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MicroRNA Alterations in Induced Pluripotent Stem Cell-Derived Neurons from Bipolar Disorder Patients: Pathways Involved in Neuronal Differentiation, Axon Guidance, and Plasticity

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Bipolar disorder (BP) is a complex psychiatric condition characterized by severe fluctuations in mood for which underlying pathological mechanisms remain unclear. Family and twin studies have identified a hereditary component to the disorder, but a single causative gene (or set of genes) has not been identified. MicroRNAs (miRNAs) are small, noncoding RNAs ~20 nucleotides in length, that are responsible for the posttranslational regulation of multiple genes. They have been shown to play important roles in neural development as well as in the adult brain, and several miRNAs have been reported to be dysregulated in postmortem brain tissue isolated from bipolar patients. Because there are no viable cellular models to study BP, we have taken advantage of the recent discovery that somatic cells can be reprogrammed to pluripotency then directed to form the full complement of neural cells. Analysis of RNAs extracted from Control and BP patient-derived neurons identified 58 miRNAs that were differentially expressed between the two groups. Using quantitative polymerase chain reaction we validated six miRNAs that were elevated and two miRNAs that were expressed at lower levels in BP-derived neurons. Analysis of the targets of the miRNAs indicate that they may regulate a number of cellular pathways, including axon guidance, Mapk, Ras, Hippo, Neurotrophin, and Wnt signaling. Many are involved in processes previously implicated in BP, such as cell migration, axon guidance, dendrite and synapse development, and function. We have validated targets of several different miRNAs, including *AXIN2*, *BDNF*, *RELN*, and *ANK3* as direct targets of differentially expressed miRNAs using luciferase assays. Identification of pathways altered in patient-derived neurons suggests that disruption of these regulatory networks that may contribute to the complex phenotypes in BP.

Keywords: bipolar disorder, patient-derived, stem cell

Introduction

MicroRNAs (miRNAs) are small, conserved, noncoding RNAs typically 19–24 nucleotides in length, which are capable of regulating multiple genes simultaneously. They contain short sequences in their 5′ ends that base pair with complementary sequences in messenger RNAs (mRNAs) [1]. This interaction typically leads to gene repression by targeting the mRNA for degradation [2–4] or by blocking the binding of translation machinery to mRNAs, thereby preventing translation [5]; however, it has also been shown that miRNA binding can activate translation in certain circumstances [6–8]. miRNA-binding sites on target RNAs are somewhat repetitive in the genome and can be found in many different mRNAs, giving each miRNA the ability to regulate multiple genes simultaneously [9] and allowing multiple miRNAs to target a single gene.

Since their discovery in *Caenorhabditis elegans* in 1993 [10], over 2,500 miRNAs have been identified in humans, and are believed to regulate 70%–90% of all human genes [11]. They have been shown to play roles in neural development [12–15], as well as in the adult brain [16,17], where they show region-specific expression patterns [18]. Expression changes in response to neuronal activity [19] and to stress [20], can regulate dendritic spine number [21] and circadian rhythm [17], and promote long-term potentiation [22]. miRNA levels have been shown to be dysregulated in postmortem brain tissue [23,24] and plasma [25] of patients with bipolar disorder (BP), and the mood stabilizer lithium alters the expression of several miRNAs both in vivo [25,26] and in vitro [27].

BP is a complex, chronic psychiatric illness characterized by pathological mood fluctuations ranging from mania/hypomania to depression, interspersed with periods of

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euthymia. The underlying biological mechanisms remain unclear [28]. Disease onset typically occurs in early adulthood or adolescence [29] and brain imaging studies of children and adolescents diagnosed with BP have identified decreases in the volume of several brain regions compared with unaffected individuals, including the dorsolateral prefrontal cortex [30], ventrolateral prefrontal cortex [31], anterior cingulate cortex [32–34], amygdala [30,34–39], and hippocampus [39,40]. The exact causes of these structural abnormalities are unclear, but they are likely to result from a complex interaction of environmental factors and multiple genes that affect neural development.

Family and twin studies have identified a hereditary component to BP [41–50], estimated to be >70%, but a causative gene has not been identified [51]. In fact, genome-wide association studies have identified numerous loci that confer susceptibility to BP although each with an odds ratio of 1.05–1.13 [52]. This suggests that many genes in different pathways each with a small effect, may contribute to the phenotype. Since miRNAs have the ability to regulate multiple genes simultaneously and their expression changes in response to stress, neuronal activity, and psychiatric drugs, miRNA dysregulation may be a potential mechanistic link between genetic anatomy and clinical manifestation.

Materials and Methods

Stem cell derivation

Skin biopsies (3 mm) were taken from patients diagnosed with BP and from control individuals with no psychiatric diagnosis. Briefly, patient fibroblasts were derived, expanded, and reprogrammed to pluripotency [53]. Clonal lines were selected, tested for karyotype, germ lineage differentiation capacity, and for mycoplasma. Selected induced pluripotent stem cell (iPSC) lines were expanded to freezer stock, differentiated into neural precursor cells (NPCs), then into neurons [53]. All procedures were approved by the relevant University of Michigan oversight committees. Skin biopsies were obtained with informed consent using procedures approved by the Institutional Review Board-Medicine (IRBMED-HUM00043228). Protocols for reprogramming skin cells to pluripotency were approved by the Human Pluripotent Stem Cell Review Oversight Committee (No. 1048). All materials were deidentified before transferring them to the laboratory.

Neural differentiation of iPSCs

iPSCs were grown on mouse embryonic fibroblasts (MEFs) in human embryonic stem cell (hESC) medium (Dulbecco's modified Eagle's medium: nutrient mixture F-12 [DMEM/F12], 20% KOSR, 1×nonessential amino acid [NEAA], 0.5×GlutaMAX, and 20 ng/mL FGF2) until they reached 80% confluence. iPSC colonies were then manually cut and scraped to remove them from MEFs. Cells were pelleted by centrifugation, resuspended in hESC medium without FGF2, containing 2 μM dorsomorphin dihydrochloride and 5 μM SB431542, and grown in suspension to form embryoid bodies (EBs). After 4 days, EBs were switched to Neural Induction Medium (NIM; DMEM/F12, 1×N2 Supplement, 1×NEAA, 1×GlutaMAX, 1×Pen/Strep, 20 ng/mL FGF2) for 3 days. On the following day (day 8 of differentiation), EBs were plated on tissue culture dishes coated with poly-L-ornithine (20 μg/mL) and laminin (10 μg/mL) in NIM. Within 5–7 days of plating EBs, neural rosettes formed. Rosettes were manually passaged twice to enrich for NPCs. After the second passage, rosettes were dissociated into single cells in 25% Accutase (diluted in Dulbecco's phosphate-buffered saline without calcium and magnesium) and plated onto dishes coated with poly-ornithine and laminin in NPC medium (Neurobasal Medium, 1×B27 Supplement, 1×NEAA, 1×GlutaMAX, 1×Pen/Strep, 20 ng/mL FGF2) to form a monolayer of NPCs, which were expanded and banked. For differentiation into neurons, NPCs were dissociated in Accutase and plated onto dishes coated with poly-ornithine and laminin at a density of 50,000 cells/cm^2 in NPC medium to form a monolayer of NPCs. One to two days after plating, NPC medium was switched to Neural Differentiation Medium (NDM; Neurobasal Medium, 1×B27 Supplement, 1×NEAA, 1×GlutaMAX) and cells were differentiated for 28 days.

Phenotype analysis

Transcript expression in neurons differentiated from NPC for 4 weeks was examined in quantitative polymerase chain reaction (qPCR) and using immunocytochemistry to assess protein expression. For immunocytochemistry, cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed in phosphate-buffered saline (PBS) and stored at 4°C in PBS containing 0.1% sodium azide. Cells were then exposed to primary antibodies (βIII tubulin, nestin, etc.), followed by secondary antibody conjugated to Cy3 or FITC. The extent of differentiation was assessed and cells photographed in a Leitz inverted fluorescence microscope.

RNA extraction and PCR analysis

After 28 days of neuronal differentiation, total RNA was extracted from neurons using the mirVana miRNA Isolation Kit from Thermo Fisher (AM1560) following the manufacturer's instructions. RNA concentrations were calculated spectrophotometrically and samples stored at −80°C in 4 μg aliquots. For qPCR, complementary DNA (cDNA) was prepared from RNA aliquots by treatment with Dnase I (Thermo Fisher Scientific) and reverse transcribing it using Superscript III (Invitrogen) and random nonamers. The cDNA was then used with iTAq Universal SYBR Green Supermix in qPCR reactions to examine the expression of stem cell or neuronal markers. Primer sequences are available on request.

Analysis and qPCR validation of miRNAs

RNA aliquots from three control and three BP patient-derived neuron samples were submitted to the Microarray Core Facility at the University of Michigan, where RNAs were labeled using the Flashtag Biotin RNA-Labeling Kit and analyzed using miRNA 4.1 (Illumina) microarrays. After hybridization, RMA was used to calculate expression values for each probe set. TaqMan mRNA Assays (Life Technologies) or the MystiCq miRNA Quantitation System (Sigma) was used to validate expression of three miRNAs elevated in control neurons and six miRNAs elevated in BP-derived neurons.

Identification of differentially expressed miRNAs

Two hundred and seventy-five probe sets with a threshold fold change of ≥1.5 were selected, then *P* values adjusted for multiple comparisons using an false discovery rate (FDR) of 0.05. These were selected for additional analysis using the Limma package of bioconductor implemented in the R statistical environment and using Database for Annotation, Visualization, and Integrated Discovery (DAVID) [54].

Target identification and analysis

To identify and analyze potential gene targets of each differentially expressed miRNA, we used four miRNA databases, including miRDB (http://mirdb.org/miRDB)—which ranks targets based on the likelihood that they contain a complementary seed sequence for a specific miRNA—to generate the final list of target genes potentially regulated by the miRNAs. Using an 80% probability cut off produced 1,263 targets predicted to be upregulated by miRNAs downregulated in BP neurons and 2,724 targets predicted to be downregulated by miRNAs upregulated in BP neurons. Hierarchical Cluster Analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and Gene Ontology (GO) classifications were then carried out in DAVID on targets of significantly altered miRNAs.

Construction of miRNA expression vectors

Genomic DNA sequences for miRNAs were obtained from the UCSC Genome Browser (https://genome.ucsc.edu). Primers containing *Eco*R1 (5′ end) and *Xba*1 (3′ end) restriction sites were used to clone each miRNA (with 100 base pairs flanking both ends of the primary miRNA sequence) from H1 (WA01) human embryonic stem cell genomic DNA. miRNAs were cloned into a modified version of the pUS2 vector (D. Turner, University of Michigan) containing an mCherry coding sequence upstream of the miRNA cloning site and a puromycin resistance cassette cloned into the second multiple cloning site (MCS).

Construction of miRNA target expression vectors

Gene-specific primers for selected targets were designed to make cDNA corresponding to a region containing the miRNA-binding site for each potential target. An 800-base pair portion of this cDNA with the miRNA-binding site in the center was then amplified by PCR using primers containing *Pme*I (5′ end) and *Sbf*I (3′ end) restriction sites. Potential gene targets were cloned from human fetal brain RNA (Invitrogen), except *BDNF* which was cloned from human iPSC-derived neurons and *NLGN1*, which was cloned from H1 hESC genomic DNA. The potential miRNA targets were then cloned into the 3′ untranslated region (UTR) of firefly luciferase in the pmirGLO dual-luciferase miRNA target expression vector (No. E1330; Promega). Once the miRNA target expression vectors were complete, site-directed mutagenesis by primer extension was used to mutate potential miRNA-binding sites into random sequences to use as negative controls. The mutated PCR products were gel extracted, digested, with *Pme*I and *Sbf*I restriction enzymes, and ligated back into the 3′ UTR of firefly luciferase in the pmirGLO vector to create scrambled control vectors.

Target validation

A dual luciferase assay (pmirGLO; Promega) was employed to test selected potential miRNA targets. The 3′ UTR (containing the complementary seed sequence) of each was cloned into the MCS of the pmirGLO vector. This vector was cotransfected into NIH 3T3 cells with a miRNA overexpression vector containing mCherry, pPUS2c. On binding of the miRNA to the target, transcription of firefly luciferase, *luc2*, is blocked and no signal is detected. The humanized *Renilla* luciferase-neomycin resistance casette, *hRluc*-neo, was used as a control reporter to normalize gene expression. To determine the peak expression of the miRNAs, NIH3T3 cells were transfected with the pPUS2c vector containing each miRNA and mCherry expression was monitored over 72 h. Peak expression occurred ~48 h posttransfection, which was selected for qPCR to examine expression of each miRNA from each miRNA expression vector. The miRNA overexpression vectors significantly increased the levels of each miRNA. Controls in which 3T3 cells received the pmirGLO vector containing a 3′ UTR in which the complementary seed sequence was mutated to prevent binding of the miRNA were also carried out. Detection of firefly luciferase activity indicated that the miRNA seed sequence was unable to bind the target, confirming that the miRNA has a binding site within that target.

For each experimental replicate ($n = 4$–5), transfections were performed in duplicate and cell lysates were combined, aliquoted into 4 wells of a 96-well plate and luciferase activity determined by averaging firefly luciferase and normalizing to the average Renilla luciferase activity. Each experimental replicate was repeated at least four times and experimental values (miRNA+target) were compared with control values (miRNA+scrambled target) and data analyzed using *t*-test.

Results

Phenotype analysis

After 4 weeks of differentiation of NPC, neurons from control as well as those from bipolar patients had differentiated extensively, forming many branching processes (Fig. 1A–F), and cultures were largely free of other cell types. Consistent with our previous observations [53], NPC from BP patients differentiated for 4 weeks into neurons express slightly higher levels of genes associated with ventral telencephalic cell types, including the GABAergic interneuron marker *NKX2.1*, and the vesicular GABA transporter *vGAT* (Fig. 1G, H). Neurons derived from control iPSC expressed somewhat higher levels of the vesicular glutamate transporter *vGLUT1* (Fig. 1I), a transcript present in excitatory, glutamatergic neurons of the cortex.

Enrichment analysis

After hybridization to Illumina 4.1 miRNA microarrays, RMA was used to calculate expression values for each probe set. Principal component analysis was carried out on the expression values and the first two principal components plotted. The bipolar patients grouped together but one control did not cluster with the others, suggesting that differences between the groups were not large. Group

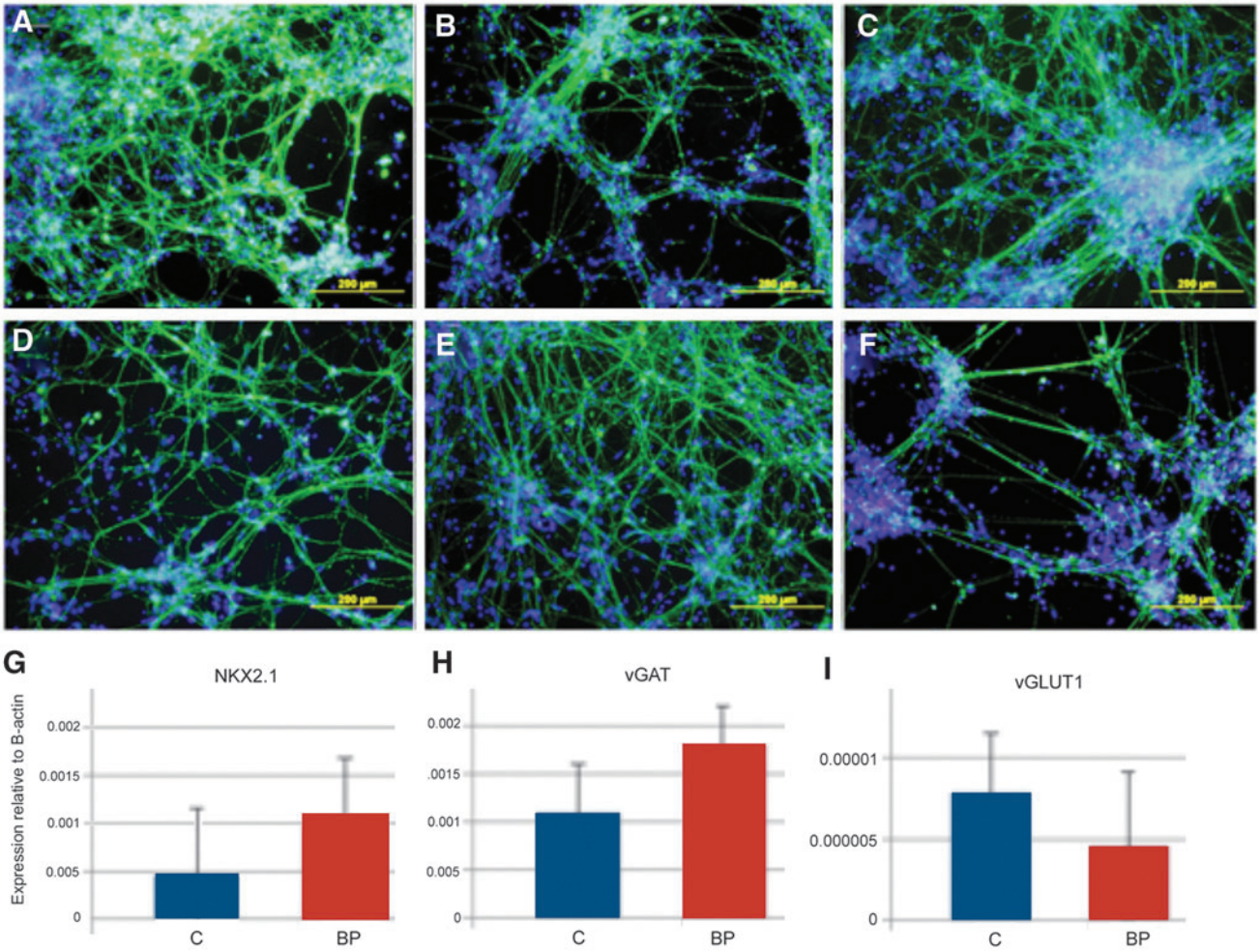

FIG. 1. Neuronal differentiation in cultures from control **(A–C)** or BP patient **(D–F)** iPSC. Cells were exposed to antibodies to βIII tubulin (FITC) followed by FITC-labeled secondary antibody and with Hoechst dye (*blue*). Scale bar = 200 μm. Neurons from BP iPSC lines expressed slightly higher levels (nsd) of the ventral forebrain neuronal marker *NKX2.1* **(G)** and of *vGAT* **(H)** and somewhat lower levels of *vGLUT1* **(I)** than controls in qPCR. Graphs represent gene expression relative to a β-actin control **(C)**. BP, bipolar disorder; iPSC, induced pluripotent stem cell; nsd, no significant difference; qPCR, quantitative polymerase chain reaction.

comparisons were carried out and probe sets with a fold change of 1.5 or greater were selected for additional analysis. *P* values were adjusted for multiple comparisons using an FDR of 0.05. Table 1 lists transcripts with significantly higher expression in neurons from bipolar individuals ($n = 34$, right columns) and those with significantly lower expression in BP neurons ($n = 24$, left columns). Several miRNAs upregulated in the bipolar group have previously been associated with neuropsychiatric or neurological conditions or with central nervous system (CNS) development (Table 1). Many are expressed in the brain, and several are regulated by lithium. Conversely, many of the miRNAs downregulated in the BP neurons have been implicated in proliferation and in cancer, but only one, miR874–5p, was previously associated with schizophrenia and BP.

Validation

Of the nine differentially expressed miRNAs examined in qPCR, eight were concordant in the direction of their fold change with the microarray data and one miRNA, miR874–5p, was discordant, being elevated in the qPCR analysis of BP miRNAs (Fig. 2). Pearson Correlation = 0.879, $P < 0.05$, two-tailed test.

Target analysis

Predicted targets of miRNAs upregulated and downregulated (with 80% confidence) in control and BP patient neurons were imported into DAVID and GO, KEGG pathway, and hierarchical cluster analyses were carried out (Tables 2 and 3). Briefly, pathways including axon guidance, pathways in cancer, as well as Mapk, Neurotrophin, and Hippo signaling, were identified in both groups, whereas Endocytosis, Ubiquitination, Proteoglycans in cancer, Phosphatidylinositol, and FoxO signaling, were present in the targets predicted to be downregulated in BP. Focal adhesion, Wnt, and Ampk signaling pathways were identified in the targets predicted to be upregulated in BP.

Gene ontology

Cellular component analysis identified a number of similar terms in both groups, including Cytosol/cytoplasm, Membrane,

Table 1. MicroRNA Expression

miRNA down in BP			*miRNA up in BP*		
miRNA	*ASSN*	*Region*	*miRNA*	*ASSN*	*Region*
MIR-10B-3P	GBM, MB, HD, S [147,148]		MIR-124-2-5P	D, NG [158–160]	
MIR-10B-5P	HD, S [149,150]		MIR-1244-4		
MIR-1269B			MIR-128-1-3P	CE, AS [161,162]	
MIR-1270	GBM [151]		MIR-1343-3P		
MIR-1233-1			MIR-138-2-3P	PD, BP [163]	BP-BA9 [24]
MIR-1306			MIR-139-5P		
MIR-378C	NSC, GBM [152,153]		MIR-181C-3P	SZ, INF [80,164–166]	
MIR-378D			MIR-195-5	EB, SZ, ASD [157,164,167]	
MIR-378F			MIR-219B-5P	EB, SZ, O [157,168,169]	SZ-BA10 [64]
MIR-378G			MIR-29C-5P	EB, ALZ, SZ [157,170,171]	BP-BA10, SZ/BP-BA9 [62,63]
MIR-422A	S [154]		MIR-3156-1-5P		
MIR-4260			MIR-3174		
MIR-4444-1			MIR-330-5P	EB, BP, NSC [157,172,173]	BP, SZ-BA9 [24]
MIR-4632-3P			MIR-382-5P		SZ-OE [174]
MIR-4736			MIR-3918		SZ-BA46 [175]
MIR-4793-5P			MIR-3939		
MIR-5002			MIR-4304		
MIR-6500-3P	GBM [155]		MIR-4327		
MIR-6740			MIR-4454		
MIR-6756			MIR-4521	FCD, GBM [176,177]	
MIR-6775-3P			MIR-4666B		
MIR-6805			MIR-4750-3P		
MIR-6821	ALZ [156]		MIR-4773-2		
MIR-874-5P	hESC [157]	BP/SZ-BA46 [23]	MIR-485-5P	EB, SZ, GBM [157,178,179]	BA10 [64]
			MIR-487B-3P	EB, GBM [157,180,181]	
			MIR-584-5P	GBM [182]	
			MIR-593-5P	GBM [183]	
			MIR-6072		
			MIR-6509-3P		
			MIR-654-3P		
			MIR-6801-5P		
			MIR-6890		
			MIR-744-3P	ND, ESC, GABA [184]	
			MIR-766-5P	hESC, ALZ [157,185]	

miRNAs expressed at significantly higher levels in control neurons (ie, downregulated in BP; *left three columns*), compared with those upregulated in BP patient-derived neurons (*right three columns*). miRNAs previously associated with stem cells, nervous system development, or mood disorders are listed (ASSN) and localization data from postmortem brain (Region). Increased expression of miRNA suggests targets should be downregulated and vice versa.

ALZ, Alzheimer's disease; AS, astrocytoma; ASD, autism spectrum disorder; ASSN, association; BA, Brodmann's area; BP, bipolar disorder; CE, cortical excitability; D, dendrites; EB, embryoid body; FCD, focal cortical dysplasia; GBM, glioblastoma; HD, Huntington's disease; hESC, human embryonic stem cell; INF, inflammation; MB, medulloblastoma; miRNA, microRNA; ND, neural differentiation; NG, neurogenesis; NSC, neural stem cells; O, oligodendrocytes; OE, olfactory epithelium; PD, panic disorder; S, synaptogenesis; SZ, schizophrenia.

and Nucleoplasm. Interestingly, targets associated with Synapse and Dendrite were also identified in both groups. Transgolgi, RNA processing body, Tight junction, Endosome, Nuclear speck, and Ruffle were present in the group of miRNA targets predicted to be downregulated in BP. Growth cone and Neuronal cell body were the only unique components in the targets predicted to be upregulated in BP.

Target validation

The binding of selected targets by differentially expressed miRNAs is summarized in Fig. 3, shown as percentage of the control (set to 100%). Of the targets of miR195–5p (which was increased in BP neurons) *AXIN2*, *BDNF*, *CACNA1E*, *MIB1*, *NLGN1*, and *RELN* were confirmed, with luciferase activity decreasing 53%–76%. Of the miR382–5p targets (also increased in expression in BP neurons), *SYT4* was significantly downregulated by the miRNA, while *NFIA* was downregulated, but levels did not reach significance. miR10b-5p (downregulated in BP neurons) targeted *ANK3*, *BDNF*, *CAMK2G*, *DLGAP2*, and *NFASC* significantly reducing luciferase activity 50%–78%.

Discussion

Current research suggests that neuropsychiatric disorders may result from alterations in pathways that affect neuronal differentiation and function [55,56], producing a spectrum

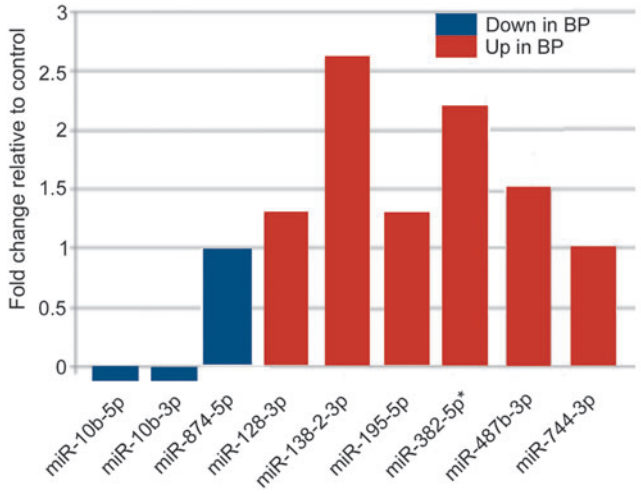

FIG. 2. Microarray validation. qPCR analysis of the expression of three miRNAs identified as downregulated in BP neurons (miR-10b-5p, miR-10b-3p, and miR-874-5p) and six miRNAs upregulated in BP neurons. Eight are consistent with the direction of change observed in the microarray. However, miR-984-5p was decreased in the BP sample in the microarray, but in qPCR was increased in BP neurons. Pearson correlation = 0.879, $P < 0.05$. miRNA, microRNA.

of conditions that reflect the sequelae of aberrant neural development [55]. Since small alterations in the expression of miRNAs can fine-tune the expression of multiple genes in regulatory networks, they are uniquely positioned to influence development and function in a cell type-specific and spatiotemporally restricted manner and are further subject to epigenetic regulation and disease-specific mutation.

Postmortem analysis of miRNAs in the BP CNS

Before the availability of iPSC-derived neurons, data regarding miRNA expression in BP has relied on the analysis of postmortem brain tissue, cerebral spinal fluid (CSF), or peripheral blood. While there is no clear consensus regarding affected brain region or neuronal cell type and miRNA dysregulation in BP [57,58], studies have often examined the frontal/prefrontal cortex (Brodmann's area [BA] 8, 9,10, 46). Of the studies of miRNA expression in the bipolar brain (reviewed in Fries et al. [58] and Geaghan and Cairns [59]), several miRNAs were coordinately differentially expressed in our sample (Table 1). These include miR-124 (increased in both BP brain and iPSC-derived BP neurons) [60], which regulates glial versus neuronal gene expression [61]. miR-219 (increased in both), which is involved in the circadian clock and also targets *CAMKII* [60,62] which is expressed in forebrain glutamatergic neurons. MiR-29c (upregulated in our BP neurons) was also upregulated in several other studies of BP: in the prefrontal cortex (PFC) [58], in extracellular vesicles from both the PFC [62,63] as well as from BA10 neuropil [64]. In another study [65], both miRNAs whose expression was significantly increased (miR-330, miR-138) in BA9 of BP patients were also significantly upregulated in our sample. miR-874, which was significantly downregulated in BP-derived neurons in the current study, was also downregulated in the dorsolateral prefrontal cortex (BA46) of BP patients [66]. Finally, of the 43 dysregulated miRNAs from the BP PFC [58], only miR-29c (up in both), and mirR-874 (decreased in both) were significantly altered in the current investigation.

Several miRNAs previously associated with BP, including miR-137 [67], miR-34a [68], and miR-9 [69], were not significantly altered in neurons from BP individuals in the current study. This may be due to the small sample size, method, or extent of differentiation, or there may be individual variation in their expression in BP. However, miR-29c (which was significantly increased in BP neurons) was previously identified in three additional studies. As with many of the other miRNAs, GO terms associated with the targets of miR-29c are related to extracellular matrix (ECM), and KEGG pathway analysis suggests roles in ECM/receptor interactions, in focal adhesions, and in pathways in cancer.

TABLE 2. KEGG PATHWAY ANALYSIS OF PREDICTED TARGETS

Targets predicted to be D/R in BP[a]		*Targets predicted to be U/R in BP*[b]	
GO term	P *value*	*GO term*	P *value*
Axon guidance	$1.7\ e^{-3}$	Axon guidance	$3.5\ e^{-3}$
Phosphatidylinositol signaling	$2.5\ e^{-3}$	Mapk signaling	$1.3\ e^{-2}$
Endocytosis	$2.5\ e^{-3}$	Pathways in cancer	$1.8\ e^{-2}$
Proteoglycans in cancer	$9.0\ e^{-3}$	Ras signaling	$3.1\ e^{-2}$
Ubiquitination	$9.4\ e^{-3}$	Neurotrophin signaling	$4.5\ e^{-2}$
FoxO signaling	$1.0\ e^{-2}$	Hippo signaling	$4.8\ e^{-2}$
Mapk signaling	$1.0\ e^{-2}$	Ampk signaling	$4.8\ e^{-2}$
Neurotrophin signaling	$1.7\ e^{-2}$	Focal adhesion	$5.4\ e^{-2}$
Pathways in cancer	$3.0\ e^{-2}$	Wnt signaling	$5.5\ e^{-2}$
Hippo signaling	$3.2\ e^{-2}$		
mTor signaling	$3.4\ e^{-2}$		
Ras signaling	$4.8\ e^{-2}$		

KEGG pathway analysis of targets predicted to be downregulated in BP neurons (*left two columns*) and targets predicted to be upregulated in BP (*right two columns*). *P* values are Benjamini adjusted for large sample size. Several signaling pathways were identified in both groups, including: Axon guidance, *Mapk*, *Ras*, Hippo, and neurotrophin signaling. *P* values were adjusted for large samples.

[a]That is, miRNAs increased in BP.

[b]miRNAs decreased in BP.

D/R, downregulated; GO, Gene Ontology; U/R, upregulated.

TABLE 3. GENE ONTOLOGY ANALYSIS OF PREDICTED TARGETS: CELLULAR COMPONENT

Targets predicted to be D/R in BP[a]		*Targets predicted to be U/R in BP*[b]	
GO term	P *value*	*GO term*	P *value*
Cytosol	$9.9\ e^{-10}$	Cytoplasm	$1.1\ e^{-3}$
Membrane	$3.9\ e^{-8}$	Dendrite	$4.3\ e^{-3}$
Nucleoplasm	$1.2\ e^{-7}$	Nucleoplasm	$5.3\ e^{-3}$
Trans-golgi	$1.6\ e^{-4}$	Membrane	$7.0\ e^{-3}$
RNA processing body	$1.8\ e^{-3}$	Synapse	$2.6\ e^{-2}$
Synapse	$3.5\ e^{-3}$	Membrane component	$2.9\ e^{-2}$
Endosome	$7.7\ e^{-3}$	Growth cone	$3.0\ e^{-2}$
Dendrite	$1.0\ e^{-2}$	Neuronal cell body	$4.5\ e^{-2}$
Tight junction	$3.8\ e^{-2}$		
Nuclear speck	$3.8\ e^{-2}$		
Ruffle	$4.8\ e^{-2}$		

GO analysis of predicted targets: cellular component. Cytosol/cytoplasm, membrane, nucleoplasm, synapse, dendrite, pathways in cancer were common to both groups, while Trans-golgi, RNA processing body, endosome, tight junction, nuclear speck and ruffle were unique to targets predicted to be downregulated in BP. Growth cone, neuronal cell body were unique to the targets predicted to be upregulated in BP.
[a]That is, miRNAs increased in BP.
[b]miRNAs decreased in BP.

miRNAs obtained from peripheral tissues, including several body fluids [70] have been suggested as possible biomarkers of disease (eg, [71]). Of the 10 miRNAs identified in that study of CSF, several were also coordinately dysregulated in our samples: miR-330 and miR-4521, which were upregulated in both, and miR-4793 which was downregulated. Sampling of extracellular vesicles (exosomes) released from neurons or glia has been suggested to provide an opportunity for peripheral sampling of brain-derived proteins. These vesicles are also important in cell/cell communication and gene expression [63] and are often loaded with miRNAs and other noncoding RNAs. As in Alzheimer's disease and Tauopathies [72], exosomes may ultimately be a ''dump'' for toxic proteins to be removed from the cell and may therefore be a new therapeutic target in BP. Exosomes form through the endosomal pathway—several components of which were identified in GO pathway analysis of targets significantly downregulated in BP in this

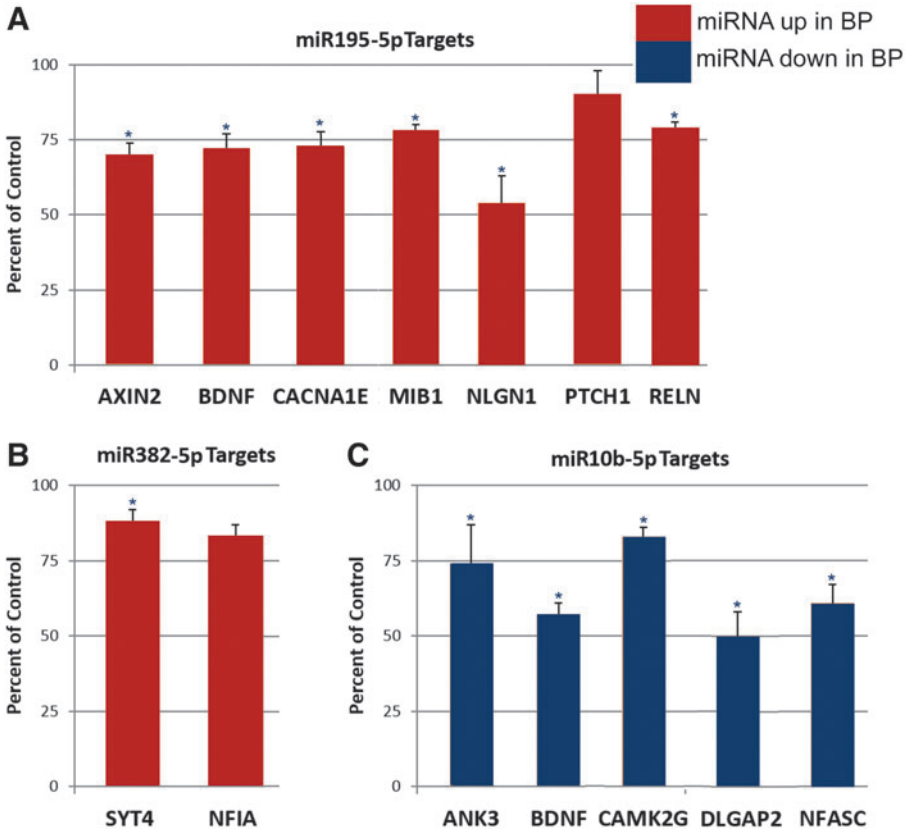

FIG. 3. Target validation **(A)** Downregulation of six of seven genes targeted by miR195-5p (up in BP). **(B)** Downregulation of two targets of miR382-5p (up in BP). **(C)** Upregulation of all five targets of miR10B-5p (down in BP). Targets were downregulated 20%–50%. $*P < 0.05$.

study. Interestingly, in a recent RNA-seq analysis of highly expressed transcripts in BP and C astrocytes (DeLong, in preparation), hierarchical cluster analysis identified ''Exosome'' as the first and most significant cluster. Of potential relevance to BP, exosomal transport and transfer has been suggested to contribute to the transgenerational inheritance of environment-induced phenotypes [73,74], and alterations in the formation, content, or release of these vesicles could have a profound effect on BP.

Drug responsiveness

miRNAs may both mediate the effects of antipsychotic drugs [60,75,76], and also be regulated by them, including lithium [75,77], valproate [75,78], as well as antipsychotics [79,80]. In fact, valproate has been shown to degrade *DICER*, which would globally decrease mature miRNA expression [81], and fluoxetine to inhibit neurogenesis by decreasing the expression of miR-16 [82]. miRNAs may also be useful markers of antipsychotic drug response [76], and miRNA targets themselves are often genetic risk factors for BP [75]. Ultimately, medicines may mediate treatment response by targeting signaling pathways, particularly β-catenin/Wnt, Mapk, Notch, and mTor, which are involved in neurogenesis, plasticity, proliferation, and synaptic function [75], as well as previously unsuspected pathways identified in this analysis; Hippo, FoxO, Ras, and Ampk signaling.

Genetics

Increasingly, evidence suggests that epigenetic alterations may play a role in BP [83]; in the case of miRNAs, their transcription is often regulated by DNA methylation [84,85]. Copy number variants and polymorphisms in genes in miRNA biogenesis pathways and in miRNA targets are overrepresented in BP [86], and miRNAs are often located in BP susceptibility loci [58,67,87–89], which may contribute to risk/transmission of risk for BP. For example, of the ~42 miRNAs that target the BP risk gene *CACNA1C*, miR-1343 was significantly increased in our sample and expression of its target, *CACNA1C*, decreased [53]. Finally, polygenic risk scores based on miRNA genes have predicted suicide behavior in BP patients [90].

miRNA targets that had a ≥80% probability of miRNA binding were analyzed using DAVID. KEGG Pathway analysis of the miRNA targets identified a number of significantly altered signaling pathways (Table 2), some of which have previously been associated with BP. Most are involved in cell growth, division, cell migration, and differentiation, emphasizing the role of neural development in BP. Common pathways include: Axon guidance, Pathways in cancer, Mapk signaling, which is critical in synaptic plasticity, and which is affected by lithium and valproic acid [91]. The Ras signaling pathway, which controls cell growth, differentiation, and survival can activate both Mapk and mTor signaling. Alterations in Ras pathway signaling have previously been reported to be involved in aspects of social behavior [92]. Hippo pathway signaling, which is involved in control of cell division and organ size, has also previously been implicated in BP [94,95]. Neurotrophin signaling was identified in both groups, and has also been previously been associated with BP [96,97]. An additional pathway predicted to be downregulated in BP was Foxo signaling, which is involved in proliferation, lifespan, and stress response. The Foxo pathway is suppressed by both lithium and BDNF and may be a target for the treatment of anxiety and depression [98]. Alterations in mTor signaling, which regulates cell growth, motility, survival, protein synthesis, and transcription, have previously been associated with depressive episodes in BP [93,94].

Three signaling pathways were identified in targets expected to be upregulated in BP: Wnt signaling, which is involved in cell division, migration, and differentiation and is often reported to be altered in BP (reviewed in Hoseth et al. [99]), Focal adhesion, and Ampk pathway signaling, which is involved in energy homeostasis and glucose metabolism, but has not previously been implicated in BP. While there is considerable evidence for alterations in intracellular signaling cascades in BP disorder, these results suggest novel pathways and new molecular targets.

GO analysis of the targets identified structural components: Cytoplasm, Membrane, and Nucleoplasm as top GO terms in both groups (Table 3). Synapse and Dendrite were also identified in both groups. Targets of the group predicted to be upregulated in BP (ie, miRNAs decreased) were associated with additional structural elements: Growth cone and Neuronal cell body, perhaps influencing neuronal morphology and thereby function. Interestingly, targets predicted to be downregulated in expression included: Trans-golgi, RNA processing body, Endosome, Nuclear speck, Tight junction, and Ruffle. Of these, the RNA processing body (P-body) is thought to play a role in miRNA gene silencing since a number of proteins necessary for miRNA gene silencing are localized to P-bodies. Several significant GO terms: Endosome, Early endosome, and Trans-golgi network suggests tight control of vesicle packaging within the neurons.

Validated targets of miR-10b (upregulated in BP) include transcripts involved in fundamental aspects of neuronal differentiation such as axon outgrowth and fasciculation, *NFASC* [100,101]; in postsynaptic density organization and signaling, *DLGAP2* [102,103] and *CAMK2G* [103,105]; in glutamatergic neuronal differentiation, *DLGAP2* [104]; organization of the axon initial segment, *ANK3* [106] and *NFASC* [101]; and in white matter organization, *ANK3* [107]. Genetic alterations in the NGF family member/neuronal survival factor *BDNF* [108,109] and in *ANK3* [110,111] have been associated with BP, and *DLGAP2* and *BDNF* with schizophrenia [112,113]. Of these, *CAMK2G* may be of particular interest in early CNS development as it is prominently expressed in the forebrain, where it plays a critical role in glutamatergic synaptic plasticity and function.

Validated targets of miR-195 include the E3 ubiquitin ligase *MIB1*, which controls neuronal differentiation and synaptic plasticity by positively regulating Notch [114] and Wnt/βCat [115] pathway signaling. *MIB1* is present in the postsynaptic density [116], is involved in dendritic spine outgrowth [117], can inhibit neurite outgrowth [116], and is overexpressed in astrocytoma [118]. Another target, *AXIN2*, is a negative regulator of the canonical Wnt pathway [119]; however, low levels are required to maintain stemness of neural stem cell [120,121]. Consistent with this, activation of the Wnt/βCat pathway plays an important role in rostral forebrain differentiation of ESC [122], while mutation and overexpression of *AXIN2* can contribute to medulloblastoma

[123]. *CACNA1E*, a BP risk factor, plays a role in synaptic plasticity and dendritic spine formation [124] through its ability to regulate cellular calcium influx; *NLGN1* promotes synaptogenesis [125] and synaptic maturation [126], and has been implicated in schizophrenia (SZ) [127] and autism spectrum disorder (ASD) [128]. *RELN* is involved in cortical neuronal migration and positioning during CNS development [129,130], and has been implicated in SZ, BP, ASD, and major depressive disorder (reviewed in Ishii et al. [131]). It is critically involved in neuronal positioning, and in the maturation and refinement of GABA and glutamatergic synaptic circuits, thereby controlling the excitatory/inhibitory balance of the developing cortex [132], which is often disrupted in BP [133].

Only *SYT4* was validated in luciferase assays as a direct target of miR-382, which was upregulated in BP. *NFIA*, a key regulator of astrocyte differentiation [134], did not meet statistical significance, although it has previously been associated with BP [135,136]. Interestingly, many of the miRNA targets examined in this study, including *SYT4*, *DLGAP2*, *MIB1*, and *CACNA1E* are involved in synaptic organization and function. A surprising number are involved in organization of the postsynaptic density: *DLGAP2/PSD95* [102,103,112]; *CAMKII* [105]; *NFASC* [137]; and *SYT* [138], where they may regulate patterning, synaptic plasticity, and function.

Overall, the validated targets of miRNAs dysregulated in BP neurons are heavily represented with transcripts involved in glutamatergic/GABAergic neurotransmission, postsynaptic associated factors, and essential contributors to cortical histogenesis and maturation/function.

miRNAs and developmental contributions to neuropsychiatric conditions

Of the >2,500 human miRNAs identified to date, over 70% are expressed in the CNS [139], where they play critical roles in lineage specification from preimplantation development [140], through neural induction [141], adult neurogenesis [142], in pathfinding, proliferation, and cell migration [143], to plasticity and synaptogenesis in the adult brain [56]. The ability of a single miRNA to regulate potentially hundreds of transcripts positions these non-coding RNAs to play critical roles in controlling signaling pathways involved in both normal and abnormal CNS development, and therefore in complex multigenic conditions, such as neuropsychiatric disease. The widespread switch in miRNA expression that occurs in the adolescent brain [144], a period which often corresponds to the identification of the first neuropsychiatric symptoms, the fact that differentially expressed targets are enriched in neurodevelopmental genes [145], as well as cell-type-specific [146] and spatiotemporal patterns of miRNA expression in the CNS, suggest that understanding how miRNA expression is controlled may be critical in recognizing their contribution to normal development and therefore to BP. In fact, Beveridge [144] has shown that many of the miRNAs that are expressed early in development and are normally downregulated shortly after birth, continue to be expressed inappropriately in the schizophrenic brain. That 9/140 of the miRNA that decrease with increasing age in the CNS (miR-124a, miR-138a, miR-139, miR-181c, miR-29c, miR-382, miR-422b, miR-487b, miR-625), but **none** of the miRNAs that increase with age, identified in the BP neurons, may suggest an important role in the maturation or plasticity of the BP CNS.

Patient-derived iPSC may be particularly useful in understanding the role(s) of miRNAs in CNS development and errors in development, because they provide an opportunity to probe and alter the expression of miRNAs, their targets, singly and in combination, in various cell types of the CNS. Targeted alteration will be important since miRNAs can affect genes in multiple pathways, with the resulting concern of off-target effects [82]. Interestingly, miRNA targets are overrepresented in genes involved in glutamatergic/GABAergic neuronal differentiation and function, in dendritic and synaptic factors, as well as in proteins that are essential contributors to cortical histogenesis. These results implicate neural development as key in neuropsychiatric disorders, and importantly, they also identify intracellular signaling cascades both novel and known to be involved in BP.

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The authors declare no competing interests.

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