normalize the mRNA abundances, and the expression of RNU48 was used as an endogenous control to normalize the expression of miRs.

Western Blotting—To quantify the *DRD2* expression at the protein level, we plated NT2 cells at 2.5×10^5 cells per well on a 6-well plate 24 h before transfection with different miRs or anti-miRs as described above. We lysed cell cultures after 48 h and extracted proteins using the mirVana PARIS kit from each cell culture. We prepared cell lysate with SDS and then ran the cell lysate on a 4–20% precast linear gradient polyacrylamide Bio-Rad Ready Gel (Bio-Rad). We transferred protein bands onto a nitrocellulose membrane via the semidry transfer method. We then blocked the membrane with 5% nonfat milk (Bio-Rad) in 5% PBS-Tween for 1 h and then incubated sequentially with mouse monoclonal antibody against human *DRD2* receptor (sc-5303) and with goat anti-mouse HRP (horseradish peroxidase) (sc-2005) with washing between incubations. We probed the expression of the endogenous control β-actin by HRP-conjugated goat polyclonal IgG antibody against actin (C-11) (sc-1615-HRP). We purchased all antibodies from Santa Cruz Biotechnology and used them at the manufacturer's recommended dilution. We exposed the membrane with Super-Signal West Femto Chemiluminescent Substrate (Thermo Scientific). The amounts of DRD2 and β-actin on gel images from Western blots were quantified by ImageJ 1.47v (54).

Transcriptome Data of Developing Human Brain Tissues—We obtained the miRNA sequencing data and RNA sequencing (RNA-seq) data of developing brain tissues from the Allen Institute for Brain Science-BrainSpan Atlas of the Developing Human Brain. The full data set includes 1,620 miRNAs of 215 brain samples and 52,376 genes (Gencode v10) of 524 brain samples. These samples are from various postmortem brain regions of developmental stages of different individuals. We extracted 185 samples (for 16 brain regions) with expression data available for both the *DRD2* and the two miRNAs, miR-326 and miR-9 (supplemental Table 1). Details of tissue acquisition, RNA-seq and miRNA-seq data processing, and data normalization can be found in the technical white paper BrainSpan Atlas of the Developing Human Brain.

Statistics and Bioinformatics—We used Student's *t* test to assess the significance of differential expression of reporter gene and endogenously expressed *DRD2* between different experimental conditions. Two-tailed *p* values were reported. In addition, for each miR with >3 experimental groups, we also performed ANOVA to test for differences across different groups. We extracted genomic sequences from the UCSC genome browser and the miR target sites on the 3′-UTRs of *DRD2* from the UCSC genome browser tracks annotated by TargetScan (55) and by PicTar (56). We predicted the RNA folding structures between miRs and targeting sequences (*i.e.* miR/target duplexes) and the minimum folding energy (ΔG) using RNAhybrid (57). We calculated LD between different SNPs of *DRD2* gene with Haploview (58).

RESULTS

Both miR-9 and miR-326 Interact with the 3′-UTR of DRD2 and Repress Reporter Gene Expression—We used the Dual-Luciferase Reporter Assay System (psiCHECK™-2) to determine whether miR-9 and miR-326 interact with the 3′-UTR of *DRD2* mRNA, thereby inhibiting the reporter gene (luciferase) expression. In the recombinant reporter gene construct, *DRD2*

3′-UTR sequence spanning the predicted binding sites of miR-9 and miR-326 was positioned downstream of the firefly luciferase open reading frame in the psiCHECK™-2 vector (Fig. 2*A*). Transfected pre-miR-9 or pre-miR-326 exhibited a dose-responsive negative effect on reporter gene expression in both NT2 and CHO cell lines. Pre-miR-9 at 1 nM induced ~40% reduction of luciferase activity, and pre-miR-326 at 50 nM induced ~50% reduction of luciferase activity (Fig. 2, *B* and *C*).

miR-9 and miR-326 Inhibit Endogenously Expressed DRD2 in NT2 Cells—A reporter gene assay proved the direct targeting of the two miRs to the 3′-UTRs of *DRD2*. We next examined whether the endogenously expressed *DRD2* is subject to the regulation by the two miRs in NT2, a cell line that endogenously expresses miR-9, miR-326, and *DRD2* (data not shown) and has been used as a model of dopaminergic neurons for studying dopamine signaling (59–62). For each miR, we tested whether overexpression of exogenous pre-miRs can inhibit endogenous expression of *DRD2* and whether knocking down the endogenous miRs can increase expression of *DRD2*. Consistent with the reporter gene assay results, overexpression of pre-miR-326 in NT2 cells showed an inhibitory effect on endogenously expressed *DRD2* mRNA, with ~40% reduction of the *DRD2* mRNA level with 50 nM pre-miR-326 (Fig. 3*A*). At the protein level, overexpression of pre-miR-326 (50 nM) also led to reduced DRD2 receptor expression (Fig. 3*B*). As expected, transfection of antisense 2′-*O*-methyl-modified oligoribonucleotide (63, 64) for miR-326 (anti-miR-326; at 45 nM) in NT2 cells increased DRD2 protein abundances, presumably through blocking the inhibitory effect of endogenously expressed miR-326 on *DRD2* expression (Fig. 3*B*).

Surprisingly, overexpression of pre-miR-9 in NT2 cells did not reduce endogenously expressed *DRD2* either at the mRNA or the protein levels (Fig. 3, *A* and *B*). Intuitively, this seemed to contradict to the observed high efficacy of miR-9 in inhibiting reporter gene expression (Fig. 2). However, the high efficacy of exogenous miR-9 as measured in the reporter gene assay (Fig. 2) does not necessarily translate into effective inhibition of endogenously expressed *DRD2* by exogenous miR-9 in Fig. 3. It is known that the inhibitory effect of an exogenous miR on its target gene expression may not be detectable in cells where the same miR is endogenously expressed at a high levels, whereas its target gene has relatively low expression (65). In our cellular model miR-9 is expressed at a high level (~8-fold more than miR-326) and *DRD2* at a much lower level (data not shown). The endogenous miR-9 is expressed in such a high level that it may have already saturated all the miR-9 targeting site of the lowly expressed *DRD2*. Consequently, the transfected exogenous miR-9, even at a high concentration (50 nM), did not show any additive effect on the endogenous expression of *DRD2*. To test for the endogenous miR-9-mediated repression of DRD2, we transfected the cells by antisense 2′-*O*-methyl-modified oligoribonucleotide (63, 64) of miR-9 (anti-miR-9). As predicted, overexpression of anti-miR-9 (45 nM) in NT2 cells led to a substantial increase of *DRD2* protein abundances (Fig. 3*B*), suggesting a strong endogenous inhibitory effect of miR-9 on *DRD2* expression in NT2 cells.

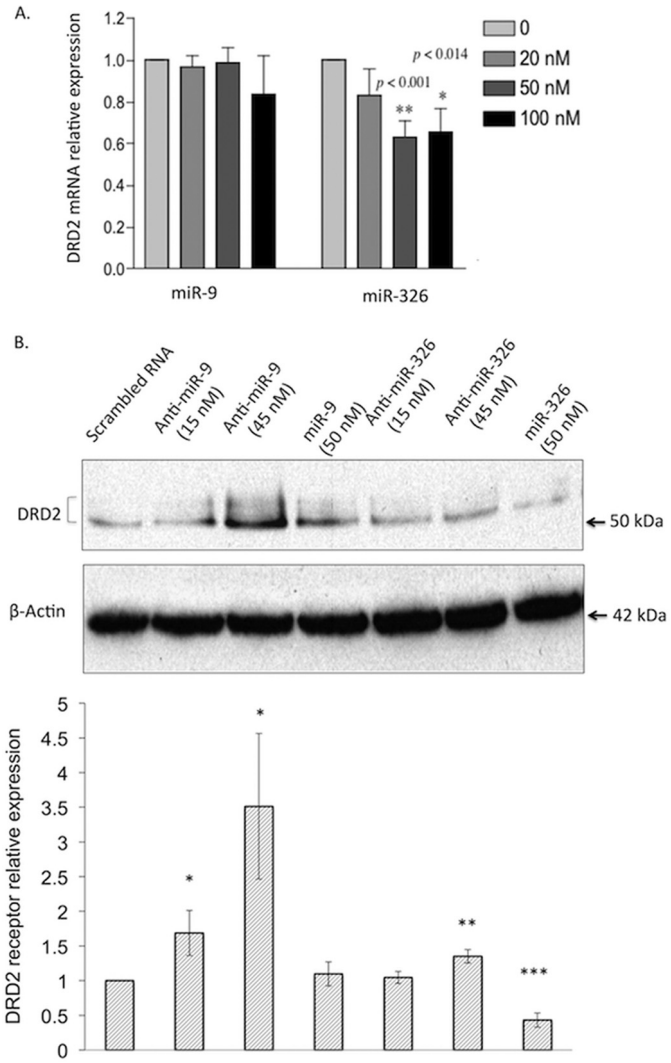

FIGURE 3. **miR-9 and miR-326 inhibited endogenous *DRD2* expression in NT2 cells.** *A*, effect of different concentrations of exogenous miR-9 and miR-326 on endogenously expressed *DRD2* mRNAs, with data presented as the average ±S.D. from at least three independent experiments each with 3 measurements for each condition. Different concentrations of exogenous miR-9 did not have significant effects on DRD2 expression (ANOVA $p = 0.35$), but concentration of miR-326 significantly influenced DRD2 expression (ANOVA $p < 0.0001$). *B*, Western blot showing the repression of DRD2 expression at the protein level by endogenously expressed miR-9 and miR-326. We harvested cell lysates 48 h post-transfection with miRs or anti-miRs, directly followed by Western blot, using β-actin as a control to normalize the DRD2 expression. The *top panel* shows a representative Western blot, and the *bottom panel* shows the relative DRD2 expression (*i.e.* ratio to the DRD2 expression in cells transfected with scrambled small RNAs). Anti-miR-9 (15 and 45 nM) and anti-miR-326 (45 nM) increase the endogenous expression of DRD2 receptors. Overexpression of miR-326 but not miR-9 inhibits the endogenous expression of DRD2 receptors. Data are presented as the average ± S.D. from four independent experiments. * indicates $p < 0.05$; ** indicates $p < 0.01$, and *** indicates $p < 0.001$ from Student's *t* test.

Genetic Variation in the Seed Sequence of the DRD2 3′-UTR Alters the Effect of miR-326—rs1130354 is within the 7-bp "seed sequence" shared by miR-9 and miR-326, whereas rs6274 is outside the seed sequence (Fig. 1); both SNPs thus could possibly affect miR interactions. For each SNP, we transfected a reporter gene construct containing the *DRD2* 3′-UTR sequence with one of the two alleles together with synthetic precursor miRs into CHO and NT2 cell lines (Fig. 2). As shown in Fig. 4, *A* and *B*, the effect of exogenous miR-9 or miR-326 on reporter

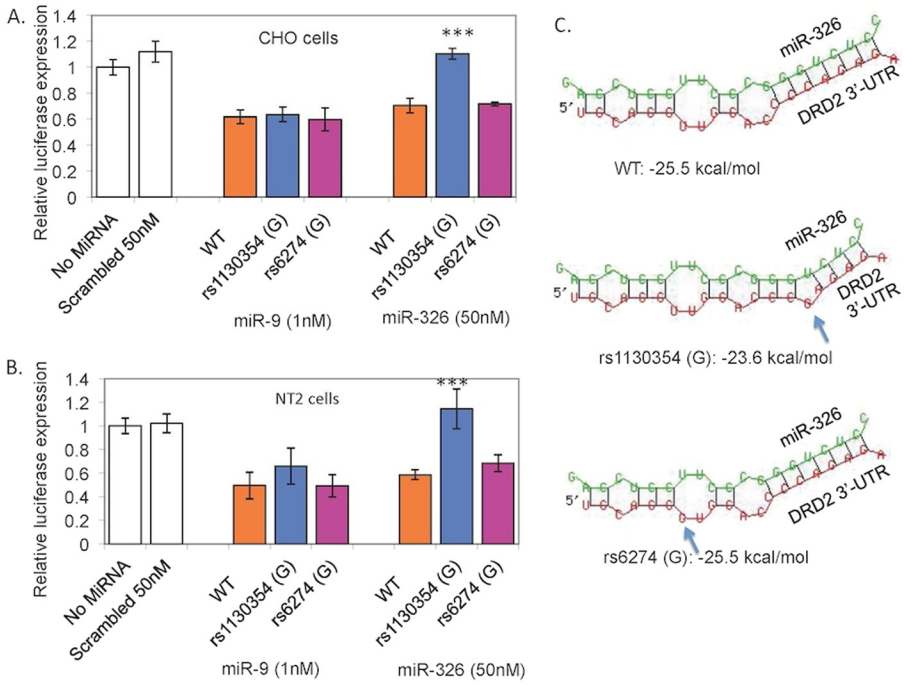

FIGURE 4. **Both reporter gene assay and *in silico* RNA-folding structure analysis supported functionality of the allele G of rs1130354 (but not rs6274).** Allele G of rs1130354 abolished the miR-326 (but not miR-9)-mediated inhibition of luciferase activity in transfected CHO cells (*A*) and NT2 cells (*B*). For both CHO and NT2 cells, SNP did not affect the inhibitory effect of miR-9 on reporter gene expression (ANOVA $p > 0.05$), but it did influence the inhibitory effect of miR-326 on reporter gene expression (ANOVA $p < 0.0001$). *** indicates $p < 0.0001$ from Student's *t* test. *C*, effects of minor alleles of rs1130354 and rs6274 on RNA-folding structures between miR-326 and *DRD2* 3′-UTR. Allele G of rs1130354, but not rs6274, disrupts the stem part of the typical stem-loop RNA-folding structure required for miR function. The *arrow* indicates the SNP site on *DRD2* 3′-UTRs in each folding structure.

gene expression in cells carrying the minor allele G of rs6274 (pD2UTR-rs6274-G) was similar to that in cells transfected with the T allele (pD2UTR-wt), suggesting rs6274 did not disrupt the base pairing between either of the miRs and 3′-UTR of *DRD2* mRNA. However, the minor allele G of rs1130354 (pD2UTR-rs1130354-G) abolished the inhibitory effect of miR-326. Furthermore, we observed a complete reversal of the miR-326-mediated repression of the reporter gene expression by allele G of rs1130354, indicating that the sequence flanking rs1130354 is likely the only miR-326 target site at the 3′-UTR of *DRD2* mRNA, consistent with the bioinformatics prediction by TargetScan (Fig. 1*B*) but not by PicTar (Fig. 1*A*). The fact that rs1130354 only influences the effect of miR-326 but no miR-9 on *DRD2* suggests miR-9 and miR-326 do not share the seed sequence for targeting *DRD2* 3′-UTR, *i.e.* both RNAs do not compete with each other at the SNP site.

The observed effect of rs1130354 and rs6274 was consistent with *in silico* analysis (57) of the SNP effect on RNA-folding structures formed by miR326 and its target sequence in *DRD2* (Fig. 4*C*). With the major alleles of both SNPs, the base-pairing between *DRD2* 3′-UTR mRNA and the mature miR-326 results in a typical "stem-loop" structure essential for miR-mediated expression repression. The minor (or "mutant") allele G of rs1130354 disrupts the stem part of this essential structure, and the minimum folding energy (ΔG) of the structure increases from −25.5 kcal/mol (major allele) to −23.6 kcal/mol (*i.e.* a less stable secondary RNA folding structure). rs6274 does not show any allelic effect on the stem-loop folding structure or the minimum folding energy.

Next, we attempted to determine whether rs1130354 functions independently from other known regulatory variants of DRD2, namely, the promoter SNP rs1799732 (−141C Ins/Del) (28), the missense SNP rs1801028 ($Ser^{311}\rightarrow Cys$) (23), the functional synonymous SNP rs6277 (C975T) (32), and the intronic splicing SNPs rs2283265 and rs1076560 (30). We genotyped rs1130354 in our MGS European ancestry sample in which genotype data were already available (48, 49) for the other reported functional SNPs listed above. We found that rs1130354 is a rare SNP in our MGS sample (minor allele frequency = 0.4%) and is not in LD with any other known functional variant (Fig. 5). This indicates that the functional allele of rs1130354 is not specifically in the same haplotype background with any other reported functional alleles in an individual and thus functions independently of them.

miRNA Regulation of DRD2 in Differentiating Dopaminergic Neurons and in Human Brains—In human neurons and brain tissues, we further evaluated the physiological relevance of the observed post-transcriptional regulation of *DRD2* by miR-326 and miR-9. Given the predominate role of the classical DA hypothesis of SZ pathogenesis, we first examined the dynamic expression changes of *DRD2* and the two miRs during DA neuron differentiation. We generated three iPSC lines from different individuals and then differentiated the iPSCs into DA neurons (Fig. 6, *A*--*C*). We have achieved high efficiency (80%) of dopaminergic neuron differentiation as previously reported (53). In a single cell gene expression analysis (quantitative PCR on Fluidigm BioMark system) of 36 manually picked single cells from the day 30 DA neurons, 35 are MAP2 positive, of which 28 are TH+ (80%). This allowed us to measure miRs and DRD2 in a relatively homogenous DA neuron population. We found a substantial increase of *DRD2* expression in early stage of DA neurons (day 20) compared with iPSCs (Fig. 6*D*) (~95-fold

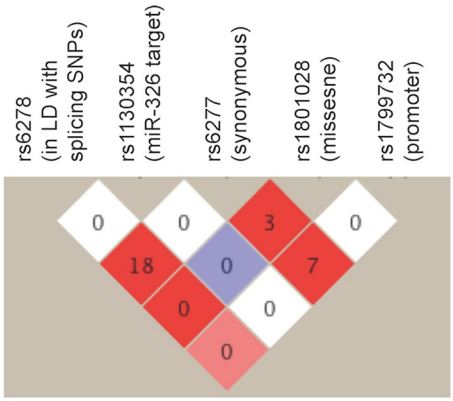

FIGURE 5. **LD between the functional rs1130354 and other previously reported regulatory variants in *DRD2*.** The *number in each square* is the pair-wise r^2 value between SNPs, and the color represents the D′ of the comparison (*white* = 0 to *red* = 1).

increase; $p = 4.7 \times 10^{-5}$); thereafter, the *DRD2* expression gradually decreased overtime with a 3.5-fold of decrease in day-45/50 neurons compared with day-20 neurons; $p = 0.047$). Interestingly, both miR-9 and miR-326 showed an inverse correlation of expression changes with the *DRD2* (Spearman's rank correlation $r = -0.4$) during the DA neuronal differentiation, suggesting a contribution of post-transcriptional regulation of *DRD2* by both miRs.

Such inverse expression correlation between *DRD2* and both miRs (miR-9 and miR-326) also exists in human brains. Among 16 brain regions with digital expression data available for *DRD2* and both miRs (BrainSpan Atlas of the Developing Human Brain), we found negative Spearman's rank correlation of expression between *DRD2* and both miRs in six brain regions (dorsolateral prefrontal cortex, inferolateral temporal cortex, ventrolateral prefrontal cortex, amygdaloid complex, cerebellar cortex, and striate cortex) (Fig. 7). There were 4 additional brain regions (hippocampus, anterior medial prefrontal cortex, orbital frontal cortex, posterior superior temporal cortex) that showed negative correlation of *DRD2* expression with only miR-326 ($r = -0.09$ to -0.48; supplemental Table 2). Albeit the limited statistical power due to small sample size ($n = \sim 10$) for each brain region, the inverse correlation between *DRD2* and miR-326 was found statistically significant in three brain regions, dorsolateral prefrontal cortex ($r = -0.90$, $p = 0.0003$), inferolateral temporal cortex ($r = -0.57$, $p = 0.033$), and ventrolateral prefrontal cortex ($r = -0.58$, $p = 0.039$) (Fig. 7), suggesting a stronger effect of miR-326 than miR-9 on *DRD2* expression changes.

DISCUSSION

DRD2 plays a central role in dysregulation of dopamine signaling pertinent to SZ (5–9). Here, we show that miR-326 and miR-9 can affect DRD2 expression and that the targeting of miR-326 to *DRD2* mRNA is disrupted by a rare functional SNP (rs1130354) within the seed sequence of *DRD2* 3′-UTR. We have demonstrated the miR-mediated post-transcriptional regulatory mechanism of *DRD2* in a neuronal cell model (NT2) co-expressing miR-9, miR-326, and *DRD2* mRNA/protein. We further showed the pathophysiological relevance of the miR-mediated regulation of *DRD2* in differentiating human DA neurons derived from iPSCs and in developing human brains. Given the central role of *DRD2* in classic hyperdopaminergic hypothesis of SZ and its involvement in multiple other neuropsychiatric disorders (2–9), our study suggests a possible role of miR-9 and miR-326 in the pathophysiology of these disorders through modulating the *DRD2* expression. Furthermore, as most antipsychotic drugs influence dopaminergic transmission through blocking DRD2 receptors, the demonstrated regulation of DRD2 by the two miRs may provide potential drug targets for treatment of disorders involving abnormal DRD2 function.

With reporter gene assays in two different cell lines, we confirmed the interactions between the 3′-UTR of *DRD2* mRNA and exogenous miR-9 and miR-326. We also confirm the targeting of endogenous *DRD2* mRNA by endogenously expressed miR-9 and miR-326 in NT2 cells. Our results contradict some bioinformatic predictions. For instance, as opposed to the PicTar prediction that there are multiple targeting sites of miR-326 in the 3′-UTR of *DRD2*, this study suggests the existence of a single miR-326 targeting site, because allele G of the functional rs1130354 completely reverses the miR-326-mediated repression of reporter gene expression (Fig. 4, *A* and *B*). Furthermore, although both miR-326 and miR-9 are predicted (PicTar) to share the same seed sequence, the seed-disrupting allele G of rs1130354 is found to only affect targeting of miR-326 to *DRD2* 3′-UTR. Thus, our results highlight the limitations of bioinformatic prediction of miR targets and support the previously proposed (65, 66) need for empirical validation of putative regulatory effects of an miR on its important target genes.

To explore the pathophysiological relevance of the observed miR-mediated DRD2 expression, we examined the co-expression of DRD2 and both miRs in differentiating DA neurons and in developing human brains. Co-expression (miRNA and targeted mRNA) analysis has becoming a general paradigm to identify *bona fide* targets and infer biological roles of miRNAs (67). We identified inverse expression correlations between *DRD2* and the two miRs (miR-9 and miR-326) in the course of DA neuron differentiation from iPSC (Fig. 6) and in developing human brains (Fig. 7), suggesting a naturally occurring miR-9- and miR-326-mediated post-transcriptional regulation of *DRD2* expression. Although miR-326 was expressed in a much lower level than miR-9 across different brain regions (~676-fold less in average; Fig. 7 and supplemental Table 1), it showed an overall stronger effect than miR-9 on *DRD2* expression variations between different individuals. This was reflected by the observation of more brain regions showing negative expression correlation of *DRD2* with miR-326 ($n = 10$) than with miR-9 ($n = 6$) and with a bigger magnitude of effect from miR-326 (Fig. 7). The weaker expression correlation of *DRD2* and miR-9 could be explained by the unparalleled high level of miR-9 expression than *DRD2* expression, which may lead to the saturated binding of miR-9 on *DRD2* 3′-UTR and consequently makes the *DRD2* expression less sensitive to the expression changes of miR-9. The less pronouncing effect of miR-9 on *DRD2* expression variation in the brain is consistent with our observation from the *in vitro* cellular model NT2 cells, where overexpression of exogenous miR-9 did not result in significant changes of DRD2 expression (Fig. 3, *A* and *B*). It is noteworthy

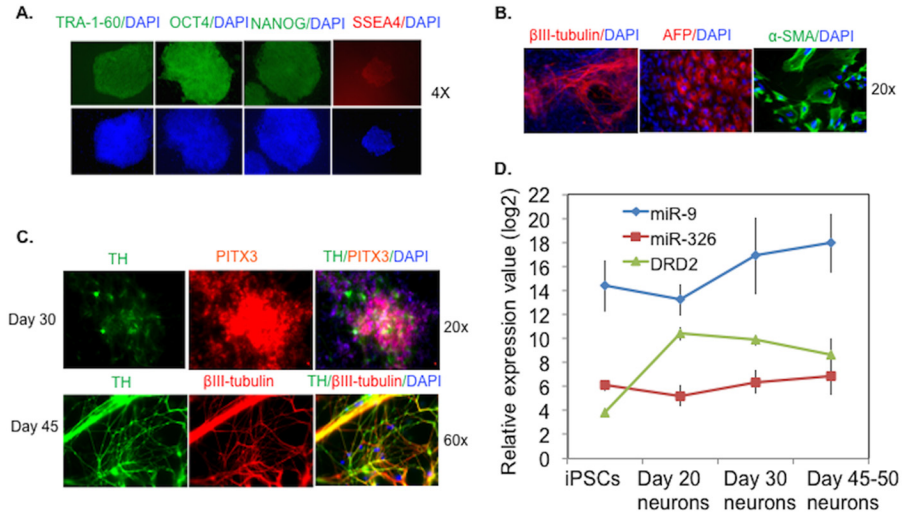

FIGURE 6. **Dynamic expression changes of *DRD2*, miR-9 and miR-326 during DA neuron differentiation from human iPSCs.** *A*, human iPSCs were characterized by positive immunofluorescence staining for pluripotent stem cell markers TRA-1–60, OCT4, NANOG, and SSEA4 (4× magnification). Three iPSC lines were generated from three different subjects. *B*, iPSCs were subjected to DA neuronal differentiation for 30 days (20× magnification) and 45 days (60× magnification). Neurons are βIII-tubulin+, and DA neurons are TH+ and PITX3+. DAPI stains nuclei. *C*, inverse correlation of *DRD2* expression with miR-9 and miR-326 expressions during DA neuronal differentiation. *DRD2* expression values were normalized to endogenous control *GAPDH*. Expression values of the two miRs were normalized to endogenous small RNA control RNU48. The *y* axis shows the relative expression values in log 2 scale. Multiplex quantitative PCR was used for expression quantification with three technical replicates per iPSC line, and the graphed data are the averages of the three iPSC lines.

that the strongest negative correlation of *DRD2* expression was found with miR-326 ($r = -0.90, p = 0.0003$) in the dorsolateral prefrontal cortex, a brain region where disturbances of glutamate, GABA, and dopamine neurotransmissions contribute to the deficits of working memory in schizophrenia (68). These results suggest the pathophysiological relevance of the miR-326-mediated post-transcriptional regulation of *DRD2*.

The demonstration of *DRD2* regulation by miR-9 and miR-326 may also have implications in understanding the biological relevance of the genetic associations recently identified in genome-wide association studies of SZ. It is noteworthy that the associations of *DRD2* and both miRs with SZ are nominally significant ($10^{-6} < p < 10^{-3}$) in the recent meta-analysis of genome-wide association studies of SZ (Psychiatric Genetics Consortium; PGC1) (69) and a combined analysis with Swedish sample (PGC2) (70). The nominally associated SNPs (and their LD proxies) in *MIR9–2* and *MIR9–3* (two of the three *MIR9* genes) are within ENCODE-annotated enhancers (histone methylation mark H3K4Me1) immediately upstream the *MIR9* genes and thus likely affect *MIR9* expression and may subsequently alter *DRD2* expression. Furthermore, *DRD2* and miR-9 have indirect functional connections to other genome-wide association study-implicated SZ loci. For instance, one of the expression repressors of miR-9, nuclear receptor subfamily 2 group E member 1 (*NR2E1*; also known as *TLX*) (71), is a target of miR-137 (72), the latter being one of the most strongly SZ-associated loci (69). Moreover, another genome-wide association study-implicated SZ susceptibility gene, *ZNF804A*, is a target of miR-137 (69, 73, 74) and also regulates transcription of *DRD2* (75). We[3] and others (76) have shown that the SZ risk allele of *MIR137* locus likely reduces *MIR137* expression. The SZ-risk allele at *MIR137* locus thus would increase *DRD2* expression through an intracellular regulation cascade of *MIR137-TLX-MIR9-DRD2*, which is congruent with the classic hyperdopaminergic hypothesis of SZ. Our empirical validation of the post-transcriptional regulation of *DRD2* by miR-326 and miR-9 thus suggests that miR-mediated dysregulation of a gene network involving *DRD2* may play an important role in SZ pathogenesis.

SNPs in miR target regions are implicated in the pathogenesis of, or susceptibility to some human disorders, *e.g.* hypertension (77), asthma (78), cancer (79, 80), and Tourette syndrome (81). The functional rs1130354 was not found associated with SZ in our MGS European ancestry sample (data not shown). However, rs1130354 (as well as the demonstrated effect of miRs on the expression of *DRD2*) might be important for other neuropsychiatric disorders and behavioral traits such as social detachment (82), neuroticism (27), and anxiety disorders (26), where dopamine and *DRD2* play a central role in their pathophysiology. The demonstration of the functionality of rs1130354 in altering miRNA326 targeting of *DRD2* thus warrants further an association test of rs1130354 with other neuropsychiatry disorders and behavioral phenotypes involving dysregulation of dopamine.

We acknowledge the limitation of our simplified cellular model for studying *DRD2* regulation. Despite the demonstrated post-transcriptional regulation of *DRD2* by both miR-9 and miR-326, transcriptional regulation likely remains a predominate mechanism in shaping the cellular RNA abundance (43). Furthermore, we only examined the post-transcriptional regulatory mechanism mediated by the 3′-UTR of *DRD2* without considering the possible combined functional effects from other known regulatory variants, such as the functional promoter, splicing, and coding synonymous SNPs (28, 30–32). Nonetheless, we revealed an miR-based (miR-9 and miR-326) post-transcriptional regulatory mechanism of *DRD2* expression. As *DRD2* activation in the brain initiates a cascade of intracellular signaling that modulates neurotransmission of

[3] J. Duan, J. Shi, C. Leites, D. He, W. Moy, and A. R. Sanders, manuscript in preparation.

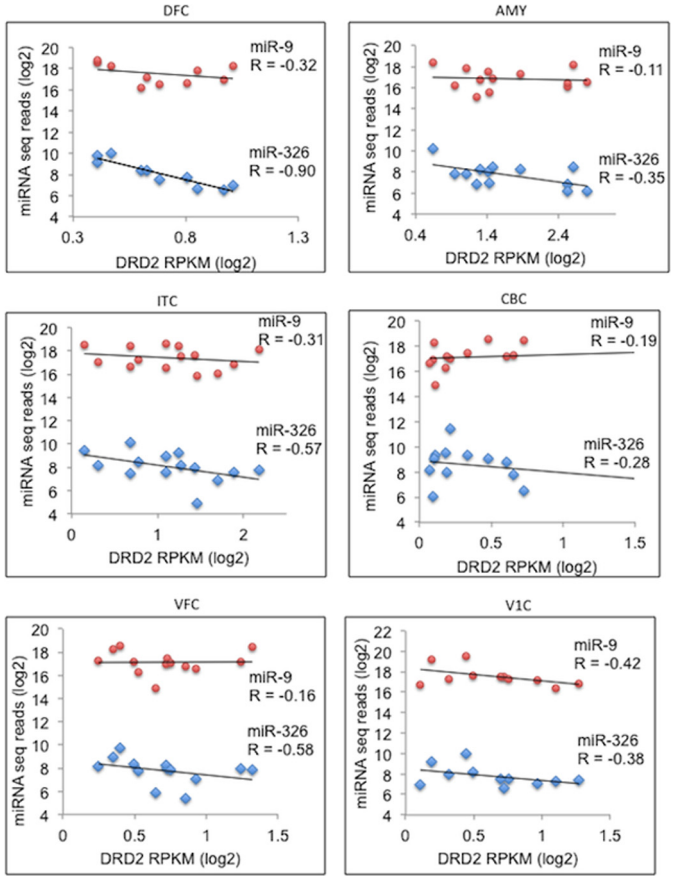

FIGURE 7. **Spearman's rank expression correlation between *DRD2* and miR-9 and miR-326 in developing human brains.** Listed are brain regions (*DFC*, dorsolateral prefrontal cortex; *ITC*, inferolateral temporal cortex; *VFC*, ventrolateral prefrontal cortex; *AMY*, amygdaloid complex; *CBC*, cerebellar cortex; *V1C*, striate cortex) that showed inverse expression correlation (*i.e.* negative R) between *DRD2* and miRs. In each panel, the *x* axis shows the expression values of *DRD2* (*RPKM*, reads/kb transcript/million reads; in log 2 scale), and the *y* axis shows the expression values of miR-9 (*red dots*) or miR-326 (*light blue dots*). Each *dot* represents a unique brain sample with age ranging from 4 months to 23 years old.

importance to the pathogenesis of several neuropsychiatric diseases (for review see Ref. 83), our study may help to understand how *DRD2* expression regulation is relevant to the pathogenesis of SZ and other neuropsychiatric disorders as well as to develop more effective clinical treatment of these disorders by modulating with the effect of miRs on *DRD2* expression.

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